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Whole-body substrate metabolism is associated with disease severity in patients with non-alcoholic fatty liver disease

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List of abreviations: adipo-IR, adipose tissue insulin resistance index; BMI, body mass index; CHO_{ox}, carbohydrate oxidation; FFM, fat-free mass; Fat_{max} , exercise intensity eliciting maximal fat oxidation; Fat_{ox}, fat oxidation; FFA, free fatty acids; IR, insulin resistance; MFO, maximal fat oxidation; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; RQ, respiratory quotient; TG, triglycerides; $\dot{V}O_{2\,peak}$, peak oxygen uptake.

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ABSTRACT

Objectives: In non-alcoholic fatty liver disease (NAFLD), hepatic steatosis is intricately linked with a number of metabolic alterations. We studied substrate utilization in NAFLD during basal, insulin-stimulated and exercise conditions, and correlated these outcomes with disease severity.

Methods: 20 patients with NAFLD (BMI 34.1±6.7 kg/m^2) and 15 healthy controls (23.4±2.7 kg/m^2) were assessed. Respiratory quotient (RQ), whole-body fat (Fat_{ox}) and carbohydrate (CHO_{ox}) oxidation rates were determined by indirect-calorimetry in three conditions: basal (resting and fasted), insulin-stimulated (hyperinsulinemic-euglycemic clamp) and exercise (cycling at the intensity eliciting maximal Fat_{ox}). Severity of disease and steatosis was determined by liver histology; hepatic Fat_{ox} from plasma β-hydroxybutyrate concentrations; aerobic fitness as $\dot{V}O_{2\,peak}$; visceral adipose tissue (VAT) by computed tomography.

Results: Within the overweight/obese NAFLD cohort, basal RQ was positively correlated with steatosis (r=0.57, P=0.01) and was higher (indicating smaller contribution of Fat_{ox} to energy expenditure) in patients with NAFLD activity score ≥5 *vs.* <5 (P=.008). Both results were independent of VAT, %body fat and BMI. Compared to the lean control group, patients with NAFLD had lower basal whole-body Fat_{ox} (P=0.024) and lower basal hepatic Fat_{ox} (i.e β-hydroxybutyrate, P=0.004). During exercise they achieved lower maximal Fat_{ox} (P=0.002) and lower $\dot{V}O_{2\,peak}$ (P<0.001) than controls. Fat_{ox} during exercise was not associated with disease severity (P=0.79).

Conclusions: Overweight/obese patients with NAFLD had reduced hepatic Fat_{ox} and reduced whole-body Fat_{ox} under basal and exercise conditions. There was an inverse relationship between ability to oxidize fat in basal conditions and histological features of NAFLD including severity of steatosis and NAFLD activity score.

SIGNIFICANCE OF THIS STUDY

What is already known about this subject?

- NAFLD is the most prevalent liver disease in industrialized countries and is associated with a number of metabolic alterations.
- In NAFLD, studies investigating whole-body and hepatic fat oxidation reported conflicting results. Further, it is not known whether the severity of NAFLD is associated with whole-body substrate oxidation rates.
- Maximal fat oxidation achieved during exercise has not been studied in NAFLD.

What are the new findings?

- Whole-body fat oxidation at rest and during exercise is reduced in overweight/obese patients with NAFLD.
- In overweight/obese patients with NAFLD, reduced whole-body fat oxidation in basal conditions is associated with degree of steatosis and histological severity of disease, independent of BMI, body fatness and visceral adipose tissue.
- Basal hepatic fat oxidation is reduced in overweight/obese patients with NAFLD.

How might it impact on clinical practice in the forseeable future?

- Behavioural or pharmacological therapies that can promote whole-body and hepatic fat oxidation in basal and exercise conditions could be useful for the treatment of NAFLD.
- Exercise training could be a suitable treatment option for NAFLD because, in addition to improving aerobic fitness and insulin sensitivity, it also promotes fat oxidation in basal and exercise conditions.

INTRODUCTION

The most prevalent liver disease in industrialized countries is non-alcoholic fatty liver disease (NAFLD).[1] NAFLD encompasses a spectrum of histological features ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Development and progression of NAFLD are intricately linked with a number of factors including genetic predisposition,[2, 3] physical inactivity, obesity and insulin resistance (IR).[4, 5] Hepatic steatosis appears to be a prerequisite for more severe liver injury and occurs when the rates of *de novo* hepatic fatty acid synthesis and of hepatic fatty acid uptake from plasma exceed the rate of hepatic fat oxidation and triglyceride (TG) export.[6] There is evidence that patients with NAFLD have increased free fatty acid delivery from the adipose tissue,[7] increased *de novo* hepatic fatty acid synthesis[8] and increased TG export.[9] On the other hand, it is less clear whether patients with NAFLD have altered whole-body and hepatic fat oxidation (Fat_{ox}).

