Impaired Glucagon-Mediated Suppression of VLDL-Triglyceride Secretion in Individuals With Metabolic Dysfunction–Associated Fatty Liver Disease (MAFLD)

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PATHOPHYSIOLOGY

Individuals with metabolic dysfunction–associated fatty liver disease (MAFLD) have elevated plasma lipids as well as glucagon, although glucagon suppresses hepatic VLDL-triglyceride (TG) secretion. We hypothesize that the sensitivity to glucagon in hepatic lipid metabolism is impaired in MAFLD. We recruited 11 subjects with severe MAFLD (MAFLD+), 10 with mild MAFLD (MAFLD−), and 7 overweight control (CON) subjects. We performed a pancreatic clamp with a somatostatin analog (octreotide) to suppress endogenous hormone production, combined with infusion of low-dose glucagon (0.65 ng/kg/min, *t* = 0–270 min, LowGlucagon), followed by high-dose glucagon (1.5 ng/kg/min, *t* = 270–450 min, HighGlucagon). VLDL-TG and glucose tracers were used to evaluate VLDL-TG kinetics and endogenous glucose production (EGP). HighGlucagon suppressed VLDL-TG secretion compared with LowGlucagon. This suppression was markedly attenuated in MAFLD subjects compared with CON subjects (MAFLD+: 13% ± [SEM] 5%; MAFLD−: 10% ± 3%; CON: 36% ± 7%, *P* < 0.01), with no difference between MAFLD groups. VLDL-TG concentration and VLDL-TG oxidation rate increased between LowGlucagon and HighGlucagon in MAFLD+ subjects compared with CON subjects. EGP transiently increased during HighGlucagon without any difference between the three groups. Individuals with MAFLD have a reduced sensitivity to glucagon in the hepatic TG metabolism, which could contribute to the dyslipidemia seen in MAFLD patients. ClinicalTrials.gov: NCT04042142.

Metabolic dysfunction–associated fatty liver disease (MAFLD) is a leading cause of liver disease (1). MAFLD is observed in individuals with dysmetabolic conditions in close association with insulin resistance (IR), abnormal glucose and fatty acid (FA) metabolism, and dyslipidemia (2). In addition, MAFLD is an independent risk factor for cardiovascular disease (CVD) (3,4). It has been widely documented that subjects with MAFLD have higher plasma concentrations of VLDL, triglyceride (TG), and higher hepatic VLDL-TG secretion (5,6), promoted by an increased de novo lipogenesis and FA supply from adipose tissue lipolysis. This may contribute to the increased risk of CVD associated with MAFLD (7–9). It is established that individuals with MAFLD with inflammatory and ballooning activity (MAFLD+, formerly denoted as nonalcoholic steatohepatitis) have a higher risk for type 2 diabetes, liver-related disease progression, and morbidity than individuals with simple steatosis (MAFLD−) (7,8) and that MAFLD with severe fibrosis confers a higher risk of CVD. Yet, there is no clarity about what factors drive MAFLD pathogenesis and atherogenesis or whether individuals with MAFLD+ and MAFLD− per se have similar cardiovascular risk (8).

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Fasting and postprandial hyperglucagonemia are recognized as important components of diabetes pathophysiology (10). Recent studies reported that subjects with MAFLD have elevated fasting and postprandial levels of plasma glucagon (11,12), even to a higher extent than patients with diabetes but without MAFLD. Glucagon and glucagon agonists are known to increase hepatic FA oxidation and reduce the secretion of VLDL-TG (13–16), whereas disruption of glucagon signaling with receptor antagonists may lead to disturbances in hepatic lipid metabolism (17). However, detailed in vivo human studies of the effect of glucagon on lipid metabolism are scarce (reviewed in Galsgaard et al. [18]) and most often restricted to healthy lean men (19). The question therefore remains whether glucagon is able to reduce VLDL-TG secretion effectively in MAFLD and especially in MAFLD with inflammation and ballooning.

Therefore, we aimed to examine the effects of glucagon on hepatic TG and glucose metabolism in subjects with MAFLD. We hypothesize that the glucagon-mediated suppression of VLDL-TG secretion is impaired in subjects with MAFLD, with greater impairment in subjects with MAFLD+ compared with MAFLD−. If this is the case, this could help to explain the dyslipidemia observed in MAFLD and the increased risk of CVD.

RESEARCH DESIGN AND METHODS

Study Approval

The regional Ethics Committee (Central Denmark Region) approved the study protocol (no. 1-10-72-110-19), and written informed consent was obtained from all subjects prior to participation. We conducted investigations according to The Declaration of Helsinki. The study is registered at www.clinicaltrials.gov (NCT04042142).

Study Subjects

We recruited 24 overweight/obese (BMI >28 kg/m^2) subjects with MAFLD and 8 overweight/obese control (CON) subjects, matched for age, sex, and BMI through advertisements. Additional inclusion criteria were age 38–72 years, nonsmokers, with no alcohol abuse and no history of diabetes, liver disease, or other chronic systemic disease, except hypertension and hypercholesterolemia.

Three subjects in the MAFLD groups and one subject in CON group were excluded due to the coronavirus disease 2019 pandemic or withdrew consent due to personal reasons; thus, 21 MAFLD subjects and 7 CON subjects were included.

