

Fasting plasma insulin concentrations are associated with changes in hepatic fatty acid synthesis and partitioning prior to changes in liver fat content in healthy adults

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Resistance to the action of insulin impacts on fatty acid delivery to the liver, fatty acid synthesis and oxidation within the liver and triglyceride export from the liver. To understand the metabolic consequences of hepatic fatty acid synthesis, partitioning, oxidation and net liver fat content in the fasted and postprandial states we studied healthy men and women with varying degrees of insulin resistance before and after consumption of a mixed meal using stable-isotope tracer methodologies. Subjects were classified as being either normoinsulinemic (NI) (fasting plasma insulin <11.2 mU/L, $n=18$) or hyperinsulinemic (HI) (fasting plasma insulin >11.2 mU/L, $n=19$). There was no difference in liver fat content between HI and NI individuals, despite HI subjects having marginally more visceral fat. However, compared with NI subjects *de novo* lipogenesis (DNL) was higher and fatty acid oxidation was lower in HI individuals. These data suggest that metabolic pathways promoting fat accumulation are enhanced in HI but paradoxically without any significant impact on liver fat content when observed in healthy people. This is likely to be explained by increased triglyceride secretion as observed by hypertriglyceridaemia.

Introduction

Non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome (1) encompasses a spectrum of conditions from hepatic steatosis through to cirrhosis (2); obesity is a known risk factor. It remains unclear why intra-hepatocellular fat starts to accumulate but it is likely to involve an imbalance between fatty acid (FA) delivery to the liver, FA synthesis and oxidation within the liver and triglyceride (TG) export from the liver (3); insulin plays a key role in all of these processes.

Within the liver, insulin has dual action: 1) it stimulates the phosphorylation of the transcription factor Forkhead box protein O1 (FoxO1) that activates gluconeogenesis and 2) it activates the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) which enhances the transcription of genes required for FA and TG synthesis (4, 5). The induction of FA synthesis (*de novo* lipogenesis (DNL)) may contribute to insulin resistance (6). In insulin resistance the FoxO1 pathway becomes resistant to insulin so gluconeogenesis continues whilst insulin sensitivity is maintained in the SREBP-1c pathway leading to accelerated DNL (4). Enhanced hepatic DNL may have significant qualitative implications as the primary FA product is saturated (palmitoyl-CoA) (7, 8) which may interfere with cellular function (9), and the entry of fatty acyl-CoA into the mitochondrion is dependent on carnitine palmitoyltransferase 1 (CPT1); malonyl-CoA, an intermediate in the DNL pathway, is a potent inhibitor of this (10). Taken together, this may lead to enhanced very low density lipoprotein (VLDL)-TG production and a net retention of intra-hepatocellular TG (4, 5). Positive associations between hepatic DNL and VLDL-TG production rates have reported (11, 12).

Insulin plays a key role in regulating FA delivery to the liver. In the fasting state, plasma non-esterified fatty acids (NEFA) arise predominantly from the hydrolysis of adipose tissue TG; fasting plasma insulin concentrations have been inversely associated with NEFA release from subcutaneous abdominal adipose tissue (13). Plasma NEFA concentrations decrease after the consumption of a mixed meal due to the antilipolytic action of insulin suppressing the hydrolysis of adipose tissue TG; spillover FA derived from the peripheral lipoprotein lipase mediated lipolysis of chylomicron-TG (14-16) may somewhat reduce, but do not override this effect (13).

Hepatic steatosis is often seen in the context of hepatic insulin resistance but it is unclear whether hepatic steatosis causes insulin resistance or if insulin resistance cause hepatic steatosis (17). As insulin has the potential to influence hepatic FA synthesis and postprandial partitioning we aimed to determine the effect of global insulin resistance on hepatic FA synthesis and partitioning in healthy men and women in the fasted state and after the consumption of a mixed test meal.

Research Design and Methods

Participants and protocol

Thirty-seven subjects were recruited from the Oxford BioBank (www.oxfordbiobank.org.uk) (18) and by advertisement. Nineteen individuals were considered hyperinsulinemic (HI), with a fasting plasma insulin concentration greater than the 75th centile (11.2mU/L) of the Oxford Biobank (14) and 18 individuals were considered normoinsulinemic (NI). All volunteers were considered non-diabetic and free from any known disease, had a body mass index $<30\text{kg/m}^2$, were not taking medication known to affect lipid or glucose metabolism, did not smoke, and did not consume alcohol above recommended limits (2). The study was approved by Portsmouth Clinical Research Ethics Committee; all subjects gave written informed consent. Data from a portion of subjects reported in this work were obtained as part of another previously published study (19).

Liver fat and body composition

Intra-hepatic lipid content was measured after an overnight fast and within two weeks of the metabolic study day by proton magnetic resonance spectroscopy (¹H-MRS) (20). As part of the ¹H-MRS spectra of liver metabolites, hepatic glycogen was measured (one peak, chemical shift 3.984) and content as a percentage of the liver water determined. Whole body composition and fat distribution were measured using dual-energy X-ray absorptiometry (DEXA) (21).

Metabolic study day

Prior to the study day, subjects were asked to avoid foods naturally enriched in ¹³C, alcohol, and strenuous exercise. The evening prior to the study day, subjects consumed deuterated

water ($^2\text{H}_2\text{O}$) (3 g/kg body water) and continued to consume $^2\text{H}_2\text{O}$ during the course of the study day for the measurement of fasting and postprandial hepatic DNL (19). On the study day, after an overnight fast and consumption of $^2\text{H}_2\text{O}$, subjects came to the clinical research unit and a cannula was inserted into an antecubital vein, and baseline (time 0) blood and breath samples taken. Participants were then fed a mixed test meal containing 40g carbohydrate and 40g fat, with 200 mg of $[\text{U}^{13}\text{C}]$ palmitic acid to trace the fate of the dietary FA (19) and at 360 min were given a glucose drink (75g glucose) to assess the second meal effect (22). Repeated blood and breath samples were taken over the study period. Indirect calorimetry was performed in the fasting state and then 120 min after meal consumption using a GEM calorimeter to determine whole body CO_2 production and whole body respiratory exchange ratio (RER) and basal energy expenditure (GEMNutrition Ltd, Daresbury, Cheshire, UK).