An ideal cadre to study whole-body substrate metabolism is to assess substrate oxidation rates under a number of physiological conditions including the basal state (resting and fasting conditions), after a meal or insulin stimulation and during exercise.[10] Studies investigating whole-body substrate oxidation of NAFLD patients in the basal state have reported conflicting results. Perseghin *et al.*[11] found lower rates of whole-body Fat_{ox} in obese adolescents with NAFLD compared to counterparts without fatty liver. In contrast, Bugianesi *et al.*[12] reported a tendency for higher rates of whole-body Fat_{ox} in 12 non-obese patients with NAFLD when compared with 6 body mass index (BMI)-matched controls. Similarly, Sanyal *et al.*[13] found higher Fat_{ox} in 6 obese NAFLD compared to 6 obese NASH, although both NAFLD and NASH groups did not differ from 6 lean controls. Krotonen *et al.*[14] found no significant difference between 29 moderately overweight individuals with NAFLD and 29 leaner healthy control. In the latter group of studies[12, 13, 14] between-group differences in

hepatic Fat_{ox} mirrored those for whole-body Fat_{ox}. In the insulin-stimulated state, results are more uniform with NAFLD patients showing a reduced insulin-mediated suppression of Fat_{ox} compared to controls.[11, 12, 14] To date, substrate oxidation during exercise has not been compared between patients with NAFLD and counterparts without fatty liver. It is important to better understand substrate metabolism during exercise because whole-body metabolic demands are increased and potential abnomalities not seen in the resting state may become apparent. Further, exercise training is increasingly recommended clinically as a component of lifesyle interventions.[15, 16]

Differences in severity of liver disease in previous cohorts may have contributed to the contrasting results reported on basal substrate metabolism in NAFLD. However, the relationship between severity of disease (which can only be assessed by liver histology) and substrate oxidation under various metabolic conditions has not been investigated to date in adults with NAFLD. In obese adolescents with NAFLD, hepatic steatosis (measured by magnetic resonance spectroscopy) and impairment in basal whole-body Fat_{ox} were shown to be positively correlated, independent of BMI.[11]

The objective of this study was to measure substrate utilization under basal, insulin-stimulated and exercise conditions in adult patients with NAFLD and to explore whether these outcome measures were correlated with degree of steatosis and severity of liver disease.

METHODS

Participants

Twenty overweight/obese patients with NAFLD and 15 lean healthy controls participated in the study. Patients were recruited from outpatient hospital clinics and NAFLD was diagnosed clinically and on liver biopsy. Exclusion criteria included the presence of other causes of liver

disease (serologically and on history), evidence of cirrhosis or decompensated liver disease, alcohol consumption >40 g/day in males or >20 g/day in females (assessed by detailed clinical history) and type 2 diabetes. Control participants were healthy non-obese adults with: normal liver enzymes (alanine transaminase <35 U/L; aspartate aminotransferase <35 U/L), no evidence of liver disease (serologically and on history), no hepatomegaly on clinical examination and no features of the metabolic syndrome.[17] Controls were non-smoking, not taking regular medications and had minimal alcohol intake. In individuals meeting these same criteria, the prevalence of steatosis has been shown to be 5%[18] or lower.[19] The study was approved by the Human Research Ethics Committees of the Princess Alexandra Hospital and the University of Queensland. Informed written consent was obtained from all participants.

While we considered the benefit of an additional obese non-NAFLD control group, the prospective liver biopsy of control participants for the purpose of this study was deemed unethical by the ethics committee and therefore exclusion of NAFLD in an obese control was not possible for this study. Further, it could be argued that obese individuals without steatosis are metabolically atypical[20] and therefore not an appropriate control group. Instead, it has been proposed that healthier physically active individuals should be assigned as a control group.[21] Accordingly, this study compares measurements in NAFLD to a healthy reference and then further explores study aims regarding disease severity within the NAFLD group alone.

General design

Each participant undertook testing in the morning after a 10-12 hour overnight fast on two occasions within a 7-day period. The first testing session involved a hyperinsulinemic-euglycemic clamp with indirect calorimetry measurements to assess substrate oxidation rates in two conditions: basal (in resting and fasted conditions) and insulin-stimulated (during the steady state of a hyperinsulinemic-euglycemic clamp). The second testing session involved

indirect calorimetry measurement during a graded exercise test on a cycle ergometer to assess substrate oxidation rate and $\dot{V}O_{2\,max}$ (aerobic fitness).

