FACTORS INDEPENDENTLY ASSOCIATED WITH CARDIORESPIRATORY FITNESS IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE.

Paul R. Afolabi [1,2], Eleonora Scorletti [1,2,3], Philip C. Calder [1,2], Christopher D. Byrne [1,2,*]

Affiliations:

[1] *National Institute for Health Research Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, Southampton, UK.*

[2] *School of Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK.*

[3]*. Department of Gastroenterology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, USA.*

***Corresponding author:** Professor Christopher D. Byrne,
Human Development and Health Academic Unit,
Faculty of Medicine, University of Southampton,
Southampton General Hospital, Southampton, UK.
Telephone number: +44 (0) 23 8120 5006.
E-mail: C.D.Byrne@soton.ac.uk

Keywords: Non-alcoholic fatty liver disease, cardiorespiratory fitness, hepatic mitochondrial function, type 2 diabetes, serum γ-glutamyl transferase, cardiovascular disease.

ABSTRACT

Low cardiorespiratory fitness (CRF) is associated with non-alcoholic fatty liver disease (NAFLD) and low CRF is an important risk factor for cardiovascular disease (CVD). The factors that influence CRF in NAFLD are poorly understood and it has been suggested that reduced hepatic mitochondrial function (HMF) may be linked to low CRF. Therefore, our aim was to determine the factors associated with CRF in NAFLD.

Methods: 97 patients with NAFLD were studied. CRF was assessed by treadmill testing and expressed as maximal O_2 consumption (VO_2 peak) per lean body mass. HMF was assessed by the ^{13}C-ketoisocaproate breath test. Multi-variable linear regression modelling was undertaken to test the independence of associations with CRF.

Results: Mean (SD) age was 51 (13) years and 61% were men. With CRF as the outcome, age (B coefficient -0.3, 95%CI -0.4, -0.2, p<0.0001), total body fat mass (B coefficient -0.2, 95%CI -0.3, -0.05, p=0.01), type 2 diabetes mellitus (T2DM) (B coefficient -3.6, 95%CI -1.1, -6.1, p=0.005), smoking status (B coefficient -5.7, 95%CI -1.9, -9.5, p=0.004), serum γ-glutamyl transferase (GGT) (B coefficient -0.04, 95%CI -0.05, -0.02, p<0.0001), HMF (B coefficient -0.5, 95%CI -0.8, -0.1, p=0.01) and diastolic function (B coefficient 0.1, 95%CI 0.05, 0.13, p<0.0001) were independently associated with CRF. This model explained 60% of the total variance in CRF (R^2=0.6, p<0.0001); and this model GGT alone explained 24% of the variance in CRF.

Conclusions: In patients with NAFLD, HMF is independently associated with CRF and a model with GGT alone explained most of the variance in CRF.

LAY SUMMARY

1. Patients with non-alcoholic fatty liver disease (NAFLD) die more frequently from cardiovascular disease than from liver disease. Low cardiorespiratory fitness (CRF) is an important risk factor for cardiovascular morbidity and mortality and low CRF occurs frequently with non-alcoholic fatty liver disease NAFLD.
2. The factors that influence low CRF in NAFLD are still poorly understood.
3. In patients with NAFLD, hepatic mitochondrial function and serum γ-glutamyl transferase (GGT) levels were both independently associated with CRF and a predictive model for CRF with GGT alone explained 24% of the variance in CRF.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a wide spectrum of liver disease from simple steatosis, to steatohepatitis (both with and without liver fibrosis), cirrhosis and end-stage liver failure that occurs in the absence of significant alcohol consumption. Epidemiological studies have shown that patients with NAFLD often have low cardiorespiratory fitness (CRF) [1-3], which is a very important cardiovascular risk factor [4]. Since patients with NAFLD die as frequently from CVD events as from end-stage liver disease [5-8], it is important to understand the factors that contribute to low CRF because low levels of fitness might explain (at least in part) the increased risk of CVD in such patients. It has been suggested that reduced hepatic mitochondrial function may be linked to low CRF as hepatic mitochondrial dysfunction is an important feature of NAFLD [9]. However, there are no studies to date that have examined the relationship between CRF and hepatic mitochondrial function in patients with NAFLD. Furthermore, it is important to elucidate the key factors that contribute to CRF in NAFLD, since a better understanding of these factors would provide a better insight into the potential for improving CRF, to reduce CVD risk.

The aim of this study was to determine the factors independently associated with CRF in patients with NAFLD and to determine the proportion of the variance in CRF that could be explained by these factors. We tested the hypothesis that reduced hepatic mitochondrial function is associated with low CRF in patients with NAFLD while adjusting for potential confounders such as age, sex, increased adiposity and physical inactivity.

MATERIALS AND METHODS

97 patients with NAFLD were studied; they were recruited as part of the INSYTE trial (**I**nvestigation of **SY**nbiotic **T**reatm**E**nt in NAFLD) (www.clinicaltrials.gov registration number NCT01680640) that is described in detail elsewhere[10] . This trial was approved by the Southampton and South West Hampshire local research ethics committee (12/SC/0614).

Inclusion and exclusion criteria for recruitment of patients with NAFLD

The inclusion and exclusion for participation in the INSYTE trial have been described in detail in the protocol paper[10]**.**

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

Patients with confirmed 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. The values for the lipid and water peak integrals were produced for each VOI and recorded for each subject. Liver fat percentage was estimated as the mean value of the liver fat percentages in the three liver regions.

