THE CHARACTERISATION OF HEPATIC MITOCHONDRIAL FUNCTION IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) USING THE ^{13}C-KETOISOCAPROATE BREATH TEST.

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ABSTRACT

Hepatic mitochondrial function (HMF) assessed by the ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT) has been previously shown to be significantly associated with the severity of biopsy proven non-alcoholic fatty liver disease (NAFLD). However, it is uncertain whether any perturbation in HMF relates specifically to severity of liver disease or factors associated with metabolic syndrome within non-alcoholic fatty liver disease (NAFLD). Our aim was to investigate whether there was any change in HMF assessed by ^{13}C-KICA BT in patients with NAFLD compared to control subjects, and to assess the factors that are independently associated with HMF. **Methods:** 77 patients with NAFLD and 11 healthy control subjects were studied. HMF was assessed using ^{13}C-KICA BT and expressed as cumulative % ^{13}C-dose recovered on breath over 1hr (cPDR over 1hr). Liver fat and fibrosis was assessed by transient elastography. Multi-variable linear regression modelling was undertaken to test the independence of associations with HMF. **Results:** HMF (cPDR over 1hr) was lower in NAFLD compared to controls [13.4% (4.8) v. 21.0% (6.3); p< 0.0001)]. In NAFLD, HMF was lower in patients with diabetes versus no diabetes [12.7% (3.4) v. 14.3% (6.1); p=0.003)]. Regression modelling showed age (β= -0.08; p=0.01), waist circumference (β= -0.08; p=0.01), hip circumference (β= -0.04; p=0.01), aspartate aminotransferase (AST) (β= -0.05; p=0.01) and diabetes status (β= -1.81; p=0.01) were independently associated with HMF (R^2= 41.5%; p<0.0001). **Conclusions:** In patients with NAFLD (compared to healthy subjects), there was a reduction in HMF assessed by the ^{13}C-KICA BT. Furthermore, in patients with NAFLD, HMF is independent and inversely associated with age, waist and hip circumference, AST and diabetes status.

INTRODUCTION

Emerging evidence suggests that hepatic mitochondrial dysfunction could play an important role in the development of type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) (1-4). Furthermore, recent studies using different non-invasive and dynamic ^{13}C-liver function breath tests in patients with NAFLD showed that although hepatic mitochondrial dysfunction is associated with NAFLD, it is uncertain whether any perturbation of hepatic mitochondrial function (HMF) relates specifically to severity of liver disease or factors associated with NAFLD, such as metabolic syndrome and type 2 diabetes (5-7). Therefore, further studies are needed to characterise the factors, including liver disease severity that are associated with HMF. Furthermore, the ability to monitor early hepatic mitochondrial dysfunction in patients with NAFLD by the non invasive assessment of mitochondrial metabolic pathways, could also contribute useful diagnostic and prognostic information for monitoring both liver function and responses to therapy.

One such promising non invasive technique for assessing HMF in-vivo is the use of ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT) (8). The specific in-vivo assessment of HMF by ^{13}C-KICA BT is possible by measuring the proportion of $^{13}CO_2$ exhaled on breath after an oral dose and hepatic mitochondrial first-pass metabolism of ^{13}C-ketoisocaproic acid, a substrate for branched chain alpha keto-acid dehydrogenase (located in the hepatic mitochondrial matrix) (9, 10). Previous studies have used the ^{13}C-KICA BT to explore HMF in patients with alcohol-induced and non-alcoholic liver disease (11, 12) and in obese women under caloric restriction (13). Thus, a non-invasive marker of hepatic mitochondrial dysfunction such as the ^{13}C-KICA BT has the potential to be used as a predictor of developing non-alcoholic steatohepatitis

(NASH) which is the inflammatory component of NAFLD. Our aim of this study was to investigate whether there was any change in HMF in patients with NAFLD compared to controls, and to assess the factors that are independently associated with HMF.