Study Design

Potentially eligible subjects were invited for a screening visit after an overnight fast (study design in Supplementary Fig. 1). We obtained a medical history, clinical examination, FibroScan, MRI, and MRS. All subjects had a normal blood count, kidney function, thyroid function, fasting plasma glucose (PG), HbA_{1c} and electrocardiogram. Subjects on statins (MAFLD− = 2, MAFLD+ = 1) paused treatment 2 weeks prior to the study day.

One week before the study day, we obtained a blood sample under fasting and sterile conditions from the study subjects for VLDL-TG tracer preparation and performed a DEXA scan to evaluate the body composition. A dietitian designed an individualized weight-maintaining diet, containing 55% carbohydrate, 15% protein, and 30% fat, for each subject. The diet was provided by the hospital kitchen and consumed during the 3 days preceding the metabolic study day.

Intrahepatic Fat Content and Elastography

We used MRS (Siemens Skyra, 3T MR scanner) to determine the hepatic fat fraction percentage (FF%) in a single 3- × 2- × 2-cm^3 voxel of liver tissue. LCModel software package version 6.3-1 L (Stephen Provencher, 2016) was used to analyze data. The cutoff between normal and abnormal intrahepatic TG content was set at FF% >5.6% (20). FibroScan was used to measure liver elasticity (LE, kPa) as a proxy measure of hepatic fibrosis.

Liver Biopsy

All subjects with an elevated FF% were invited to and accepted a liver biopsy, planned within weeks of the study day and performed by an experienced hepatologist percutaneously. However, due to logistic challenges during the coronavirus disease 2019 pandemic, we accepted specimens from biopsies performed within 6 months of the study day, and in one subject, the liver biopsy was cancelled entirely. Based on MRS and LE results, this subject was classified as a MAFLD− patient. Liver sections were evaluated by an experienced pathologist and scored according to the generally accepted histological criteria (21). Presence of hepatocyte ballooning along with inflammation defined MAFLD+ and no ballooning defined MAFLD−.

Body Composition

Total body weight, body fat percentage, and fat-free mass were determined by a DEXA scan QDR-2000 (Hologic, Marlborough, MA). Visceral and subcutaneous fat depots from caput femoris to the upper rim of the left kidney were quantified using MRI and software developed in-house (Siswin).

VLDL-TG Tracer Preparation

One week before the study day, an 80-mL blood sample was obtained under sterile conditions and labeled as described elsewhere, with minor modifications (22). Plasma was immediately separated and sonication with 20 μCi of [1-^{14}C]triolein (PerkinElmer, Waltham, MA) at 5°C for 2 h in a sterile tube and then transferred to sterile tubes, covered with sterile saline (d = 1.006 g/cm^3), and ultracentrifuged (50.3 Ti rotor [40,000 rpm] or 50.4 Ti rotor [37,000 rpm], Beckman Instruments, Palo Alto, CA)

for 18 h at 4°C. The supernatant was collected with a sterile Pasteur pipette, passed through a Millipore filter, and stored at 5°C.

Protocol

Subjects arrived at the research unit at 8 P.M. on the evening before the metabolic study day for an overnight stay. Glucose (1.4 g/kg body wt) was administered and finished 8 h before study start to load hepatic glycogen stores and minimize confounding from different glycogen stores between subjects.

The next morning, catheters were placed in an antecubital vein for infusions and in a vein of the contralateral heated hand to obtain arterialized blood. To suppress endogenous production of glucagon and insulin and thereby inhibit counterregulatory mechanisms, we performed a pancreatic clamp. After collection of baseline samples at 7 A.M. (t = 0 min) (Fig. 1), we initiated an infusion of a somatostatin analog (octreotide, 60 ng/kg/min), and a replacement dose of growth hormone (Genotropin, 3 ng/kg/min) and insulin (Humulin, 0.2 mU/kg/min) (23). Replacing glucagon, a 4.5-h low-dose glucagon period was started (GlucaGen, 0.65 ng/kg/min, t = 0–270 min) (LowGlucagon), followed by a 3-h high-dose glucagon period (1.5 ng/kg/min, t = 270–450 min) (HighGlucagon). We collected blood samples for plasma VLDL-TG concentration and ^{14}C specific activity (SA) concurrently with $^{14}CO_2$ in breath samples at t = 0 and at 10-min intervals during LowGlucagon (t = 240–270 min) and every 20 min during HighGlucagon (t = 410–450 min), where a steady-state in SA was observed. After reviewing results from the first nine participants, we did not detect an elevation in endogenous glucose production (EGP) at the end of HighGlucagon as expected, which may be due to glucagon evanescence. Hence, we decided to collect blood samples during t = 310–390 min in the remaining 19 participants (3 CON; 8 MAFLD−, 8 MAFLD+).

Plasma VLDL-TG Concentration and SA

VLDL-TG was separated from 3 mL weighted plasma samples by ultracentrifugation as described above. VLDL was obtained by tube slicing 1 cm from the top, and the weight was recorded. TG content was analyzed in 300-µL aliquots, and VLDL-TG plasma concentration was calculated. The remaining VLDL-TG was transferred to scintillation glasses, scintillations fluid added, and [^3H] and [^{14}C] activity measured by dual-channel liquid scintillation counting to <2% counting error.

