



High-protein diet more effectively reduces hepatic fat than low-protein diet despite lower autophagy and FGF21 levels

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Abbreviations: ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; ALT, alanine transaminase; AMPK γ 1, AMP-activated protein kinase γ 1; AST, aspartate transaminase; ATF4, activating transcription factor 4; Atg, autophagy-related protein; BCA, bicinchoninic acid; BioPS, biopsy preservation solution; BiP, binding immunoglobulin protein; BMI, body mass index; BSA, bovine serum albumin; ChREBP, carbohydrate-responsive element-binding protein; CPM, count per million; CPT1A, carnitine palmitoyltransferase 1A; CS, citrate synthase; DDIT3, DNA damage inducible transcript 3; DMEM, Dulbecco's modified eagle medium; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N',N'-tetraacetic acid; ELISA, enzyme linked immunosorbent assay; ER, endoplasmic reticulum; ETS, mitochondrial electron transport system; FA, fatty acid; FASN, fatty acid synthase; FBS, fetal bovine serum; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; FGF21, fibroblast growth factor 21; GGT, γ -Glutamyl transpeptidase; HbA_{1c}, glycated hemoglobin A_{1c}; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid); HP, high-protein diet; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IHL, intrahepatic lipids; LC3A, microtubule-associated protein 1A-light chain 3; LC3B, microtubule-associated protein 1B-light chain 3; LP, low-protein diet; LPL, lipoprotein lipase; MCAD, medium-chain acyl-CoA dehydrogenase; MRS, proton magnetic resonance spectroscopy; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PBS, phosphate buffered saline; PPAR, peroxisome proliferator activated receptor; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real-time polymerase chain reaction; RIPA, radioimmunoprecipitation assay; RPLP0, ribosomal protein lateral stalk subunit P0; SAF, steatosis-activity-fibrosis; SCD1, stearoyl-CoA desaturase 1; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SREBP1c, sterol regulatory element-binding protein 1c; SUIT, substrate-uncoupler-inhibitor titration; TBS, tris-buffered saline; Tris, 2-Amino-2-(hydroxymethyl)propane-1,3-diol; XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1.

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Abstract

Background and aims: Non-alcoholic fatty liver disease (NAFLD) is becoming increasingly prevalent and nutrition intervention remains the most important therapeutic approach for NAFLD. Our aim was to investigate whether low- (LP) or high-protein (HP) diets are more effective in reducing liver fat and reversing NAFLD and which mechanisms are involved.

Methods: 19 participants with morbid obesity undergoing bariatric surgery were randomized into two hypocaloric (1500-1600 kcal/day) diet groups, a low protein (10E% protein) and a high protein (30E% protein), for three weeks prior to surgery. Intrahepatic lipid levels (IHL) and serum fibroblast growth factor 21 (FGF21) were measured before and after the dietary intervention. Autophagy flux, histology, mitochondrial activity and gene expression analyses were performed in liver samples collected during surgery.

Results: IHL levels decreased by 42.6% in the HP group, but were not significantly changed in the LP group despite similar weight loss. Hepatic autophagy flux and serum FGF21 increased by 66.7% and 42.2%, respectively, after 3 weeks in the LP group only. Expression levels of fat uptake and lipid biosynthesis genes were lower in the HP group compared with those in the LP group. RNA-seq analysis revealed lower activity of inflammatory pathways upon HP diet. Hepatic mitochondrial activity and expression of β -oxidation genes did not increase in the HP group.

Conclusions: HP diet more effectively reduces hepatic fat than LP diet despite of lower autophagy and FGF21. Our data suggest that liver fat reduction upon HP diets result primarily from suppression of fat uptake and lipid biosynthesis.

KEYWORDS

autophagy, dietary protein, ER-stress, FGF21, liver fat, mitochondria, nutrition, obesity

1 | INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), which is defined by excessive triglyceride accumulation in more than 5% of the hepatocytes, is becoming increasingly prevalent and causes serious global health problem as it affects 20%-30% of Western populations.¹ The chronic and progressive stage of NAFLD, non-alcoholic steatohepatitis (NASH), dramatically increases the risks of irreversible fibrosis, cirrhosis and even hepatocellular carcinoma (HCC).² Nowadays, caloric restriction is a widely accepted treatment for NAFLD. However, it has not been fully resolved which macronutrient composition is most effective in reducing liver fat content. For instance, high-protein (HP) diets have been proven to increase energy expenditure, decrease blood glucose levels, promote fat oxidation and consequently support weight loss, and reduce liver fat in mouse and human studies.³⁻⁶ Low-protein (LP) diets, on the other hand, were also shown to increase energy expenditure and improve glucose homeostasis, despite an elevated food intake, in several mouse studies.⁷⁻¹⁰