MATERIALS AND METHODS

77 patients with NAFLD and 11 healthy control subjects were studied. The patients with NAFLD were recruited as part of the INSYTE (**I**nvestigation of **SY**nbiotic **T**reatm**E**nt in NAFLD) trial (www.clinicaltrials.gov registration number NCT01680640). This trial was approved by the Southampton and South West Hampshire local research ethics committee (12/SC/0614). The healthy subjects were recruited from the local community to assess hepatic mitochondrial function and this part of the study was approved by the Southampton and South West Hampshire local research ethics committee (15/SC/0619). All participants gave informed written consent.

Inclusion and exclusion criteria for recruitment of patients with NAFLD

The inclusion criteria for participation in the INSYTE trial were age >18 years and, after an initial clinical diagnosis of NAFLD and identification of liver fat (by ultrasound, computed tomography, transient elastography or histological examination of liver), confirmation of >5% liver fat by Magnetic Resonance Spectroscopy (MRS) (details below) was undertaken (14). Patients also must have had no known aetiological factors for liver fat accumulation, including alcohol consumption ≤14 units /week for women, or ≤21 units / week for men (14 units of alcohol is equivalent to six pints of average-strength beer or 10 small glasses of low-strength wine) (15). Patients also had no evidence of prior hepatitis A, B or C, primary biliary cirrhosis, autoimmune hepatitis, or haemochromatosis. The exclusion criteria also included: a history of diarrhoea, diverticulosis, actively symptomatic irritable bowel syndrome, inflammatory bowel diseases, coeliac disease (seropositivity for anti-endomysial immunoglobulin A antibodies; IgA EMA); decompensated acute or chronic liver disease; previous bariatric or other abdominal surgery; continuous use of antibiotics;

use of probiotics within the 2 months preceding enrolment; or evidence of immunoglobulin A or immunoglobulin deficiency. Participants weighing > 155 kg were also excluded because this was the maximum weight threshold for the magnetic resonance imaging scanner.

Inclusion and exclusion criteria for the healthy control group

The inclusion criteria for recruitment of healthy volunteers were men and women (>18 years of age) with no medical history of chronic liver disease or taking any prescribed medications. The exclusion criteria included alcohol consumption (>14 units/week for women and >21 units/week for men), surgery affecting the digestive tract anatomy, with the exception of appendectomy.

Assessment of liver fat percentage by Magnetic Resonance Spectroscopy (MRS)

Only patients with NAFLD underwent MRS of the liver to measure the quantity of liver fat accumulated in three discrete liver zones. Three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margins of the liver. Liver fat percentage was estimated as the mean value of the liver fat percentages in the three liver regions.

Assessment of liver fibrosis by transient elastography

The presence of liver fat or liver fibrosis was ascertained by use of transient elastography (FibroScan, Echosens, Paris, France) in patients with NAFLD and healthy subjects. The Controlled Attenuation Parameter (CAP) (db/M) was assessed as a proxy measure of liver fat and used to exclude a diagnosis of NAFLD in the control subjects. The technical background has been previously described in detail [16] and the results are expressed in dB/m. The liver stiffness measure (kPa) was assessed as a proxy measure of liver fibrosis. Details of the technical description and examination procedure have been described previously [17]. Results are expressed as the median value in kilopascals (kPa) [18]. Both measurements were obtained by a trained clinician (ES).

Anthropometric and biochemical measurements

Body mass index (BMI), hip and waist circumferences were recorded. Glucose, hemoglobulin A1c (HbA1c), total cholesterol, high density lipoprotein (HDL)-cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), albumin, bilirubin and total protein were measured in fasting serum of patients with NAFLD, whilst only ALT, ALP, albumin, bilirubin and total protein were measured in healthy control subjects using commercially available kits according to the manufacturers' instructions.

