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Dietary Fatty Acid Oxidation is Decreased in Non-Alcoholic Fatty Liver Disease: A Palmitate Breath Test Study

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Abstract

Background/Aim: Hepatic fat excess in non-alcoholic fatty liver disease (NAFLD) reflects an imbalance between fat accumulation and disposal. Conflicting data exist for the role of fatty acid oxidation (FAO), one of the disposal pathways, and have mostly come from studies delivering fatty acids (FA) intravenously. Whether FAO of orally-provided FA is affected in NAFLD is unknown.

Methods: We performed a breath-test study to measure FAO in subjects with NAFLD and healthy controls. Subjects ingested [1-^{13}C] palmitic acid (PA, 10 mg/kg) in a liquid meal and the rate of $^{13}CO_2$ appearance in expired air was measured over 6 hours by a BreathID device (Exalenz) to obtain the cumulative percent dose recovered (CPDR), the total amount of ingested ^{13}C recovered. CPDR was corrected by the results of a [1-^{13}C] acetate breath test, performed 1–4 weeks later, to calculate the rate of PA β-oxidation.

Results: PA oxidation was 27% lower in 43 subjects with NAFLD compared to 11 controls (CPDR 9.5±2.4% vs. 13.1±3.7%, p=0.0001) and this persisted after correcting for acetate (29.3±10.5 vs. 36.6 ±13.9, p=0.03). The decrease in FAO was not due to delayed transit as the time to peak ^{13}C detection did not differ between groups (4.9±1.2 vs. 4.7±0.8 hours, p=0.7). Rates of PA oxidation were not correlated with obesity, hepatic or adipose insulin resistance, ALT, liver fat content and NAFLD histology.

Conclusion: FAO of orally-delivered FA is decreased in NAFLD compared to healthy controls, likely reflecting decreased β-oxidation. The use of a breath test offers noninvasive dynamic assessment of FAO.

Lay summary:

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We used a breath test to study the metabolism of dietary fatty acids, the building blocks of fat, in individuals with fatty liver and compared them to normal weight individuals. We found that individuals with fatty liver break down less dietary fat, which can contribute to fat storage in the liver.

Keywords

β-oxidation; stable isotope; palmitic acid; acetate

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is estimated to affect 30 % of the US population[1]; it commonly co-exists with features of the metabolic syndrome (obesity, hypertension, diabetes mellitus and hypertriglyceridemia) and its rising prevalence seems to accompany the increasing rates of obesity[2].

Hepatocyte triglyceride content reflects a balance between lipid acquisition (fatty acid (FA) uptake or de-novo lipogenesis and esterification) and removal (β oxidation or metabolism, and export as a component of VLDL particles). In NAFLD, insulin resistance is associated with increased de-novo lipogenesis and adipose tissue lipolysis, both driving an increase of FA availability to hepatocytes[2]. Fabbrini et al[3] found that the increase in very low density lipoprotein (VLDL) triglyceride secretion rate in subjects with NAFLD is not able to adequately compensate for the increased rate of intrahepatic triglyceride production. Whether FA oxidation (FAO) is also dysregulated in patients with NAFLD is unclear. We aim to determine if FAO of an oral fat load is different between subjects with NAFLD and healthy normal-weight controls.

Materials and methods

Subjects and study design

Forty-three subjects with NAFLD and 11 healthy normal-weight controls were enrolled in this prospective study (clinicaltrials.gov). The study was approved by the NIDDK/NIAMS Institutional Review Board and all subjects gave written informed consent. All authors had access to study data and approved the final manuscript.

NAFLD group

Included were adults (>=21 years) of both genders with NAFLD defined as having imaging of the liver within 6 months of enrollment consistent with excess liver fat and at least one of:

1. elevated transaminases (ALT>31 U/L for men or >19 U/L for women, or AST >30 U/L) or
2. metabolic syndrome or diabetes.

Laboratory tests were obtained within 12 weeks of the breath test. Subjects were excluded if they had a history of excessive alcohol consumption (> 30 g/d for men or > 20 g/d for women), other causes of liver disease (e.g. viral hepatitis, autoimmune or genetic liver

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disease), decompensated cirrhosis, advanced lung or heart disease, insulin-dependent diabetes, allergy to milk or soy, pregnancy or nursing, and history of prior gastrointestinal resection.