Assessment of cardiorespiratory fitness

Cardiorespiratory fitness (CRF) was measured in terms of maximal oxygen uptake (VO_2 peak) and determined from breath-by-breath analysis of O_2 consumption and CO_2 production using a Cortex metalyser 3B instrument (Cortex Biophysik, Germany) during maximal treadmill exercise (Woodway P55 treadmill) with 12-lead ECG monitoring throughout the test. Participants were advised to avoid strenuous exercise for 24 h and alcohol on the day prior to testing. The treadmill exercise was undertaken on the morning of the study and beforehand participants were asked not take any medications such as beta-blockers for the duration of the study activity and had a standardized breakfast without tea or coffee. Participants were fitted with an air-tight facemask, which allowed the analysis of expired air. To allow participants to become acclimated to the facemask and to determine resting energy expenditure, resting measurements was taken for 3 min prior to commencement of activity-induced measurements. Participants were encouraged to continue until the respiratory exchange ratio was >1.1 and they reached at least 90% of their predicted maximum heart rate (as determined by 220-age in a previous study)[11] unless they experienced chest pain or felt unwell. CRF was then measured by peak VO_2 adjusted for lean body mass.

We chose to express CRF relative to lean body mass instead of total body weight as we wanted to assess the relationship between CRF and other metabolic parameters of NAFLD independently of adiposity. Furthermore, recent studies have suggested that the expression of CRF relative to total body weight can introduce confounding by body adiposity when assessing the relationship between CRF and other

metabolic parameters of NAFLD [12-14]. We undertook an ECG before and after the treadmill test.

Assessment of physical activity

Physical activity was assessed using an activity monitor (Sense Wear Pro2 Armband, Bodymedia Inc, Pittsburgh, PA, USA) [15].The Sensewear Pro2 armband is a compact and lightweight 82 g device worn around the upper arm that is well tolerated and contains accelerometers that sense movement in two planes, a galvanic skin sensor, a temperature sensor, and a near-patient temperature sensor. The SenseWear Pro2 armband allows reliable measurement of physical activity levels and subjects wore the activity monitor for 7-10 days to gain a reliable estimate of mean metabolic equivalents of tasks (METS) and time spent in physical activity, lying down and sleeping for a 24-h period while the device was worn. All subjects were instructed that the armband was to be worn at all possible times and to remove the armband only for bathing / showering purposes or any water-based activity. The inclusion criterion for accepting a subject's armband data was $\geqslant$80% wear time. Data collected from the armband included: time spent in physical activity, lying down and sleeping at sedentary levels (<1.5 METS) using the software developed by the manufacturer (Innerview Professional Research Software V.5.1, BodyMedia).

Assessment of body fat and lean body mass quantitation

Dual-energy X-ray absorptiometry (DEXA) using a Delfia W 4500 instrument (Hologic, Bedford, MA; coefficient of variation = 0.68%) was used to measure lean body mass (DEXA lean body mass) and total fat mass (DEXA total fat mass).

Assessment of pulse-wave analysis-derived measures of wave reflection and diastolic function.

Participants underwent an overnight fast were asked not to take any alcohol during the fasted period. Furthermore, participants were asked not to take any medications that may affect the results of pulse wave analysis for the duration of the study activity. The pulse-wave analysis (PWA) was undertaken by a single observer by radial artery applanation tonometry (SphygmoCor, version 7, Atcor, Sydney, Australia) to obtain measures of wave reflection [augmentation index at 75 beats/min heart rate (AIx@HR75)], which is a measure of arterial stiffness and peripheral arteriolar resistance, and measures of diastolic function/myocardial perfusion (subendocardial viability ratio-SEVR%). Waveforms were processed using specialised software to calculate an averaged radial artery waveform and to derive a corresponding central aortic pressure waveform using a previously validated generalised transfer function [16,17]. PWA has been validated as a non-invasive technique for estimating diastolic cardiac function, and myocardial perfusion relative to the left ventricular workload can be estimated by the SEVR [18,19]. As the invasive nature of cardiac catheterization restricts its use in research on healthy volunteers, arterial tonometry has gained popularity in research studies [20]. The wave format at the radial artery is easily and reproducibly measured at the wrist, and this non-invasive technique allows the estimation of diastolic function (SEVR%) and wave reflection [augmentation index at 75beats/min (AIx@HR75)] as a proxy for arterial stiffness. Although SEVR% does not take into account the left ventricular end-diastolic pressure, SEVR% is a good estimate of the sub-endocardial viability index (derived from cardiac catheterization) in individuals without evidence of ischemic heart disease in whom the left ventricular end-diastolic pressure is normal [21,22].

SEVR% is not related to aortic pulse pressure but, rather, solely to the diastolic time: systolic time in middle-aged individuals and is, therefore, a measure of diastolic function [21].

Assessment of liver fibrosis by transient elastography

The presence of liver fibrosis was ascertained by use of transient elastography (FibroScan, Echosens, Paris, France). 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 [23]. Results are expressed as the median value in kilopascals (kPa)[24].

Anthropometric and biochemical measurements

Body mass index (BMI), hip and waist circumferences were recorded. Hemoglobulin A1c (HbA1c), total cholesterol, high density lipoprotein (HDL)-cholesterol, triglycerides (TAG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) were measured in fasting serum using established procedures.

Assessment of hepatic mitochondrial function by ^{13}C-ketoisocaproate breath test

The ^{13}C-ketoisocaproate breath test (^{13}C-KICA BT) was used as a validated test of hepatic mitochondrial function **[25-27]**. 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 1 mg/kg body weight of 2-keto-[1-^{13}C]-isocaproic acid (99% ^{13}C; Cambridge Isotopes, USA) along with 20 mg/kg body weight of L-leucine (Sigma) in 200 ml 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 hepatic mitochondrial function [28].

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 26 (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 whereby correlation coefficients are indicated by r. Based on a previous study using a rule of thumb for interpreting the strength of correlation coefficients, values of correlation coefficients between 0 and 0.3 (0 and -0.3) indicate weak, 0.3 and 0.7 (−0.3 and −0.7) indicate moderate, values between 0.7 and 1.0 (−0.7 and −1.0) indicate strong positive (negative) associations [29]. Pearson's partial correlation coefficients between variables were calculated with adjustment for potential confounding factors in order to adjust for the effects of these variables.

