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

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Abstract
Non-alcoholic fatty liver disease (NAFLD), characterized by lipid deposition within the liver (intrahepatocellular lipid, IHCL), is associated with insulin resistance and the metabolic syndrome. It has been suggested that impaired skeletal muscle mitochondrial function may contribute to ectopic lipid deposition, and the associated metabolic syndrome, by altering post-prandial energy storage. To test this hypothesis, we performed a cross-sectional study of 17 patients with NAFLD (mean ± SD; age 45±11 y; BMI 31.6±3.4 kg/m^2) and 18 age- and BMI-matched healthy controls (age 44±11 y; BMI 30.5±5.2 kg/m^2). We determined body composition by magnetic resonance imaging (MRI), IHCL and intramyocellular (soleus and tibialis anterior) lipids (IMCL) by proton magnetic resonance spectroscopy (^{1}H-MRS) and skeletal muscle mitochondrial function by dynamic phosphorus magnetic resonance spectroscopy (^{31}P-MRS) of quadriceps muscle. Although matched for BMI and total adiposity, after statistical adjustment for gender, patients with NAFLD (defined by IHCL $\geq$ 5.5%) had higher IHCL (25±16 vs. 2±2 %; p<0.0005), and a higher prevalence of the metabolic syndrome (76 vs. 28%) compared with healthy controls. Despite this, visceral: subcutaneous fat ratio, IMCL and muscle mitochondrial function were similar between the NAFLD and control groups, with no significant difference in the rate constants of post-exercise PCr recovery (1.55±0.4 vs. 1.51±0.4 min^{-1}), a measure of muscle mitochondrial function. Impaired muscle mitochondrial function does not appear to underlie ectopic lipid deposition, or the accompanying features of the metabolic syndrome, in patients with NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) describes a pathological spectrum in which lipid excessively accumulates within the liver, with or without associated necroinflammatory changes. The association of NAFLD with insulin resistance, the metabolic syndrome and type 2 diabetes mellitus is well established [1]. Petersen *et al.* [2] highlighted the role played by skeletal muscle, and associated insulin resistance, in the development of the metabolic syndrome and NAFLD, showing that reduced muscle glycogen synthesis and compensatory increased hepatic *de novo* lipogenesis result in a characteristic dyslipidaemia (increased plasma triglycerides and lower HDL) and increased hepatic triglyceride synthesis; furthermore, exercise-induced reversal of muscle insulin resistance, which dramatically increased postprandial net muscle glycogen synthesis, reduced hepatic triglyceride synthesis (by ~40%) [3].

Although it is well established that intracellular lipid accumulation within muscle and liver accompanies systemic insulin resistance, and mechanisms have been proposed for causal pathophysiological links between lipid accumulation and impaired insulin signaling [4, 5] the extent to which mitochondrial dysfunction contributes to accumulation of lipid in non-adipose sites, including skeletal muscle and liver, remains contentious. Some studies have suggested that defects in skeletal muscle mitochondrial oxidative capacity might contribute to ectopic lipid accumulation [2, 3, 6, 7] while others have found no significant association between ectopic fat deposition and altered mitochondrial function [8, 9]. The experimental literature in this area has been complicated by the variety of ways in which mitochondrial function can be assessed [10].

A way to study skeletal muscle mitochondrial function noninvasively *in vivo* is phosphorus magnetic resonance spectroscopy (^{31}P MRS) [10]. We set out in this study to use ^{31}P MRS, together with MR-based assessments of muscle and liver cell triglyceride and whole-body adipose tissue distribution, to study the relationship between these in patients with NAFLD compared to normal subjects matched for age, gender, BMI and whole body fitness.

Methods

Participants

All participants were sedentary (<2h low intensity physical activity per week) non-smokers with no history of excessive alcohol intake (average weekly consumption <14 units for females and <21 units for males). None had a history of ischaemic heart disease or type 2 diabetes mellitus, and their fasting glucose levels were normal. There were no contraindications to exercise or MRI scanning.

NAFLD patients: 17 patients with NAFLD (mean±SD; age 45±11 y; BMI 31.6±3.4 kg m^{-2}) were recruited by a single, experienced hepatologist at each of two tertiary referral specialist liver clinics. The diagnosis of NAFLD was made, in patients who had been referred for investigation of raised plasma transaminase levels, after careful exclusion of drug causes, viral hepatitis (negative hepatitis B and C serology), autoimmune hepatitis and primary biliary cirrhosis (negative auto-antibody screen) or metabolic disorders such as α_1-antitrypin deficiency or Wilson's disease (normal $\alpha1$ antitrypsin and caeruloplasmin concentrations). Allocation to the NAFLD group was confirmed once liver triglyceride content was measured as $\geq 5.5\%$ by proton magnetic resonance spectroscopy (^{1}H-MRS) [11].