Analytical methods

Whole blood was collected into heparinized syringes (Starstedt, Leicester, UK) and plasma was rapidly separated by centrifugation at 4°C for the measurement of plasma metabolite and insulin concentrations as described (14).

Separations of chylomicron-Svedberg flotation rate (S_f) >400 and VLDL-rich fraction (S_f 20-400) were made by sequential flotation using density gradient ultracentrifugation as previously described (14). The S_f 20-400 fraction was further separated by immunoaffinity chromatography to obtain a fraction completely devoid of apoB48 and will hereafter be called VLDL (14).

Samples were taken at 0, 30, 60, 90, 120, 180, 240, 300, 360, 390 and 420 min after the consumption of the test meal for the measurement of plasma glucose, insulin, TG, NEFA, 3-hydroxybutyrate (3OHB), chylomicron-TG and TG-rich lipoproteins (TRL)-TG and at 0, 180, 240, 300, 360, and 420 min for the analysis of VLDL-TG. Breath samples were collected at 0, 60, 90, 120, 180, 240, 300, 360, 390 and 420 min into EXETAINER[®] tubes (Labco Ltd, High Wycombe, Bucks, UK) for measurement of ¹³CO₂ enrichment.

Fatty acid and isotopic enrichment

To determine the specific FA composition and isotopic enrichment, total lipids were extracted from plasma and lipoproteins and FA methyl esters (FAMES) prepared (19, 23). The FA compositions (μmol/100 μmol total FA) in these fractions were determined by gas chromatography (GC), and palmitate concentrations calculated (14).

¹³C/¹²C ratios in [U-¹³C]palmitate were measured in plasma NEFA, TG, S_f >400 (chylomicron-TG), S_f 20-400-TG and VLDL-TG FAMES derivatives using a Delta Plus XP gas chromatography-combustion isotope ratio mass spectrometer (GC-C-IRMS) (Thermo electron, Bremen, Germany) (24). The tracer to tracee ratio (TTR) of a baseline measurement (before administration of [U-¹³C]palmitate) was subtracted from the TTR of each sample to account for natural abundance. The TTRs for [U-¹³C]palmitate were multiplied by the corresponding palmitate concentrations to give plasma and lipoprotein tracer concentrations (25).

¹³C/¹²C ratios in breath samples and the relative rate of whole-body meal-derived FA oxidation were calculated as previously described (24). The rate of expiration of ¹³CO₂ in breath was calculated by multiplying the CO₂ production (VCO₂, mmol/min) by the TTR of

$^{13}\text{CO}_2/^{12}\text{CO}_2$ (24). To allow for sequestration of label into the bicarbonate pool a dietary acetate recovery factor of 51% was applied (26). The data was corrected for lean mass (determined by DEXA) to account for individual differences between the NI and HI groups. Hepatic ketone body production was assessed by measuring the isotopic enrichment from $[\text{U}^{13}\text{C}]$ palmitate in 3OHB in deproteinised plasma (27).

Fasting and postprandial hepatic DNL was assessed based on the incorporation of deuterium from $^2\text{H}_2\text{O}$ in plasma water (Finnigan GasBench-II, ThermoFisher Scientific, UK) into VLDL-TG palmitate using GC-MS with monitoring ions with mass-to-charge ratios (m/z) of 270 ($\text{M}+0$) and 271 ($\text{M}+1$) (28). For simplicity, 'DNL' refers to the proportion of newly synthesized palmitate in VLDL-TG, this represents the synthesis of FA from non-lipid precursors (29). To assess the partitioning of DNL palmitate into desaturation pathways the ratio of $[\text{H}_2]16:1\text{n-7}/[\text{H}_2]16:0$ in VLDL-TG was determined as a marker of hepatic stearoyl-CoA desaturase1 (SCD1) activity (30).

Statistical methods

Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated (31). The relative and absolute contribution of meal FA to VLDL-TG was calculated at the end of the study (Time 420 min) (22).

Data were analysed using SPSS for Windows v22 (SPSS, UK, Chertsey, UK). All data are presented as means \pm SEM unless otherwise stated. Areas under the curve (AUCs) were calculated by the trapezoid method. AUCs have been divided by the relevant time period to give time-averaged values. All data sets were tested for normality according to the Shapiro-Wilk test. For anthropometric data, comparisons between the groups were made using a

general linear univariate model with sex as a covariate. Comparisons between the NI and HI group were made using an independent t-test or Mann Whitney U tests for non-parametric data independent t-test. Repeated measures ANOVA, with time and group as factors was used to investigate the change between groups over time for specific metabolites. Associations between variables were carried out using Spearman's rank correlation coefficient for the respective groups.

Results

Participant characteristics

NI subjects were slightly older, with marginally lower amounts of total, android and visceral fat masses ($P<0.05$), despite a similar BMI, than HI subjects (Table 1). Liver fat and glycogen content was not different between the groups (Table 1). The HI group had significantly higher ($P<0.05$) fasting concentrations of plasma glucose and TG than the NI group (Table 1). Fasting plasma VLDL-TG concentrations tended ($P=0.07$) to be higher in the HI compared to the NI group (Table 1).

Postprandial plasma biochemical parameters

Consumption of the mixed test meal exacerbated the differences in fasting plasma glucose and insulin concentrations, with the HI group having significantly ($P<0.05$) higher postprandial excursions compared to the NI group (Figure 1A-B). Fasting differences in plasma TG concentrations were not maintained over the postprandial period, with no difference between the groups (Figure 1D). In line with the fasting data, there were no notable differences in the postprandial response in plasma NEFA or 3OHB concentrations (Figure 1C, E). Whole-body RER tended ($P=0.07$) to be higher in HI compared to NI individuals in the fasting state but was significantly ($P<0.05$) higher in HI compared to NI subjects during the postprandial period, indicative of lower FA oxidation (Figure 1F).

