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ORIGINAL ARTICLE

Metformin does not affect postabsorptive hepatic free fatty acid uptake, oxidation or resecretion in humans: A 3-month placebo-controlled clinical trial in patients with type 2 diabetes and healthy controls

Lars C. Gormsen MD[1] | Esben Søndergaard MD[2] | Nana L. Christensen MLS[1] | Steen Jakobsen PhD[1] | Erik H. T. Nielsen MSc[1] | Ole L. Munk PhD[1] | Lars P. Tolbod PhD[1] | Niels Jessen MD[3,4] | Søren Nielsen MD[2]

[1]Department of Nuclear Medicine and PET Centre, Aarhus University Hospital, Aarhus C, Denmark

[2]Department of Endocrinology, Aarhus University Hospital, Aarhus C, Denmark

[3]Department of Clinical Pharmacology, Aarhus University Hospital, Aarhus C, Denmark

[4]Department of Biomedicine, Aarhus University, Aarhus C, Denmark

Correspondence
Lars C. Gormsen MD PhD, Department of Nuclear Medicine and PET Centre, Aarhus University Hospital, DK 8000 Aarhus C, Denmark.
Email: lars.christian.gormsen@clin.au.dk

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Aims: To explore whether the pre-clinical findings that metformin improves lipid metabolism, possibly through modulation of intrahepatic partitioning of fatty acids towards oxidation and away from re-esterification and resecretion as triglycerides (TGs), can be translated to a human setting.

Materials and methods: We performed a 3-month randomized, placebo-controlled, parallel-group clinical trial in patients with type 2 diabetes (T2D; *n* = 24) and healthy controls (*n* = 12). Patients with T2D received either placebo (placebo group) or 1000 mg metformin twice daily (metformin group), while healthy subjects were all treated with metformin (control group). Hepatic fatty acid metabolism was measured by [^{11}C]palmitate positron-emission tomography, hepatic TG secretion and peripheral oxidation by ex vivo labelled [1-^{14}C]VLDL-TG and VLDL particle size by TG/apolipoprotein B ratio. Body composition was assessed by dual-energy X-ray and whole-body lipid oxidation by indirect calorimetry.

Results: Metformin treatment for 3 months produced the anticipated decrease in fasting plasma glucose (FPG) in the metformin group (FPG 7.9 ± 1.8 mM [study day 1] vs 6.4 ± 1.1 mM [study day 2]), whereas patients in the placebo group and healthy controls had similar FPG levels before and after the trial (mixed model group vs time interaction; *P* = .003); however, contrary to our hypothesis, metformin treatment did not affect hepatic lipid metabolism or peripheral oxidation.

Conclusion: The observed beneficial effects on lipid metabolism during metformin treatment in humans appear to be secondary to long-term alterations in body composition or glucose homeostasis.

KEYWORDS

clinical trial, liver, metformin

1 | INTRODUCTION

Metformin is the preferred first-line drug used to treat hyperglycaemia in patients with type 2 diabetes (T2D). This is primarily because of its efficacy in reducing overall cardiovascular morbidity and mortality[1] while favourably affecting lipid profile.[2] Metformin treatment has thus been estimated to reduce circulating triglycerides (TGs) by 10%,[3–5] ameliorating T2D-related hypertriglyceridaemia.[6]

Whereas the clinical effect of metformin treatment is now firmly established, the mechanisms by which the drug affects both glucose and lipid metabolism are still poorly understood. Metformin is thought to activate the 5′ adenosine monophosphate-activated protein kinase (AMPK),[7] resulting in reduced hepatic gluconeogenesis,[8] but may also have AMPK-independent glucose-lowering effects.[9] Activated AMPK partitions lipids away from lipogenesis towards oxidation,[10–12] and metformin could therefore potentially affect lipid

metabolism favourably through AMPK. AMPK activation may also directly inhibit TG formation through inhibition of glycerol-3-phosphate acyltransferase,[13] resulting in TG-poorer VLDL particles being formed.

Although there is evidence to suggest that metformin may increase hepatic fatty acid oxidation and decrease TG secretion through AMPK, the hypothesis is based on preclinical findings and has, to our knowledge, not been tested in human studies. This may be explained by the relative inaccessibility of the liver and the resultant lack of robust methods to measure hepatic lipid partitioning. Human studies of the lipid-lowering effects of metformin have therefore primarily entailed measurements of whole-body lipid oxidation assessed by indirect calorimetry[14] or simple measurements of circulating lipid concentrations[3,4] with only scant attempts made to describe involved mechanisms. Nevertheless, more organ-specific methods do exist.

Positron-emission tomography (PET) is a non-invasive, image-based radiotracer technique allowing continuous measurements of the intrahepatic fate of the fatty acid radiotracer [^{11}C]palmitate. Based on liver tissue time vs radioactivity curves, the fraction of palmitate directed towards oxidation or re-esterification can be calculated in both animals[15] and humans.[16] In addition, hepatic TG secretion can be measured by our in-house developed ex vivo-labelled [1-^{14}C]VLDL-TG radiotracer.[17]

The aim of the present study, therefore, was to determine whether metformin alters hepatic fatty acid channelling in humans by directing fatty acids towards oxidation rather than re-esterification and resecretion as VLDL-TG. We did this by conducting a randomized, double-blind, clinical trial in patients with relatively recent-onset T2D with a control cohort of healthy age- and body composition-matched participants. It was our primary hypothesis that treatment with metformin would increase hepatic fatty acid oxidation (as measured by [^{11}C]palmitate PET) and decrease VLDL-TG secretion (as measured by [1-^{14}C]VLDL-TG rate of appearance). It was a secondary hypothesis that metformin treatment would result in the formation of TG-poorer VLDL particles and a more rapid clearance of VLDL particles from the circulation (as measured by fractional [1-^{14}C]VLDL-TG oxidation).