Breath $^{14}CO_2$ SA

Breath samples were collected (IRIS-breath-bags; Wagner Analysen Technik) to calculate [1-^{14}C]VLDL-TG FA oxidation from $^{14}CO_2$ activity. The air was passed through a solution containing 0.5 mL hyamine hydroxide in 1 mol/L methanol, 2 mL 96% ethanol, and two drops of phenolphthalein. A color change occurred when 0.5 mmol CO_2 was trapped in the solution, and then [^{14}C] activity was measured by liquid scintillation.

Glucose Kinetics

A [3-^3H]glucose (12 µCi bolus, 0.12 µCi/min constant infusion; Department of Nuclear Medicine and PET-Centre, Aarhus University Hospital, Aarhus, Denmark) was used to estimate EGP and R_d. We collected blood samples for plasma glucose and [3-^3H]glucose SA at baseline and at 10-min intervals during LowGlucagon and every 20 min during HighGlucagon, as described above. In case of PG <4.5 mmol/L, we initiated and titrated the glucose infusion to avoid hypoglycemia; [3-^3H]glucose was added to

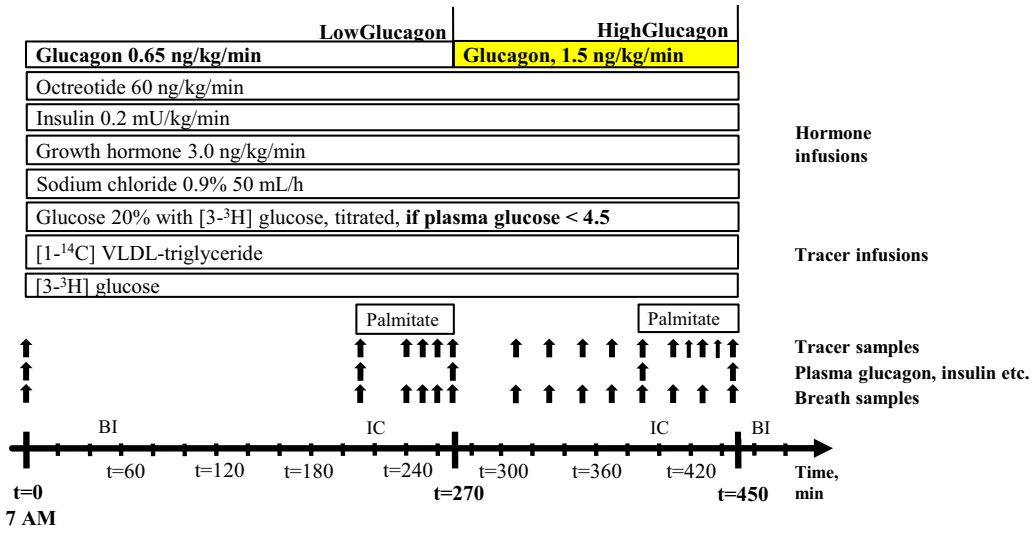

Figure 1—Study day protocol. BI, adipose tissue and muscle biopsy; IC, indirect calorimetry; palmitate, [9,10-^3H]palmitate.

the infused glucose (100 μCi/500 mL glucose 20%) to minimize rapid dilution of the labeled glucose pool.

Palmitate Flux

A 1-h constant infusion of [9,10-^{3}H]palmitate (Department of Nuclear Medicine and PET-Centre, Aarhus University Hospital) was given at t = 210–270 min and t = 390–450 min, 0.3 μCi/min. Blood samples for measurements of plasma palmitate concentration and SA were collected before the infusion and every 10 min during the last 30 min of each infusion period. Plasma palmitate concentration and SA were measured by high-performance liquid chromatography using [$^2H_{31}$]palmitate as the internal standard. Steady-state SA was verified for each individual.

Calculations

Hepatic VLDL-TG secretion rate was calculated as [1-^{14}C]VLDL-TG infusion rate divided by the average steady-state VLDL-TG SA. To calculate VLDL-TG oxidation, the VLDL-TG secretion rate was multiplied with the fractional oxidation of the infused VLDL-TG tracer as ($^{14}CO_2$SA · VCO_2)/(k · 0.56 · F), in which k is the volume of CO_2 at 20°C and 1 atm pressure (22.4 L/mol), 0.56 is the fractional acetate carbon recovery factor in breath CO_2 (24), and F is the tracer infusion rate.

EGP/R_a and R_d were determined by the Steele non-steady-state equations as modified by deBodo (25,26). If glucose 20% was infused, EGP was calculated by subtracting the mean glucose infusion rate from the isotopically determined R_a.

Palmitate flux (μmol/min) was calculated as the [9,10-^{3}H]palmitate infusion rate divided by the steady-state SA.

Indirect Calorimetry and Oxidation Rates

Indirect calorimetry (Deltatrac monitor, Datex Instrumentarium, Helsinki, Finland) was used to measure resting respiratory exchange ratio (RER) and substrate oxidation rates from t = 210–230 min, and t = 390–410 min. Collected urine was used to calculate protein oxidation from urea excretion. Lipid and glucose oxidation were calculated after correction for protein oxidation (27).

Laboratory Procedures

A YSI 2300 STAT Plus glucose analyzer was used to analyze PG.