^{13}C-ketoisocaproate breath test protocol

The ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT) protocol was undertaken in patients with NAFLD and healthy control subjects. Subjects refrained from alcohol and had fasted overnight for at least 12 hours prior to each test. All subjects were at rest for the duration of the study and remained fasted throughout. On the morning of the study, to standardize CO_2 production, subjects were asked to lie down on a bed and carbon dioxide produced (VCO_2) at rest was measured by indirect calorimetry (GEM Nutrition, UK) for 25 minutes prior to the start of the ^{13}C-KICA BT. Paired breath samples for baseline measurement of isotopic abundance were collected from each subject exhaling directly into 12-ml Exetainer breath tubes (LABCO Ltd, High Wycombe, UK) via straws. Each subject then ingested a solution containing 1mg/kg body weight of 2-keto-[1-^{13}C]-isocaproic acid (99% -^{13}C; Cambridge Isotopes, USA) along with 20mg/kg body weight of L-leucine (Sigma) in 200ml of water. Further paired breath samples were then collected every 10 min for 60 min. The enrichment of ^{13}C (atom % excess) in CO_2 in breath at time (t) was calculated from the ^{13}C abundance measurements by Continuous Flow Isotope Ratio Mass Spectrometry (CF-IRMS, ABCA System, SERCON, Crewe, UK) and used with a measure of the total CO_2 production to calculate the cumulative per cent ^{13}C-dose recovered over 1hr (cPDR over 1hr). The cPDR over 1 hour has been previously validated as a measure of HMF (8-10).

Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test. Data are presented as means ± SDs for normally distributed data and medians and interquartile ranges (IQRs) for non-normally distributed data. Data were analysed using Statistical Package for the Social Sciences (SPSS) Version 21.0 (IBM, New York, USA). Comparison of continuous variables between groups was performed with Mann Whitney U-tests for non-normally distributed data or Students t-tests for normally distributed data, and differences in proportions were analysed using the Chi-squared-test. Univariate associations between variables were investigated using Spearman's rank correlation for non-normally distributed data or Pearson's correlation for normally distributed data. To test for the independence of associations between explanatory factors and cPDR over 1hr, these factors were entered into a multiple linear regression model with ^{13}C-KICA BT as the outcome variable. Models were run with all the explanatory factors entered; or stepwise, in order to investigate the proportion of the variance in cPDR over 1hr that could be explained by individual factors in the model.

RESULTS

Characteristics of the study participants

Table 1 shows the baseline characteristics of patients with NAFLD and healthy subjects. The median age (interquartile range) was 51 (15) years for 77 patients with NAFLD and 40 (27) years for 11 healthy individuals. In the healthy versus patients with NAFLD groups, there were significant differences between weight, BMI, CAP score, ^{13}C-KICA BT, liver stiffness score and ALT (all $p < 0.0001$) and ALP ($p = 0.003$).

Patients with NAFLD were stratified by type 2 diabetes (T2DM) status, as shown in **Table 2**. Liver stiffness was higher ($p = 0.01$) along with concentrations of Hb1Ac and fasting plasma glucose (both $p < 0.0001$) in patients with T2DM compared with those without T2DM. Concentrations of total cholesterol were lower ($p = 0.01$) in patients with T2DM compared those without T2DM. However, there was no significant differences between those with or without T2DM, in both BMI and % liver fat. The cumulative percent $^{13}CO_2$-dose recovered over 1hr (cPDR over 1hr) for the ^{13}C-KICA-BT in patients with T2DM was lower than in patients with NAFLD who did not have T2DM ($p=0.003$).