Healthy controls

Included were adults (>= 21years) of both genders with BMI <= 25 kg/m^2 and no history of excessive alcohol consumption, medication use (except oral contraceptives), diabetes or liver disease and with normal liver transaminases and fasting glucose on screening laboratory tests.

Palmitate Breath test (PBT)

After an overnight fast, subjects were given 10mg/kg of [1-^{13}C] palmitic acid (PA, Cambridge Isotope Laboratories, Tewksbury, MA) in a standardized heated liquid meal (Ensure®, Abbott Laboratories), to be consumed within 5 minutes. The appearance of ^{13}C in expired CO_2 was assayed by the BreathID (Exalenz Bioscience, Modiin, Israel) molecular correlation spectrometer device[4,5]. Subjects wore a nasal cannula connected to the device and the expired $^{13}CO_2/^{12}CO_2$ ratio was measured continuously. After measurement of baseline levels, subjects ingested the test meal and measurements continued for 6 hours after that. Real-time graphical representation of the results was displayed during the test and the final results were downloaded as a computer file for off-line analysis.

During the test, subjects remained sedentary in a comfortable chair. Physical activity and talking were minimized. A 15-minute bathroom break was allowed after the first 3 hours of the test.

Acetate Correction

The rates of label appearance in expired CO_2 after ingestion of labeled PA reflect the total rate of its metabolism. Although the labeled carbon can be metabolized to CO_2 directly through β-oxidation and the tricarboxylic acid (TCA) cycle, there is also a possibility of label fixation during the exchange reactions of the TCA cycle and label retention in the bicarbonate pool. To control for this, all subjects also underwent an acetate breath test (ABT) to provide a correction factor for use in oxidation calculations[6]. ABT was performed in a similar manner to PBT with the test meal containing 2mg/kg ^{1}[1-^{13}C] acetate (AA, Cambridge Isotope Laboratories, Tewksbury, MA). PBT and ABT were performed 1–4 weeks apart to prevent tracer carry-over.

Dietary Assessment

To control for dietary habits, a 24-hour dietary recall was obtained by nutrition staff on the day of the study. Participants were asked to provide information on intake during the 24 hours before their visit, using a multi-pass approach to limit the extent of under reporting.

Measurement of insulin resistance

Insulin resistance was estimated by the homeostatic model assessment (HOMA-IR) calculated as plasma glucose (mg/dL) * plasma insulin (μU/mL)/405[7]. Adipo-IR, a measure

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of adipose tissue insulin resistance, was calculated as plasma free fatty acids (mmol/L) * plasma insulin (μU/mL)[8]. All values were collected in the fasting state.

Additional Data

Liver fat quantitation by ^{1}H-magnetic resonance spectroscopy (MRS), liver stiffness measurement (LSM), and liver biopsies were not included in the study, but results were available for a subset of subjects who participated in other clinical trials. MRS was performed as previously described[9]. LSM was measured using vibration controlled transient elastography (VCTE, Fibroscan) with the M- or XL-probe as appropriate. The median result of 10 valid measurements is reported. Liver biopsies were scored according to the NASH-CRN scoring system[10].

Patients were defined as having cirrhosis based on histology, LSM >= 10.3 or based on clinical and radiological findings.

Analysis and Statistical Methods

Breath test results are reported as the percent dose recovered (PDR) and cumulative percent dose recovered (CPDR). PDR reflects the rate of appearance of ^{13}C in the expired air at any given time point, normalized to weight, height and dose. CPDR at any time point reflects the total percentage of ^{13}C dose that was collected up to that time. The PA CPDR was divided by the AA CPDR for the same subject, to obtain the corrected PA CPDR.

Descriptive statistics are shown as frequencies or described with appropriate measures of central tendency. The Mann-Whitney test or Chi Square test were used to determine significance of the differences between the two subject groups where appropriate. Spearman rank test for correlation was used for secondary analyses of the association of the breath test results with baseline variables within the NAFLD groups. All p-values reported are two-sided with a level of significance of 0.05.

Primary Analysis

The study primary end point was the 6-hour PA CPDR. The primary analysis was the comparison of the primary end point between NAFLD subjects and controls.

Secondary Analyses

The timing of the peak PDR was compared between NAFLD subjects and controls.