Insulin, C-peptide, glucagon, and free FA (FFA) concentrations were measured at t = 0, 210, 270, 390, and 450 min. Insulin (no. 10-1113-01), glucagon (no. 10-1271-01) and C-peptide concentrations (no. 10-1136-01) were measured by ELISA (Mercodia, Uppsala, Sweden). Serum FFA concentrations were measured by a colorimetric method (Wako Pure Chemical Industries, Osaka, Japan). VLDL-TG and total TG concentrations were analyzed on a cobas 111 (F. Hoffmann–La Roche), using a glycerol blanked kit.

Statistical Analyses

The data were analyzed with SPSS 27 software and plotted with GraphPad Prism 9. Normal distribution of data was tested with a Shapiro-Wilk normality test, and data were log-transformed when appropriate.

When possible after due test of assumptions, we used a two-way ANOVA for repeated measurements (RM-ANOVA), with time and group as factor variables. Relevant between-group comparisons of baseline data (t = 0) and Δ values (HighGlucagon − LowGlucagon) were analyzed using parametric (one-way ANOVA) or nonparametric methods (Kruskal-Wallis). Between-time point comparisons of within-group data were analyzed using paired sample t test, one-way RM-ANOVA, or corresponding nonparametric test (Wilcoxon signed-rank test), depending on the setting. Cross-tabulations were analyzed using the Pearson χ^2 test, and correlations were performed using the Pearson r. A P value of <0.05 was considered significant.

Data and Resource Availability

The data sets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Subject Characteristics

Table 1 reports subject characteristics of the 28 well-matched study subjects at inclusion. Of note, 8 of 11 of MAFLD+ patients were men compared with 5 of 10 for MAFLD− patients and 3 of 7 for CON subjects (P = 0.390, χ^2). Per design, FF% was higher in the MAFLD groups than the CON group, with no difference between the MAFLD+ and MAFLD− groups. Visceral fat mass, LE, and HbA_{1c} were significantly greater in the MAFLD+ compared with the CON group. Details on histology can be found in Supplementary Table 1.

Hormone and Metabolite Concentrations

C-peptide was significantly higher in the MAFLD+ than in the CON group at t = 0 (P = 0.014). After initiation of the octreotide infusion, endogenous insulin secretion was similarly suppressed in all groups, illustrated by the expected C-peptide decay curve (Fig. 2*A*).

Glucagon concentrations were similar at baseline, increasing slightly at LowGlucagon and markedly at HighGlucagon, with no difference between groups (Fig. 2*B*). The glucagon concentrations during LowGlucagon and HighGlucagon were within the normal range of obese subjects after short-term and prolonged fasting, respectively (28–30).

Insulin was significantly higher in the MAFLD groups than in the CON group at baseline (P = 0.02). Levels increased in all groups after initiation of replacement insulin infusion and were higher in the MAFLD groups ($P < 0.01$), with no difference between MAFLD+ and MAFLD− (Fig. 2*C*).

Table 1—Baseline subject characteristics

	MAFLD+ subjects $n = 11$	MAFLD− subjects $n = 10$	CON subjects $n = 7$
Age, years	61 ± 2	61 ± 2	58 ± 5
Sex			
Female	3	5	4
Male	8	5	3
Body composition and plasma lipoproteins			
BMI, kg/m^2	35 ± 1	34 ± 1	33 ± 1
Total body mass, kg	105.56 ± 3.9	101.5 ± 4.1	103.5 ± 6.1
Body fat, %	40 ± 2	44 ± 3	44 ± 3
Fat-free mass, kg	61 ± 3	55 ± 3	55 ± 4
Subcutaneous fat, L	12.0 ± 1.3	14.4 ± 1.7	14.2 ± 1.5
Visceral fat, L	8.3 (4.8–17.0)*	7.1 (4.8–14.6)	5.5 (3.2–6.5)
Waist-to-hip ratio	1.03 (0.96–1.31)*	1.01 (0.91–1.08)	0.93 (0.87–1.04)
HDL, mmol/L	1.20 (0.97–1.9)	1.35 (0.93–2.1)	1.30 (0.9–2.2)
LDL, mmol/L	3.3 ± 0.3	3.5 ± 0.2	3.2 ± 0.3
Hepatic fat content and damage			
Hepatic FF, %	21.3 ± 3.0*	17.0 ± 2.8*	3.7 ± 0.5
LE, kPa	6.1 (4.3–14.5)*	5.2 (4.0–7.8)	5.0 (3.55–6.0)
ALT, units/L	31 (17–106)	33 (15–55)	25 (17–35)
Glucose and insulin sensitivity			
HbA_{1c}, %	5.6 (5.3–6.3)	5.4 (5.3–6.4)	5.3 (4.9–5.6)
HbA_{1c}, mmol/mol	38 (34–45)*	36 (34–46)	34 (30–38)
Plasma glucose, mmol/L	6.0 ± 0.2	5.8 ± 0.3	5.5 ± 0.1

Data are shown as mean (± SEM) or median (range). *$P < 0.05$ when compared with CON subjects.