Relationship between ^{13}C-KICA BT and anthropometric and biochemical parameters

Univariate associations between ^{13}C-KICA BT values (expressed as cPDR over 1hr) and anthropometric and biochemical parameters measured in patients with NAFLD are shown in **Table 3.** Multiple linear regression modelling was undertaken to test the independence of associations between significant factors selected from univariate associations and tests of comparisons, and the ^{13}C-KICA BT derived

cumulative per cent ^{13}C-dose recovered over 1hr (cPDR over 1hr) as the outcome (**Table 4**). In a linear regression model that included age, sex, waist and hip circumference, aspartate aminotransferase (AST), liver stiffness measurement and type 2 diabetes (T2DM) as key explanatory variables and cPDR over 1hr as the outcome, age (β= -0.08; p=0.003), waist circumference (β= -0.08; p=0.01), hip circumference (β= -0.04; p=0.01), AST (β= -0.05; p=0.009) and T2DM (β= -1.81; p=0.01) were independently associated with cPDR over 1hr (Model fit r^2 =0.41; p <0.0001). In this model T2DM status explained 12.0% of the variance in cPDR over 1hr; and hip circumference, AST, age and waist circumference explained 11.2%, 6.1%, 6.4%, and 5.7%, of the variance in cPDR over 1hr, respectively.

DISCUSSION

The results of our study show that in patients with NAFLD, there was a decrease in markers of liver damage (such as ALT and ALP), liver stiffness severity and HMF (assessed by ^{13}C-KICA BT) compared to healthy subjects. When patients with NAFLD were stratified according to their T2DM status; liver stiffness severity, cholesterol and HMF, were significantly different between both groups. In patients with NAFLD, age, waist and hip circumference, AST and T2DM status (but not liver fat percentage nor liver stiffness severity) were independently associated with HMF. In regression modelling, T2DM status explained 12.0% of the variance in cPDR over 1hr; and hip circumference, AST, age and waist circumference explained 11.2%, 6.1%, 6.4%, and 5.7% (respectively), of the variance in cPDR over 1hr. Thus, these combined data suggest that the ^{13}C-KICA BT could be useful in clinical practice to assess HMF.

Our findings show an association between age and HMF and these data suggest that ageing may have a role in indirectly influencing HMF, perhaps due to decreased mitochondrial mass with age, as previously suggested in animal studies (16). AST is known to be present in both the cytosol and mitochondria of hepatocytes. However, the cytosolic and mitochondrial forms of AST are immunologically distinct isoenzymes and about 80% of AST activity in the liver is due to the mitochondrial isoenzyme (17). The significant relationship between serum AST and HMF observed in our study is also consistent with other studies that showed the elevation of AST associated with NAFLD (18, 19). Our findings also provide further evidence to highlight the important role mitochondrial AST plays in aerobic glycolysis (via the malate/aspartate shuttle) within the hepatocytes (20) and is plausible that any

disruption to mitochondrial function or mitochondrial AST activity could lead to an increased risk of increasing hepatic glucose production and insulin resistance (21), that are often observed in NAFLD (22).

Recent literature reviews (23, 24) have suggested that HMF could play a key role in NAFLD as well as T2DM. We suggest that the independent associations we observed between T2DM status, and both high waist and hip circumference, with lower levels of HMF could plausibly reflect the fact that T2DM and increased waist and hip circumferences are all linked by insulin resistance. As speculated in **Figure 1**, it is plausible that increased branched chain amino acids (BCAAs) could mediate the link between these risk factors and lower hepatic mitochondrial function, but addressing this speculation requires further study.

We suggest that the ^{13}C-KICA BT could be useful to identify patients with moderate to severe NASH and further research in this patient group is needed. Currently, liver biopsy remains the gold standard for diagnosing NASH and having a non-invasive relatively simple diagnostic test for NASH in the clinic would be valuable both as a diagnostic marker of disease and also potentially to monitor responses to therapy.

Our study had some limitations that should be considered. Our results show that although liver stiffness was associated with the ^{13}C-KICA BT in univariate analysis, liver stiffness was not associated with the ^{13}C-KICA BT in multivariable regression modelling. A limitation however, is that we used transient elastography instead of liver biopsy to characterise liver fibrosis. Liver biopsies are not being undertaken to test outcomes in the INSYTE trial which is focussed on testing an intervention to

ameliorate liver fat in patients with relatively early liver disease. Therefore, we used transient elastography to characterise liver stiffness as a proxy measure of liver fibrosis. Furthermore, we suggest that the relationship between plasma BCAAs, insulin resistance and HMF needs to be explored further, as measurements of BCAAs were not undertaken in our study.