Isotopic enrichment of plasma and breath

Inclusion of [^{13}C]palmitate into the mixed test meal provided the opportunity to trace the fate of dietary FA. There was no difference in the appearance of [^{13}C]palmitate in plasma chylomicron-TG between the groups (Figure 2A). Following the consumption of the second meal (at 360 min) the amount of [^{13}C]palmitate (from the first meal) incorporated into

chylomicron-TG at 420 min was similar between groups, suggesting no difference in the second meal effect. The appearance of [^{13}C]palmitate in the plasma NEFA pool was significantly higher ($P<0.05$) in the NI compared to HI group (Figure 2B) but there was no difference between the groups in the appearance of [^{13}C] in VLDL-TG (Figure 2C). We calculated the contribution of meal-derived FA to VLDL-TG at 420 min and found a significantly ($P<0.05$) lower relative contribution in the HI compared to NI group (11 (1)% vs 14 (1)%, (mean (sem)) $P<0.05$); this difference disappeared when expressed as an absolute concentration. In the fasting state, NI individuals had a significantly ($P<0.05$) lower relative contribution of DNL to VLDL-TG compared to HI individuals. The contribution of DNL to VLDL-TG increased over the postprandial period ($P<0.01$) in both groups, with the difference observed in the fasting state between the groups becoming less obvious ($P=0.07$) (Figure 2D). We assessed the [$^2\text{H}_2$]16:1n-7/[$^2\text{H}_2$]16:0 ratio in VLDL-TG, as a marker of the desaturation of DNL palmitate and found it to be significantly ($P<0.01$) higher in NI compared to HI individuals in both the fasting state and at the end of the postprandial period (420 min) (Table 1).

We assessed dietary FA oxidation by measuring the incorporation of ^{13}C (from dietary fat) in plasma 3OHB as a marker of hepatic FA oxidation and in breath CO_2 as a marker of whole-body dietary FA oxidation. We found a significantly ($P<0.05$) greater incorporation ^{13}C into plasma 3OHB in NI compared to HI individuals over the postprandial period ($P<0.05$) (Figure 2E). In line with this and the difference in postprandial RER, we found the production of $^{13}\text{CO}_2$ (per unit lean mass) tended ($P=0.07$) to be higher in NI compared to HI group individuals (time by group interaction, $P=0.05$) (Figure 2F).

To assess the effect of increased liver fat content on fasting and postprandial FA synthesis and partitioning we compared 10 NI and 9 HI individuals with a liver fat content >3.4%, the median of groups. Fasting plasma insulin was significantly ($P<0.01$) higher in the HI compared to NI group, as was postprandial plasma insulin and glucose concentrations (Supplementary Table 1). There was no difference between the groups in the appearance of [^{13}C] from the dietary fat into plasma chylomicron-TG, VLDL-TG and 3OHB, nor in fasting or postprandial hepatic DNL. Incorporation of [^{13}C] into the plasma NEFA pool was higher ($P<0.05$) in the NI compared to HI group (Supplementary Table 1).

Associations between plasma insulin, liver fat, hepatic FA synthesis and oxidation

We found a positive association between fasting plasma insulin and liver fat content in both the NI and HI groups (Figure 3A). Fasting insulin concentrations were also positively associated with liver glycogen content, but only in the NI ($r_s=0.61$, $P<0.05$) and not the HI ($r_s=0.24$, $P=\text{NS}$) group. When combined, we found a positive association between liver fat and glycogen content ($r_s=0.38$, $P<0.05$, $n=35$ (NI $r_s=0.35$ and HI $r_s=0.39$, $P=\text{NS}$ for both)). There was a significant positive association between fasting plasma insulin concentrations and fasting hepatic DNL in the HI but not the NI group (Figure 3B). In both groups we found robust inverse associations between fasting hepatic DNL and fasting plasma 3OHB concentrations, $r_s=-0.65$, $P<0.01$ (NI) and $r_s=-0.54$ (HI), $P<0.05$, respectively.

We observed a positive association between the postprandial response in plasma VLDL-TG concentrations and hepatic DNL in the NI but not the HI group (Figure 3C). There was a robust inverse association between the postprandial response in hepatic DNL and the incorporation of ^{13}C , representing recently ingested dietary fat, in plasma 3OHB in the NI group whilst this association was diminished in the HI group (Figure 3D).

Discussion

Hepatic steatosis is often accompanied by hepatic insulin resistance; it remains unclear whether hepatic steatosis causes insulin resistance or vice versa (17). We defined individuals as HI on the basis of their fasting plasma insulin concentration (14). We found HI individuals had marginally more total and visceral fat than NI individuals but there was no difference in liver fat content between the groups. Despite NI and HI individuals having a similar amount of liver fat, we observed profound differences in fasting plasma glucose, insulin and TG concentrations. We found fasting and postprandial hepatic DNL to be notably higher in the HI compared to NI group. The HI subjects had significantly lower dietary FA oxidation and the difference between the groups was augmented in the postprandial state. These observations were not evident in NI and HI individuals with a liver fat content >3.4%. Our findings demonstrate that hepatic steatosis does not need to be present to induce changes in intrahepatic FA metabolism; HI induces changes in FA partitioning that would, if maintained over a period of time, lead to accumulation of liver fat.

Within the liver, insulin integrates carbohydrate and lipid metabolism where they are directed to storage as TG and glycogen. We measured liver fat and glycogen content and found no difference between the groups in either; we did find liver fat content to be positively associated with liver glycogen content. Animal studies have suggested in insulin resistance, portal hyperinsulinaemia drives FoxO inactivation leading to a decrease in the hepatic glucose 6-phosphatase catalytic subunit to glucokinase (G6pc/Gck) ratio and increased hepatic DNL, TG, diacylglycerol and glycogen content (32). Our findings of higher hepatic DNL in HI compared to NI individuals are in line with this concept. We found a positive association between fasting plasma insulin concentrations and fasting hepatic DNL in the HI group only. Enhanced DNL leads to an increase in newly formed TG that will either reside

within the liver or be exported within VLDL (7, 8). We found hepatic DNL to be positively associated with VLDL-TG concentrations in the NI but not the HI group. It could be speculated that DNL FA were preferentially channelled toward secretion in VLDL in the NI group and channelled toward storage in the HI group. Animal work has suggested DNL FA exit the liver immediately as VLDL-TG, rather than being stored (33); although evidence for this is sparse in humans (34, 35) hepatic DNL has been positively associated with VLDL-TG production rates (11, 12).