Histological analysis of liver biopsy

Liver biopsy specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and were subsequently scored by an expert hepatopathologist (AC). The percentage of hepatocytes with steatosis was estimated. The severity of liver injury was assessed using the NAFLD activity score (NAS)[22] and the criteria described by Brunt.[23] A diagnosis of steatosis alone or NASH was made using conventional histologic criteria, independent of NAS.[24]

Body composition

Fat mass and fat-free mass (FFM) were measured by dual-energy X-ray absorptiometry (GE Lunar Prodigy enCore 2005, General Electric, Madison, WI). In the NAFLD group distribution of abdominal fat (visceral and subcutaneous) was determined by computed tomography (Philips Brilliance 16, Cleveland, OH) as previously described.[25]

Hyperinsulinemic-euglycemic clamp

Insulin sensitivity was evaluated by the hyperinsulinemic-euglycemic clamp technique,[26] with a protocol previously described.[12] Teflon catheters were placed into an antecubital vein for infusions, and into a dorsal hand vein (heated to 55°C to achieve arterialization of venous blood) for sampling. After obtaining a basal blood sample, primed insulin infusion was initiated at a rate of 1 $mU \cdot kg^{-1} \cdot min^{-1}$ (Humulin R; Eli Lilly, Indianapolis, IN) and was maintained at a constant rate throughout the procedure (120 minutes). Plasma glucose concentration was monitored every 5 minutes using an automated glucose analyzer (YSI 2300 Stat Plus, YSI Life Sciences, Yellow Springs, OH). Euglycemia was maintained infusing a 25% glucose solution at a variable rate.[26]

The glucose infusion rate in the steady-state of the hyperinsulinemic-euglycemic clamp (M-value) represented whole-body glucose disposal rate. Non-oxidative glucose disposal rate was calculated by subtracting the oxidative glucose disposal rate (CHO_{ox} during the insulin-stimulated state determined by indirect calorimetry) from the M-value. The insulin sensitivity index (M/I), a measure of the quantity of glucose metabolized per unit of insulin concentration, was calculated by dividing M-value by the insulin concentation reached in the insulin-stimulated state.[26] An index of adipocyte IR (adipo-IR) was calculated as the product of the fasting plasma free fatty acids (FFA) and insulin concentration.[27]

Biochemical analysis

Blood samples were drawn at 10-minute intervals during the last 40 minutes of the hyperinsulinemic-euglycemic clamp. Glucose was analyzed using an automated glucose analyzer [interassay coefficient of variation (CV) 2%]. Insulin was assayed using an immunoenzymatic assay with chemiluminescence detection (Unicel DxI 800 Immunoassay System, Beckman Coulter, Brea, CA). Total cholesterol, high-density lipoprotein-cholesterol and TG were assayed by an enzymatic colorimetric assay with Roche Modular Chemistry Analyzer (South San Francisco, CA). Low-density lipoprotein-cholesterol and very low density lipoprotein were calculated using the Friedewald equation.[28] Serum FFA concentrations were measured with an *in vitro* enzymatic colorimetric method (Wako NEFA assay, Wako chemicals, Richmond, VA, CV 2.3%). Plasma β-hydroxybutyrate concentrations, an index of hepatic ketogenesis,[29] were measured enzymatically (Stanbio, Boerne, TX, CV 2.2%).

Graded exercise test

Maximal aerobic power and substrate utilization were assessed with a graded exercise test on a cycle ergometer. Testing included a sub-maximal phase to assess energy expenditure, Fat_{ox} and CHO_{ox} at various intensities, and a maximal phase to determine peak oxygen

consumption ($\dot{V}O_{2\,peak}$). The starting workload for the submaximal phase was individualized at 20% of the theoretical maximal mechanical work.[30] Workload was increased by 10% at each stage until the respiratory exchange ratio was above 1.0 during the last minute of the stage. Stages lasted 5 minutes and were separated by 2-minute rest intervals. The maximal phase started at a workload corresponding to two stages below the intensity reached at the end of the submaximal phase, and workload was incremented by 10% every minute until volitional exhaustion.

Indirect calorimetry

Indirect calorimetry measurements (TrueOne 2400 Metabolic Measurement System, Parvo Medics, UT) to determine oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were performed in three conditions: 1) basal, 2) insulin-stimulated and 3) exercise. Basal and insulin-stimulated measurements lasted 20 minutes with participants lying supine and breathing through a ventilated hood. Measurements during the graded exercise test were performed continuously, with participants wearing mouthpiece and nose clip.

Whole-body respiratory quotient (RQ) was calculated as $\dot{V}CO_2/\dot{V}O_2$. Whole-body Fat_{ox} and CHO_{ox} were calculated using stoichiometric equations and appropriate energy equivalents, with the assumption that the urinary nitrogen excretion rate was negligible.[31] Average values of $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated during the last 10 minutes of basal and insulin-stimulated periods, and during the last minute of each submaximal exercise stage. Subsequently, Fat_{ox} values determined at each stage of the exercise test were graphically depicted as a function of exercise intensity. The stage at which the value of measured Fat_{ox} rate was maximal (maximal fat oxidation, MFO) was determined and the corresponding intensity identified (Fat_{max}).[32] Data measured at Fat_{max} were employed for comparison

between groups. M-value, energy expenditure and substrate oxidation rates were corrected for FFM.