Control subjects: 18 age- and BMI-matched healthy controls (age 44±11 y; BMI 30.5±5.2 kg m^{-2}) were recruited via local advertisement. None were taking any prescribed medication and all had normal plasma levels of liver transaminases. Allocation to the control group was confirmed once liver triglyceride content was measured as ≤ 5.5%.

Ethical considerations
The study conformed to the *Declaration of Helsinki* and was approved by the local NHS research ethics committee. Participants were informed of the methods verbally and in writing before providing written informed consent.

Research Design
This was a cross-sectional study. Participants reported to the laboratory on two occasions. All measurements were performed following an overnight fast, 12h abstinence from caffeine and 24h abstinence from alcohol and strenuous exercise. The first visit entailed anthropometric and blood pressure measurements, biochemical measurements and a maximal oxygen consumption test. The second visit was for all MR-based measurements: abdominal MRI, ^{1}H-MRS of liver, and ^{31}P-MRS and ^{1}H-MRS of leg muscle.

Anthropometric and blood pressure measurements: After a full medical history, a single observer (AI) performed all the anthropometric assessments (weight, height, waist and hip circumference) and blood pressure measurements.

Biochemical measurements: Following an overnight fast a blood sample was taken. Plasma was analysed using the Olympus AU2700 analyser (Beckman Coulter, High Wycombe, UK) with standard proprietary reagents as follows: glucose with hexokinase, total cholesterol and high-density lipoprotein (HDL) with cholesterol esterase/oxidase, triglyceride with glycerol kinase and liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) with International Federation of Clinical Chemistry (IFCC) kinetic UV (without pyridoxal phosphate activation). The intra- and inter-assay coefficient of variation was ≤10%. Low-density lipoprotein (LDL) was calculated according to the Friedewald formula. All laboratory assays were performed in the clinical biochemistry laboratory at University Hospital Aintree.

Metabolic syndrome: The diagnosis of the metabolic syndrome was based on the national Cholesterol Education Program Adult Treatment Panel III criteria [12] when ≥3 of the following are present: i) central obesity: waist circumference ≥ 102 cm (male), ≥ 88 cm (female); dyslipidemia: ii) TG ≥ 1.7 mmol/L (150 mg/dl) and iii) HDL-C < 40 mg/dL (male), < 50 mg/dL (female); iv) hypertension: systolic blood pressure ≥ 130/85 mmHg; v) hyperglycaemia: fasting plasma glucose ≥ 6.1 mmol/L (110 mg/dl).

Cardiorespiratory fitness (VO$_{2peak}$) *test:* Fitness was assessed on a treadmill ergometer following the Bruce protocol [13]. After an initial 2 min warm up at 2.2 km/h on the flat, the initial workload was set at 2.7 km/h at 5° grade; step-wise increments in speed and grade were made every minute thereafter. Heart rate (Polar Electro Oy, Kempele, Finland) and rate of perceived exertion were monitored continuously [14]. VO$_{2peak}$ was calculated from expired gas fractions (Oxycon Pro, Jaegar, Hochberg, Germany) as the highest consecutive 15s period of gas exchange data occurring in the last minute before volitional exhaustion.

MR measurements Measurements of total abdominal, abdominal subcutaneous and visceral fat by whole-body MRI, and of intrahepatocellular lipid (IHCL) and intramyocellular lipid (IMCL) by ^{1}H MRS, were carried out using a Siemens 1.5T Symphony MR scanner (Siemens AG, Erlangen, Germany) as previously described [15-18]. ^{31}P MRS assessments of muscle mitochondrial function in quadriceps were carried out using a Siemens 3T Trio MR scanner (Siemens AG, Erlangen, Germany) using an isometric knee extension exercise protocol similar to one we employed previously for studies of calf muscle during plantar flexion [19]. Subjects lay supine (secured with a Velcro strap across the hips) with the right knee flexed 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 aluminum bar fitted with a strain gauge. ^{31}P MRS data were acquired from right quadriceps muscle using a dual-tuned 18cm/15cm 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=15s) spectrum and a 32-scan partially saturated (TR=2s) resting spectrum were collected. The exercise protocol consisted of 5 min rest followed by 2 bouts of isometric exercise each followed by 7 min recovery periods, while spectra were collected (TR=2) every 8 s paced at 0.5 Hz (1 s on, 1 s off) by an audible cue, exercise force being fed back visually 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). All spectra were acquired with Nuclear Overhauser enhancement (NOE): ^{1}H spins were saturated by 10 radiofrequency pulses (900 flip angle), 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. 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 lineshapes for PCr, Pi, PDE, PME, NADP and ATP (β-ATP a 1:2:1 triplet, α-ATP and γ-ATP both 1:1 doublets). The chemical shift of the inorganic phosphate (Pi) peak relative to phosphocreatine (PCr) (σ parts per million) was used by standard means to determine intracellular pH. PCr recovery time courses were fitted to a monoexponential function to estimate 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 [20]. As the PCr recovery rate constant did not differ significantly between the two exercise intensities, and as pH changes were small throughout, values of k are presented as mean of the two intensities to reduce variability [19].