Increased fibroblast growth factor 21 (FGF21) expression and secretion,⁷ and increased hepatic autophagy^{11,12} may contribute to

Key points

We investigated whether the hypocaloric low- (LP) or high-protein (HP) diets are more effective in reducing liver fat and which mechanisms are involved. Liver samples were collected during bariatric surgery after three weeks of diet. HP diet strongly reduced hepatic fat than LP diet despite lower autophagy and FGF21, and thus, primarily resulted from suppression of fat uptake and lipid biosynthesis.

ameliorate metabolic responses to LP diets. At present, there are contradictory results regarding autophagy and lipid degradation. Singh et al¹³ found that 'lipophagy' represents an essential pathway for the degradation of lipid droplets, while Shibata et al¹⁴ reported that autophagy was involved in the formation and growth of lipid droplets resulting in potentially opposite effects. In addition, the elevation of endogenous FGF21 secretion has also been shown to prevent diet-induced obesity and insulin resistance in skeletal muscle-specific autophagy-deficient mice¹⁵ and has been proposed to

reflect a protective response in order to improve metabolic parameters in metabolic disorders.¹⁶ On the other hand, HP diets might suppress liver lipogenesis at the molecular level¹⁷⁻¹⁹ to reduce liver fat. Furthermore, mitochondrial dysfunction may contribute to developing NAFLD through the formation of ROS²⁰ and the depletion of ATP levels.²¹

In this clinical study, we aimed to clarify whether LP or HP diets exert greater effects on liver fat reduction and to investigate their molecular mechanisms during dietary intervention. For this, we performed a 3-week dietary intervention in 19 participants with morbid obesity and an indication of NAFLD prior to bariatric surgery and collected blood and liver tissue samples during surgery. We hypothesized that LP and HP diets might induce liver fat reduction via different mechanisms. We suggested that LP diets in contrast with HP intakes would induce autophagy, FGF21, and thus reduce liver fat content, and that HP diets might trigger increased mitochondrial activity and fatty acid β -oxidation, or decrease liver lipogenesis.

2 | MATERIALS AND METHODS

For a detailed description of methods and materials, please refer to the SI Methods.

2.1 | Study design, dietary intervention, blood and tissue samples, and proton magnetic resonance spectroscopy

In cohort I, 19 participants with morbid obesity undergoing bariatric surgery were divided into two hypocaloric (1500-1600 kcal/d) diet groups (HP: 30 E% protein, 25-30 E% fat, 35-45 E% carbohydrates, $n = 9$; LP: 10 E% protein, 25-35 E% fat, 55-65 E% carbohydrates, $n = 10$), for three weeks prior to surgery (Table S1). Groups were matched for age, gender and body mass index (BMI). Study participants obtained food plans given as 10-d rotating menus (Table S2) and included recipes. HP food plans were rich in low-fat dairy products (cheese, milk, yoghurt), meat, eggs, fruits and vegetables, whereas LP (low-methionine) food plans were rich in bread, rice, potatoes, soy products, fruits and vegetables. Sweets, cookies and sweet beverages were excluded in both groups. Part of the food (protein shakes in the HP and vegan spreads in the LP group) was provided to the participants.

The primary outcome of the study was change in liver fat content after three weeks of dietary intervention; secondary outcomes were anthropometric measurements, routine biochemistry of liver parameters, serum markers of glucose metabolism and liver fat (FGF21), autophagy flux, gene expression and mitochondrial activity in the liver.

At the beginning and at the end of the intervention (3-5 days before surgery), anthropometric measurements, fasting blood samples, body composition determination via BOD POD (Cosmed,

Rome, Italy) were performed. Assessment of intrahepatic lipid (IHL) content, measured by proton magnetic resonance spectroscopy (¹H-MRS) was performed in the early afternoon, at the end of each clinical investigation day. On the day of surgery, blood, liver and adipose tissue samples were collected. Study participants were advised to follow the diets during this gap and not to change their physical activity.

In cohort II, 10 obese patients, who consumed a 3-week hypocaloric (1200-1500 kcal/d) diet with a moderately increased protein intake (20-22 E% protein) and did not undergo bariatric surgery, were selected as a reference protein (RP) group (Table 1, Table S1). The serum data and IHL content from these patients were used as reference. The register and ethics approval of the study are shown in the SI Methods.