Statistical analysis

Data are expressed as the mean ± standard deviation for all variables. Student t-tests for independent samples were used to compare the mean values between groups categorised according to cohort (NAFLD *vs.* controls), disease severity (NAFLD with NAS<5 *vs.* NAFLD with NAS≥5), and BMI (NAFLD with BMI < or ≥33 kg/m^2). Paired Student t-tests were used to compare energy expenditure and substrate oxidation rates in different conditions within groups. Analysis of covariance was used to adjust for FFM. Association between continuous variables was assessed using Spearman's non-parametric rank correlation coefficient and multivariate analysis. Statistical analysis was performed with the software SPSS 17.0 for Windows (SPSS, Chicago, IL) and Graph Pad Prism version 5.0 for Mac (GraphPad Software, San Diego, CA). For all statistical analyses, the level of significance was set at $P<0.05$.

RESULTS

Participant characteristics

Characteristics of study groups are presented in Table 1. Liver histology from patients with NAFLD showed macrovesicular steatosis ranging from 10 to 100%, with an average of 71±31%. Sixteen patients were diagnosed with NASH, while four with steatosis alone. Fourteen patients had a NAS≥5, while six patients had a NAS<5. Fibrosis was observed in 10 patients (stage 1 in 3, stage 2 in 4, and stage 3 in 3).

Age and gender were not significantly different between NAFLD and controls. BMI and percentage of body fat were higher in the NAFLD group in comparison with controls. Patients with NAFLD had higher fasting plasma TG, insulin and glucose, while fasting plasma FFA

were not different between groups. In the NAFLD cohort, visceral adipose tissue area was 194 ± 94 cm^2, while subcutaneous adipose tissue area was 384 ± 197 cm^2.

Table 1. Demographic, anthropometric, and laboratory characteristics of the study groups

	Control (n=15)	NAFLD (n=20)	*P-value*
Age (years)	41 ± 11	48 ±11	0.07
Gender (M:F)	10:5	12:8	0.92
BMI (kg/m^2)	23.4 ± 2.7	34.1 ± 6.7	< 0.001
Weight (kg)	71.2 ± 12.4	101.2 ± 26.9	< 0.001
Fat mass (%)	25.0 ± 6.3	38.5 ± 8.0	< 0.001
Fat-free mass (kg)	53.4 ± 10.4	61.5 ± 15.3	0.08
Waist (cm)	82.0 ± 7.5	112.7 ± 18.0	< 0.001
Triglycerides (mmol/L)	0.51 ± 0.24	1.9 ± 1.03	< 0.001
HDL cholesterol (mmol/L)	1.37 ± 0.33	0.92 ± 0.28	< 0.001
LDL cholesterol (mmol/L)	2.77 ± 0.89	3.22 ± 1.0	0.17
VLDL cholesterol (mmol/L)	0.23 ± 0.11	0.86 ± 0.47	< 0.001
β-Hydroxybutyrate (mmol/L)	0.14 ± 0.07	0.09 ± 0.03	0.004
Fasting free fatty acids (mmol/L)	0.63 ± 0.18	0.58 ± 0.18	0.43
Fasting glucose (mmol/L)	5.0 ± 0.4	5.5 ± 0.4	0.002
Fasting insulin (mU/L)	3.4 ± 2.0	20.2 ± 16.7	< 0.001
Fasting C-peptide (nmol/L)	0.51 ± 0.18	1.37 ± 0.73	< 0.001
Alanine aminotransferase (U/L)	19.9 ± 6.5	65.7 ± 42.2	< 0.001
Aspartate aminotransferase (U/L)	21.8 ± 7.1	42.0 ± 22.2	0.002

BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

Insulin resistance

Patients with NAFLD were severely insulin resistant (Table 2), with lower M-value compared to lean controls (4.1±1.5 *vs.* 9.1±2.2 $mg \cdot kgFFM^{-1} \cdot min^{-1}$, $P<0.001$), and demonstrated