Secondary post hoc analysis was done to assess the impact of other variables including age, BMI, lab results (ALT, AST, glucose, insulin, HOMA-IR, adipo-IR, triglycerides) on PA oxidation within the NAFLD group.

Results

Study participants

Forty-three NALFD subjects, with steatosis demonstrated by ultrasound, were enrolled in the study between March 2015 and May 2018. Baseline characteristics and metabolic

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features consistent with NAFLD shown in (Table 1). Although liver fat quantitation by ^{1}H-magnetic resonance spectroscopy (MRS) and LSM were not included in the study, results were available for 14 (33%) and 35 (81%) of the NAFLD subjects, respectively (Table 1). Similarly, liver biopsies were not included in the study, but results were available for 11 (26%) of the NAFLD subjects, of whom 6 had evidence of steatohepatitis. Overall, 11(%) patients had compensated cirrhotic.

Healthy controls had normal BMI, ALT, glucose and insulin, and absence of clear metabolic abnormalities (Table 1).

Rates of oral palmitic acid oxidation

The rate of appearance of ^{13}C in expired breath in NAFLD subjects within 6 hours after ingestion of labeled PA was 27% less in NAFLD subjects compared to healthy controls (CPDR 9.5±2.4% vs 13.1±3.7%, p=0.0001, Figure 1a–b), consistent with a lower rate of FAO.

ABT results did not differ between groups (34.4±7.5% vs 38.1±9.7%, p=0.2, Figure 1c–d). After controlling for AA, the corrected PA 6-hour CPDR was also significantly lower in subjects with NAFLD compared to controls (29.3±10.5 vs. 36.6 ±13.9, p=0.03, Figure 1e).

Absorption of substrates

To ensure that the difference between the groups was not due to delayed absorption of PA in NAFLD subjects, time to the peak PA PDR was compared, and did not differ between groups (4.9±1.2 hours in NAFLD vs. 4.7±0.8 in controls, p=0.7, Figure 2a). AA was absorbed and metabolized more rapidly than PA and the timing of the peak of AA PDR was also similar between the groups (1.03±0.5 vs. 1.07±0.3 hours, p=0.9, Figure 2b).

Effect of Dietary Intake

Twenty-four-hour dietary recall data showed that total energy intake in the preceding day as well as the relative macronutrient contribution to the caloric intake did not differ between NAFLD and healthy controls (Table 2). The PA 6-hour CPDR did not correlate with dietary fat intake or other macronutrients (Table 3).

Effect of metabolic syndrome on PA oxidation

There was no correlation of PA CPDR with BMI or with indices of insulin resistance - HOMA-IR and Adipo-IR. PA CPDR also did not corelate with glucose, or TG levels (Figure 3).

Effect of features of liver disease on PA oxidation

PA CPDR was not associated with ALT levels (Figure 3). There was no correlation of PA CPDR with liver fat content assessed by ^{1}H-MRS in the 14 subjects that had available data. In the subset of subjects with available histology, PA CPDR was not associated with histological scores or with the presence of steatohepatitis, although we are underpowered in this subgroup analysis to detect a statistically-significant difference (Supplementary Figure). PA CPDR was not associated with LSM assessed by VCTE (Figure 3).

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Patients with cirrhosis had PA CPDR that was not different from non-cirrhotic (6.2 vs. 9.6, p=0.2)

Effect of race and age on PA oxidation

Beyond metabolic parameters, the NAFLD cohort differed from the controls in age and Hispanic ethnicity. However, PA CPDR did not differ between Hispanic and non-Hispanic NAFLD subjects (8.8±2.5vs. 9.5±2.3, p=0.38) and did not correlate with age (R=0.2, P=0.19), ruling them out as confounders.

Discussion

In this study we aimed to determine if oxidation of an orally-delivered FA differs between NAFLD and healthy normal weight controls, utilizing an oral PBT. We found that subjects with NAFLD had a marked reduction of PA oxidation compared to healthy controls and this difference persisted after acetate correction. Reduced PA oxidation was observed irrespective of NAFLD severity.