After initiation of the pancreatic clamp, three CON subjects needed an infusion of 20% glucose to maintain PG >4.5 mmol/L; 3.9, 0, and 6.4 μmol/kg/min during LowGlucagon and 6.7, 3.7, and 8.4 μmol/kg/min during HighGlucagon. We found a similar course of PG in all groups during the pancreatic clamp (interaction $P = 0.215$, time effect $P < 0.01$, group effect $P = 0.063$, RM-ANOVA) (Fig. 2*D*). Focusing only on the HighGlucagon period, the increase in the glucagon infusion rate elicited similar minor and transient increments in PG in all groups (interaction $P = 0.191$, time effect $P < 0.01$, group effect $P = 0.085$). Results were similar if excluding the three CON subjects in need of the glucose infusion from the analysis.

FFA concentrations were equal in all groups at $t = 0$ (Fig. 2*E*) but decreased after initiation of the pancreatic clamp (interaction $P = 0.026$, time effect $P < 0.01$, group effect $P = 0.123$). Whereas the FFA concentration stayed suppressed in the CON group, the FFA increased in the HighGlucagon period in both MAFLD groups (both $P < 0.03$, paired t test as post hoc).

VLDL-TG Kinetics and Concentrations

HighGlucagon suppressed the VLDL-TG secretion rate compared with LowGlucagon in all groups, but with an attenuated suppression in both MAFLD groups (interaction $P = 0.004$, time effect $P < 0.01$, group effect $P = 0.316$) (Fig. 3*A*). Expressed as a percentage change, MAFLD groups had a reduced suppression of the VLDL-TG secretion rate compared with the CON group, with no difference between MAFLD groups (MAFLD+ 13% ± 5%, MAFLD− 10% ± 3%, CON 36% ± 7%; $P = 0.002$) (Fig. 3*B*). In the subset of 19 subjects for whom we had early HighGlucagon VLDL-TG data, we found no significant difference between the early and late VLDL-TG secretion rate values (Supplementary Fig. 2), indicating that the reduction in VLDL-TG secretion rate occurred rapidly.

We found no significant differences in plasma VLDL-TG concentrations at $t = 0$ (Fig. 2*F*). Comparing LowGlucagon to HighGlucagon, there was a significant difference in the change in VLDL-TG concentrations between groups as well as a significant group effect (interaction $P = 0.005$, no significant time effect, group effect $P = 0.034$). Correspondingly, we observed an insignificant decrease in VLDL-TG concentrations in the CON group ($P = 0.084$, paired t test as post hoc), a significant increase in VLDL-TG levels in the MAFLD+ group ($P < 0.01$, paired t test), and stable conditions for the MAFLD− group. The significant group effect was due to generally higher VLDL-TG concentrations in the MAFLD+ group than in the CON group (post hoc RM-ANOVA, CON vs. MAFLD+, $P = 0.03$). Results for total TG concentrations are found in Supplementary Fig. 3.

VLDL-TG FA Oxidation

Whole-body VLDL-TG FA oxidation rates were similar at LowGlucagon but increased slightly during HighGlucagon in the MAFLD groups, whereas a reduction was observed in the CON group (interaction $P = 0.039$, no time or group effect) (Supplementary Fig. 4).