Statistical analysis
The primary outcome variable for this study was the PCr recovery rate constant k. Groups were compared using the two-sample t-test or Mann-Whitney U tests if distributional assumptions were not met following logarithmic or Box-Cox transformation. In order to assess the sensitivity of some results to the gender imbalance between groups we also compared groups using linear regression/ANCOVA, adjusting for sex. Poisson 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

mean(95% confidence intervals), 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 wree conducted using Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

Results
Clinical characteristics (Table 1)
Patients with NAFLD and control subjects were matched with no significant differences in age, body mass index, fat mass and cardiorespiratory fitness (even when normalised for body weight) (Table 1). The NAFLD group had a significantly greater waist circumference [7.9cm (95% CI=0.6, 15.1); *P*=0.03], although when adjusted for total body weight this difference was no longer significant.

Biochemical characteristics and components of the metabolic syndrome (Table 1)
Patients with NAFLD had higher ALT [61 mU/l (95% CI= 30, 83) vs. 21 mU/l (95% CI= 16, 32); *P*<0.001] and GGT [48 mU/l (95% CI= 42, 79) vs. 28 mU/l (95% CI= 23, 40); *P*=0.03] when compared with controls but AST was not significantly different [24 mU/l (95% CI= 10, 43) vs. 21 mU/l (95% CI= 16, 25); *P*<0.001]. Although total cholesterol was not significantly different, patients with NAFLD had a significantly higher triglyceride [2.3 mU/l (95% CI= 1.6, 3.4) vs. 1.3 mU/l (95% CI= 1.0, 2.3); *P*=0.007] and lower HDL cholesterol [1.0 mmol/l (95% CI= 0.9, 1.2) vs. 1.2 mmol/l (95% CI= 1.1, 1.5); *P*=0.007]. Fasting glucose did not differ between the two groups (Table 1). The prevalence of metabolic syndrome was greater in the NAFLD group (76% vs. 28%, *P*=0.004) and the patients with NAFLD had a higher mean number of components of the metabolic syndrome (2.9±1.0 vs. 1.8 ±1.2; *P*=0.05) (Figure 1).

MR-derived measures of adipose tissue mass (Figure 1)
After statistical adjustments for gender, total abdominal fat did not differ between the two groups, nor did the amount visceral fat in patients with NAFLD (Figure 1). VAT: SAT ratio was similar in NAFLD patients compared to controls. As expected, liver fat was higher in NAFLD patients (Figure 1) but IMCL in both soleus and tibialis anterior was not significantly different between the two groups (Figure 1).

Cytosolic pH and phosphorus metabolites in resting muscle (Table 1)
There were no significant differences in resting muscle intracellular pH (pH_i) between the two groups and there was no significant cytosolic acidification during exercise in either group. The resting phosphate metabolite ratios inorganic phosphate:ATP and PCr:ATP did not differ significantly between the two groups nor was there any significant difference in the amount of PCr depletion during exercise.

Assessment of muscle mitochondrial function (Figure 2)
The post exercise PCr recovery rate constant did not differ significantly between the two groups [1.62 min^{-1} (95% CI= 0.34, 1.86) vs. 1.50 min^{-1} (95% CI= 1.18, 1.77); *P*=0.81].

Relationship of muscle mitochondrial function to the metabolic syndrome (MS)
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 [0.03 min^{-1} (95% CI=-0.24, 0.30), *P*=0.83] (Figure 3). Similarly,

examining the number of components of the metabolic syndrome in both controls and NAFLD patients we found no correlation with the PCr recovery rate constant (r= -0.03, P=0.83).

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_{2peak}, any biochemical measure or any MR-derived measure of body composition.

Discussion
The major finding of this study is that skeletal muscle mitochondrial function, determined using dynamic ^{31}P-MRS measurements after exercise, is similar in patients with NAFLD to age-, BMI- and cardiorespiratory fitness-matched controls. The similarity in mitochondrial function was observed despite greater liver (but not visceral) fat deposition and more numerous components of the metabolic syndrome in patients with NAFLD suggesting that factors other than skeletal muscle mitochondrial dysfunction play a pathophysiological role in the metabolic phenotype of NAFLD.