The β-oxidation pathway is a cyclic process in which acyl-CoAs are shortened, and the two carboxy-terminal carbon atoms are released as acetyl-CoA each time a cycle is fully completed[11]. Acetyl-CoA can later be converted into ketone bodies (β-hydroxybutyrate or acetoacetate) or can be incorporated into the TCA for full oxidation[12]. Since acetate enters the TCA cycle directly as acetyl-CoA, the results of the ABT can serve to control for label fixation in the exchange reactions of the TCA and for retention in the bicarbonate pool[6]. By correcting the PA oxidation by the acetate correction factor, we are measuring predominantly the rates of β-oxidation.

NAFLD commonly occurs together with the metabolic syndrome[13], and this accounts for many of the differences in baseline characteristics between our NAFLD subjects and healthy controls. It is thus unclear if the difference between the NAFLD and controls in PA oxidation is specific to NAFLD or due to the presence of coexisting metabolic syndrome. However, since the CPDR did not correlate with obesity (BMI), HOMA-IR, or adipose tissue insulin resistance, which are common features of the metabolic syndrome, it is likely to be specific to NAFLD. A study comparing FAO between subjects with metabolic syndrome with or without NAFLD would help answer that question and assess the impact of metabolic syndrome exclusive of NAFLD, but this would be difficult to perform. Studies have shown that the prevalence of NAFLD in subjects with metabolic syndrome can be 70–80%[14], rendering it difficult to find subjects with metabolic syndrome who have absolutely no evidence of NAFLD.

To ensure that the difference in PA oxidation was not due to a delay in absorption in the NAFLD group, as subjects with insulin resistance and obesity can have impaired gastrointestinal motility, we compared the time to peak PDR between groups and found no difference that explains the decrease in FAO.

Previously Lambert et al[15] described a “second meal effect” that refers to the impact of previous macronutrient consumption on the metabolism of meal triglyceride. To rule this out

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as a cause for our findings we evaluated meal composition in both groups and found that total caloric and macronutrient intake was similar in the 24 hours preceding the study. Furthermore, total energy and macronutrient composition did not correlate with PA oxidation.

Age and the proportion of Hispanic subjects were the only non-metabolic parameters that differed between the NAFLD and healthy control groups. The healthy controls were younger than the NAFLD group and as expected the NAFLD subjects were predominantly Hispanic. However, within the NAFLD group, age did not correlate with PA oxidation and there was no difference in FAO between Hispanics and non-Hispanics. Thus, the difference between the groups in rates of PA oxidation are not due to difference in baseline parameters.

The results of breath testing with the BreathID device are comparable to those of “classic” off-line breath testing using isotope ratio mass spectroscopy[16] but allow for real-time assessment of test validity and results. The same device with a different substrate (^{13}C-Urea) is approved by the FDA for the diagnosis of H. Pylori infection[17].

Our study assessed total body FAO, but the actual site of oxidation that accounts for the difference between NAFLD and controls is unclear. Fatty acids can be oxidized in most tissues (except the brain and red blood cells) but three are quantitatively more important than others: adipose tissue, skeletal muscle and liver.

Given that our study hints at NAFLD as the cause for the impaired FAO, it is plausible that the difference is predominantly driven by reduction in hepatic FAO; however, this cannot be definitively proven with our study design.

There are currently no reliable methods for measuring hepatic FAO in vivo. Ketone bodies are produced mainly in liver mitochondria since few other tissues in the body are capable of ketogenesis and indirect measurement of hepatic FAO, assessed by plasma ketone body concentrations, suggests that hepatic FAO is either increased or normal in subjects with NAFLD[3]. However, this is an indirect measurement and could be affected by other parameters.

Mitochondrial dysfunction plays a key role in the pathogenesis of NAFLD. Data from patients with NASH show loss of hepatic mitochondrial cristae and a paracrystalline inclusion of unknown composition[18–20]. In vivo data in humans using various ^{13}C-labeled substrates (methionine and ketoisocaproic acid) suggested a lower rate of mitochondrial oxidation in patients with NAFLD[20–22]. These results are in line with our findings.