2 | MATERIALS AND METHODS

2.1 | Study participants

In total, 30 patients with T2D and 12 healthy controls were recruited through advertisements in local newspapers, as well as from the outpatient clinic. Of these, 24 patients with T2D met the following inclusion criteria: age > 50 years, body mass index (BMI) <40 kg/m^2 and T2D as defined by American Diabetes Association criteria. If patients with T2D were receiving metformin treatment at the time of their initial screening, treatment was discontinued for 4 weeks before initiation of the study drug and study day 1. Twelve healthy control subjects who met the following criteria were also included: age > 50 years, BMI < 40 kg/m^2 and normal glucose tolerance. Two patients with T2D did not complete the second study day because of hospital admissions unrelated to the study drug (1 patient fell and had a vertebral column fracture, and 1 patient was hospitalized with febrile illness and had her study medication discontinued during the stay in hospital). New patients with T2D replaced the drop-outs. One healthy control subject did not meet for the second study day because of intolerable gastrointestinal side effects from metformin treatment.

The study protocol was performed in accordance with the Declaration of Helsinki and was approved by the Central Denmark Committee on Health Research (no. 1-10-72-522-12) and the Danish Medicines Agency (EudraCT 2012-000808-16). The local Good Clinical Practice unit at Aarhus University Hospital monitored the study. All participants signed written informed consent before entering the study. The study was registered with ClinicalTrials.gov identifier NCT01729156.

2.2 | Design and intervention

The study was a randomized, placebo-controlled, parallel-group study, in which patients with T2D were randomized to receive either placebo or 1000 mg metformin twice daily (Figure 1A). All healthy controls were administered 1000 mg metformin twice daily. Metformin dose was uptitrated from 500 mg twice daily to 1000 mg twice daily over a 4-week period. Participants attended 2 study days during the course of the study: immediately prior to the first dose (study day 1) and after completion (study day 2) of the study period (90 $\pm$ 3 days of study drug). Study day outline is provided in Figure 1B. Anticipated changes in hepatic lipid metabolism are outlined in Figure 1C.

Randomization to the placebo group or metformin group was performed using random permuted blocks of 4 participants with 1:1 allocation ratio, resulting in 12 participants in the placebo and 12 in the metformin group. Both study participants and investigator were blinded to the allocation until the end of the study.

2.3 | Study days 1 and 2 protocol

Overnight fasted participants were admitted at 8:30 AM to the PET Centre at Aarhus University Hospital for the initial PET/computed tomography (CT) study. At t = −30 minutes, intravenous catheters were placed in an antecubital vein for administration of radiotracers and in a contralateral dorsal heated hand vein for blood sampling. At t = 0 minutes, low-dose CT was performed to correct for attenuation and anatomical localization of the PET data. At t = 10 minutes, 351 $\pm$ 58 MBq [^{11}C]palmitate was administered, followed by a 50-minute list-mode PET scan centred on the liver. Blood samples to correct for radioactive metabolites were drawn at t = 20, 30, 40, 50 and 60 minutes. During the PET study, blood samples were also drawn at t = 0, 30 and 60 minutes to measure circulating free fatty acids (FFAs). Immediately after the PET scan, participants were transferred to the Clinical Research Centre at Aarhus University Hospital, where they were placed in a bed. At t = 90 minutes, a bolus injection of 43 $\pm$ 32 kBq ex vivo-labelled [1-^{14}C]VLDL-TG was administered, followed by blood samples at t = 90, 95, 100, 110, 120, 150, 180, 240 and 300 minutes to determine plasma [1-^{14}C]VLDL-TG-specific activity. At the same time points, breath samples were taken to