Analysis of the post-exercise PCr recovery kinetics in the vastus lateralis muscle quantifies the *in vivo* mitochondrial oxidative capacity of skeletal muscle because, during recovery from exercise, PCr is resynthesized purely as a consequence of oxidative ATP synthesis. Such measures correlate with *ex vivo* measurements of muscle mitochondrial function and whole-body measures of aerobic fitness in type 2 diabetic patients [21, 22], similarly to healthy controls [22]. We observed no correlation with aerobic fitness in the current study, which could potentially be due to the small range of cardiorespiratory fitness observed in both study groups.

A number of reports [10] have implicated mitochondrial dysfunction in skeletal muscle in the development of insulin resistance. In a cohort of healthy, lean, insulin-resistant offspring of patients with type 2 diabetes, a ^{31}P MRS-derived measurement taken to reflect mitochondrial ATP production in skeletal muscle [10] was found to be ~30% lower (associated with ~60% lower insulin-stimulated glucose uptake by muscle and a ~80% greater IMCL than in healthy controls [23]. In a related study in a cohort of insulin-resistant offspring, a reduction in muscle mitochondrial density (measured in muscle biopsy specimens) was accompanied by increased IMCL, reduced insulin-stimulated glucose uptake and impairment of insulin signal transduction [24]. In the elderly, the same relationship was observed between claimed measures of mitochondrial dysfunction, ectopic fat accumulation and insulin resistance [6]. However, proposals of a causal relationship between muscle mitochondrial dysfunction, increased muscle cellular triglyceride accumulation and disordered post-receptor insulin signaling have been called into question by animal [25] and human [26] studies. Slowed PCr recovery is evident relative to controls matched for age, gender, BMI and fitness in e.g. primary congenital insulin resistance (7 patients vs. 12 controls) [27] and congenital lipodystrophy (7 patients vs. 15 controls) [20]. These studies show that studies of the present kind and size are capable of demonstrating defects of muscle oxidative capacity, relative to fitness-matched controls; this supports the inference from our failure to detect any such difference that no such abnormalities exists in our NAFLD patients. In both primary congenital insulin resistance and congenital lipodystrophy this impaired muscle oxidative function must be a consequence or simply an accompaniment of insulin resistance, since it

cannot be its cause ADDIN EN.CITE []). Our present negative finding suggests that muscle mitochondrial dysfunction is not a primary factor in the development of NAFLD and its cardiometabolic sequelae, but rather may occur as a secondary phenomenon.

We did not observe any difference in IMCL between NAFLD and control groups. The significance of IMCL is controversial, with some studies suggesting that muscle lipid accumulation is an early and critical event in the development of insulin resistance [28]. In contrast, similar to Thomas *et al*. [29], we found no correlation between IMCL and liver fat and, like Machado and colleagues [9] we found no relationship between IMCL and muscle mitochondrial dysfunction [6, 26].

There is good evidence that skeletal muscle insulin resistance is implicated in the development of NAFLD and the metabolic syndrome, and that this may be reversed with exercise [2, 3]. As the primary outcome measure of the current study was the rate constant of PCr recovery i.e. mitochondrial oxidative capacity, in relation to liver fat content, we were less concerned with measures of insulin sensitivity. The NAFLD patients had a higher mean fasting insulin concentration and HOMA-IR, but this only approached statistical significance (P=0.08). However, the limitations of HOMA-IR have been previously discussed and it has been suggested that surrogate estimates of insulin sensitivity (including HOMA-IR) can lead to conclusions that are totally different from those based on the results of euglycaemic–hyperinsulinaemic clamp measurements. Thus in the current study, in the absence of measures of insulin sensitivity using more sensitive, gold-standard techniques such as the hyperinsulinaemic, euglycaemic clamp, it would be misleading to draw conclusions on the relative degrees of insulin sensitivity/resistance of each individual/group.