impairment in both the oxidative (3.0±0.7 *vs.* 3.6±0.7 mg·kgFFM^{-1}·min^{-1}, *P*=0.04) and the non-oxidative glucose disposal pathways (1.8±1.3 *vs.* 5.1±2.4 mg·kgFFM^{-1}·min^{-1}, *P*<0.001). The insulin sensitivity index M/I was also significanly lower in NAFLD (5.9±3.6 *vs.* 17.1±5.1 (mg·kgFFM^{-1}·min^{-1})·(mU·L^{-1})$^{-1}$, *P*<0.001), showing that the differences between groups were maintained after adjusting for the insulin levels reached (80.6±26.9 in NAFLD *vs.* 51.2±7.9 mU/L in control, *P*<0.001). Adipo-IR was more severe in NAFLD patients compared to controls (11.1±8.6 *vs*. 2.2±1.3 mmol·mU·L^{-2}, *P*<0.001) and in NAFLD patients with fibrosis compared to those without (17.5±10.2 *vs*. 7.2±4.1 mmol·mU·L^{-2}, *P*=0.013). Within the NAFLD cohort, adipo-IR was associated with BMI (*r*=0.70, *P*<0.001), visceral adipose tissue (*r*=0.53, *P*=0.02), M-value (*r*=-0.50, *P*=0.003), but not with hepatic steatosis (*r*=0.23, *P*=0.35).

Table 2. Metabolic parameters during basal, insulin-stimulated and exercise conditions in NAFLD *vs.* control

	Control (n=15)	NAFLD (n=20)	*P*-value
Insulin resistance and substrate oxidation rates			
M-value (mg·kgFFM^{-1}·min^{-1})	9.1 ± 2.2	4.1 ± 1.5	< 0.001
M/I (mg·kgFFM^{-1}·min^{-1})·(mU·L^{-1})$^{-1}$	17.1 ± 5.1	5.9 ± 3.6	<0.001
Adipo-IR (mmol·mU·L^{-2})	2.2 ± 1.3	11.1 ± 8.6	0.001
Basal Fat_{ox} (mg·kgFFM^{-1}·min^{-1})	1.5 ± 0.4	1.2 ± 0.3	0.024
Basal CHO_{ox} (mg·kgFFM^{-1}·min^{-1})	1.6 ± 0.8	2.4 ± 0.7	0.004
Insulin-stimulated Fat_{ox} (mg·kgFFM^{-1}·min^{-1})	0.8 ± 0.3	1.0 ± 0.3	0.17
Insulin-stimulated CHO_{ox} (mg·kgFFM^{-1}·min^{-1})	3.6 ± 0.7	3.0 ± 0.7	0.036
Maximal fat oxidation and aerobic fitness			
$\dot{V}O_{2\,peak}$ (ml·kg^{-1}·min^{-1})	43.8 ± 9.6	21.0 ± 6.0	< 0.001
$\dot{V}O_{2\,peak}$ (ml·kgFFM^{-1}·min^{-1})	52.7 ± 19.0	33.6 ± 6.7	< 0.001

Workload at $\dot{V}O_{2\,peak}$ (W)	285 ± 72	155 ± 68	< 0.001
MFO (mg·kgFFM^{-1}·min^{-1})	5.8 ± 3.7	2.5 ± 1.4	0.002
Workload at MFO (W)	103.4 ± 47.3	45.7 ± 17	< 0.001
Fat_{max} (% $\dot{V}O_{2\,peak}$)	48.9 ± 11.2	50.2 ± 15.9	0.80

M-value, glucose infusion rate in the steady-state of the hyperinsulinemic-euglycemic clamp; M/I, insulin sensitivity index; Adipo-IR, adipose tissue insulin resistance index; Fat_{ox}, fat oxidation rates, CHO_{ox}, carbohydrate oxidation rates, $\dot{V}O_{2\,peak}$, peak oxygen uptake; MFO, maximal fat oxidation; W, Watts; Fat_{max}, exercise intensity eliciting maximal fat oxidation.

Substrate oxidation under basal conditions

After adjusting for FFM, total energy expenditure in the basal state was not different between groups (*P*=0.26). However, the proportion of energy derived from fat and CHO did differ between groups, with NAFLD patients oxidizing more CHO (2.41±0.73 *vs.* 1.6±0.77 mg·kgFFM^{-1}·min^{-1}, *P*=0.004) and less fat (1.22±0.28 *vs.* 1.49±0.39 mg·kgFFM^{-1} ·min^{-1}, *P*=0.024) than controls (Figure 1A and 1B). This was confirmed by the higher RQ in patients with NAFLD (0.82±0.04 *vs.* 0.78±0.03, *P*=0.007, Figure 1C).

Within the overweight/obese NAFLD group, basal RQ was positively correlated with hepatic steatosis (*r*=0.57, *P*=0.01, Figure 2A). This association was confirmed by linear regression multivariate analysis, after controlling for BMI, % body fat, visceral adipose tissue, subcutaneous adipose tissue, age and gender (standardized β=0.56, *P*=0.021). Indeed, basal RQ was not correlated to visceral adipose tissue (*r*=0.07, *P*=0.77), % body fat (*r*=0.31, *P*=0.19) and BMI (*r*=0.29, *P*=0.23, Figure 2B), and was not significantly different in patients with NAFLD with BMI < or ≥33 kg/m^2 (supplementary material). Further, basal RQ was significantly lower in the 6 patients with a NAS of <5 compared to the 14 patients with NAS≥5 (0.79±0.02 *vs.* 0.83±0.03, *P*=0.008, Figure 2C), and this difference also persisted after adjusting for visceral adipose tissue (*P*=0.01), % body fat (*P*=0.01) and BMI (*P*=0.02).