Other studies investigating whole body and hepatic fat oxidation have reported conflicting results, which may be affected by the method of measurement. FAO has been studied using labeled fatty acids, indirect calorimetry or indirectly using measurement of plasma ketone bodies, as discussed above. In a study by Croci et al, obese/overweight patients with NAFLD showed reduced whole-body FAO compared with healthy controls when assessed with indirect calorimetry under basal, insulin stimulation (hyper-insulinemic-euglycemic clamp) and exercise[23]. These results are consistent with our findings. However, several studies using tracer methodology showed an increase in FAO in NAFLD subjects[24,25]. Most

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of these studies have been done using labeled FA given as intravenous infusions with frequent blood sampling and prolonged duration of testing to reach an isotopic steady state. The prolonged infusion of fatty acid to a peripheral vein at a constant rate does not reflect physiologically the fluctuating levels of fatty acids in the circulation and the complex hormonal and metabolic response to an oral source of caloric load. Furthermore, there may be inherent differences in the fate of FA derived from peripheral adipose tissue (mimicked by an IV infusion) and those derived from an oral load. Although PBT with oral loading has been used in the past to evaluate FAO in healthy subjects[26], to the best of our knowledge our study is the first to utilize it to assess FAO in subjects with NAFLD and compare them to healthy controls.

The strengths of our study include firstly, the measurement of FAO in a physiologic manner by providing the substrate orally in a mixed meal. Secondly, we applied stringent selection of healthy controls by using strict ALT and BMI cutoffs to avoid inadvertent recruitment of NAFLD subjects in this group[27]. Thirdly, unlike isotopic ratio mass spectroscopy in which breath samples are collected intermittently, the BreathID device continuously senses exhaled breath in real-time, providing continuous data measurement throughout the 6-hour study.

An important limitation of our study is that not all subjects with NAFLD had liver biopsies or liver fat quantification by H^1-MRS. This limits our ability to infer a correlation between liver fat content and FAO and to assess whether FAO differs between NAFL and non-alcoholic steatohepatitis. Future studies may address this.

Although the healthy controls were not required to have liver imaging to prove absence of steatosis, their likelihood of having it is very low given the strict exclusion criteria[27]. To validate this assumption, we examined available data from the NHANES III survey. We identified 539 participants with interpretable ultrasound who met our healthy control criteria by reporting excellent health, taking no medications, drinking <= 30 drinks/month, having normal BMI and ALT, and no evidence for viral hepatitis, diabetes, hypertension or stroke. Only 7% of these participants had significant steatosis. Furthermore, 7 (64%) of the healthy controls in our study had liver ultrasounds in another clinical trial, and all were negative for steatosis. Thus, we believe these subjects are adequate as healthy controls

Secondly, it is possible that full oxidation was not captured during the 6-hour study period. Additionally, we evaluated a single fatty acid, and results of PA oxidation might not be generalizable to oxidation of other FA or triglycerides. However, palmitic acid has been the standard tracer used in FAO studies. Lastly, our study is specifically underpowered for H^1-MRS and liver biopsy data analysis as this was not part of the study design.

In conclusion, we found that subjects with NAFLD have decreased oxidation of orally-delivered palmitic acid, likely reflecting decreased β-oxidation. The use of a breath test offers a noninvasive dynamic tool for the assessment of fatty acid oxidation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations:

NAFLD	Non-alcoholic fatty liver disease
FAO	fatty acid oxidation
PA	palmitic acid
CPDR	cumulative percent dose recovered
AA	acetate
FA	fatty acid
PBT	palmitate breath test
TCA	tricarboxylic acid cycle
ABT	acetate breath test
PDR	percent dose recovered
LSM	liver stiffness measurement
VCTE	vibration controlled transient elastography