In conclusion, in patients with NAFLD (compared to healthy subjects), there was a reduction in HMF assessed by the ^{13}C-KICA BT. Furthermore, in patients with NAFLD, HMF is independent and inversely associated with age, waist and hip circumference, AST and diabetes status.

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Conflict of interest: None to declare.

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Table 1. Anthropometric, clinical and biochemical characteristics of the healthy controls and NAFLD patients.

Parameter	Healthy subjects (n = 11)	Patients with NAFLD (n = 77)	p-value
Age (years)	40 (27)	51 (15)	0.15
Sex (Female/Male, n)	7/4	29/48	0.11**
Weight (kg)	71.3 (22)	97.7 (28.2)	< 0.0001
BMI (kg. m^{-2})	23.3 (4.9)	34 (8)	< 0.0001
CAP (dB/m)	184 (23)	321 (69)	< 0.0001
Liver stiffness (kPa)	3.9 (1.1)	6.2 (4.2)	< 0.0001
^{13}C-KICA BT (cPDR over 1h-%)	21 (6.3)	13.4 (4.8)	< 0.0001
ALT (IU/L)	16 (6)	59 (43.7)	<0.0001
ALP (IU/L)	62 (33)	84 (42)	0.003
Albumin (g/L)	40 (5)	41 (4)	0.73
Bilirubin (µmol/L)	12 (4)	10 (5)	0.07
Total protein (g/L)	70 (6)	70 (5)	0.83

Values are expressed as median (interquartile range). Significant differences between groups ($p < 0.05$) were determined using either the Mann-Whitney U-test or the chi-square test **. Abbreviations: BMI, body mass index; CAP, controlled attenuation parameter; ^{13}C-KICA BT, ^{13}C-ketoisocaproate breath test; ALT, alanine aminotransferase; ALP, alkaline phosphatase; kPa, kiloPascals; n, number of subjects.

Table 2. Anthropometric and biochemical characteristics of NAFLD patients stratified by type 2 diabetes status.

	NAFLD		
Parameter	**No T2DM (n=48)**	**T2DM (n =29)**	**p-value**
Age (years)	50 (23)	51 (14)	0.30
Female/male (n)	19/29	10/19	0.31**
Weight (kg)	103.6 (36.4)	97.8 (23)	0.68
BMI (kg.m^{-2})	33.8 (8.3)	35 (5.3)	0.17
Waist circumference (cm)	110.6 (19.8)	114.4 (16.1)	0.18
Hip circumference (cm)	118.4 (15.6)	113.6 (11.4)	0.86
CAP (dB/M)	318 (72)	322 (81.5)	0.3
MRS liver fat (%)	33.5 (36)	31(29.2)	0.74
Liver stiffness (kPa)	6.1 (3.3)	8.8 (4.6)	0.01
^{13}C-KICA BT (cPDR over 1h-%)	14.3 (6.1)	12.7 (3.4)	0.003
Hb1Ac (% Total Hb)	37 (7)	58 (18)	<0.0001
Fasting plasma glucose (mmol/L)	5.5 (0.8)	7.6 (2.6)	<0.0001
Cholesterol (mmol/L)	5.05 (1.5)	4.4 (1.3)	0.01
HDL cholesterol (mmol/L)	1.2 (0.4)	1.2 (0.4)	0.47
Cholesterol/HDL ratio	4.1 (1.1)	3.9 (1.2)	0.20
TAG (mmol/L)	1.9 (1.0)	1.8 (1.0)	0.67
AST (IU/L)	32 (22)	41 (28.5)	0.06
ALT (IU/L)	56 (46.5)	63 (43.5)	0.40
ALP (IU/L)	90.5 (49)	75 (22)	0.54
GGT (IU/L)	53.5 (53)	65 (80)	0.07
Albumin (g/L)	40.5 (4)	42 (4)	0.96
Bilirubin (µmol/L)	10 (5)	9.0 (5.5)	0.13
Total protein (g/L)	70.5 (5.7)	70 (3)	0.61

Data are expressed as median (interquartile range). Significant differences between groups ($p < 0.05$) were determined using either the Mann-Whitney U-test or the chi-square test **.