Patients with NAFLD showed evidence of reduced hepatic ketogenesis with lower basal plasma concentrations of β-hydroxybutyrate than lean controls (0.09±0.03 *vs* 0.14±0.07 mmol/L, *P*=0.004, Figure 3A). Within the NAFLD cohort, fasting concentrations of β-hydroxybutyrate were inversely correlated with fasting plasma TG (*r*=-0.64, *P*=0.002, Figure 3B) and very low-density lipoprotein (*r*=-0.67, *P*=0.002), but not correlated with fasting insulin (*P*=0.42), fasting FFA (*P*=0.42), % hepatic steatosis (*P*=0.96) or basal RQ (*P*=0.40). β-hydroxybutyrate concentrations were not different between patients with NAS <5 and those with NAS ≥5 (0.10±0.04 *vs.* 0.09±0.2, *P*=0.60), nor between patients with BMI <33 kg/m^2 and those with BMI ≥33 kg/m^2 (supplementary material). When combining NAFLD and control groups, fasting concentrations of β-hydroxybutyrate were also inversely correlated with fasting plasma TG (*r*=-0.64, *P*<0.001) and very low-density lipoprotein (*r*=-0.63, *P*<0.001).

Substrate oxidation under insulin-stimulated conditions

Under insulin-stimulated conditions there was no apparent difference between groups in total energy expenditure (*P*=0.27). However, patients with NAFLD had lower CHO_{ox} (*P*=0.036, Figure 1C) and a lower RQ (*P*=0.037, Figure 1C). The switch in substrate oxidation in response to insulin stimulation was different between groups: NAFLD patients increased CHO_{ox} and suppressed Fat_{ox} to a lesser extent than controls (0.67±0.81 *vs.* 2.05±0.68 mg·kgFFM^{-1}·min^{-1}, *P*<0.001, and -0.26±0.35 *vs.* -0.7±0.33 mg·kgFFM^{-1}·min^{-1}, *P*=0.001, respectively, Figure 1D). Consistently, the change in RQ from the basal state was smaller in NAFLD (0.04±0.03 *vs.* 0.11±0.04, *P*<0.001, Figure 1C). Hepatic steatosis was not correlated with change in RQ from basal to insulin-stimulated conditions (*P*=0.29), however there was a trend for the % hepatic steatosis to be correlated with the insulin sensitivity index (*r*=-0.40, *P*=0.09).

Substrate oxidation during exercise

Aerobic fitness, as measured by $\dot{V}O_{2\,peak}$, was lower in the NAFLD group (33.6±6.7 *vs.* 52.7±19.0 ml·kgFFM^{-1}·min^{-1}, P<0.001, Table 2). When cycling at the intensity eliciting maximal fat oxidation (Figure 4), patients with NAFLD had a lower MFO (2.54±1.43 *vs.* 5.87±3.71 mg·kgFFM^{-1}·min^{-1}, P=0.002) than the control group. CHO_{ox} was not significantly different, but tended to be lower in NAFLD (P=0.06), while total energy expenditure (P<0.001) and increase in both Fat_{ox} (P=0.002) and CHO_{ox} (P=0.023) from basal to exercise were significantly lower in NAFLD. The intensity at which MFO was reached (Fat_{max}) was lower in NAFLD when expressed in absolute terms (45.7±17 *vs.* 103.4±47.3 Watts, P<0.001), but not in relative terms (50.2±15.9 *vs.* 48.9±11.2 % of $\dot{V}O_{2\,peak}$, P=0.80). After adjusting for $\dot{V}O_{2\,peak}$ by covariate analysis, MFO was not different between groups (P=0.13). MFO during acute exercise was not correlated with degree of steatosis (P=0.26) and was not significantly different in patients with NAS of <5 compared to patients with NAS≥5 (P=0.79).

All the results of this study were confirmed by covariate analysis, with FFM as covariate.

DISCUSSION

In NAFLD, hepatic steatosis is intricately linked with a number of metabolic alterations. In the current study, overweight/obese patients with NAFLD demonstrated reduced whole-body Fat_{ox} in basal conditions and during acute exercise compared to lean controls. Within the overweight/obese NAFLD group, alterations in basal substrate metabolism were associated with more severe steatosis and more severe disease, independent of BMI and fat topography.