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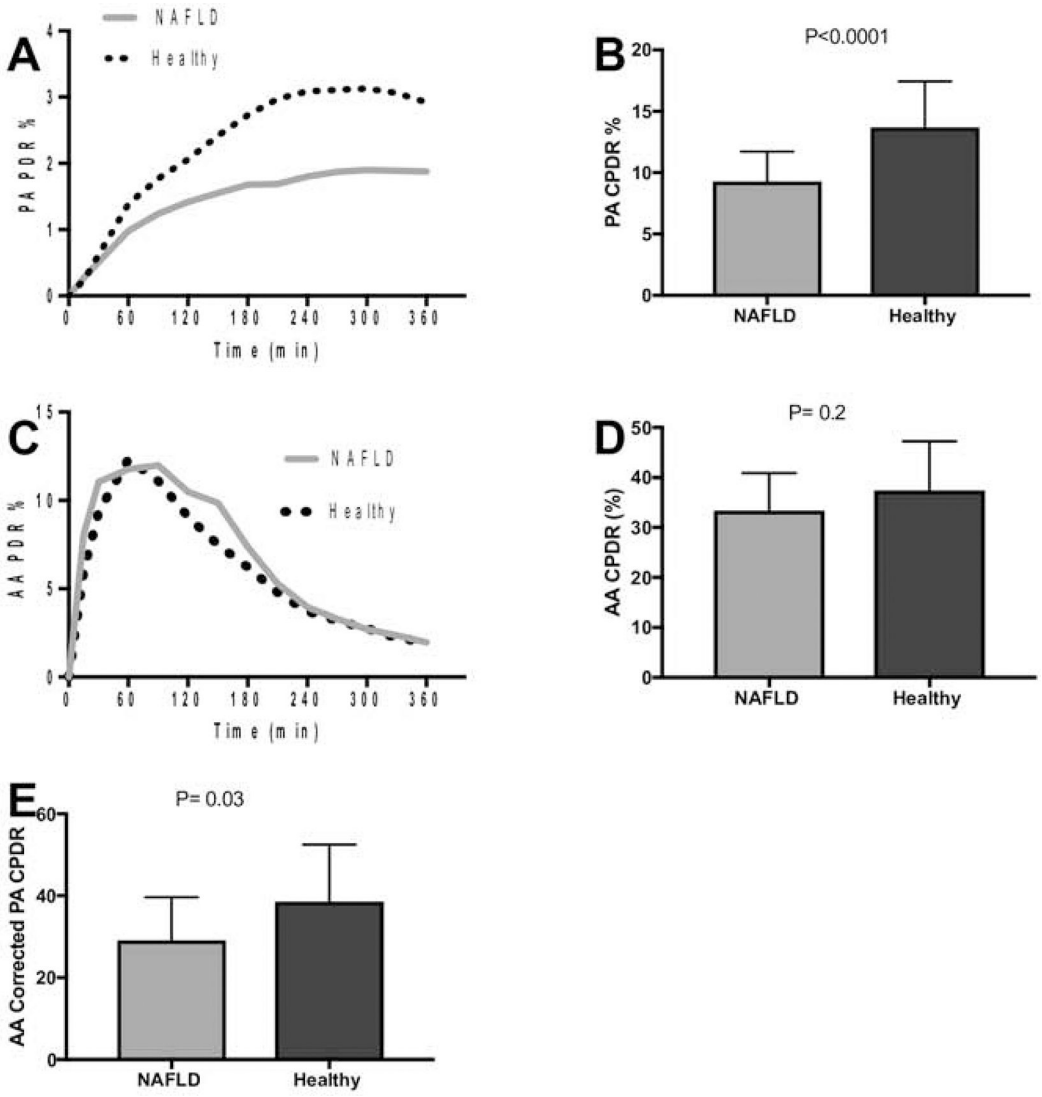

Figure 1. Rates of PA and AA oxidation

(A) Subjects with NAFLD have decreased recovery of ^{13}C label from PA compared to healthy controls. (**B)** Cumulative 6-hour recovery of ^{13}C from PA oxidation is significantly decreased in subjects with NAFLD. (**C**) Recovery of ^{13}C label from AA is similar in both groups. (**D**) Cumulative 6-hour AA oxidation is similar between NAFLD and healthy controls. (**E**) After acetate correction, subjects with NAFLD still have significantly lower PA oxidation than healthy controls. PA – palmitic acid, AA – acetate, PDR – percent dose recovered, CPDR – cumulative percent dose recovered. Results presented as median with IQR