To test for the independence of associations between explanatory factors and VO_2 peak relative to lean body mass, factors were entered into a multiple linear regression model with VO_2 peak relative to lean body mass as the outcome variable. Unstandardized B coefficients represent the amount of change in the dependent variable due to a change of 1 unit in the independent variable. Models were run with all the explanatory factors entered simultaneously and also stepwise, in order to

investigate the proportion of the variance in VO_2 peak relative to lean body mass that could be explained by each of the independent factors in the model. The independent variables entered into final regression model were age, sex, DEXA total body fat mass, Type 2 diabetes status, smoking status, GGT, hepatic mitochondrial function, SEVR% and AIx@HR75. The same model was then re-run stepwise to assess the contribution of each variable to the overall variance in VO_2 peak and five predictive models were generated: model 1 (GGT alone), model 2 (GGT and SEVR), model 3 (GGT, SEVR and age), model 4 (GGT, SEVR, age and smoking status) and model 5 (GGT, SEVR, age and smoking status and type 2 diabetes status). A p value < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ SD unless otherwise stated.

RESULTS

Characteristics of the study participants

Table 1 shows the baseline characteristics of patients with NAFLD stratified by sex. There were significant differences between men and women for age, body weight, BMI, lean body mass, total fat mass, ALP, HDL-cholesterol, augmentation index (Alx@HR75), diastolic function (SEVR), duration of physical activity and VO_2 peak relative to lean body mass.

Patients were then stratified by tertile of VO_2 peak relative to lean body mass **(Table 2)**. Age, DEXA total body fat mass, HbA1c, hepatic mitochondrial function, Alx@HR75, and SEVR all showed significant differences between tertiles **(Table 2)**. There were no significant differences in smoking status between the tertiles or differences in VO_2peak relative to lean body mass between smokers and non-smokers (29.8$\pm$6.0 ml/kg/min versus 32.7$\pm$8.1ml/kg/min, p=0.2). The relationship between VO_2 peak relative to lean body mass and GGT was further examined and, although no significant difference in GGT was observed between tertiles, a significant linear trend was observed across tertiles (p=0.01).

Univariate associations between VO_2 peak relative to lean body mass and anthropometric and metabolic parameters and measures of cardiovascular function in patients with NAFLD are shown in **Table 3**. VO_2 peak relative to lean body mass showed a significant moderate negative association with age and HbA1c and a significant moderate positive association with SEVR. Furthermore, VO_2 peak relative to lean body mass also showed a significant moderate negative association with log GGT and Alx@75 along with a significant weak positive association with hepatic

mitochondrial function and the duration of physical activity. **Figure** 1 shows the significant association between VO_2 peak relative to lean body mass and GGT. Partial correlation coefficients were analysed to further assess the relationship between VO_2 peak relative to lean body mass and hepatic mitochondrial function while controlling for age and DEXA total body fat mass. After controlling for age and DEXA total body fat mass, there was a non-significant partial correlation between VO_2 peak relative to lean body mass and hepatic mitochondrial function (Pearson correlation coefficient r=0.1; p=0.5) and (Pearson correlation coefficient r= 0.12; p=0.3) respectively.

Multiple linear regression modelling was undertaken to test the independence of associations between significant factors selected from univariate associations and the VO_2 peak relative to lean body mass as the outcome **(Table 4).** Smoking status was also included in this model as there was a trend towards higher VO_2 peak relative to lean body mass in non-smokers (32.7±8.1ml/kg/min) compared with smokers 29.8±6.0 ml/kg/min, (p=0.2). The regression model was then re-run stepwise (**Table 5**) to estimate how much of the variance in VO_2 peak relative to lean body mass could be explained by each of the factors in the model.

The key explanatory factors in the model were age, sex, DEXA total body fat mass, T2DM, smoking status, GGT, hepatic mitochondrial function, SEVR and AIx@HR75. Of these factors, only age (unstandardized B coefficient = -0.3; p<0.0001), DEXA total body fat mass (unstandardized B coefficient = -0.2; p=0.01), T2DM (unstandardized B coefficient = -3.6; p=0.005), smoking status (unstandardized B coefficient = -5.7; p=0.004), GGT (unstandardized B coefficient = -0.04; p< 0.0001), hepatic mitochondrial function (unstandardized B coefficient = -0.5 ; p=0.01) and

SEVR (unstandardized B coefficient = 0.1; p<0.0001) were independently associated with VO_2 peak relative to lean body mass. The full predictive model of VO_2 peak relative to lean body mass containing the explanatory variables was statistically significant (model fit $R^2 = 0.60$, $p < .0001$) and identified 60% of the total variance in VO_2 peak relative to lean body mass.

In the stepwise multiple linear regression analysis **(Table 5),** GGT alone (model 1) explained 24% of the variance in VO_2 peak relative to lean body mass. The addition of SEVR to the model (model 2) led to a statistically significant increase in R^2 of 0.13, p<0.0001. Addition of age (model 3) led to a further increase in R^2 of 0.11, p<0.0001); addition of smoking status (model 4) led to a further increase in R^2 of 0.052, p = 0.002) and T2DM (model 5) a further increase in R^2 of 0.023, p=0.03). Thus adding each of these factors in turn led to a statistically significant increase in R^2; and models 2, 3, 4 and 5 explained a further variance of 13%, 11%, 5% and 2.3%, compared to the 24% of the variance explained by GGT alone (as shown in model 1) **(Table 5)**.

As duration of physical activity was also significantly associated with VO_2 peak relative to lean body mass **(Table 3)**, we also added duration of physical activity to the final regression model as shown in **Table 4**. The addition of duration of physical activity and the use of other medications such as statins, beta-blockers, antihypertensive drugs and metformin did not affect the associations between VO_2 peak relative to lean body mass and the independent factors in the final regression model.