2.2 | Histology

After haematoxylin and eosin (H&E) staining, liver biopsies were assessed for hepatocellular ballooning, lobular inflammation, fibrosis and hepatic triglyceride content. Study subjects were divided into three groups according to the steatosis-activity-fibrosis (SAF) score^{22,23} into "normal", "steatosis" and "NASH" groups. The histological diagnoses by SAF score in the LP and HP groups are shown in Table S3.

2.3 | Ex vivo examinations, protein extraction and Western blotting for autophagy flux

Fresh liver tissue was incubated with or without 0.1 μ M bafilomycin A1 and 10 μ g/mL leupeptin (both from Sigma Aldrich Chemie) for 2 hours as described.²⁴ Protein extraction was performed by lysing frozen liver tissue in RIPA buffer. Western blotting and chemiluminescent detection were carried out on Fusion SL (PeqLab Biotechnologie GmbH).

2.4 | RNA extraction and gene expression analysis

Followed by RNA extraction from liver samples, gene expression profiling was performed by RNA-Seq as described in SI Methods. Expression of genes involved in autophagy, ER-stress, FGF21 pathway and lipid metabolism was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) as described before.²⁵ Primer sequences are shown in Table S4.

2.5 | Hepatic triglyceride and citrate synthase activity analysis

Frozen liver tissue was powdered, lysed and homogenized for further analyses. Triglyceride content analysis was performed according to

TABLE 1 Anthropometric measurements and routine blood parameters before and after 3 weeks of dietary intervention

	LP group		HP group		RP group		P value			
	Week 0	Week 3	Week 0	Week 3	Week 0	Week 3				
	P _{LP}		P _{HP}		P _{RP}					
Anthropometry										
Weight [kg]	133.19 ± 7.48	127.88 ± 6.98	4.43 × 10⁻⁴	139.61 ± 8.13	134.92 ± 8.36	.001	109.53 ± 5.10	104.19 ± 4.96	3.6 × 10⁻⁵	.324
BMI [kg/m ²]	45.18 ± 1.24	43.45 ± 1.30	2.36 × 10⁻⁴	44.48 ± 1.29	42.94 ± 1.38	.001	38.49 ± 1.70	36.60 ± 1.62	3.5 × 10⁻⁵	.324
Fat mass [%]	53.02 ± 2.60	51.59 ± 2.09	.858	54.84 ± 2.68	55.60 ± 2.80	.839	43.24 ± 3.99	38.43 ± 5.45	.490	.774
Fat mass [kg]	69.8 ± 5.9	64.9 ± 4.9	.052	77.4 ± 9.6	77.8 ± 8.1	.252	46.7 ± 6.9	35.3 ± 6.9	.230	.748
Fat-free mass [kg]	61.0 ± 4.5	60.9 ± 4.2	.978	62.2 ± 5.2	60.6 ± 4.9	.203	59.1 ± 3.1	55.0 ± 2.2	.057	.606
Serum parameters										
AST [U/l]	32.32 ± 6.58	34.68 ± 4.99	.240	24.36 ± 3.44	36.58 ± 11.82	.386	22.50 ± 0.93	24.10 ± 1.97	.439	.491
ALT [U/l]	44.31 ± 10.76	43.83 ± 5.85	.944	36.68 ± 8.28	50.28 ± 9.82	.002	28.50 ± 3.07	28.70 ± 2.62	.942	.401
GGT [U/l]	66.18 ± 24.08	41.82 ± 12.71	.001	34.59 ± 10.91	56.13 ± 31.35	.007	26.30 ± 2.53	18.70 ± 1.61	.014	.008
Creatinine [μmol/l]	77.61 ± 7.65	85.21 ± 8.66	.013	77.13 ± 6.05	75.55 ± 5.29	.473	80.80 ± 4.59	70.54 ± 4.71	.425	.016
Urea [mmol/l]	5.26 ± 0.39	3.17 ± 0.26	1.63 × 10⁻⁴	4.69 ± 0.47	6.04 ± 0.62	.031	4.94 ± 0.34	4.50 ± 0.42	.180	3 × 10⁻⁶
Uric acid [μmol/l]	362.30 ± 30.26	398.90 ± 18.33	.050	389.38 ± 39.27	362.75 ± 23.89	.120	373.53 ± 17.99	330.11 ± 12.96	.130	.035
Cholesterol [mmol/l]	4.81 ± 0.35	4.46 ± 0.21	.121	4.87 ± 0.44	4.17 ± 0.41	.040	5.38 ± 0.26	4.55 ± 0.31	.012	.067
HDL-c [mmol/l]	0.98 ± 0.07	0.92 ± 0.04	.138	1.11 ± 0.10	1.02 ± 0.06	.578	1.21 ± 0.08	1.06 ± 0.05	.012	.206
LDL-c [mmol/l]	2.87 ± 0.30	2.50 ± 0.25	.052	3.09 ± 0.38	2.58 ± 0.38	.033	3.37 ± 0.25	2.97 ± 0.24	3 × 10⁻⁴	.463
Triglyceride [mmol/l]	2.11 ± 0.22	2.31 ± 0.28	.515	1.48 ± 0.11	1.23 ± 0.08	.077	2.12 ± 0.27	1.29 ± 0.15	.043	.029
NEFA [mmol/l]	0.60 ± 0.06	0.72 ± 0.08	.214	0.69 ± 0.06	0.69 ± 0.09	.956	0.77 ± 0.05	0.84 ± 0.06	.491	.404
Glucose [mmol/l]	7.94 ± 1.04	6.24 ± 0.49	.002	6.39 ± 1.19	6.17 ± 0.28	.734	5.80 ± 0.14	5.60 ± 0.17	.092	.018
Insulin [mU/l]	32.75 ± 6.47	15.53 ± 1.94	4.25 × 10⁻⁴	21.08 ± 3.23	13.36 ± 2.09	.042	15.91 ± 1.83	9.64 ± 1.07	.010	.470
HOMA-IR	11.66 ± 2.41	4.33 ± 0.63	.004	6.10 ± 1.06	3.67 ± 0.58	.081	4.07 ± 0.44	2.25 ± 0.36	.028	.022
HbA _{1c} [%]	6.34 ± 0.62	6.42 ± 0.56	.683	5.60 ± 0.07	5.43 ± 0.06	.116	5.87 ± 0.11	5.45 ± 0.14	.131	.125