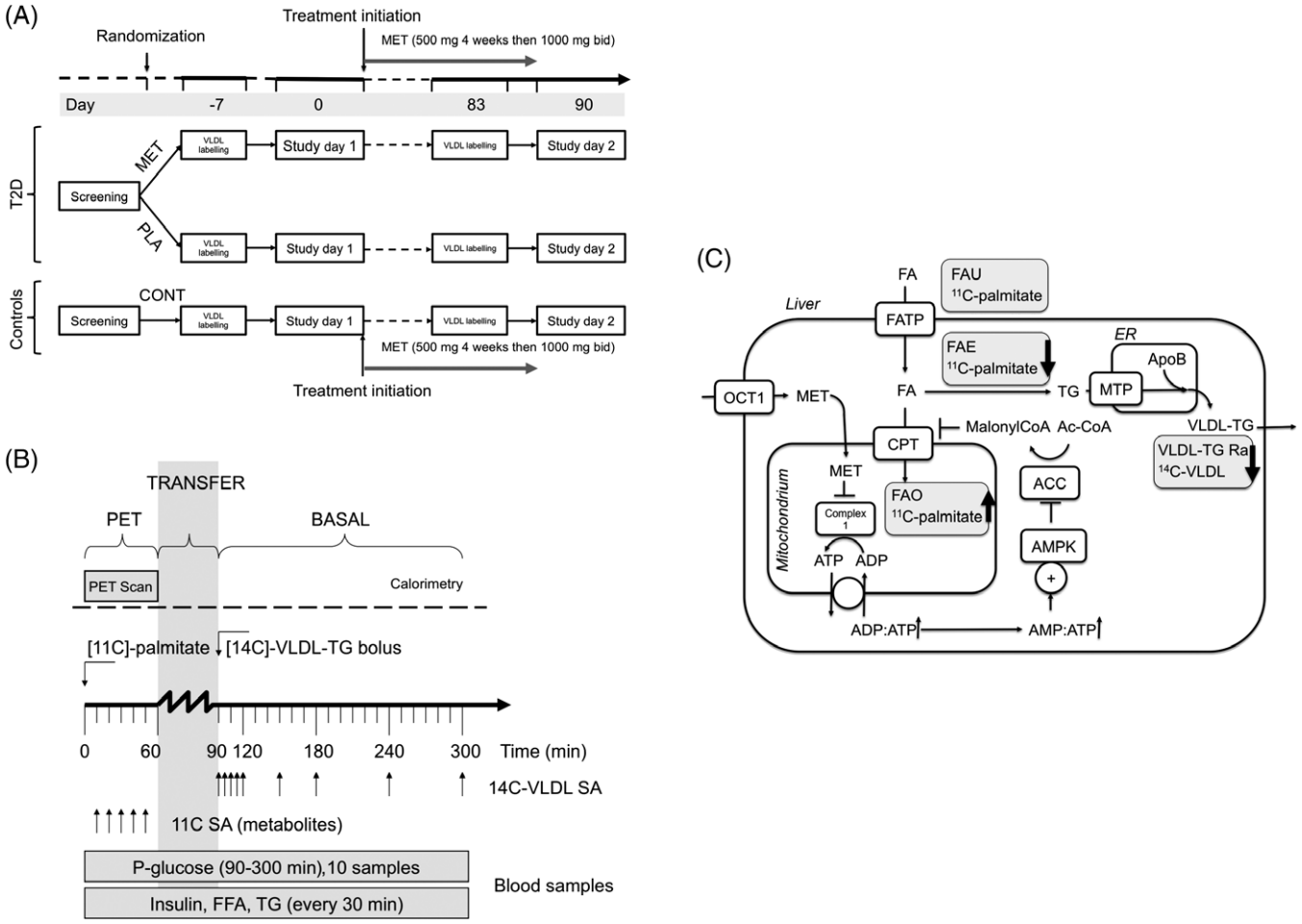

FIGURE 1 A, Study design. B, Study days. C, Putative effects of metformin in the liver. The various fatty acid pathways that metformin was hypothesized to affect are highlighted in grey shaded boxes, with arrows denoting whether fluxes were thought to decrease or increase. ACC, acetyl-CoA carboxylase; ApoB, apolipoprotein B; CONT, healthy control subjects; CPT, carnitin-palmitoyl transferase-1; ER, endoplasmatic reticulum; FA, Fatty acids; FAE, Fatty acid esterification; FAO, Fatty acid oxidation; FAU, Fatty acid uptake; FATP, fatty acid transport proteins; MET, metformin; MTP, mitochondrial transfer protein; OCT1, organic cationic transporter 1; PET, positron-emission tomography; PLA, placebo; SA, specific activity; TG, triglycerides; T2D, type 2 diabetes

determine [^{14}C]CO2. Indirect calorimetry was performed at t = 150 to 180 minutes.

2.4 | Blood samples

Plasma glucose concentrations were measured immediately using a YSI 2300 STAT Plus glucose analyser (YSI, Yellow Springs, Ohio). Blood samples were placed on ice and separated as quickly as possible by centrifugation (2753 × g at 4°C for 10 minutes). Aliquots of plasma were stored at 4°C for isolation of VLDL after completion of the study day. Remaining samples were stored at −20°C for later analysis. FFAs were quantified using the in vitro enzymatic colorimetric method assay NEFA-HR(2) (Wako Chemicals GmbH, Neuss, Germany) and insulin was measured by ELISA.

2.5 | PET acquisition and image reconstruction

The PET/CT scans were conducted using a Siemens Biograph 64 PET/CT (Siemens, Erlangen, Germany). List-mode-acquired PET data were divided into 20 frames with the following frame structure: 8 × 15 seconds, 2 × 30 seconds, 2 × 120 seconds, 1 × 180 seconds, 6 × 300 seconds, 1 × 600 seconds. PET images were reconstructed using a 3-dimensional iterative algorithm (3 iterations, 21 subsets, 5-mm Gaussian post-filter). List-mode PET data were decay-corrected to scan start.

2.6 | [^{14}C]VLDL-TG procedures

In brief, VLDL-particles were isolated by ultracentrifugation 7 days prior to study days and ex vivo-labelled with [1-^{14}C]trioleine. On study days, plasma samples were drawn and the VLDL fraction was isolated by ultracentrifugation for 18 hours. VLDL-TG specific activity was subsequently calculated based on VLDL-TG concentrations and ^{14}C–activity measured in a scintillation counter (Beckman Coulter, Brea, California). Breath samples were collected in IRIS breath bags (Wagner Analysen Technik, Bremen, Germany) and ^{14}CO2 activity was determined as previously described.[18] Measurements of breath 14C activity were later used for calculations of VLDL-TG oxidation.

2.7 | Indirect calorimetry

Resting energy expenditure and respiratory exchange ratio were measured by indirect calorimetry (Deltatrac monitor; Datex Instruments,

Baldwin Park, California) during 30-minute periods, starting at 150 minutes. The protein oxidation rate was estimated from urinary urea excretion, and net lipid and glucose oxidation rates were calculated from indirect calorimetry measurements with correction for protein oxidation.[19]

2.8 | Calculations

2.8.1 | Hepatic [^{11}C]palmitate metabolism

The input function was image-derived and calculated based on the dual input from the hepatic artery (aorta volume of interest) and portal vein. Hepatic arterial supply was assumed to constitute 20% of total liver blood supply. Plasma input was individually corrected for haematocrit and radioactive metabolites, as described in detail in File S1 and elsewhere.[20]

Hepatic palmitate kinetics were analysed using two different approaches: a linear approach (modified Patlak allowing for loss of radiotracer back into the circulation) yielding hepatic uptake rate (Ki) and a non-linear approach: a reversible 3-tissue-compartment model described and validated by Iozzo et al.[16] All procedures and calculations are described in detail in File S1.

2.8.2 | Fatty acid rate of appearance

Plasma concentrations of [^{11}C]palmitate over time were used to calculate the whole-body substrate clearance, as ratio of the injected dose to the area under the tracer concentration curve. The clearance of [^{11}C]palmitate was multiplied by serum FFA levels to obtain the rate of fatty acid appearance (μmol/min). The assumption was made that the clearance of palmitate was representative of the overall FFA clearance.[21]

2.8.3 | VLDL-TG metabolism

The VLDL-TG rate of appearance and fractional oxidation were calculated based on the decay curves of [^{14}C]VLDL-TG in plasma and the appearance of ^{14}CO2 in exhaled air. In brief, VLDL-TG Ra can be calculated as the area under the plasma VLDL-TG SA vs time curve divided by the injected dose, and fractional oxidation as the area under the exhaled ^{14}CO2 (in dpm/min) vs time curve divided by the dose.[18] All calculations are provided in File S1.