DISCUSSION

The novel findings of our study are that there are independent associations between CRF and GGT, diastolic function, age, total body fat mass, smoking status, type 2 diabetes mellitus (T2DM) and hepatic mitochondrial function; but there was not an independent association between CRF and a measure of liver fibrosis as a marker of severity of NAFLD. Our study also shows that there is an independent but weak negative association between hepatic mitochondrial function and CRF. To the best of our knowledge, this is the first study that has shown such an association between CRF and hepatic mitochondrial function in patients with NAFLD.

In the current study, we also showed for the first time that GGT was inversely associated with CRF and GGT alone explained most of the variance (i.e. 24%) in CRF (as shown in **Table 5**). These findings support observations from recent epidemiological studies showing that lower CRF is associated with elevated GGT [3,30]. That said, the biological mechanisms involved in the relationship between CRF, GGT and NAFLD are unclear. One possible mechanism that could explain the relationship between GGT and CRF is the key role that oxidative stress plays during the progression of NAFLD. Studies have shown that GGT is elevated during oxidative stress [31-33] and it has been suggested that the increase in serum GGT may be a metabolic adaptive response to increase the de-novo synthesis of intracellular reduced glutathione (GSH) which is major anti-oxidant in skeletal muscle cells and hepatocytes [34-36]. Our findings suggest that low CRF in patients with NAFLD could lead to an increase in oxidative stress and therefore an increase in GGT activity may be a metabolic adaptive response to the resulting oxidative stress.

Univariate correlation analysis showed that there was a weak but significant positive association between CRF and hepatic mitochondrial function. However, as many previous studies have suggested that CRF and hepatic mitochondrial function may be confounded by age and adiposity [28,37], we therefore performed a partial correlation analysis to further examine the relationship between CRF and hepatic mitochondrial function. Our results show that age and adiposity have potential confounding effects on the association between CRF and hepatic mitochondrial function. Furthermore, our study also showed that when the association between CRF and hepatic mitochondrial function was controlled for other potential confounding factors in regression analysis, such as type 2 diabetes, GGT and diastolic function, in addition to age and adiposity; there was an independent and significant albeit weak negative association between CRF and hepatic mitochondrial function. This observation is in contrast with the association suggested in previous studies [38,39]. One possible explanation for our observation is that there is an increased metabolic adaptive response of the hepatic mitochondria, with increased mitochondrial function, in patients with NAFLD who have a low CRF. However, the precise underlying mechanisms involved in the association between CRF and hepatic mitochondrial function in patients with NAFLD need further investigation.

In our cohort only two patients had evidence of prior CVD and in both these patients their condition was stable. Our findings provide further evidence to highlight the role of diastolic function in CRF and importantly the association between CRF and diastolic function that we observed was not confounded by duration of physical activity at sedentary levels as observed in a previous study testing the relationship between SEVR and CRF in subjects with central obesity [40].

Overall, recent literature reviews have suggested that using exercise to increase CRF could be a therapeutic tool for NAFLD as well as T2DM [41,42]. We suggest that the independent associations we observed between age, T2DM status, smoking status, GGT, hepatic mitochondrial function and diastolic function with CRF, could plausibly reflect their roles as cardiovascular risk factors in patients with NAFLD. From examining the unstandardized B coefficients in **Table 4** for each of the explanatory factors independently associated with CRF; the presence of T2DM (-3.6 ml/min/kg lean body mass) and smoking status (-5.7 ml/min/kg lean body mass) are clinically meaningful, since a decrease in CRF of only 1 ml/min/kg has been shown to be associated with a significant 9% increase in all-cause mortality and cardiovascular mortality[43,44]. As can be seen from the data in **Tables 1** and **2**, the IQRs for GGT levels in each group are large, reflecting the wide range of GGT concentrations that we observed in the INSYTE participants. From the data in Figure 1 (where GGT was $\log_{10}$ transformed to show the linear relationship between $\log_{10}$ GGT and CRF), there was a 1.5 $\log_{10}$ difference between the minimum and maximum GGT values (16IUL to 529 IU/L) in participants in the cohort. Using the unstandardized B coefficient from Table 4 (-0.04), this range (513 IU/L) in GGT is therefore associated with a 513 x 0.04 = 20.5 ml/min/kg decrease in VO_2 peak. We reason that a decrease of 20.5 ml/min/kg VO_2 peak could also be highly clinically relevant [43,44].

The present study has some limitations that should be considered. We estimated diastolic function and arterial stiffness indirectly using a non-invasive device (SphygmoCor), which has been used in a sub-study of the Anglo-Scandinavian Cardiac Outcomes Trial in the Conduit Artery Function Evaluation study [45], and was

approved by the US Food and Drug Administration in 2001. However, a potential weakness of this technology is that the calibration of central aortic pressures depends on the accuracy of the brachial pressure measurements [46]. Other limitations of our study were that we used transient elastography instead of liver biopsy to characterise liver fibrosis and we did not have a healthy control group to compare measurements of CRF or other metabolic markers with those of patients with NAFLD. In addition, our relatively small study was undertaking in a predominantly white ethnic group and our results may not be applicable to other ethnic groups.

In conclusion, our study shows that HMF and GGT, together with diastolic function, age, total body fat mass, smoking status and T2DM were all independently associated with CRF. These factors together explained 60% of the total variance in CRF. Of these factors in the model, most of the variance was explained by GGT alone (24%); followed by the measure of diastolic function (SEVR), which explained a further 13%; age, which explained a further 11%; smoking status, which explained a further 5%; and type 2 diabetes, which explained a further 2% of the total variance in CRF.

ACKNOWLEDGEMENTS

The authors will like to thank the National Institute for Health Research Southampton Biomedical Research Centre for funding this study and supporting the authors throughout the study. The authors also thank Sanchia Triggs, Gemma Rood and Jennifer Hedges without whose help this study would not have been possible and Lucinda England for research governance administration. Finally, the authors thank all the study volunteers for their invaluable contribution towards furthering knowledge about NAFLD.