It is proposed that DNL is a pathway for sustaining metabolic homeostasis and although an energetically inefficient way to store excess energy, it is an important mechanism for glucose disposal (36). In the present study, despite subjects consuming two test meals, we did not observe a marked divergence in postprandial hepatic DNL between the groups. It is plausible, if followed for longer the pattern of hepatic DNL would notably differ between the groups with the HI group having a greater lipogenic response to the second meal. The induction of hepatic DNL has been suggested to contribute towards insulin resistance (6); we cannot distinguish whether an increase in DNL caused insulin resistance or vice versa.

Enhanced DNL increases the production of long chain saturated fatty acyl-CoAs (e.g. palmitoyl-CoA) (7, 8). A potential fate of newly synthesised palmitoyl-CoA is partitioning towards desaturation by SCD1 (30). We measured the ratio of $[^2\text{H}_2]16:1\text{n}-7/[^2\text{H}_2]16:0$ in VLDL-TG as a marker of SCD1 activity and found the ratio to be significantly higher in the fasting and postprandial states in NI, compared to HI individuals. It is plausible greater desaturation of newly formed palmitate to palmitoleate would prevent accumulation of intra-hepatocellular palmitoyl-CoA. Evidence from animal and cellular studies (37, 38) suggests that lipotoxicity arising from the accumulation of long chain FA is specific to saturated FA

with increased accumulation causing cell dysfunction (9). Palmitoyl-CoA can be utilized for the synthesis of intracellular ceramide; both saturated FA and ceramides have been suggested to upregulate pro-inflammatory pathways and pro-insulin resistance factors (6). The factors influencing the partitioning of newly-synthesised palmitoyl-CoA toward specific metabolic pathways remain unclear.

The appearance of [^{13}C]palmitate in the systemic NEFA pool, consistent with spillover from chylomicron-TG hydrolysis (39), was higher in the NI compared to HI group despite no difference in systemic NEFA concentrations. This is consistent with our observation that adipose tissue FA trafficking was downregulated in abdominally-obese, HI males when compared to lean, NI males (15). The contribution of systemic NEFA to VLDL-TG production has been reported to be similar between insulin-sensitive and insulin-resistant individuals (14) and those with and without NAFLD (40, 41).

Removal of FA within the liver occurs by secretion as TG in VLDL or via oxidation. Although we did not measure VLDL-TG production rates others (42, 43) have reported VLDL apoB and TG production rates to be higher in obese, insulin-resistant compared to lean, insulin-sensitive individuals. In contrast, acute induction of hyperinsulinemia, in healthy insulin-sensitive males suppresses the total production rate of VLDL apoB and TG (44), even in the presence of excess NEFA concentrations (45). On the basis of these observations, it could be speculated that the HI individuals in the present study had a higher VLDL-TG production rate than NI individuals. Others have reported individuals with NAFLD to have a higher VLDL-TG secretion rate than those without (41, 46). We did not observe a difference in the appearance of [U^{13}C]palmitate (from dietary fat) in VLDL-TG between the groups however our findings demonstrate a proportion of dietary-derived FA

entering the liver undergo β -oxidation and the acetyl-CoA liberated enters a pool that is used for ketogenesis and this occurred to a greater extent in NI compared to HI individuals. We did not measure 3OHB production rate thus the absolute contribution of dietary FA into the ketogenic pathway cannot be determined. $^{13}\text{CO}_2$ production was also lower in HI compared to NI individuals. In support of these differences, we found NI compared to HI individuals to have a significantly lower fasting and postprandial whole-body RER, which was indicative of FA oxidation. We found a strong inverse association between postprandial hepatic DNL and the plasma [^{13}C]3OHB in NI which was not evident in the HI group. The findings from the NI group clearly highlight a divergence in FA partitioning suggesting intracellular metabolism is being moved away from esterification towards oxidation. The switch in intracellular metabolism was not so evident in the HI group; it could be speculated that the lack of association between these pathways signaling an attempt to dispose of excess intrahepatic glucose and FA.

There are some limitations to our study. Although subjects consumed two test meals it likely, due to the short duration of the study, peak hepatic DNL was not achieved as it occurs approximately 4-5 hours after consumption of a second meal (47). We did not determine the VLDL-TG, apoB or DNL production rate, therefore quantitative differences in the contribution of dietary and DNL FA to VLDL-TG production cannot be determined. The production rate from dietary fat and DNL to VLDL-TG has been reported to be 0.46 $\mu\text{mol}/\text{min}$ and 0.78 $\mu\text{mol}/\text{l}$ in individuals without NAFLD and 0.56 $\mu\text{mol}/\text{min}$ and 2.57 $\mu\text{mol}/\text{min}$ in individuals with NAFLD (41). In contrast, the absolute contribution of dietary fat to VLDL-TG was notably higher than the contribution from DNL when healthy males were given a liquid formula (32% fat) via duodenal infusion over a period of 11 hours

(48) demonstrating the contribution of dietary fat to VLDL-TG production will be dependent on the amount and regularity of consumption.

Insulin resistance is often associated with hepatic steatosis and therefore is suggested to have a central role in the development of hepatic steatosis (49) however it remains unclear if insulin resistance causes the development of steatosis or vice versa (17). Our data demonstrate that notable differences in hepatic fatty acid metabolism are evident between NI and HI individuals across a spectrum of liver fat contents. It is plausible that if maintained over a period of time or further augmented, the alterations in intra-hepatic fatty acid synthesis and partitioning reported here, may lead to net liver fat accumulation.

Author contributions

CP, MP, RB, CAM and LH conducted the study, CP, MP, RB, and CAM carried out analyses. All authors contributed to data analysis and wrote the manuscript. LH secured funding (BHF FS/11/18/28633). LH 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.

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Disclosure

None of the authors has any conflict of interest to declare.

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Figure legends

Figure 1. Plasma concentrations of (A) glucose (effect of time $P < 0.001$, group $P < 0.01$); (B) insulin (effect of time $P < 0.001$, group $P < 0.001$, time x group interaction $P < 0.01$); (C) non-esterified fatty acids (NEFAs) (effect of time $P < 0.001$); (D) triglyceride (TG) (effect of time $P < 0.001$); (E) 3-hydroxybutyrate (3OHB) (effect of time $P < 0.001$); in normoinsulinemic (NI) (●) and hyperinsulinemic (HI) (○) subjects and (F) the fasting (Time 0) and postprandial (Time 120 min) whole-body respiratory exchange ratio (RER) ($*P < 0.05$ between groups) after a mixed test meal in NI (■) and HI (□) subjects.