Although we could not directly examine the inter-relationship between PCr recovery constant and insulin resistance, we compared instead the groups with or without the metabolic syndrome, and according to the number of components of the metabolic syndrome. We found no difference in PCr rate constant between those with or without the metabolic syndrome nor did we observe any relationship between the number of components and the PCr rate constant. A recent paper [30] has addressed the issue of a connection (possibly causal) between muscle energy metabolism, liver steatosis and insulin resistance by reanalysing data collected together from 6 previously-published studies [8, 31-35] of the research group. The total study population comprised 113 (52 male and 61 female) participants of whom 87 had normal glucose tolerance, 16 had impaired fasting glucose and/or glucose intolerance, and 10 had frank type 2 diabetes. This large study population permitted the identification of some interesting correlations; the rate of muscle Pi-ATP exchange, measured by magnetisation transfer, correlated negatively with hepatic lipid content and haemoglobin A1c (HbA1c), and positively with insulin sensitivity; there was no relation to body mass, muscle triglyceride. However, as alluded to above, there are serious problems with Pi-ATP exchange as a metabolic marker of mitochondrial metabolism [10]. It is now generally acknowledged that no measure of resting ATP *turnover* can be interpreted in terms of muscle oxidative *capacity,* which is by contrast the standard interpretation of measures derived from post-exercise PCr kinetics (the simplest of which is the rate constant, as used in the present work). In the light of this, the Pi-ATP exchange rate is sometimes regarded as a measure of 'mitochondrial activity' and' ATP synthase flux' [30]. However, the rate of Pi-ATP exchange is approximately an order of magnitude larger than the actual rate of oxidative ATP turnover in resting muscle, the difference being likely due non-mitochondrial exchange catalysed by two enzymes of glycolysis [10]. Interestingly, a cross-sectional study of 15 normal subjects [36],

significant correlations were found between the resting Pi-ATP exchange rate and a resting-ischaemia ^{31}P MRS measure of resting ATP synthesis rate (despite the order-of-magnitude difference in absolute numbers), and PCr-recovery based measures of mitochondrial function (despite the logical distinctness of ATP *turnover* and oxidative *capacity,* as mentioned above, and for reasons which are so far unexplained [10]. This first of these correlations, together with some observations of clinical or experimental changes in Pi-ATP exchange in physiologically expected directions [37] (again, notwithstanding the order-of-magnitude error), is sometimes described as supporting [37] or even validating [30] the Pi-ATP exchange measurement. However, as almost nothing is known about what determines the overwhelmingly dominant non-mitochondrial component of the measurement [10], this interpretation remains problematic. The important point for the present work is that, although we cannot exclude the possibility that a larger study (nearer the 113 subjects of [10] than the 35 we studied) would show a relationship between liver fat and a measure of muscle mitochondrial function, the correlation reported in [30] gives no reason to expect it.

In addition to skeletal muscle, the properties of adipose tissue and adipose tissue dysfunction are also implicated in the deposition of ectopic fat (although not examined in this study). Insulin resistance in adipose tissue results in a reduced ability of insulin to suppress fatty acid release from adipose tissue (leading to increased free fatty acid flux to the liver) and the magnitude of adipose insulin resistance is inversely correlated with liver fat [38, 39]. Furthermore, characteristics of subcutaneous fat and defective regulation of lipid storage genes in subcutaneous adipose tissue may limit its expandability [40].

Clinical Perspectives
It has been suggested that impaired skeletal muscle mitochondrial function may contribute to ectopic lipid deposition, and the associated metabolic syndrome, by altering post-prandial energy storage. We provide evidence that skeletal muscle mitochondrial function is preserved in patients with NAFLD, compared to age, BMI and fitness-matched healthy controls, and cannot account for ectopic fat deposition or the high prevalence of metabolic syndrome. Thus it would appear that factors other than skeletal muscle mitochondrial dysfunction play a pathophysiological role in the metabolic phenotype of NAFLD, with available data suggesting skeletal muscle and adipose tissue insulin resistance play a major role.

Authors Contributions
GJK together with **DJC** contributed to the conception of the project and experimental design. **GJK** provided significant contribution in relation to the interpretation of this data. **CJAP, HJ and VSS** contributed to the data collection process, recruitment and made critical revisions to the manuscript. **AI**, **WEB** and **VLA** were involved in all aspects of data collection. **CD, FSM** and **MU** made critical revisions to the manuscript prior to submission. **JPW** contributed to the intellectual content of the manuscript and made critical revisions prior to submission. All authors approved this version of the manuscript.

Table 1. Baseline characteristics of participants reported as mean+/- SD, median(lower quartile, upper quartile) or frequency(%).

		NAFLD (*n*=17)	Controls (*n*=18)	*P*
Anthropometric	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(10,43)	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 (mmol/l)*	1.0(0.9,1.2)	1.2(1.1,1.5)	**0.007**
	Triglyceride (mU/l)*	2.3(1.6,3.4)	1.3(1.0,2.3)	**0.007**
	Fasting glucose (mmol/l)*	5.0(4.7,5.3)	4.8(4.4,5.1)	0.18
	Fasting insulin *	91.2(67.2,114.6) (*n*=13)	78.8(55.4,86.4) (*n*=14)	0.11
	HOMA-IR *	4.1(3.0,7.6)	3.2(2.7,4.3)	0.08
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**
Cardiorespiratory fitness	V̇O$_2$ max (ml/kg/min)*	23.5(20,31.3)	25.75(22.9,30)	0.69
	V̇O$_2$ max/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 fall	0.14±0.12/	0.12±0.13/	0.07/

	-0.06±0.09	-0.01±0.09	0.62
Resting PCr/ATP	4.25±0.68	4.07±0.57	0.40
End-exercise PCr depletion (%)*	0.19(0.14, 0.26)/	0.20(0.08, 0.29)/	0.82/
	0.35(0.24, 0.50)	0.32(0.12, 0.44)	0.75
PCr recovery rate constant (min^{-1})*	1.62(0.34, 1.86)	1.50(1.18, 1.77)	0.81

Pairs of results represent 70%/90% MVC separately.