Conflict of interest: None to declare.

Financial support: This study received funding from the National Institute for Health Research Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK.

Table 1. Baseline characteristics of patients with NAFLD.

Variables	Men (n = 59)	Women (n = 38)	p-value
Age (yr)	47.7±12.9	55.0±12	0.005
Body weight (kg)	102.5±16	93.5±16.5	0.01
BMI (kg/m^2)	32.2±4.5	35.0±5.5	0.01
DEXA lean body mass (kg)	70±9.3	54±9.0	<0.0001
DEXA total body fat mass (kg)	32.0±8.4	39.5±9.3	<0.0001
HbA1c (mmol/mol)	40.0(24.2)	39.0(21.2)	0.1
Type 2 diabetes * (Yes) (n, %)	18.0(30.5%)	18.0(47.4%)	0.1
Smoking status * (No) (n, %)	55.0(93.2%)	30.0(81.1%)	0.1
Beta-blockers use* (Yes) (n, %)	3.0(5.1%)	3.0(7.9%)	0.6
Anti-hypertensive drug use (Yes) (n, %)	23.0(39.0%)	19.0(50.0%)	0.3
Metformin* (Yes) (n, %)	17.0(29.0%)	11.0(30.0%)	1.0
Statins* (Yes) (n, %)	14.0(23.7%)	13.0(34.2%)	0.3
AST (Iu/L)	35.5(19.7)	37.5(18.0)	0.4
ALT (Iu/L)	57.0(33.5)	49.0(40.0)	0.1
ALP (Iu/L)	73.0(23.5)	95.0(40.5)	0.0004
GGT (U/L)	49.0(51.5)	50.0(78.0)	0.9
TAG (mmol/L)	2.0±0.9	2.6±4.1	0.2
Cholesterol (mmol/L) Total (mmol/L) LDL (mmol/L) HDL (mmol/L)	 4.8±1.1 2.6±0.9 1.1±0.3	 5.2±1.3 3.0±1.0 1.3±0.2	 0.1 0.1 0.01
Liver stiffness (kPa)	6.4 (5.0)	7.9 (4.0)	0.1
Hepatic mitochondrial function (%)	14.0±4.0	12.7±3.0	0.1
Magnetic resonance spectroscopy (MRS) liver fat (%)	25.6(32.0)	26.0(42.0)	0.3
Augmentation index at 75 mins (AIx@HR75) (%)	19.0(11.0)	24.5(10.0)	<0.0001
Sub-endocardial viability ratio (SEVR) (%)	163.5(45.0)	133.5(30.0)	0.02
Physical activity duration (hr.day^{-1})	1.2(1.3)	0.7(0.8)	0.001
Lying duration (hr.day^{-1})	8.4(1.7)	8.8(2.9)	0.5
Sleep duration (hr.day^{-1})	6.6(1.1)	6.8(1.9)	0.1
Predicted maximal heart rate (%)	77.5±11.4	79.5±14.1	0.6
VO_2peak (ml/min/kg lean body mass)	34.4±7.3	29.0±7.6	0.001

Mean ±SD or Median (inter-quartile range)
*- Cross-tab Pearson chi-squared.

Table 2. Anthropometric and biochemical characteristics of patients with NAFLD stratified by tertiles of cardiorespiratory fitness (VO_2peak relative to lean body mass).

	Maximal oxygen uptake (VO_2peak) (ml/min/kg lean body mass)			p-value	
Variables	Low tertile (5.6–30.0) (n=32)	Middle tertile (30.0-35.5) (n=32)	High tertile (35.5-54.5) (n=33)	Differences between the groups	Linear Trend
Age (yr)	56.2±12.4 ***	51.6±12.5 ****	44.1±11.3	0.0005	0.0001
Female/male (n) *	18/14	12/20	8/25	0.03	N/A
Body weight (kg)	97.0±16.4	102.8±16.5	97.2±16.5	0.3	0.1
BMI (kg/m^2)	33.8±5.1	34.4±3.3 ****	31.7±5.1	0.1	0.1
DEXA lean mass (kg)	61.0±12	66.1±12	64±12.7	0.2	0.3
DEXA total body fat mass (kg)	36.0±10.3	36.5±9.6	32.5±8.2	0.2	0.1
HbA1c (mmol/mol)	52.5(35) **, ***	41.0(17.5) ****	36.0(9.0)	0.001	<0.0001
Type 2 diabetes (Yes) (n,%)*	20(62.5%)	10(31.3%)	6(18.2%)	0.001	N/A
Smoking status (No) (n,%)*	26(84%)	29(91%)	30(91%)	0.6	N/A
AST (Iu/L)	42.5(38)	35.0(23.5)	33.5(25)	0.8	0.2
ALT (Iu/L)	61.0(49)	51.0(44.5)	59.5(39)	0.8	0.5
ALP (Iu/L)	85.5 (71.0)	93.0(33.0)****	76.0(26.5)	0.1	0.01
GGT (U/L)	62.5(78.5)	58.0(54.0)	51.0(34.0)	0.4	0.01
TAG (mmol/L)	2.9±4.5	1.9±0.6	1.7±0.8	0.1	0.1
Cholesterol (mmol/L) Total (mmol/L) LDL (mmol/L) HDL (mmol/L)	 5.1±1.5 2.6±1.0 1.3±0.3	 4.8±1.1 2.8±1.0 1.2±0.2	 5.0±1.0 2.9±1.0 1.2±0.2	 0.7 0.7 0.3	 0.8 0.5 0.3
Liver stiffness (kPa)	6.9(4.7)	7.0(5.3)	5.8(2.2)	0.1	0.05
Hepatic mitochondrial function (%)	13.0±3.3	12.7±3.6 ****	14.8±3.7	0.05	0.05
Magnetic resonance spectroscopy (MRS) liver fat %	18.5(29)	26.0(25)	32.0(38)	0.4	0.5
AIx@HR75 (%)	23.5(8.0) **	19.0(15.0)	18.0(16.0)	0.02	0.02
SEVR (%)	135.5(44.0) ***	148.0(34.0)	157.0(36.0)	0.01	0.001
Physical activity duration (hr/day)	0.8(0.9)	0.9(1.1)	1.0(0.9)	0.3	0.1
Lying duration (hr/day)	8.1(2.0)	8.6(3.6)	8.4(1.8)	0.9	0.8
Sleep duration (hr/day)	6.7(1.5)	6.6(2.8)	6.7(0.9)	0.7	0.5