2.8.4 | HOMA-IR

Homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated according to the formula: HOMA-IR = fasting glucose in mmol/L × fasting insulin in μU/mL, divided by 22.5.

2.8.5 | Body composition

Total body fat, leg fat, fat percentage and fat-free mass were assessed using a Hologic QDR-2000 dual-energy X-ray (DXA) absorptiometry densitometer (Hologic, Bedford, Massachusetts). Upper body fat and visceral fat mass were calculated from CT measurements of intra-abdominal and subcutaneous adipose tissue combined with abdominal fat mass measured by DXA scan, as previously described.[22] Abdominal subcutaneous fat was taken as upper body fat (DXA) minus visceral fat. Leg fat was measured by use of the region of interest program in the DXA instrument.

2.9 | Statistics

Sample size was calculated based on VLDL-TG Ra, as we have performed an extensive range of studies using the [1-^{14}C]VLDL tracer and consequently have access to exhaustive data on population means and variability. Sample size was based on the assumption that average VLDL-TG Ra in patients with T2D was 87 ± 31 μmol/min[23] and that metformin treatment (based on meta-analysis data) could result in a total decrease in TG levels by ~10%.[24] Total TG reflects TGs in all lipoprotein fractions, with VLDL-TG accounting for 60% to 75%,[25] also in line with our own observations. It is reasonable to assume that any decrease in total TGs will primarily reflect a decrease in the VLDL fraction because the putative target for metformin is the liver and the mean decrease in VLDL-TG Ra during metformin treatment was therefore estimated to be ~20% or 17 μmol/min. No data exist detailing the range of a metformin-induced decrease in VLDL-TG Ra, but it is reasonable to assume that it could be between 0 and 50 μmol/min. The SD of the change in VLDL-TG Ra in the metformin-treated group was therefore estimated to be 50/4 = 12.5. If we set $\alpha = 0.05$ and $\beta = 0.8$, sample size can be calculated to be 8. Accounting for missing values and unknown average data for the PET parameters, we chose to include 12 in each group.

Statistical analyses were performed using a hierarchical mixed linear model with a compound symmetry covariance structure, including one within-factor (time; indicating overall mean change between baseline and measurement after intervention), one between-factor (group; placebo, metformin and control), and interaction term (group*time; indicating whether mean change during the study was different between the groups). Missing data points were accounted for by restricted maximum likelihood estimation within the linear mixed models.

Unless otherwise stated, values are given as mean ± SD. A *P* value <.05 was taken to indicate statistical significance. All analyses were performed in SPSS version 21.

3 | RESULTS

3.1 | Anthropometry

By design, patients with T2D and healthy controls did not differ in weight (Table 1), and as measured by DXA, no difference in lean body mass, fat mass and fat percent was observed on study day 1, although healthy controls tended to have lower lean body mass. After completion of the 3-month trial, no significant changes (time vs group interaction) in fat mass (visceral and subcutaneous), weight or lean body mass were noted.

3.2 | Glucose and insulin concentrations

As expected, fasting plasma glucose (FPG) levels were elevated in patients with T2D compared with controls (Table 2, upper bracket). Patients with T2D allocated to the metformin group had slightly but non-significantly greater FPG levels and lower insulin levels compared with those allocated to the placebo group. Metformin treatment for 3 months produced the anticipated decrease in FPG in the metformin

TABLE 1 Patient baseline characteristics and body composition at inclusion

	T2D: placebo group		T2D: metformin group		Control group		P		
	Pre	Post	Pre	Post	Pre	Post	Group	Time	Group × time
Baseline characteristics									
Sex: M/F	9/3	9/3	6/6	6/6	6/6	6/5	.38		
Age, years	60 (5)		64 (5)		62 (6)		.22		
BMI, kg/m^2	31.2 (4.3)		30.3 (5.7)		27.3 (4.1)		.12		
Waist/hip	1.01 (0.11)		0.97 (0.10)		0.90 (0.13)		.10		
Diabetes duration, months	58 (54)		43 (43)				.46		
Concomitant statin treatment	8/12		7/12		2/12*		.03		
HbA1c, mmol/mmol	45 (6)		51 (6) **		37 (3)*		<.001		
HbA1c, %	6.2 (0.5)		6.8 (0.5) **		5.6 (0.3)*		<.001		
Total cholesterol, mmol/L	4.2 (0.9)		4.5 (1.4)		5.7 (1.1)*		.01		
HDL, mmol/L	1.2 (0.4)		1.4 (0.5)		1.8 (0.5)*		.01		
LDL, mmol/L	2.1 (0.9)		2.4 (1.2)		3.4 (0.9)*		.01		
Body composition (DXA)									
Weight, kg	93.8 (17.4)	93.3 (16.9)	88.6 (16.4)	88.2 (16.3)	79.3 (14.9)	79.6 (15.3)	.10	.30	.97
LBM, kg	60.6 (12.2)	60.3 (12.1)	53.9 (8.0)	53.7 (8.2)	51.0 (9.9)	51.0 (9.7)	.07	.18	.73
Fat mass, kg	28.4 (7.5)	28.1 (7.7)	29.2 (12.3)	28.7 (11.8)	23.9 (7.9)	23.5 (8.7)	.37	.26	.80
UB sc fat, kg	14.1 (3.9)	13.6 (4.3)	14.7 (6.2)	14.4 (6.1)	11.1 (4.1)	10.7 (4.0)	.19	.14	.58
Visceral fat, kg	5.9 (2.6)	6.1 (2.5)	5.4 (1.8)	5.5 (1.6)	4.4 (3.1)	4.6 (3.1)	.30	.55	.57
LB sc fat, kg	8.5 (2.1)	8.4 (2.1)	9.7 (6.0)	9.5 (6.3)	8.8 (4.1)	8.7 (4.2)	.82	.66	.68
Fat, %	32.0 (6.3)	32.0 (6.1)	34.1 (8.7)	34.0 (8.8)	31.7 (6.9)	31.5 (7.5)	.73	.74	.82

BMI, body mass index; DXA, dual-energy X-ray; HbA1c, glycated haemoglobin; LBM, lean body mass; LB sc, lower body subcutaneous; T2D, type 2 diabetes; UB sc, upper body subcutaneous.