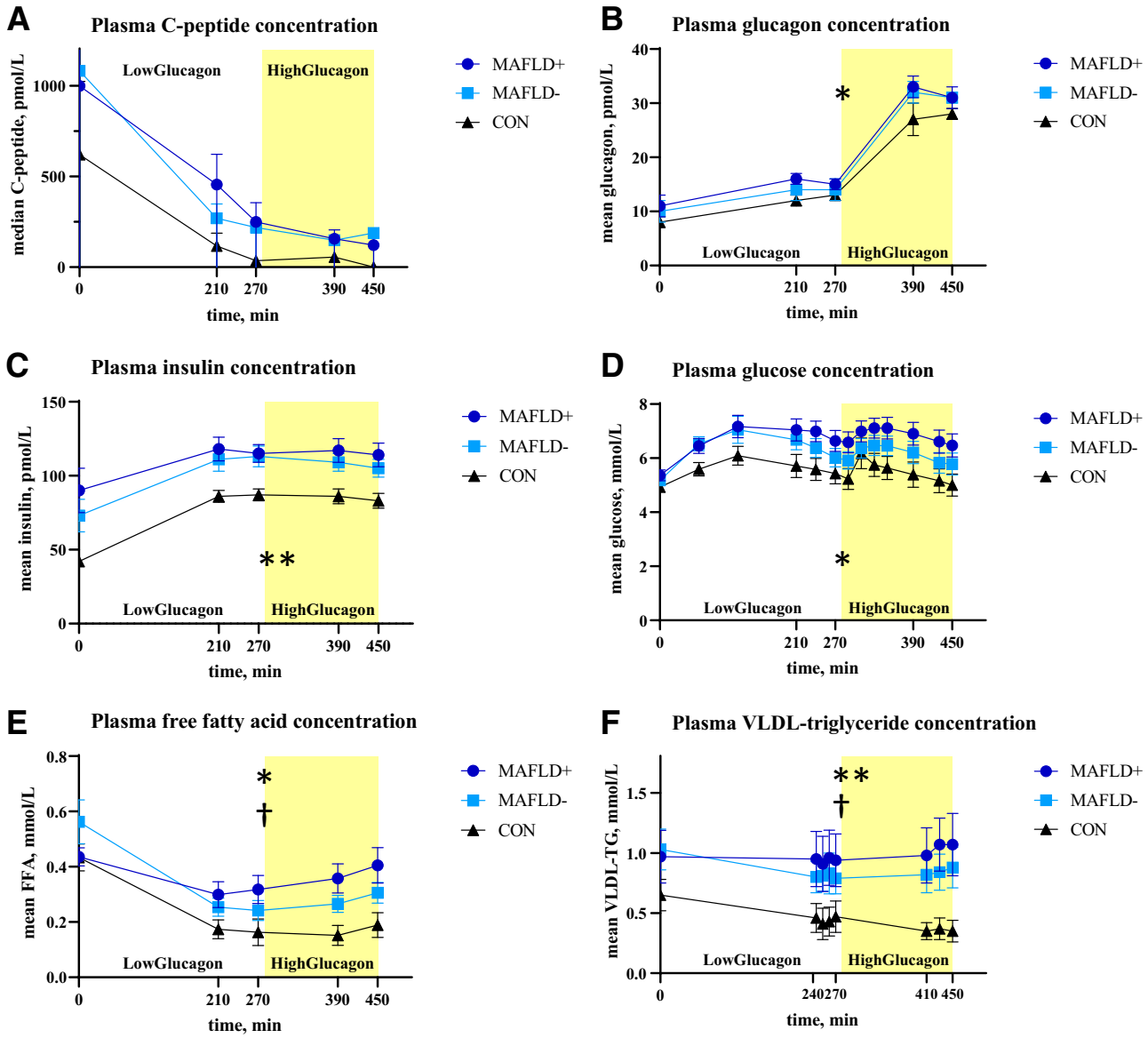

Figure 2—Plasma concentrations at baseline (t = 0) and during LowGlucagon and HighGlucagon infusion of C-peptide (*A*), glucagon (*B*), insulin (*C*), glucose (*D*), FFA (*E*), and VLDL-TG (*F*). *A*: Error bars (range). *B–F*: Error bars (SEM). RM-ANOVA, t = 0–450: *time effect $P \leq 0.05$, **group effect $P \leq 0.05$, †interaction $P \leq 0.05$.

Palmitate Flux

Palmitate flux did not change differently between groups and remained stable between LowGlucagon and HighGlucagon (Supplementary Fig. 5). However, the MAFLD+ group had a higher flux compared with the CON group (group effect, $P = 0.029$, RM-ANOVA, MAFLD+ vs. CON $P = 0.038$, post hoc).

Endogenous Glucose Production

During LowGlucagon, EGP was stable and similar between groups (Fig. 4). During HighGlucagon, an early increase in EGP was observed in the subgroup with early HighGlucagon measurements ($t = 270$ vs. $t = 330$ min, $P < 0.01$). During the late HighGlucagon period (t = 410–450 min), an EGP decrease was observed for the entire study group (time effect $P = 0.045$, no interaction or group effect, RM-ANOVA). For R_d rates, there were no significant differences over time or between groups (Supplementary Fig. 6).

Substrate Oxidation

RER was similar in all groups and decreased during HighGlucagon (time effect $P < 0.01$, no interaction or group effect) (Supplementary Table 2). Of notice, lipid oxidation rate increased (time effect $P < 0.01$, no interaction or group effect).

Correlations

The change in VLDL-TG secretion correlated positively with hepatic FF% ($r = 0.51$, $P < 0.01$) (Fig. 5*A*), visceral

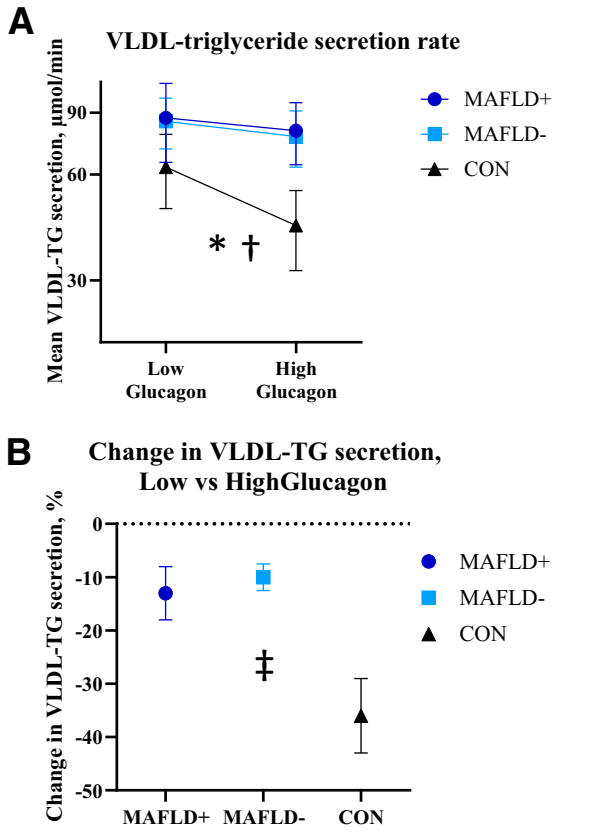

Figure 3—*A:* VLDL-TG secretion rate at LowGlucagon and HighGlucagon infusion rate. Error bars (SEM). Log_{10} scale. *B*: Percentage change in VLDL-TG secretion. Error bars (SEM). RM-ANOVA: *time effect $P \leq 0.05$, †interaction $P \leq 0.05$. One-way ANOVA: ‡$P < 0.05$.

fat mass ($r = 0.53$, $P < 0.01$) (Fig. 5*B*), and C-peptide at $t = 0$ ($r = 0.57$, $P < 0.01$). We also found moderate correlations with HbA_{1c}, LE, and BMI (Supplementary Table 3). The change in VLDL-TG secretion was moderately and positively correlated with palmitate flux at HighGlucagon ($r = 0.442$, $P = 0.009$) (Supplementary Fig. 7).