Cardiorespiratory fitness maximal oxygen uptake (VO_2peak) relative to lean body mass. Mean±SD or Median (inter-quartile range); N/A- Not applicable; *- Cross-tab Pearson chi-squared; Significant differences between variables and across the groups were determined by either the one-way ANOVA or Kruskal-Wallis tests.

- significant differences between low versus middle tertile; *- significant differences between low versus high tertile; ****-significant differences between middle versus high tertile.

Table 3. Univariate associations between cardiorespiratory fitness (VO_{2peak} relative to lean body) and measures of adiposity and cardio-metabolic markers in patients with NAFLD

	VO_2peak (ml/min/kg lean body mass)	
Parameters	**Correlation coefficient (r)**	**p-value**
Age (yr)	-0.41	<0.0001
DEXA total body fat mass (kg)	-0.19	0.05
HbA1c (mmol/mol)	-0.39	<0.0001
TAG (mmol/L)	-0.19	0.06
HDL-cholesterol (mmol/L)	-0.07	0.47
AST (Iu/L)	-0.09	0.39
ALT (Iu/L)	0.05	0.63
ALP (Iu/L)	-0.19	0.07
log GGT (Iu/L)*	-0.32	0.001
Liver stiffness (kPa)	-0.19	0.07
Hepatic mitochondrial function (%)	0.21	0.04
MRS liver fat (%)	0.09	0.34
AIx@HR75 (%)	-0.28	0.01
SEVR (%)	0.39	<0.0001
Physical activity duration (hr/day)	0.23	0.03
Lying duration (hr/day)	0.01	0.94
Sleep duration (hr/day)	-0.03	0.75

Abbreviations: BMI, Body mass índex; MRS, Magnetic resonance spectroscopy; HbA1c, hemoglobulin A1c; HDL, high density lipoprotein; TAG, triacylglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutaryl transferase. MRS, magnetic resonance spectroscopy; AIx@HR75, augmentation index at 75mins; SEVR, sub-endocardial viability ratio; PAEE, physical activity energy expenditure; METS, metabolic equivalents. (r) = Spearman correlation coefficients.* (r) = Pearson correlation coefficient.

Figure 1. The relationship between VO_2peak relative to lean body mass and gamma glutamyl transferase (GGT).

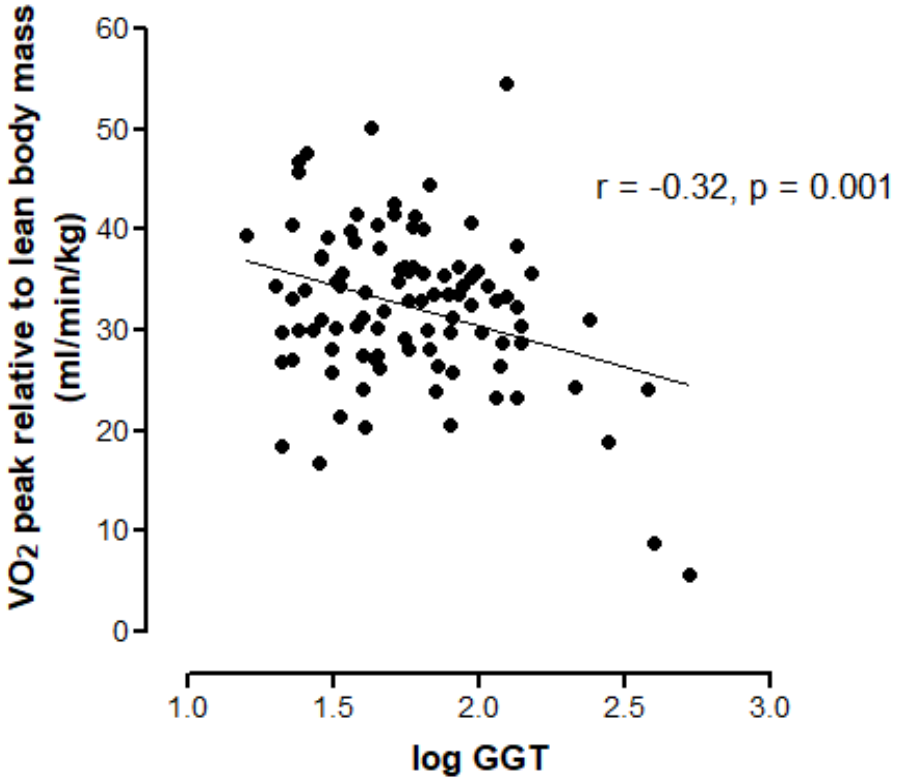

Table 4. Associations between VO_2 peak relative to lean body mass and anthropometric, cardiovascular and metabolic markers in patients with NAFLD.

	Unstandardised coefficient		
Independent variables	**B**	**95% CI**	**p-value**
Age (years)	-0.29	-0.40, -0.19	<0.0001
Sex (F)	-0.71	-2.07, 3.49	0.61
DEXA total body fat mass (kg)	-0.18	-0.32, -0.05	0.01
Type 2 diabetes	-3.64	-1.14, -6.13	0.005
Smoking status	-5.68	-1.89, -9.48	0.004
GGT (IU/L)	-0.04	-0.05, -0.02	<0.0001
Hepatic mitochondrial function (%)	-0.49	-0.84, -0.12	0.01
SEVR (%)	0.09	0.05, 0.13	<0.0001
AIx@HR75	0.05	-0.07, 0.17	0.44

Abbreviations: SE; standard error; F, female; GGT, gamma-glutamyltransferase; SEVR, sub-endocardial viability ratio; AIx@HR75, augmentation index at 75 mins. $R^2 = 0.60$, $p < 0.0001$.