Patients with NAFLD also had reduced basal hepatic Fat_{ox}, and this was associated with increased fasting circulating TG.

The present study was designed to comprehensively investigate substrate metabolism and IR. It is the largest study to assess disease severity by liver histology in conjunction with whole-body substrate metabolism and IR. Substrate oxidation was measured in three different physiological states: basal, insulin-stimulated and exercise, which forms an ideal cadre to study whole-body energy homeostasis and understand mechanisms of dysfunction. Gold standard techniques for the assessment of liver disease (liver histology), IR (hyperinsulinemic-euglycemic clamp) and body composition (dual-energy X-ray absorptiometry) were used. NAFLD patients with a broad spectrum of steatosis were studied (10-100%).

In the patient group studied, NAFLD and obesity coexisted, therefore it was not possible to establish the specific contribution of each factor to the differences observed between patients and lean controls. For this reason, we did not limit our study to a comparison between NAFLD and controls, but also performed analyses within the overweight/obese NAFLD cohort, to establish the relationship between disease severity and substrate oxidation under different physiological conditions.

In basal conditions, patients with NAFLD demonstrated an alteration in whole-body substrate metabolism with a lower Fat_{ox} and a higher CHO_{ox} compared to controls. The different outcome compared to previous studies (which showed Fat_{ox} to be lower,[11] similar,[14] or trending to be higher[12] in NAFLD versus controls) could be due to the heterogeneity of disease severity in NAFLD or to differences in plasma substrate concentrations such as fasting glucose and fasting FFA. Anthropometric characteristics of the study groups may also be implicated. Some studies have attempted to match groups for BMI by comparing lean[11] or moderately overweight individuals[14] with and without NAFLD. While this approach has

some advantages, it also has limitations. Lean individuals with NAFLD represent only a small proportion of the clinical population[18, 19] and may have different genetic characteristics.[2] In additon, BMI is a poor indicator of body composition and body fat distribution at the individual level.[33]

To determine if there was a dose effect between the severity of steatosis and basal substrate metabolism we performed analyses within the overweight/obese NAFLD cohort. We found that hepatic steatosis was positively correlated to basal RQ and that RQ was significantly higher in patients with more severe disease. A higher basal RQ indicates that a smaller proportion of whole-body total energy expenditure is derived from Fat_{ox}. These findings were independent of visceral adipose tissue, % body fat and BMI. These observations suggest that reduced whole-body Fat_{ox} in basal conditions may contribute to hepatic fat accumulation and may be implicated in the pathogenesis of NAFLD. Accordingly, a recent review proposed that alterations in fatty acid metabolism lead to an accumulation of ectopic (intrahepatic and intramuscular) TG, resulting in IR in liver and skeletal muscle.[6]

In addition to lower basal rates of whole-body Fat_{ox}, the patients with NAFLD we studied had lower basal concentrations of β-hydroxybutyrate, indicating reduced hepatic Fat_{ox}.[29] The lower basal β-hydroxybutyrate in NAFLD despite similar basal FFA concentrations for both NAFLD and control suggests differential fatty acid partitioning in the liver between groups.[34] Indeed, very low-density lipoprotein (a product of the esterification pathway) was higher in NAFLD while β-hydroxybutyrate (a product of the oxidative pathway) was lower. Basal β-hydroxybutyrate was negatively correlated with very low-density lipoprotein and TG, both when combining groups and when performing the analysis within the NAFLD cohort. In animal models, an inhibition of hepatic Fat_{ox} leads to an increase in hepatic steatosis,[35, 36] while an increase in hepatic Fat_{ox} reduces hepatic steatosis.[37, 38] In humans, lower basal β-hydroxybutyrate concentrations were found in obese compared with lean individuals[39, 40]

and in hypertriglyceridaemic compared with normolipidaemic moderately obese individuals.[41] Few studies have examined this issue in NAFLD, and results are inconsistent, with either higher[12] or similar[13, 14] β-hydroxybutyrate concentrations in NAFLD *vs.* controls. In these studies the differences in hepatic Fat_{ox} between patients and controls mirror those for fasting FFA.[12, 13, 14]

When studying substrate oxidation in insulin-stimulated conditions, we noted that patients with NAFLD increased CHO_{ox} and suppressed Fat_{ox} to a lesser extent than controls, and this was consistent with previous observations.[11, 12, 14] In other words, patients demonstrated *metabolic inflexibility,* which was defined by Kelley *et al.* as an impaired capacity to adapt fuel oxidation to fuel availability.[42] Assessment of IR revealed that the patients with NAFLD had lower M-value and lower oxidative and non-oxidative glucose disposals compared to the control group, indicating a global impairment in skeletal muscle glucose metabolism. Between-group differences in insulin sensitivity were even more apparent after normalizing for the insulin concentrations achieved. We acknowledge that M-value and non-oxidative glucose disposal rate may be underestimated in this study given that the hepatic glucose output was not considered, however, previous research has shown that with the insulin dosage that was used in the present study, hepatic glucose output is minimal even in obese[13] or NAFLD patients.[12] Further, consistent with previous observations, we showed that patients with NAFLD had more severe IR in the adipose tissue than controls[12, 43] and that severity of adipo-IR was related to the severity of hepatic fibrosis.[44, 45]