*P < 0.05 vs both groups of patients with T2D. **P < .05 metformin vs placebo. Body composition at inclusion and during the study is shown in the lower bracket. As seen, no effect of metformin on fat and lean body mass was observed (mixed model group vs time interaction P=NS in all comparisons). All values are mean (SD).

group (FPG 7.9 ± 1.8 mmol/L, study day 1 vs 6.4 ± 1.1 mmol/L, study day 2), whereas patients with T2D in the placebo group and healthy controls had similar FPG levels before and after the trial (mixed model group vs time interaction, P = .003). Basal insulin levels were unaffected in all groups.

3.3 | FFA, TG and VLDL-TG concentrations and VLDL particle size

Basal FFA levels did not differ among groups and were unaffected by treatment, whereas basal TG and apolipoprotein B (ApoB) levels as expected were lower in the healthy controls on study day 1 (Table 2, middle bracket). VLDL particle size as measured by the TG/ApoB ratio was similar in all groups. Neither VLDL-TG, TG or ApoB concentrations nor particle size changed after treatment.

3.4 | Whole-body glucose and lipid oxidation

Whole-body energy expenditure and respiratory quotient were similar in all groups during basal conditions and were unaffected by treatment. Basal lipid and glucose oxidation rates were similar in all groups before initiation of treatment and were unaltered on study day 2 (Table 2).

3.5 | Hepatic fatty acid metabolism measured by [11C]palmitate PET

The intrinsic capacity of the liver to take up fatty acids (Ki) was inversely correlated with insulin resistance, as measured by HOMA-IR (Figure 2A) and hepatic fatty acid oxidation was positively correlated with whole-body lipid oxidation (Figure 2B). Hepatic fatty acid fluxes (μmol/mL tissue/min) are shown in Figure 2C-E, with exact numbers given in File S1. There was no difference in basal hepatic fatty acid uptake, re-esterification or oxidation on study day 1 and no changes in any of these variables were observed on study day 2. Whole-body fatty acid turnover was also unaffected by metformin (FFA Ra: placebo 489 ± 139 μmol/min, day 1 vs 477 ± 150 μmol/min, day 2; metformin 497 ± 205 μmol/min, day 1 vs 559 ± 295 μmol/min, day 2; control 419 ± 202 μmol/min, day 1 vs 465 ± 127 μmol/min, day 2; group vs time interaction P = .39).

3.6 | VLDL-TG Ra and fractional oxidation

Combined VLDL-TG SA vs time bi-exponential curves normalized to peak activity are shown in Figure 3A. Basal VLDL-TG Ra on study day 1 was significantly lower in controls but treatment did not affect VLDL-TG Ra in either group (VLDL-TG Ra: placebo

TABLE 2 Metabolism

	T2D: placebo group		T2D: metformin group		Control group		P		
	Study day 1	Study day 2	Study day 1	Study day 2	Study day 1	Study day 2	Group	Time	Group × time
Glucose and insulin									
FPG, mmol/L	6.9 (1.0)	7.0 (2.0)	7.9 (1.8)	6.4 (1.1)*	5.4 (0.3)**	5.2 (0.4)	**.001**	**.01**	**.003**
Insulin basal (pM)	83 (32)	81 (46)	68 (49)	60 (38)	44 (23)	44 (33)	.06	0.30	.55
HOMA-IR	3.6 (1.4)	3.6 (2.1)	3.2 (1.7)	2.5 (1.4)	1.5 (0.9) **	1.5 (1.3)	**.006**	0.13	.08
FFAs and TGs									
FFA basal, mmol/L	0.57 (0.18)	0.52 (0.18)	0.63 (0.16)	0.64 (0.14)	0.57 (0.15)	0.63 (0.13)	.33	.84	.05
TGs, mmol/L	1.67 (0.53)	1.54 (0.63)	1.59 (0.95)	1.47 (1.07)	0.89 (0.54)**	0.89 (0.53)	**.03**	.40	.84
VLDL-TG, mmol/L	1.07 (0.46)	1.03 (0.55)	1.08 (0.79)	0.99 (0.92)	0.52 (0.49)	0.52 (0.46)	.06	.53	.92
ApoB basal, μg/L	62 (30)	76 (35)	66 (46)	61 (46)	33 (27)**	31 (24)	**.02**	.72	.31
TG/ApoB ratio	10.6 (1.7)	10.2 (2.8)	11.7 (2.9)	10.8 (2.3)	11.0 (3.7)	11.0 (3.1)	.72	.32	.72
Calorimetry									
EE basal, kcal/24 h	1755 (354)	1747 (352)	1707 (277)	1764 (352)	1588 (368)	1584 (330)	0.36	0.78	0.25
RQ basal	0.81 (0.05)	0.81 (0.03)	0.81 (0.06)	0.81 (0.06)	0.82 (0.05)	0.80 (0.06)	0.96	0.23	0.67
Basal lipidox, mg/kg/min	0.80 (0.36)	0.73 (0.44)	0.74 (0.44)	0.87 (0.30)	0.75 (0.33)	0.84 (0.28)	0.96	0.29	0.21
Basal glucoseox, mg/kg/min	0.91 (0.29)	1.09 (0.68)	1.04 (0.79)	0.82 (0.51)	1.09 (0.62)	0.85 (0.72)	0.95	0.38	0.22

ApoB, apolipoprotein B; B, EE, energy expenditure; FFA, free fatty acid; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; RQ, respiratory quotient; TG, triglycerides.

*P < .05 vs placebo and control group. **= P < .05 control vs metformin and placebo group. Lipid metabolism is presented in the middle bracket. Total TG and ApoB concentration was lower in the CONT group vs both T2D groups at inclusion, but was unaltered after treatment in all groups. All values are mean (SD).