Table 5. Stepwise multivariable linear regression models explaining variance in VO_2 peak relative to lean body mass.

Independent variables	R-square (R^2)	R^2 change	p-value
Model 1 GGT	0.240	0.240	<0.0001
Model 2 GGT and SEVR.	0.366	0.126	<0.0001
Model 3 GGT, SEVR and Age.	0.477	0.111	<0.0001
Model 4 GGT, SEVR, Age and Smoking status.	0.530	0.052	0.002
Model 5 GGT, SEVR, Age, Smoking status and Type 2 diabetes.	0.553	0.023	0.03

Abbreviations: GGT, gamma-glutamyltransferase; SEVR, sub-endocardial viability ratio.

REFERENCES

1. Palve KS, Pahkala K, Suomela E, et al. Cardiorespiratory Fitness and Risk of Fatty Liver: The Young Finns Study. *Med Sci Sports Exerc.* 2017;49(9):1834-1841.
2. Shaw A, Houghton D, Hallsworth K, Jakovljevic DG, Trenell MI, Cassidy S. Adiposity predicts low cardiorespiratory fitness in individuals with metabolic diseases. *Diabetes Res Clin Pract.* 2018;146:300-304.
3. Croci I, Coombes JS, Bucher Sandbakk S, et al. Non-alcoholic fatty liver disease: Prevalence and all-cause mortality according to sedentary behaviour and cardiorespiratory fitness. The HUNT Study. *Prog Cardiovasc Dis.* 2019;62(2):127-134.
4. Imboden MT, Harber MP, Whaley MH, Finch WH, Bishop DL, Kaminsky LA. Cardiorespiratory Fitness and Mortality in Healthy Men and Women. *J Am Coll Cardiol.* 2018;72(19):2283-2292.
5. Arslan N. Obesity, fatty liver disease and intestinal microbiota. *World J Gastroenterol.* 2014;20(44):16452-16463.
6. Wong CR, Lim JK. The Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease Outcomes. *Clin Liver Dis (Hoboken).* 2018;12(2):39-44.
7. Mirbagheri SA, Rashidi A, Abdi S, Saedi D, Abouzari M. Liver: an alarm for the heart? *Liver Int.* 2007;27(7):891-894.
8. Arslan U, Turkoglu S, Balcioglu S, Tavil Y, Karakan T, Cengel A. Association between nonalcoholic fatty liver disease and coronary artery disease. *Coron Artery Dis.* 2007;18(6):433-436.
9. Cuthbertson DJ, Bowden Davies K. Higher levels of cardiorespiratory fitness keep liver mitochondria happy! *J Physiol.* 2017;595(17):5719-5720.
10. Scorletti E, Afolabi PR, Miles EA, et al. Design and rationale of the INSYTE study: A randomised, placebo controlled study to test the efficacy of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease. *Contemp Clin Trials.* 2018;71:113-123.
11. Fox SM, Naughton JP, Haskell WL. Physical activity and the prevention of coronary heart disease. *Ann Clin Res.* 1971;3(6):404-432.
12. Savonen K, Krachler B, Hassinen M, et al. The current standard measure of cardiorespiratory fitness introduces confounding by body mass: the DR's EXTRA study. *Int J Obes (Lond).* 2012;36(8):1135-1140.
13. Tompuri T, Lintu N, Savonen K, et al. Measures of cardiorespiratory fitness in relation to measures of body size and composition among children. *Clin Physiol Funct Imaging.* 2015;35(6):469-477.
14. Krachler B, Savonen K, Komulainen P, Hassinen M, Lakka TA, Rauramaa R. Cardiopulmonary fitness is a function of lean mass, not total body weight: The DR's EXTRA study. *Eur J Prev Cardiol.* 2015;22(9):1171-1179.
15. Mignault D, St-Onge M, Karelis AD, Allison DB, Rabasa-Lhoret R. Evaluation of the Portable HealthWear Armband: a device to measure total daily energy expenditure in free-living type 2 diabetic individuals. *Diabetes Care.* 2005;28(1):225-227.
16. Chen CH, Nevo E, Fetics B, et al. Estimation of central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. *Circulation.* 1997;95(7):1827-1836.
17. Pauca AL, O'Rourke MF, Kon ND. Prospective evaluation of a method for estimating ascending aortic pressure from the radial artery pressure waveform. *Hypertension.* 2001;38(4):932-937.
18. Brazier J, Cooper N, Buckberg G. The adequacy of subendocardial oxygen delivery: the interaction of determinants of flow, arterial oxygen content and myocardial oxygen need. *Circulation.* 1974;49(5):968-977.