* Mann-whitney U Test, ¥Poisson regression.

Figure Legends

Figure 1. Body composition data demonstrating total abdominal fat, abdominal subcutaneous (SC) fat, visceral fat, intramyocellular fat (IMCL) of soleus muscle, liver fat and the number of components of the metabolic syndrome in patients with NAFLD (*n*=17) and age- and BMI-matched healthy controls (*n*=18).

Figure 2. Post-exercise phosphocreatine (PCr) recovery rate constant, k (min^{-1}) in patients with NAFLD (*n*=17) and age- and BMI-matched healthy controls (*n*=18).

Figure 3. Relationship between post-exercise PCr recovery rate constant, k (min^{-1}) and the number of components of the metabolic syndrome within the healthy controls (triangle) and patients with NAFLD (circle).

References

1   Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A. J., Natale, S., Forlani, G. and Melchionda, N. (2001) Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. **50**, 1844-1850

2   Petersen, K. F., Dufour, S., Savage, D. B., Bilz, S., Solomon, G., Yonemitsu, S., Cline, G. W., Befroy, D., Zemany, L., Kahn, B. B., Papademetris, X., Rothman, D. L. and Shulman, G. I. (2007) The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A. **104**, 12587-12594

3   Rabol, R., Petersen, K. F., Dufour, S., Flannery, C. and Shulman, G. I. (2011) Reversal of muscle insulin resistance with exercise reduces postprandial hepatic de novo lipogenesis in insulin resistant individuals. Proc Natl Acad Sci U S A. **108**, 13705-13709

4   Kantartzis, K., Machann, J., Schick, F., Fritsche, A., Haring, H. U. and Stefan, N. (2010) The impact of liver fat vs visceral fat in determining categories of prediabetes. Diabetologia. **53**, 882-889

5   Samuel, V. T., Petersen, K. F. and Shulman, G. I. (2010) Lipid-induced insulin resistance: unravelling the mechanism. Lancet. **375**, 2267-2277

6   Johannsen, D. L., Conley, K. E., Bajpeyi, S., Punyanitya, M., Gallagher, D., Zhang, Z., Covington, J., Smith, S. R. and Ravussin, E. (2012) Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. J Clin Endocrinol Metab. **97**, 242-250

7   Szendroedi, J., Kaul, K., Kloock, L., Strassburger, K., Schmid, A. I., Chmelik, M., Kacerovsky, M., Kacerovsky-Bielesz, G., Prikoszovich, T., Brehm, A., Krssak, M., Gruber, S., Krebs, M., Kautzky-Willer, A., Moser, E., Pacini, G. and Roden, M. (2013) Lower fasting muscle mitochondrial activity relates to hepatic steatosis in humans. Diabetes Care

8   Prikoszovich, T., Winzer, C., Schmid, A. I., Szendroedi, J., Chmelik, M., Pacini, G., Krssak, M., Moser, E., Funahashi, T., Waldhausl, W., Kautzky-Willer, A. and Roden, M.

(2011) Body and liver fat mass rather than muscle mitochondrial function determine glucose metabolism in women with a history of gestational diabetes mellitus. Diabetes Care. **34**, 430-436

9   Machado, M. V., Ferreira, D. M., Castro, R. E., Silvestre, A. R., Evangelista, T., Coutinho, J., Carepa, F., Costa, A., Rodrigues, C. M. and Cortez-Pinto, H. (2012) Liver and muscle in morbid obesity: the interplay of fatty liver and insulin resistance. PLoS One. **7**, e31738

10   Kemp, G. J. and Brindle, K. M. (2012) What do magnetic resonance-based measurements of Pi-->ATP flux tell us about skeletal muscle metabolism? Diabetes. **61**, 1927-1934

11   Browning, J. D., Szczepaniak, L. S., Dobbins, R., Nuremberg, P., Horton, J. D., Cohen, J. C., Grundy, S. M. and Hobbs, H. H. (2004) Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. **40**, 1387-1395

12   Expert Panel on DetectionEvaluation, T. H. B. C. A. (2001) Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). JOURNAL-AMERICAN MEDICAL ASSOCIATION. **285**, 2486-2497