Abbreviations: n, number of subjects; BMI, body mass index; CAP, controlled attenuation parameter; MRS, magnetic resonance spectroscopy; kPa, kiloPascals; ^{13}C-KICA BT, ^{13}C-ketoisocaproate breath test; Hb1Ac, hemoglobulin 1Ac; HDL, high density lipoprotein; TAG, triacylglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutaryl transferase.

Table 3. Univariate associations between anthropometric and biochemical parameters and ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT) in patients with NAFLD.

Parameters	Correlation coefficient (r)	p-value
Age (years)	-0.21	0.06
Sex (F)	-0.21	0.07
BMI (kg.m^{-2})	-0.40	<0.001
Waist circumference (cm)	-0.34	0.003
Hip circumference (cm)	-0.36	0.001
MRS liver fat (%)	0.09	0.46
Liver stiffness (kPa)	-0.34	0.003
Hb1Ac (% Total Hb)	-0.2	0.1
Fasting plasma glucose (mmol/L)	-0.16	0.2
Total Cholesterol (mmol/L)	0.04	0.7
HDL cholesterol (mmol/L)	-0.18	0.13
Cholesterol/HDL ratio	0.14	0.23
TAG (mmol/L)	0.06	0.62
AST (IU/L)	-0.29	0.01
ALT (IU/L)	-0.05	0.70
ALP (IU/L)	0.09	0.45
GGT (IU/L)	-0.12	0.3
Albumin (g/L)	0.2	0.07
Bilirubin (µmol/L)	0.08	0.51
Total protein (g/L)	0.1	0.4

Abbreviations: F, female, BMI, Body mass índex; MRS, Magnetic resonance spectroscopy; Hb1Ac, hemoglobulin 1Ac; HDL, high density lipoprotein; TAG, triacylglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutaryl transferase.
(r) = Spearman correlation coefficients.

Table 4. Multi-variable linear regression modelling between cumulative per cent ^{13}C-dose recovered over 1hr (cPDR over 1hr) during ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT), and anthropometric, clinical and biochemical parameters in NAFLD.

Independent variables	β-coefficient	95% CI	p-value
Age (years)	-0.08	-0.14, -0.03	0.003
Sex (F)	-0.25	-1.7, 1.2	0.73
Waist circumference (cm)	-0.08	-0.15, -0.02	0.01
Hip circumference (cm)	-0.04	-0.06, -0.01	0.01
AST (IU/L)	-0.05	-0.1, -0.01	0.01
Liver stiffness (kPa)	0.04	-0.2, 0.27	0.75
Type 2 diabetes (T2DM)	-1.81	-3.25, -0.37	0.01

Abbreviations: F, female; AST, aspartate aminotransferase.

Figure 1. A proposed mechanism showing the relationship between plasma leucine catabolism and hepatic mitochondrial function in NAFLD. BCATm is known to be highly expressed in the peripheral tissues but has very low expression in the liver. The low level of expression of BCATm in the liver allows for the initial step in leucine catabolism to take place mostly in the peripheral tissues. α-ketoisocaproate (α-KICA) generated in peripheral tissues is then released back into circulation and upon entering the liver, undergoes irreversible catabolism to CO_2 which is mediated by BCKDH located in the hepatic inner mitochondrial membrane. Abbreviations: BCATm, mitochondrial branched chain aminotransferase; BCKDH, branched chain α-ketoacid dehydrogenase.