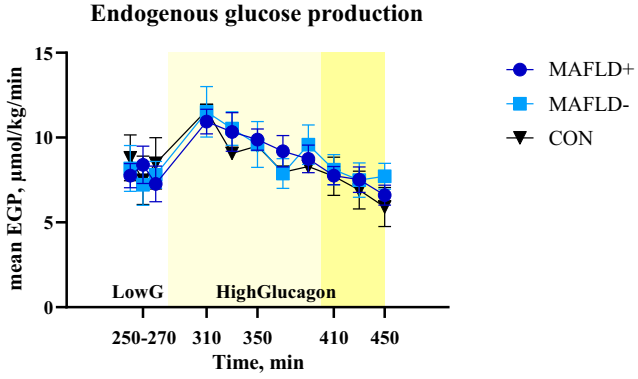

Figure 4—EGP in all subjects at LowGlucagon (LowG, $t = 250$, 260, and 270) and at late HighGlucagon ($t = 410$, 430, and 450). EGP in the subgroup of 19 subjects with early ($t = 310$–390) measurements. Error bars (SEM). For the three CON patients with EGP measurements in early HighGlucagon, SEM are not shown due to the low number of subjects.

DISCUSSION

Our primary finding is a blunted glucagon-mediated suppression of hepatic VLDL-TG secretion in subjects with MAFLD compared with CON subjects, matched for age, sex, and BMI. The difference in suppression (reduction in VLDL-TG secretion of 36% vs. 11% for CON and MAFLD subjects, respectively) is supported by an increase in plasma VLDL-TG concentration during HighGlucagon in subjects with MAFLD. Moreover, hepatic FF% correlates with a blunted decrease in VLDL-TG secretion. Therefore, we interpret these findings as an indication of MAFLD-associated hepatic glucagon resistance with respect to suppression of VLDL-TG secretion. The question remains whether the glucagon resistance is an early protective response to dysmetabolic changes in MAFLD; beneficial in the short run by promoting hepatic VLDL-TG export, but atherosclerotic in the long run.

In contrast, we found no difference in EGP in response to physiological high glucagon concentrations between overweight CON and MAFLD subjects. This novel finding points to a disproportionate effect of glucagon on hepatic VLDL-TG and glucose kinetics, suggesting that the abnormal effect of glucagon on VLDL-TG metabolism precedes abnormal effects on glucose metabolism in subjects with MAFLD. Of course, MAFLD groups, especially MAFLD+, were subject to higher plasma insulin concentrations, which might have influenced our results on EGP differently in the three groups.

We found no difference in the glucagon-mediated VLDL-TG suppression between subjects with histologically confirmed MAFLD with and without steatohepatitis. Of note, the included MAFLD subjects had rather mild disease, and findings may be different in subjects with MAFLD of greater severity, including fibrosis. Although there is an obvious need for further studies to support our findings, the equally reduced response to glucagon seems to be an early event in MAFLD pathogenesis and not a driver in the transition from simple steatosis to MAFLD with steatohepatitis.

In healthy individuals, glucagon diminishes VLDL-TG secretion (18). This is supported by thorough works of Longuet et al. (13), confirming that glucagon decreases plasma TGs, inhibits hepatic TG synthesis and secretion, and stimulates hepatic FA β-oxidation in mice in a peroxisome proliferator–activated receptor α/AMPK/p38 mitogen-activated protein kinase–dependent and insulin-independent manner. Human studies of glucagon effects on lipid metabolism are, however, scarce. So far, only one study has examined the acute effect of increased glucagon per se on lipoprotein metabolism. Xiao et al. (19) studied endogenous apolipoprotein B-100 VLDL-TG and