13   Bruce, R. A., Kusumi, F. and Hosmer, D. (1973) Maximal oxygen intake and nomographic assessment of functional aerobic impairment in cardiovascular disease* 1. Am Heart J. **85**, 546-562

14   Borg, G. (1998) Borg's perceived exertion and pain scales. Human Kinetics Publishers

15   Gardner, C. J., Irwin, A. J., Daousi, C., McFarlane, I. A., Joseph, F., Bell, J. D., Thomas, E. L., Adams, V. L., Kemp, G. J. and Cuthbertson, D. J. (2012) Hepatic steatosis, GH deficiency and the effects of GH replacement: a Liverpool magnetic resonance spectroscopy study. Eur J Endocrinol. **166**, 993-1002

16   Gardner, C. J., Richardson, P., Wong, C., Polavarapu, N., Kemp, G. J. and Cuthbertson, D. J. (2011) Hypothyroidism in a patient with non-alcoholic fatty liver disease. Bmj. **342**, c7199

17   Jones, H., Sprung, V. S., Pugh, C. J., Daousi, C., Irwin, A., Aziz, N., Adams, V. L., Thomas, E. L., Bell, J. D., Kemp, G. J. and Cuthbertson, D. J. (2012) Polycystic ovary syndrome with hyperandrogenism is characterized by an increased risk of hepatic steatosis compared to nonhyperandrogenic PCOS phenotypes and healthy controls, independent of obesity and insulin resistance. J Clin Endocrinol Metab. **97**, 3709-3716

18   Cuthbertson, D. J., Irwin, A., Gardner, C. J., Daousi, C., Purewal, T., Furlong, N., Goenka, N., Thomas, E. L., Adams, V. L., Pushpakom, S. P., Pirmohamed, M. and Kemp, G. J. (2012) Improved glycaemia correlates with liver fat reduction in obese, type 2 diabetes, patients given glucagon-like peptide-1 (GLP-1) receptor agonists. PLoS One. **7**, e50117

19   Kemp, G. J., Roberts, N., Bimson, W. E., Bakran, A., Harris, P. L., Gilling-Smith, G. L., Brennan, J., Rankin, A. and Frostick, S. P. (2001) Mitochondrial function and oxygen supply in normal and in chronically ischemic muscle: a combined 31P magnetic resonance

spectroscopy and near infrared spectroscopy study in vivo. Journal of vascular surgery. **34**, 1103-1110

20   Sleigh, A., Stears, A., Thackray, K., Watson, L., Gambineri, A., Nag, S., Campi, V. I., Schoenmakers, N., Brage, S., Carpenter, T. A., Murgatroyd, P. R., O'Rahilly, S., Kemp, G. J. and Savage, D. B. (2012) Mitochondrial oxidative phosphorylation is impaired in patients with congenital lipodystrophy. J Clin Endocrinol Metab. **97**, E438-442

21   Bajpeyi, S., Pasarica, M., Moro, C., Conley, K., Jubrias, S., Sereda, O., Burk, D. H., Zhang, Z., Gupta, A., Kjems, L. and Smith, S. R. (2011) Skeletal muscle mitochondrial capacity and insulin resistance in type 2 diabetes. J Clin Endocrinol Metab. **96**, 1160-1168

22   Praet, S. F., De Feyter, H. M., Jonkers, R. A., Nicolay, K., van Pul, C., Kuipers, H., van Loon, L. J. and Prompers, J. J. (2006) 31P MR spectroscopy and in vitro markers of oxidative capacity in type 2 diabetes patients. Magma. **19**, 321-331

23   Petersen, K. F., Dufour, S., Befroy, D., Garcia, R. and Shulman, G. I. (2004) Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. **350**, 664-671

24   Morino, K., Petersen, K. F., Dufour, S., Befroy, D., Frattini, J., Shatzkes, N., Neschen, S., White, M. F., Bilz, S., Sono, S., Pypaert, M. and Shulman, G. I. (2005) Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. The Journal of clinical investigation. **115**, 3587-3593

25   Hancock, C. R., Han, D. H., Chen, M., Terada, S., Yasuda, T., Wright, D. C. and Holloszy, J. O. (2008) High-fat diets cause insulin resistance despite an increase in muscle mitochondria. Proc Natl Acad Sci U S A. **105**, 7815-7820

26   Toledo, F. G., Menshikova, E. V., Azuma, K., Radikova, Z., Kelley, C. A., Ritov, V. B. and Kelley, D. E. (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

27   Sleigh, A., Raymond-Barker, P., Thackray, K., Porter, D., Hatunic, M., Vottero, A., Burren, C., Mitchell, C., McIntyre, M., Brage, S., Carpenter, T. A., Murgatroyd, P. R., Brindle, K. M., Kemp, G. J., O'Rahilly, S., Semple, R. K. and Savage, D. B. (2011) Mitochondrial dysfunction in patients with primary congenital insulin resistance. The Journal of clinical investigation. **121**, 2457-2461