86.2 ± 48.6 μmol/min, day 1 vs 83.6 ± 49.3 μmol/min, day 2; metformin 77.5 ± 48.8 μmol/min, day 1 vs 71.1 ± 31.7 μmol/min, day 2; control 48.6 ± 41.3 μmol/min, day 1 vs 48.3 ± 30.5 μmol/min, day 2; group vs time interaction P = .98) Figure 3B.

Combined VLDL-TG oxidation (dpm/min) vs time curves normalized to peak activity are shown in Figure 3C. Fractional VLDL-TG oxidation during the 3.5-hour observation period was remarkably similar among groups and did not change after treatment (fractional VLDL-TG oxidation: placebo 30.9 ± 6.4%, day 1 vs 29.8 ± 7.2%, day 2; metformin 31.3 ± 13.4%, day 1 vs 26.7 ± 8.8%, day 2; control 29.5 ± 5.9%, day 1 vs 29.4 ± 5.3%, day 2; group vs time interaction P = .53) (Figure 3D).

4 | DISCUSSION

As expected, 3 months of metformin treatment significantly lowered FPG concentration in patients with T2D but not in healthy controls. Contrary to our hypothesis, we did not observe any measurable effects of metformin treatment on hepatic or whole-body lipid metabolism, regardless of whether we measured concentrations, substrate fluxes or VLDL particle size.

Our primary aim was to assess the impact of metformin treatment on hepatic fatty acid metabolism, for which we used [^{11}C]palmitate PET. To our knowledge, [^{11}C]palmitate has only rarely[16,21,26] been used for this purpose because of the dual blood supply of the organ, and consequently, we analysed data using both a linear and a non-linear (multi-compartmental modelling) approach. Using the linear approach, we obtained robust estimates of hepatic palmitate uptake rate, Ki, which can be viewed as a surrogate measure of intrinsic hepatic fatty acid transport capacity, independently of portal FFA availability. We observed similar Ki values among patients with T2D and healthy controls before initiation of treatment, which were also roughly comparable (~0.2) to Ki values measured by Hames et al.[26] and by others using the fatty acid analogue 18F–FTHA.[27,28] Interestingly, the baseline Ki values were significantly and inversely correlated with insulin sensitivity (HOMA-IR). This indicates that hepatic FFA uptake capacity may be reduced in insulin-resistant states, a finding that is supported by animal studies[29–31] showing a relationship between impaired hepatic fatty acid transporters (CD36 and FATPs) and insulin resistance. By contrast, Hames et al. did not observe impaired hepatic [^{11}C]palmitate uptake in humans with CD36 deficiency, which underlines the notion that other hepatic fatty acid transporters (FATPs) act as the rate-limiting step in hepatic FFA uptake.[31,32]

We did not observe an effect of metformin treatment on Ki in either healthy controls or patients with T2D. That metformin did not affect the intrinsic capacity of the liver to take up fatty acids was to be expected because it was our initial hypothesis that metformin would affect only the *intrahepatic* partitioning of fatty acids towards oxidation. Hepatic fatty acid oxidation and esterification rates calculated by the multicompartmental approach proposed by Iozzo et al.,[16] however, resulted in similar non-significant changes after metformin treatment. Data from the indirect calorimetry measurements appear to support these observations because no effect on whole-body oxidation was observed either. It should be borne in mind, however, that whole-body lipid oxidation is in the range of ~400 μmol/min.[33] If we assume that a normal liver weighs ~1400 mL, we can estimate that