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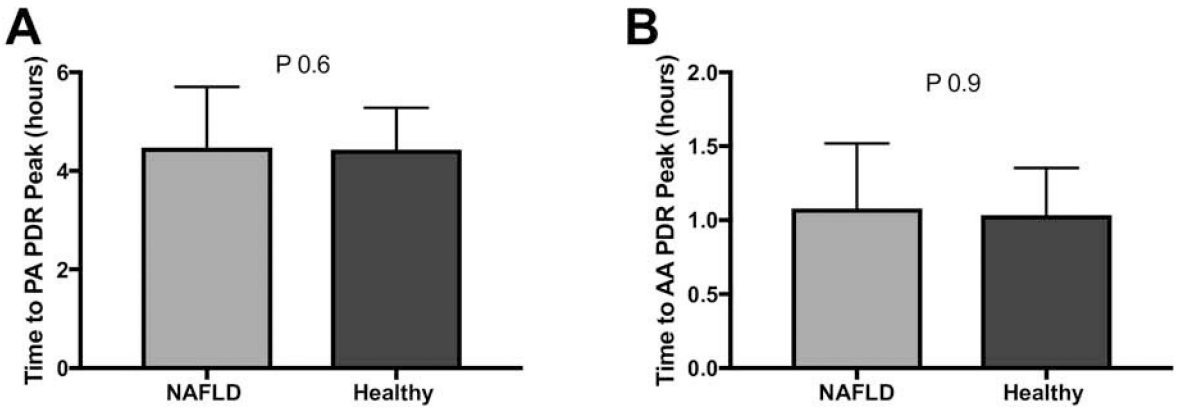

Figure 2. Time to peak ^{13}C recovery.

(A) Time to peak PA PDR is similar between subjects with NAFLD and healthy controls. **(B)** Time to peak AA PDR is also similar between the 2 groups. PA – Palmitic acid, AA-Acetate, PDR – percent dose recovered.

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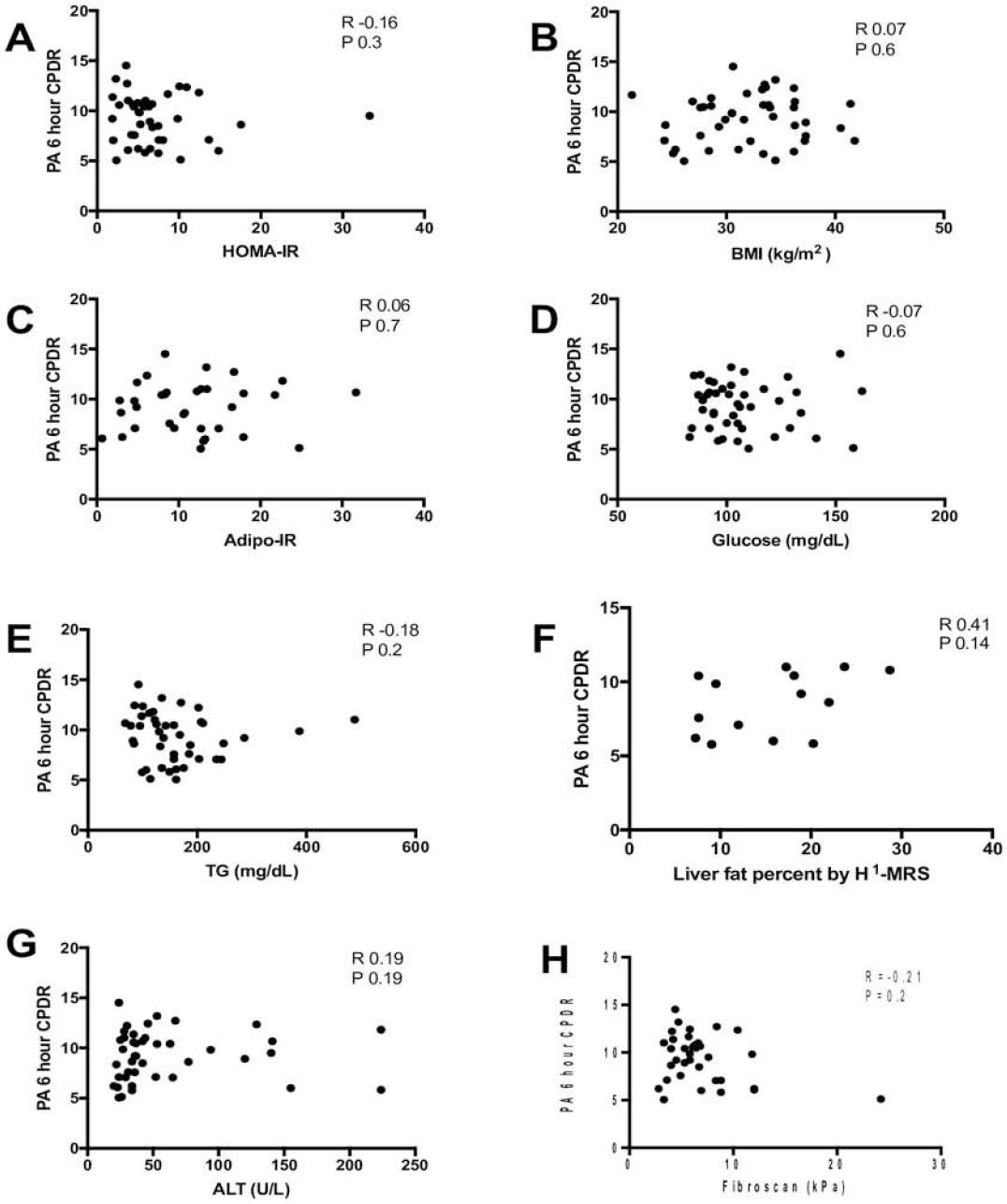