Figure 2. Postprandial enrichment of [^{13}C]palmitate in (A) plasma chylomicron-triglyceride (TG) (effect of time $P < 0.001$); (B) plasma non-esterified fatty acids (NEFAs) (effect of time $P < 0.001$, group $P < 0.05$); (C) plasma very low density lipoprotein triglyceride (VLDL-TG) (effect of time $P < 0.001$); (D) the relative contribution of newly synthesised palmitate (DNL) in plasma very low density lipoprotein triglyceride (VLDL-TG) (effect of time $P < 0.001$, group $P = 0.07$); (E) the appearance of [^{13}C] from dietary fat in plasma 3-hydroxybutyrate (3OHB) (effect of time $P < 0.001$, group $P < 0.05$); and (F) the appearance of [^{13}C] in breath CO_2 (effect of time $P < 0.001$, group $P = 0.07$) after a mixed test meal in normoinsulinemic (NI) (●) and hyperinsulinemic (HI) (○) subjects.

Figure 3. Correlations between (A) liver fat content (%) and fasting plasma insulin concentration (mU/L) in normoinsulinemic (NI) (●) ($r_s = 0.51$, $P < 0.05$) and hyperinsulinemic (HI) (○) ($r_s = 0.58$, $P < 0.05$) subjects; (B) the fasting relative contribution (%) of newly synthesised palmitate (DNL) in very low density lipoprotein triglyceride (VLDL-TG) and fasting plasma insulin concentrations (mU/L) in NI ($r_s = 0.20$, P

= NS) and HI ($r_s = 0.53$, $P < 0.05$) subjects; **(C)** the time-averaged (AUC) relative contribution (%) of newly synthesised palmitate (DNL) in very low density lipoprotein triglyceride (VLDL-TG) and the time-averaged (AUC) plasma concentration of VLDL-TG ($\mu\text{mol/L}$) in NI ($r_s = 0.56$, $P < 0.05$) and HI ($r_s = 0.09$, $P = \text{NS}$) subjects; **(D)** the time-averaged (AUC) relative contribution (%) of newly synthesised palmitate (DNL) in very low density lipoprotein triglyceride (VLDL-TG) and the time average (AUC) appearance of [^{13}C] in plasma 3-hydroxybutyrate (3OHB)($\mu\text{mol/L}$) in NI ($r_s = -0.75$, $P < 0.001$) and HI ($r_s = -0.14$, $P = \text{NS}$) subjects.

Table 1. Characteristics of study participants.

	Normoinsulinemia (n=18)	Hyperinsulinemia (n=19)
Women / Men	6 / 12	3 / 16
Age (y)	48 (39 -59)	44 (27 – 48)*
Body mass index (kg/m ²)	26.9 (21.2 – 29.9)	27.5 (22.8 – 29.7)
Waist (cm)	92 (81 – 103)	96 (86 – 106)
Total fat mass (kg)	24.9 (12.9 – 38.4)	24.5 (19.4 – 39.9)*
Total lean mass (kg)	53.5 (37.7 – 65.5)	58.3 (39.1 – 72.5)
Visceral fat (kg)	1.10 (0.05 – 1.98)	1.31 (0.57 – 2.39)*
Android fat (kg)	2.3 (0.8 – 3.2)	2.5 (1.8 – 4.3)*
Gynoid fat (kg)	3.7 (2.0 – 6.5)	3.6 (2.6 – 6.6)
Android : Gynoid ratio	0.67 (0.19 – 0.96)	0.66 (0.45 – 1.03)
Liver fat (%)	3.4 (0.7 – 24.4)	3.4 (1.4 – 27.6)
Liver glycogen (%)	0.3 (0.1 – 0.7)	0.3 (0.1 – 2.0)
HOMA-IR	1.9 (1.2 – 2.4)	3.5 (2.5 – 6.1)***
Basal Energy Expenditure (kcal)	1548 (1262 – 2015)	1788 (1448 – 2077)

Fasting Plasma Biochemical Parameters

Glucose (mmol/l)	5.1 (4.1 – 5.5)	5.5 (4.7 – 6.4)**
Insulin (mU/l)	8.7 (5.2 – 10.9)	13.6 (11.3 – 25.9)***
NEFA (μmol/l)	421 (165 – 690)	377 (172 – 661)
Total cholesterol (mmol/l)	5.4 (3.8 – 7.0)	5.5 (3.3 – 7.6)
HDL cholesterol (mmol/l)	1.1 (0.7 – 2.0)	1.0 (0.7 – 1.6)
Non-HDL cholesterol (mmol/l)	3.9 (2.3 – 5.9)	4.3 (2.6 – 6.4)

TG (μmol/l)	1191 (401 – 3933)	1905 (329 – 4085) [*]
VLDL-TG (μmol/l)	930 (150 – 2614)	1234 (169 – 1704)
3OHB (μmol/l)	49 (21 – 168)	39 (21 – 107)