Another finding of the present study was that, during exercise, MFO in patients with NAFLD was less than half those in control participants, indicating a reduced ability to increase fat oxidation when performing an acute exercise session. However, the lower aerobic fitness appeared to contribute to the lower MFO observed in patients with NAFLD. After correcting for $\dot{V}O_{2\,peak}$, the difference in MFO between NAFLD and control was no longer apparent.

Further, the exercise intensity at which MFO occurred, Fat_{max}, was significantly lower in NAFLD compared to controls when expressed in absolute terms (W), but not when expressed in relative terms ($\%\dot{V}O_{2peak}$). Outcomes of studies investigating substrate oxidation during exercise in type 2 diabetes and obesity are divided, with some showing a lower Fat_{ox} during exercise in obese[46] and type 2 diabetes patients[47, 48] compared to controls, while others find no difference.[49, 50]

We also observed that the aerobic fitness of patients with NAFLD was extremely low, with most patients falling in the lowest percentile according to the *American College of Sports Medicine* guidelines.[51] Low fitness level[52] and NAFLD[53] have been shown to be independently associated with the risk of cardiovascular events, however, few studies have assessed physical fitness quantitatively in NAFLD.

Longitudinal studies demonstrate that lifestyle interventions aimed at increasing aerobic fitness improve IR,[54] and positively impact on Fat_{ox} under basal[55] and exercise[16, 56] conditions. A recent study showed that the magnitude of reduction in steatosis after calorie restriction was negatively correlated with post-treatment plasma ketone body and negatively correlated with post-treatment basal RQ,[57] suggesting that enhanced hepatic and whole-body Fat_{ox} contribute to the reduction in steatosis. Therefore, approaches that enhance basal and exercise Fat_{ox} may have a role in the management of NAFLD. These include exercise training,[55] calorie restriction,[57] but also some pharmacological[58] and nutraceuticals agents.[59]

In conclusion, this study showed that overweight/obese patients with NAFLD have reduced basal whole-body and hepatic Fat_{ox}, and reduced Fat_{ox} during exercise compared to lean controls. Irrespective of body composition, there was an inverse relationship between ability to oxidize fat in basal conditions and histological features of NAFLD. This suggests that reduced basal Fat_{ox} may contribute to ectopic accumulation of fat in the liver and may be

implicated in the pathogenesis of NAFLD. This alteration could represent an important therapeutic target for new treatments in NAFLD. Behavioural and pharmacological approaches that promote Fat_{ox} in basal and exercise conditions warrant further investigation in this patient population.

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Figures

Figure 1. Fat oxidation **(A)** and carbohydrate oxidation **(B)** under basal and insulin stimulated conditions in 15 control *vs.* 20 NAFLD participants**. (C)** Respiratory quotient at basal and in the insulin-stimulated state. **(D)** Change in substrate utilization from basal to the insulin-stimulated state. ∗ $P<0.05$, ∗∗ $P<0.01$ and ∗∗∗ $P<0.001$ between control and NAFLD.

Figure 2. (A) Correlation between % hepatic steatosis and basal respiratory quotient (RQ) in 20 overweight/obese patients with NAFLD. The positive association between basal respiratory quotient and hepatic steatosis was maintained after controlling for BMI, % body fat, visceral adipose tissue, subcutaneous adipose tissue, age and gender (standardized ß=0.56, $P=0.021$). **(B)** Correlation between basal respiratory quotient and BMI in 20 overweight/obese patients with NAFLD. **(C)** Basal respiratory quotient in patients with NAFLD having a NAFLD activity score (NAS) <5 (n=6) *vs.* patients having a score ≥5 (n=14). The difference persisted after adjusting for visceral adipose tissue ($P=0.01$), % body fat ($P=0.01$) and BMI ($P=0.02$).

Figure 3. (A) Fasting ß-hydroxybutyrate in 15 control *vs.* 20 NAFLD participants. ** P<0.01.(**B**) Correlation between fasting ß-hydroxybutyrate and fasting tryglicerides in 20 overweight/obese patients with NAFLD.

Figure 4. Fat oxidation **(A)** and carbohydrate oxidation **(B)** during exercise in 15 control *vs.* 20 NAFLD participants. ** *P*<0.01 between control and NAFLD.

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