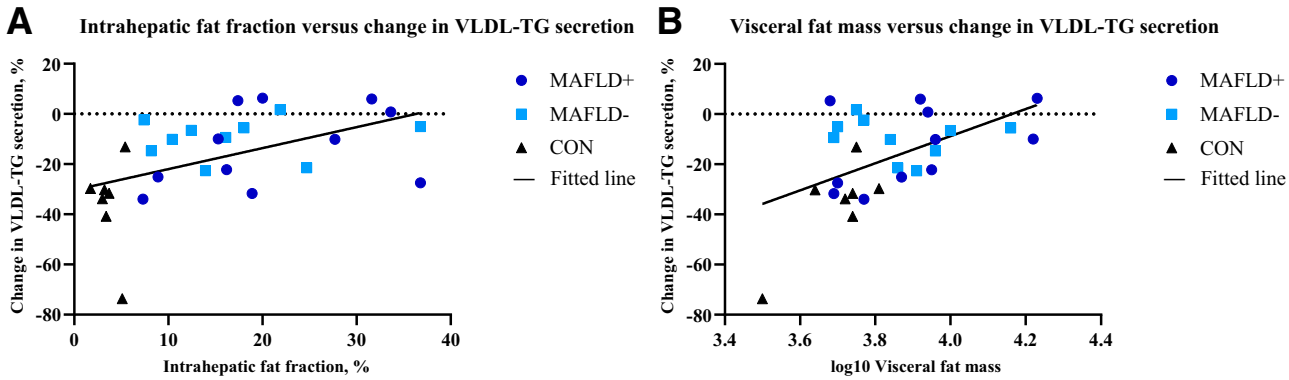

Figure 5—Correlation between change in VLDL-TG secretion and intrahepatic fat content ($r = 0.51$, $P < 0.01$) (*A*) and visceral fat mass ($\log_{10}$, $r = 0.53$, $P < 0.01$) (*B*).

chylomicron turnover during normo- and threefold hyperglucagonemia in healthy lean men. Hyperglucagonemia decreased the production rate of apolipoprotein B-100 lipoproteins, but had no effect on VLDL-TG concentrations, chylomicron metabolism, or de novo lipogenesis. These results, obtained in insulin-sensitive men, are difficult to translate to dysmetabolic conditions such as MAFLD or type 2 diabetes.

An elevated VLDL-TG secretion rate in subjects with MAFLD, not sufficiently suppressed by insulin or glucagon, may have several implications. First, it contributes to hypertriglyceridemia and encourages extrahepatic lipid uptake, which may further impair insulin sensitivity to glucose metabolism. Second, increased VLDL-TG secretion contributes to the production of small, atherogenic LDL particles and promotes HDL cholesterol depletion (31), thereby enhancing CVD risk. In line with this, trials using glucagon receptor antagonists LY2409021 and MK-0893 observed significant increases in hepatic lipid accumulation and plasma lipids in patients with type 2 diabetes (17,32).

The concept of glucagon resistance has been investigated at the level of hepatic amino acid metabolism in subjects with MAFLD, suggesting that hepatic steatosis causes resistance to glucagon in the amino acid metabolism, whereas the effect on EGP was conserved (29). Other groups have added mechanistic insights into hepatic glucagon resistance (33–35). For instance, Charbonneau et al. (34) showed that high-fat diet–induced steatosis in rats resulted in a reduced number of hepatic glucagon receptors, and a decreased number of glucagon receptors was also found in patients with MAFLD (36). In addition, glucagon action on hepatic lipid metabolism is dependent on AMPK action (13), which is suppressed in MAFLD. The difference between the glucagon-mediated effects on VLDL-TG secretion and EGP; however, emphasizes that the glucagon resistance observed may in fact be a matter of substrate availability more than "glucagon resistance" at the level of the glucagon receptor or signaling impairment.

This points to a major limitation of our study. Although we tried to isolate the glucagon effect on VLDL-TG metabolism by using a pancreatic clamp, the observed effects of glucagon cannot be seen in isolation. The effects on hepatic glucose and VLDL-TG metabolism are modulated by differences in insulin concentration and efficacy and by substrate availability (37). Insulin inhibits peripheral lipolysis and, thereby, hepatic FFA delivery, thereby lowering VLDL-TG secretion (38), although to a lesser extent in subjects with MAFLD (37). Higher insulin concentrations throughout the study day were noted in MAFLD subjects, probably due to lower insulin clearance in subjects with steatosis (39). The elevated insulin level should decrease VLDL-TG secretion more effectively in MAFLD subjects than in CON subjects. Yet, this was not observed, suggesting that the peripheral IR was not overcome despite the higher insulin level in MAFLD+ subjects. The change in VLDL-TG secretion was positively correlated to C-peptide and palmitate flux. This indicates that a blunted glucagon-mediated reduction in VLDL-TG secretion is more prevalent in subjects with IR and strongly suggests that secretion is modulated by FFA delivery. Other intrahepatic factors, with potential impact on VLDL-TG production and secretion include differences in FA oxidation, remnant lipoprotein particle uptake, and lipogenesis (38). Therefore, we can conclude that subjects with MAFLD suffer a blunted glucagon-mediated suppression of VLDL-TG secretion, although we cannot conclude how much it is influenced by hepatic and peripheral IR. An elevated palmitate flux was not noted in subjects with MAFLD−, however, and FFA availability is probably not the full explanation for the blunted suppression of VLDL-TG secretion in MAFLD.

As a second limitation, we did not include a group maintained at a low glucagon dose to address an independent time effect. Third, insulin and glucagon were infused peripherally, not portally. Fourth, we used a modest glucagon dose to be sure to investigate at a physiological relevant level of hyperglucagonemia. We cannot conclude whether the effects are different in case of even higher,

"pharmacological" glucagon levels. Further studies of the independent and combined effects of variable glucagon and insulin concentrations are obviously warranted.

In conclusion, this study shows that patients with MAFLD, with and without inflammatory and ballooning activity, have a reduced glucagon-mediated suppression of VLDL-TG secretion despite similar effect on EGP. We therefore propose that this study adds mechanistic insight into why patients with MAFLD may suffer atherogenic dyslipidemia.

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