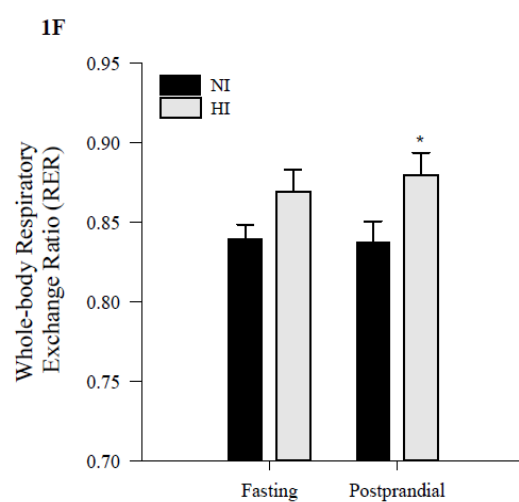
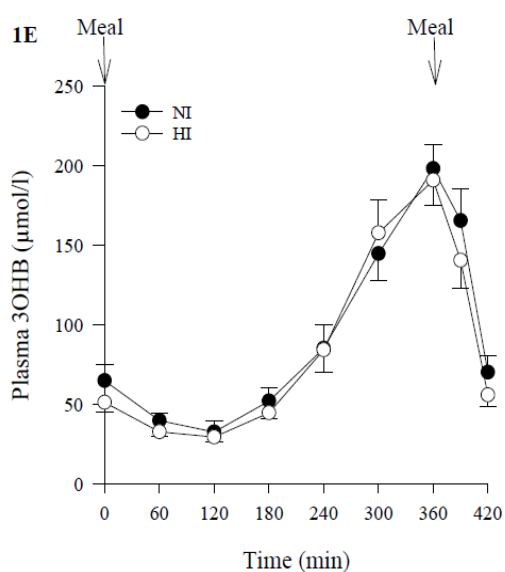
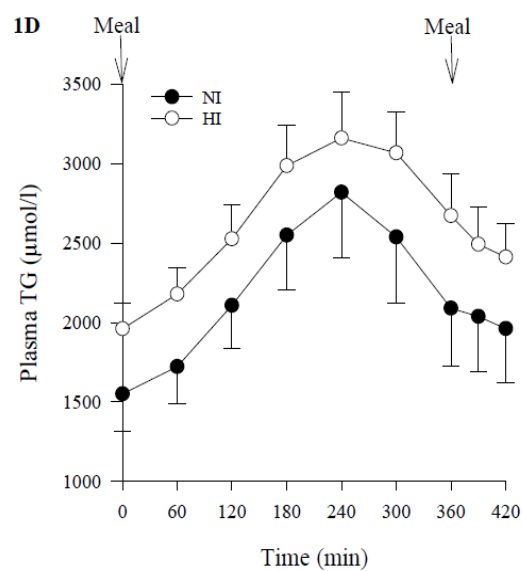
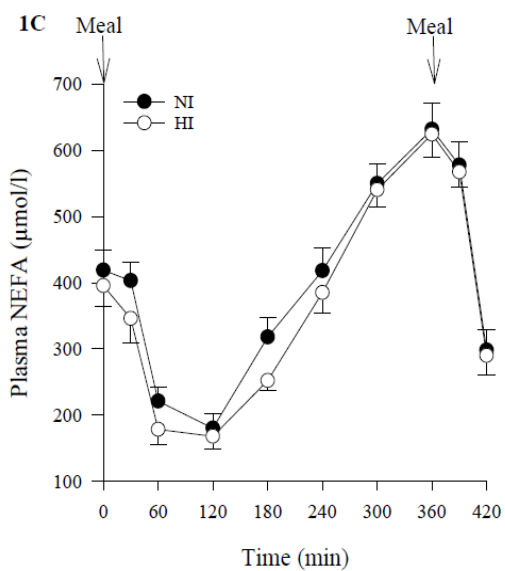
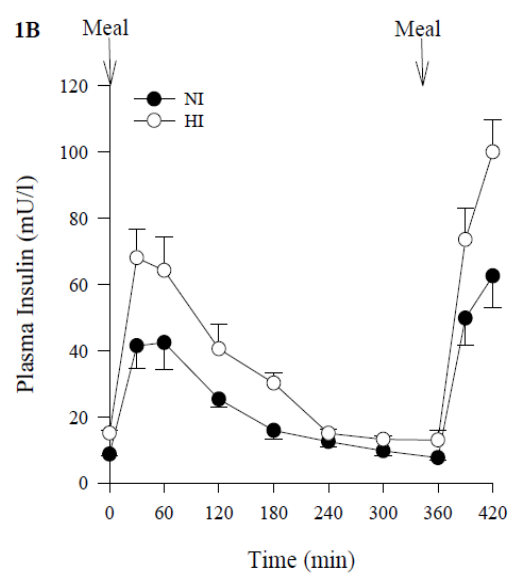
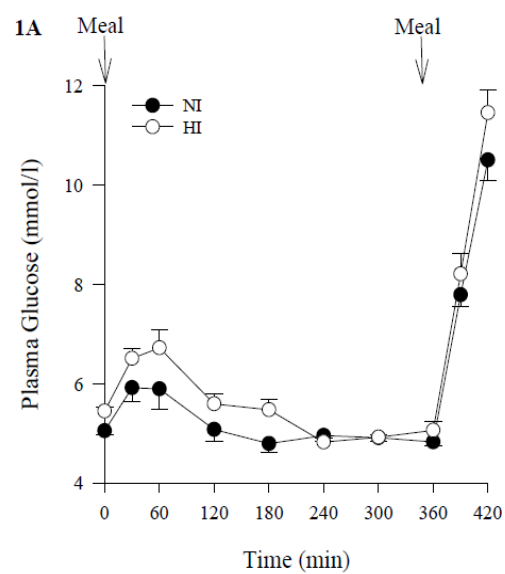
Isotopic desaturation ratio in VLDL-TG[†]