Figure 3. Correlation of PA oxidation with metabolic syndrome features and liver fat.

The 6-hour palmitic acid CPDR in NAFLD subjects is not correlated with **(A)** HOMA-IR, **(B)** BMI, **(C)** Adipo-IR, **(D)** Glucose, **(E)** Triglycerides, **(F)** Liver fat percent by ^{1}H-MRS or **(G) ALT, (H) LSM**.

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Table 1:

Baseline Demographic and Clinical Characteristics

	NAFLD (n=43)	Healthy (n=11)	P value
Age (years)	54 (42–59)	33 (25–40)	<0.0001
Male Sex (n, %)	22 (55%)	5 (45%)	0.5
Race (%)			0.72
Non-white	26 (60%)	6 (55%)	
White	17 (40%)	5 (45%)	
Hispanic ethnicity	19 (44%)	0	
BMI (kg/m^2)	32.2 (28.5–35.3)	21.8 (21–22.8)	<0.0001
ALT (U/L)	27 (28–64)	16 (17–23)	<0.0001
AST (U/L)	28 (21–49)	19 (13–19)	0.001
Platelets (K/uL)	238 (206–250)	221 (206–234)	0.29
Insulin (μU/mL) [n=42]	23.8 (16.4–30.5)	9 (5.1–10.1)	<0.0001
Glucose (mg/dL)	102 (83–162)	88 (83–94)	0.0003
HOMA-IR [n=42]	5.85 (4.2–7.9)	1.97 (1.06–2.32)	<0.0001
Triglyceride (mg/dL)	142 (109–186)	54 (44–88)	<0.0001
Diabetes (n, %)	11 (26%)	0	
Liver fat content by ^{1}H-MRS (%) [n=14]	16.6 (28–68.5)	NA	
Liver Stiffness Measurement (kPa) [n=35]	3.5 (4.5–7.9)	NA	
Liver biopsy [n=11]			
Steatohepatitis	6 (55%)	NA	
NAFLD activity score (NAS)	3 (2–5)	NA	

Results are expressed as median (IQR) or number (percentage). NA – not applicable

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Table 2:

Dietary intake and macronutrient composition in NAFLD and healthy controls

Macronutrient	NAFLD (n=35)	Healthy controls (n=11)	P value
Total energy (kcal)	1722 (1234–2163)	1677 (1315–2389)	0.9
Calories from Fat (%)	34 (29–41.8)	35.5 (32.2–41.6)	0.39
Calories from Carbohydrate (%)	45.5 (33.5–50.8)	45.3 (41.2–49.2)	0.99
Calories from Protein (%)	19 (15–23)	15.4 (13.6–19.2)	0.08

Data shown as median (IQR)

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Table 3:

Correlation of dietary total energy and macronutrient intake with PA oxidation

Macronutrient	NAFLD		Healthy Controls	
Energy (kcal)	R −0.10	P 0.56	R −0.37	P 0.26
Fat (grams)	R −0.07	P 0.70	R −0.24	P 0.46
Carbohydrates (grams)	R −0.06	P 0.73	R −0.5	P 0.12
Protein (grams)	R 0.05	P 0.77	R −0.15	P 0.65

Data shown are Spearman correlation coefficients between PA 6-hr CPDR and energy and macronutrient intake