28   Sinha, R., Dufour, S., Petersen, K. F., LeBon, V., Enoksson, S., Ma, Y. Z., Savoye, M., Rothman, D. L., Shulman, G. I. and Caprio, S. (2002) Assessment of skeletal muscle triglyceride content by (1)H nuclear magnetic resonance spectroscopy in lean and obese adolescents: relationships to insulin sensitivity, total body fat, and central adiposity. Diabetes. **51**, 1022-1027

29   Thomas, E. L., Hamilton, G., Patel, N., O'Dwyer, R., Dore, C. J., Goldin, R. D., Bell, J. D. and Taylor-Robinson, S. D. (2005) Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and proton magnetic resonance spectroscopy study. Gut. **54**, 122-127

30   Szendroedi, J., Kaul, K., Kloock, L., Straßburger, K., Schmid, A. I., Chmelik, M., Kacerovsky, M., Kacerovsky-Bielesz, G., Prikoszovich, T. and Brehm, A. (2013) Lower fasting muscle mitochondrial activity relates to hepatic steatosis in humans. Diabetes Care, DC_131359

31   Brehm, A., Krssak, M., Schmid, A. I., Nowotny, P., Waldhausl, W. and Roden, M. (2006) Increased lipid availability impairs insulin-stimulated ATP synthesis in human skeletal muscle. Diabetes. **55**, 136-140

32   Szendroedi, J., Schmid, A. I., Chmelik, M., Toth, C., Brehm, A., Krssak, M., Nowotny, P., Wolzt, M., Waldhausl, W. and Roden, M. (2007) Muscle mitochondrial ATP synthesis and glucose transport/phosphorylation in type 2 diabetes. PLoS medicine. **4**, e154

33   Kacerovsky, M., Brehm, A., Chmelik, M., Schmid, A. I., Szendroedi, J., Kacerovsky-Bielesz, G., Nowotny, P., Lettner, A., Wolzt, M., Jones, J. G. and Roden, M. (2011) Impaired insulin stimulation of muscular ATP production in patients with type 1 diabetes. Journal of internal medicine. **269**, 189-199

34   Kacerovsky-Bielesz, G., Kacerovsky, M., Chmelik, M., Farukuoye, M., Ling, C., Pokan, R., Tschan, H., Szendroedi, J., Schmid, A. I., Gruber, S., Herder, C., Wolzt, M., Moser, E., Pacini, G., Smekal, G., Groop, L. and Roden, M. (2012) A single nucleotide polymorphism associates with the response of muscle ATP synthesis to long-term exercise training in relatives of type 2 diabetic humans. Diabetes Care. **35**, 350-357

35   Szendroedi, J., Zwettler, E., Schmid, A. I., Chmelik, M., Pacini, G., Kacerovsky, G., Smekal, G., Nowotny, P., Wagner, O., Schnack, C., Schernthaner, G., Klaushofer, K. and Roden, M. (2008) Reduced basal ATP synthetic flux of skeletal muscle in patients with previous acromegaly. PLoS One. **3**, e3958

36   Schmid, A. I., Schrauwen-Hinderling, V. B., Andreas, M., Wolzt, M., Moser, E. and Roden, M. (2012) Comparison of measuring energy metabolism by different (31) P-magnetic resonance spectroscopy techniques in resting, ischemic, and exercising muscle. Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine. **67**, 898-905

37   Befroy, D. E., Rothman, D. L., Petersen, K. F. and Shulman, G. I. (2012) (3)(1)P-magnetization transfer magnetic resonance spectroscopy measurements of in vivo metabolism. Diabetes. **61**, 2669-2678

38   Korenblat, K. M., Fabbrini, E., Mohammed, B. S. and Klein, S. (2008) Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. Gastroenterology. **134**, 1369-1375

39   Lomonaco, R., Ortiz-Lopez, C., Orsak, B., Webb, A., Hardies, J., Darland, C., Finch, J., Gastaldelli, A., Harrison, S., Tio, F. and Cusi, K. (2012) Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. Hepatology. **55**, 1389-1397

40   Alligier, M., Gabert, L., Meugnier, E., Lambert-Porcheron, S., Chanseaume, E., Pilleul, F., Debard, C., Sauvinet, V., Morio, B., Vidal-Puig, A., Vidal, H. and Laville, M.

(2013) Visceral fat accumulation during lipid overfeeding is related to subcutaneous adipose tissue characteristics in healthy men. J Clin Endocrinol Metab. **98**, 802-810