19. Buckberg GD, Fixler DE, Archie JP, Hoffman JI. Experimental subendocardial ischemia in dogs with normal coronary arteries. *Circ Res.* 1972;30(1):67-81.
20. Agabiti-Rosei E, Mancia G, O'Rourke MF, et al. Central blood pressure measurements and antihypertensive therapy: a consensus document. *Hypertension.* 2007;50(1):154-160.
21. Chemla D, Nitenberg A, Teboul JL, et al. Subendocardial viability ratio estimated by arterial tonometry: a critical evaluation in elderly hypertensive patients with increased aortic stiffness. *Clin Exp Pharmacol Physiol.* 2008;35(8):909-915.
22. Chemla D, Nitenberg A, Teboul JL, et al. Subendocardial viability index is related to the diastolic/systolic time ratio and left ventricular filling pressure, not to aortic pressure: an invasive study in resting humans. *Clin Exp Pharmacol Physiol.* 2009;36(4):413-418.
23. Sandrin L, Fourquet B, Hasquenoph JM, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol.* 2003;29(12):1705-1713.
24. Castera L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology.* 2005;128(2):343-350.
25. Berthold HK, Giesen TA, Gouni-Berthold I. The stable isotope ketoisocaproic acid breath test as a measure of hepatic decarboxylation capacity: a quantitative analysis in normal subjects after oral and intravenous administration. *Liver Int.* 2009;29(9):1356-1364.
26. Witschi A, Mossi S, Meyer B, Junker E, Lauterburg BH. Mitochondrial function reflected by the decarboxylation of [13C]ketoisocaproate is impaired in alcoholics. *Alcohol Clin Exp Res.* 1994;18(4):951-955.
27. Lauterburg BH, Grattagliano I, Gmur R, Stalder M, Hildebrand P. Noninvasive assessment of the effect of xenobiotics on mitochondrial function in human beings: studies with acetylsalicylic acid and ethanol with the use of the carbon 13-labeled ketoisocaproate breath test. *J Lab Clin Med.* 1995;125(3):378-383.
28. Afolabi PR, Scorletti E, Smith DE, Almehmadi AA, Calder PC, Byrne CD. The characterisation of hepatic mitochondrial function in patients with non-alcoholic fatty liver disease (NAFLD) using the. *J Breath Res.* 2018;12(4):046002.
29. Mukaka MM. Statistics corner: A guide to appropriate use of correlation coefficient in medical research. *Malawi Med J.* 2012;24(3):69-71.
30. Medrano M, Labayen I, Ruiz JR, et al. Cardiorespiratory fitness, waist circumference and liver enzyme levels in European adolescents: The HELENA cross-sectional study. *J Sci Med Sport.* 2017;20(10):932-936.
31. Lee DH, Jacobs DR, Gross M, et al. Gamma-glutamyltransferase is a predictor of incident diabetes and hypertension: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. *Clin Chem.* 2003;49(8):1358-1366.
32. Zhang H, Forman HJ, Choi J. Gamma-glutamyl transpeptidase in glutathione biosynthesis. *Methods Enzymol.* 2005;401:468-483.
33. Irie M, Sohda T, Iwata K, et al. Levels of the oxidative stress marker γ-glutamyltranspeptidase at different stages of nonalcoholic fatty liver disease. *J Int Med Res.* 2012;40(3):924-933.
34. Irie M, Sohda T, Anan A, et al. Reduced Glutathione suppresses Oxidative Stress in Nonalcoholic Fatty Liver Disease. *Euroasian J Hepatogastroenterol.* 2016;6(1):13-18.
35. Hossain IA, Rahman Shah MM, Rahman MK, Ali L. Gamma glutamyl transferase is an independent determinant for the association of insulin resistance with nonalcoholic fatty liver disease in Bangladeshi adults: Association of GGT and HOMA-IR with NAFLD. *Diabetes Metab Syndr.* 2016;10(1 Suppl 1):S25-29.
36. Yeo YH. Redox regulation of metabolic syndrome: recent developments in skeletal muscle insulin resistance and non-alcoholic fatty liver disease (NAFLD). In: Lai Y-C, ed. *Current Opinion in Physiology.* Vol 9. Redox regulation edited by Sruti Shiva and Miriam Cortese-Krott , Volume 9, Pages 1-100 (June 2019). ed: ELSEVIER; 2019:79-86.

37. Câmara M, Browne RAV, Souto GC, et al. Independent and combined associations of cardiorespiratory fitness and muscle strength with metabolic syndrome in older adults: A cross-sectional study. *Exp Gerontol.* 2020;135:110923.
38. Morris EM, McCoin CS, Allen JA, et al. Aerobic capacity mediates susceptibility for the transition from steatosis to steatohepatitis. *J Physiol.* 2017;595(14):4909-4926.
39. Morris EM, Jackman MR, Johnson GC, et al. Intrinsic aerobic capacity impacts susceptibility to acute high-fat diet-induced hepatic steatosis. *Am J Physiol Endocrinol Metab.* 2014;307(4):E355-364.
40. Turzyniecka M, Wild SH, Krentz AJ, Chipperfield AJ, Clough GF, Byrne CD. Diastolic function is strongly and independently associated with cardiorespiratory fitness in central obesity. *J Appl Physiol (1985).* 2010;108(6):1568-1574.
41. van der Windt DJ, Sud V, Zhang H, Tsung A, Huang H. The Effects of Physical Exercise on Fatty Liver Disease. *Gene Expr.* 2018;18(2):89-101.
42. Stevanović J, Beleza J, Coxito P, Ascensão A, Magalhães J. Physical exercise and liver "fitness": Role of mitochondrial function and epigenetics-related mechanisms in non-alcoholic fatty liver disease. *Mol Metab.* 2020;32:1-14.
43. Laukkanen JA, Zaccardi F, Khan H, Kurl S, Jae SY, Rauramaa R. Long-term Change in Cardiorespiratory Fitness and All-Cause Mortality: A Population-Based Follow-up Study. *Mayo Clin Proc.* 2016;91(9):1183-1188.
44. Khan H, Kunutsor SK, Rauramaa R, Merchant FM, Laukkanen JA. Long-Term Change in Cardiorespiratory Fitness in Relation to Atrial Fibrillation and Heart Failure (from the Kuopio Ischemic Heart Disease Risk Factor Study). *Am J Cardiol.* 2018;121(8):956-960.
45. Williams B, Lacy PS, Thom SM, et al. Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: principal results of the Conduit Artery Function Evaluation (CAFE) study. *Circulation.* 2006;113(9):1213-1225.
46. Cloud GC, Rajkumar C, Kooner J, Cooke J, Bulpitt CJ. Estimation of central aortic pressure by SphygmoCor requires intra-arterial peripheral pressures. *Clin Sci (Lond).* 2003;105(2):219-225.