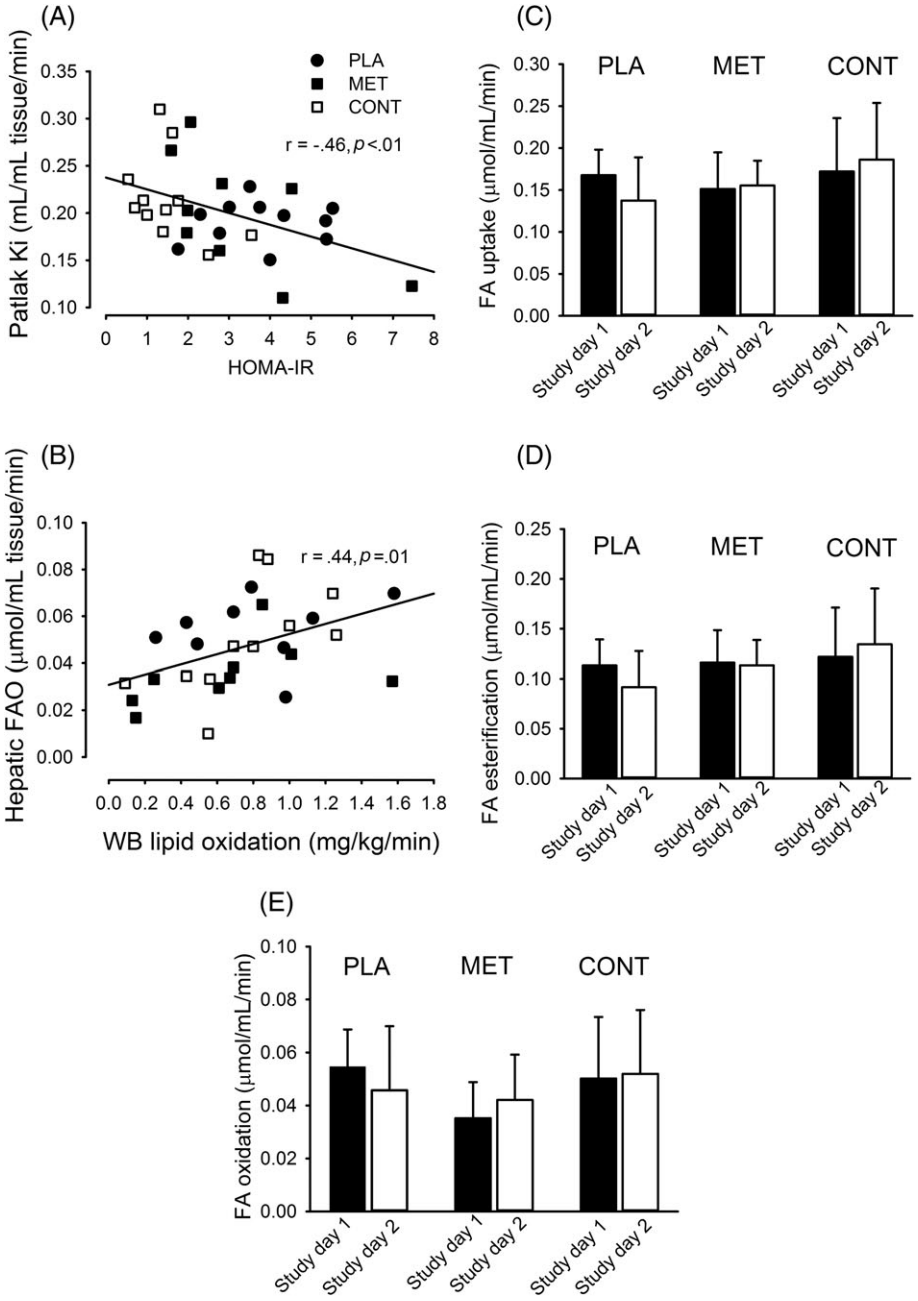

FIGURE 2 A, Significant inverse relationship between homeostatic model assessment of insulin resistance (HOMA-IR) and the intrinsic capacity of the liver (Ki) to take up fatty acids on study day 1 assessed by [^{11}C]palmitate positron-emission tomography (PET). Closed circles = placebo (PLA), closed boxes = metformin (MET), open boxes = control group (CONT). B, Significant positive association between whole-body lipid oxidation (by indirect calorimetry) and hepatic fatty acid oxidation (by [^{11}C]palmitate PET) was noted. **C, D,** and **E**, Hepatic fatty acid uptake C, reesterification D, and oxidation rates E, were similar in all groups on study day 1 and were unaltered by treatment (P = NS in all comparisons). Error bars are mean ± SD. n = 9 (PLA), n = 9 (MET) and n = 11 (CONT) on study day 1 and n = 11 (PLA), n = 10 (MET) and n = 10 (CONT) on study day 2. FA, fatty acids; WB, whole body

hepatic lipid oxidation in our group of patients with T2D treated with metformin was 0.04 µmol/mL tissue/min × 1400 mL = 56 µmol/min. A non-trivial decrease in hepatic lipid oxidation of 25% would then only translate into a decrease in whole-body oxidation of (0.25 × 56/400) × 100 (%) = 3.5%. It is highly likely that such a small decrease would not be detected by indirect calorimetry.

As in most,[23,34–36] but not all,[37] previous studies of VLDL-TG secretion, patients with T2D had greater VLDL-TG secretion and consequently elevated levels of TGs when compared with healthy controls. Our control subjects did not significantly differ from patients with T2D in terms of body composition (visceral fat, total fat mass or BMI) at inclusion and it is therefore likely that the increased VLDL-TG secretion and VLDL-TG levels were a consequence of decreased insulin sensitivity. Reflecting the lower VLDL-TG levels in healthy controls, ApoB concentrations were also significantly lower in control subjects and, consequently, TG/ApoB ratios were similar in all groups, demonstrating that VLDL particle size was unaffected by diabetes status.[23]

Metformin treatment did not affect VLDL-TG secretion or particle size in either group, which was in line with the present findings of similar VLDL-TG re-esterification rates measured by [^{11}C]palmitate PET. To our knowledge, the effect of metformin treatment on VLDL-TG secretion rates has previously only been studied in mice, where VLDL-TG production rates and particle size, as in the present study, were found to be unaltered by treatment;[38] however, contrary to our observations in humans, metformin treatment resulted in lower VLDL-TG concentrations in mice, which was caused by a 58% increased uptake in brown adipose tissue (BAT). This was associated with a striking decrease in BAT lipid droplet size, AMPK activity and hormone-sensitive lipase activity. This could indicate that metformin