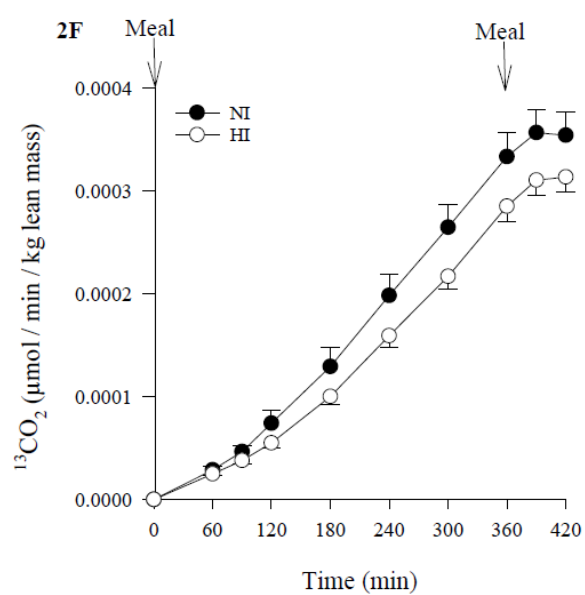
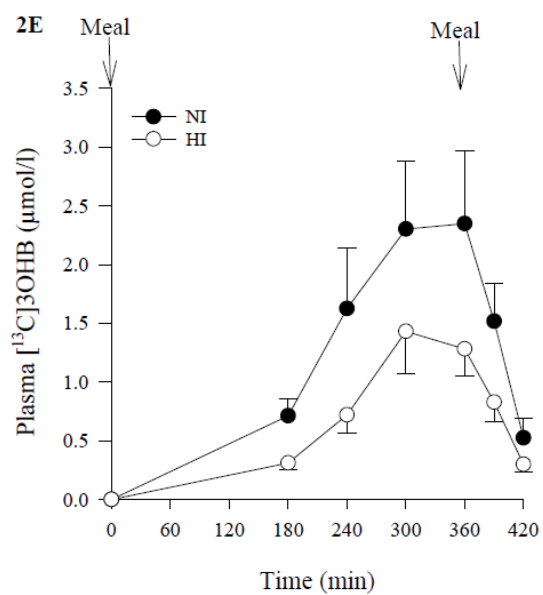
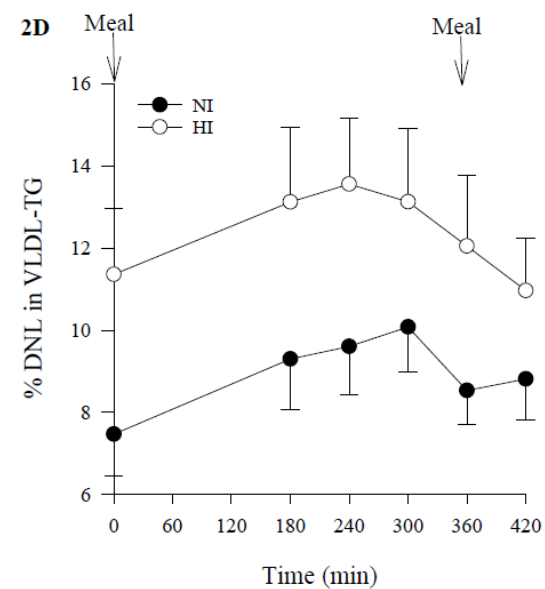
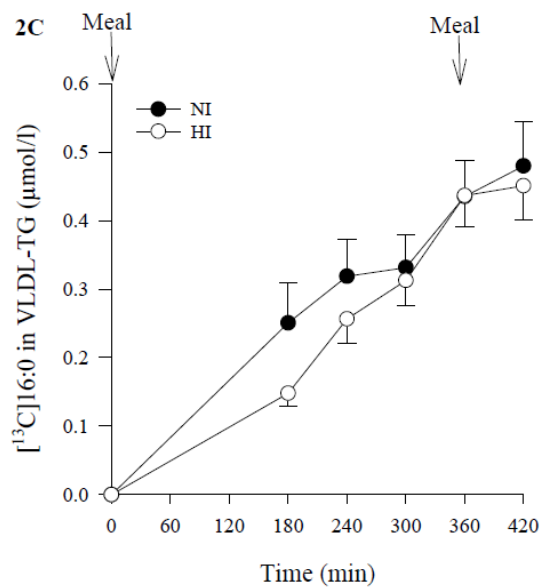
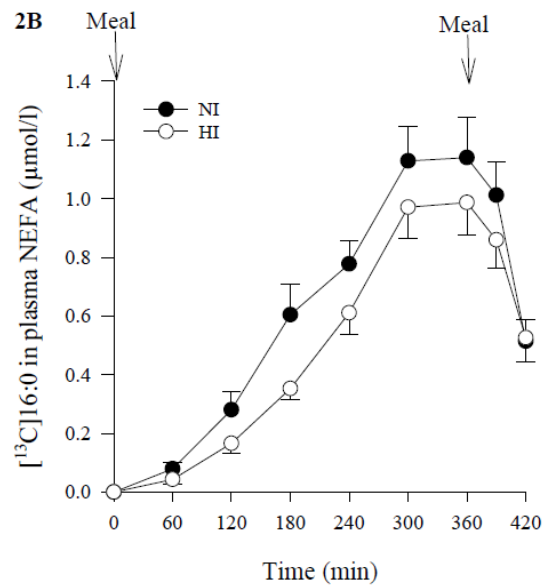
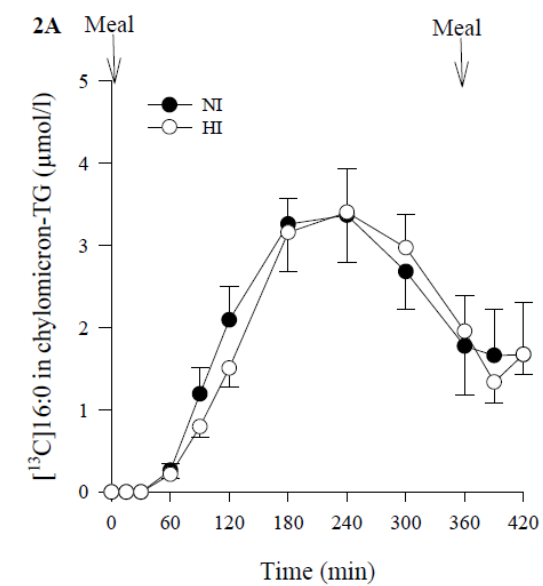
Fasting [² H ₂]16:1n-7 / [² H ₂]16:0	0.45 ± 0.13	0.10 ± 0.03 ^{**}
Postprandial [² H ₂]16:1n-7 / [² H ₂]16:0 [‡]	1.26 ± 0.82	0.20 ± 0.07 ^{**}

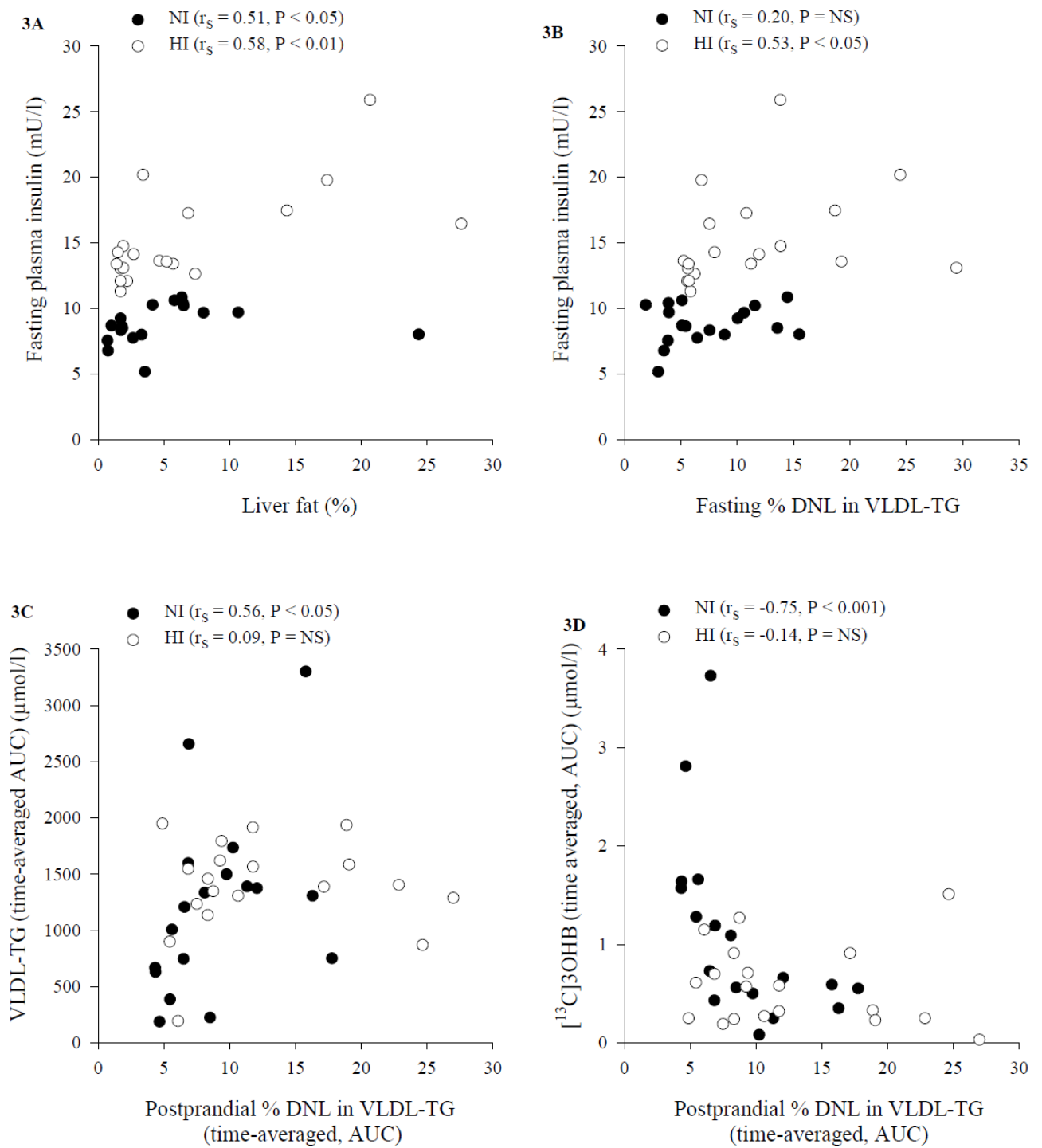
Data expressed as median (range). [†]Data expressed as mean ± sem. [‡]measured at 420 mins.

*P<0.05, **P<0.01, ***P<0.001, normoinsulinemia vs hyperinsulinemia. Abbreviations:

HOMA-IR, homeostatic model assessment of insulin resistance; NEFA, non-esterified fatty acids; TG, triglyceride; VLDL, very low density lipoprotein; HDL, high density lipoprotein; 3OHB, 3-hydroxybutyrate.







Supplementary Table 1. Characteristics of a sub-set of study participants with a liver fat content greater than 3.4%.

	Normoinsulinemia	Hyperinsulinemia Liver
	Liver fat >3.4% (n=10)	fat >3.4% (n=9)
Women / Men	1 / 9	1 / 8
Age (y)	47 ± 2	43 ± 2
Body mass index (kg/m ²)	27.4 ± 0.4	28.1 ± 0.5
Liver fat (%)	7.9 ± 2.0	12.2 ± 2.7
HOMA-IR	2.4 ± 0.3	4.1 ± 0.3**
<i>Fasting Plasma Biochemical Parameters</i>		
Glucose (mmol/l)	5.2 ± 0.1	5.4 ± 0.1
Insulin (mU/l)	10.5 ± 1.2	16.7 ± 1.4**
NEFA (μmol/l)	441 ± 39	442 ± 51
TG (μmol/l)	1976 ± 346	2062 ± 321
3OHB (μmol/l)	67 ± 16	50 ± 9
Hepatic DNL (%)	9.5 ± 2.3	11.1 ± 1.7
<i>Time-averaged postprandial plasma concentrations</i>		
Glucose (mmol/l)	5.7 ± 0.1	6.0 ± 0.1*
Insulin (mU/l)	29.2 ± 4.4	44.9 ± 5.0*
NEFA (μmol/l)	437 ± 28	401 ± 32
TG (μmol/l)	2780 ± 442	2942 ± 413
3OHB (μmol/l)	100 ± 15	74 ± 9
Hepatic DNL (%)	10.8 ± 2.3	12.6 ± 1.9
[¹³ C]16:0 in chylomicron-TG (μmol/l)	2.3 ± 0.4	1.9 ± 0.4

[¹³ C]16:0 in NEFA (μmol/l)	0.7 ± 0.1	0.5 ± 0.1 [*]
[¹³ C]16:0 in VLDL-TG (μmol/l)	1.1 ± 0.1	0.9 ± 0.1
[¹³ C]3OHB (μmol/l)	1.2 ± 0.3	0.5 ± 0.1

Data expressed as mean ± sem. ^{*}P<0.05, ^{**}P<0.01, ^{***}P<0.001, normoinsulinemia with liver fat >4% vs hyperinsulinemia with liver fat >4%.

Abbreviations: HOMA-IR, homeostatic model assessment of insulin resistance; NEFA, non-esterified fatty acids; TG, triglyceride; VLDL, very low density lipoprotein; 3OHB, 3-hydroxybutyrate; DNL, *de novo* lipogenesis.