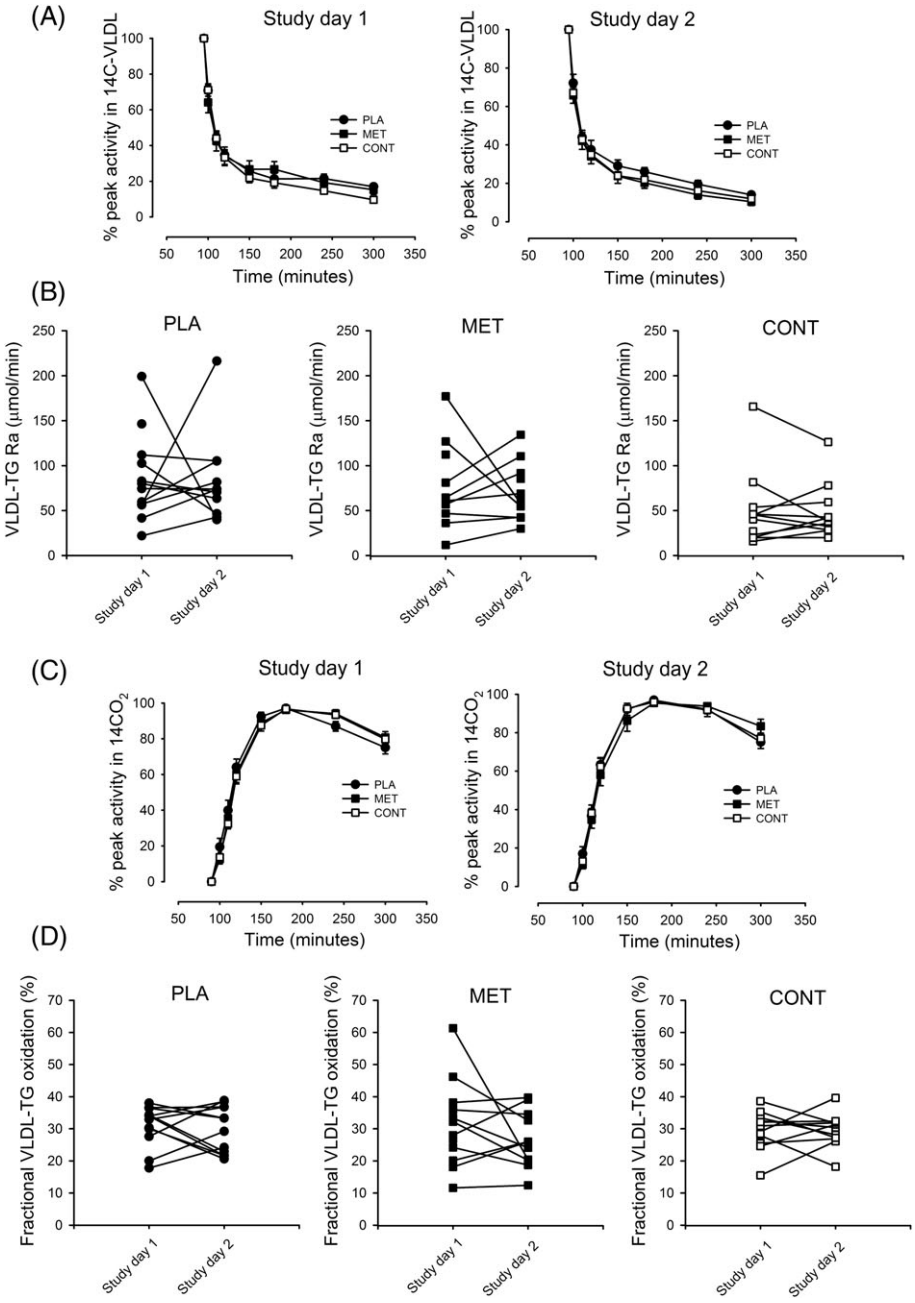

FIGURE 3 A, [^{14}C]VLDL-triglycerides (TG) specific activity normalized to peak activity on study days. As seen, specific activity decreased in a biexponential manner allowing us to fit the data to a bi-exponential decay function. B, VLDL-TG Ra was significantly lower in the CONT group (mixed model group $P = .03$) whereas metformin treatment had no effect (mixed model group vs time interaction $P = .98$). C, [^{14}C]VLDL activity in breath samples (dpm/min) normalized to peak activity. On both study days, breath ^{14}C specific activity was nearly similar in all groups and peaked at $t = 180$ minutes, after which a gradual decline was observed. D, Fractional VLDL-TG oxidation was calculated based on the area under the activity curve divided by the total injected dose. As evident from the SA curves, no difference in fractional oxidation was observed between groups and no effect of metformin treatment was found (mixed model group vs time interaction $P = .53$). Closed circles = placebo (PLA), closed boxes = metformin (MET) and open boxes = control group (CONT). Error bars are mean ± SEM. $n = 12$ (PLA), $n = 10$ (MET) and $n = 12$ (CONT) on study day 1 and $n = 11$ (PLA), $n = 11$ (MET) and $n = 11$ (CONT) on study day 2

induces a rapid increase in BAT lipid turnover, presumably as a result of increased lipid oxidation, which was corroborated by increased mitochondrial content as assessed by Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) and endothelial NOS. Although the presence of BAT in adult humans has been confirmed in numerous studies, it is at a much lower level than in rodents and with considerable variation. Our study design did not include estimates of BAT activity or BAT biopsies and we were therefore unable to investigate whether these intriguing results from animal studies translate into a human setting; however, we did measure fractional VLDL-TG oxidation, which could be a surrogate marker of the putative enhancing effects of metformin on BAT VLDL-TG oxidation. Again, our results were strikingly negative because no effect of fractional VLDL-TG oxidation was observed in either group, but this observation is prone to type 2 error because the study was not powered for this endpoint.

Some limitations to the study should be noted. First, there is evidence to suggest that even short-term metformin treatment may reduce circulating fatty acid clearance in men, resulting in increased levels of fatty acids and increased oxidation by the myocardium.[39] Although the sex distribution was not statistically different between groups in this study, it is conceivable that subtle differences in hepatic lipid oxidation may have been obscured by the slightly skewed sex ratio in the placebo group. Second, the metformin group had slightly poorer glycaemic control than the placebo group as assessed by glycated haemoglobin concentration at inclusion (51 vs 45 mmol/mol). Although both groups can be considered to be well controlled with regard to glucose homeostasis,[40] it is conceivable that the difference may have resulted in discretely different lipid metabolism before treatment initiation; however, this did not translate into different effects of metformin treatment on lipid metabolism at the end of the study. Third, it is possible that metformin may affect

postprandial and not postabsorptive lipid metabolism as suggested by other groups.[41,42] Exploring this possibility will require a completely different experimental setup.

In summary, 3 months of metformin treatment in patients with T2D and healthy controls does not affect hepatic FFA channelling, VLDL-TG secretion, particle size or peripheral VLDL-TG oxidation. It is therefore likely that the beneficial effects of metformin treatment on circulating TGs in patients with T2D are a result of subtle changes that may entail changes in body composition or more indirect effects caused by altered levels of hormones involved in glucose homeostasis.

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Conflict of interest

L.C.G., E.S., N.L.C., S.J., E.H.T.N., O.L.M., L.P.T., N.J and S.N. have nothing to disclose.

Author contributions

L.C.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. L.C.G. designed the experiments, carried out the studies, generated and analysed the data and wrote the manuscript. E.S. and S.N. designed the experiments, generated data, contributed to the discussion and reviewed/edited the manuscript. N.L.C., S.J., O.L.M., L.P.T. and E.H.T. generated and analysed the data and reviewed/edited the manuscript. N.J. contributed to designing the trial, contributed to the discussion and reviewed/edited the manuscript.

ORCID

Lars C. Gormsen http://orcid.org/0000-0002-0144-1305

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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