Note: Serum parameters were measured in the fasted state in serum collected immediately after the dietary intervention. Values are presented as means ± SEM.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; GGT, γ-glutamyl transpeptidase; HbA_{1c}, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; HP, high-protein diet; LDL-c, low-density lipoprotein cholesterol; LP, low-protein diet; NEFA, non-esterified fatty acid.

P value (Δ Change); the comparison of dietary -induced changes among the LP, HP, and RP groups. Bold font shows significant P-values (P < .05).

Triglyceride Determination Kit (Sigma Aldrich Chemie) and the absorbance changes were detected at 540 nm by spectrophotometry. Citrate synthase (CS) activity analysis was completed by adding 10 μL of 1:5 diluted supernatant, 215 μL reaction buffer, 25 μL acetyl CoA (3.6 mM) and 50 μL oxaloacetate (3 mM) in each well of the 96-well plates and the absorbance changes were monitored at 412 nm for 7 minutes by spectrophotometry.

2.6 | Hepatic mitochondrial respiration analysis

Mitochondrial respiration analysis was performed in the homogenized liver using the high-resolution respirometry OROBOROS O2k-Core (Oroboros Instruments) at 37°C in a hyperoxygenated environment. Substrates were added according to a multiple substrate-uncoupler-inhibitor titration (SUIT) protocol and shown in the online supplemental methods.²⁶⁻²⁸

2.7 | Power calculation and statistical analysis

Power calculation was completed using the G-Power software v.3.1 for the primary end point Δ liver fat content. Power calculation was based on the difference of liver fat after 6 weeks of the high-protein diet observed in [ref. 3] where the mean reduction in the liver fat content was -7.0% with standard deviation 5.4%. For the paired parametric design and $\alpha < 0.05$, the calculated sample size was seven subjects in each treatment group for statistical power of 80%, and six subjects in each treatment group for statistical power of 70%.

Statistical analysis was performed via SPSS Statistics (IBM) and figures were plotted in GraphPad Prism (GraphPad Prism Inc). Data are reported as mean \pm standard error of the mean (SEM). T test and Mann-Whitney-U test were used to compare two groups, while ANOVA with subsequent post-hoc analysis (LSD test) was applied to compare more than two groups. Correlation analysis was performed through Pearson's coefficient or Spearman's rank correlation coefficient. Statistical significance was defined as $P < .05$.

3 | RESULTS

3.1 | Clinical characteristics of subjects

In cohort I, 19 subjects completed LP (age of 47 ± 9 years, BMI at baseline of $46.4 \pm 3.4 \text{ kg/m}^2$) or HP (age of 49 ± 9 years, BMI at baseline of $44.2 \pm 1.3 \text{ kg/m}^2$) dietary intervention. In cohort II, 10 subjects, who consumed a moderately high-protein diet and did not undergo bariatric surgery, was selected as a reference protein group (RP, age of 56 ± 2 years, BMI at baseline of $38.5 \pm 1.7 \text{ kg/m}^2$).

After 3 weeks of hypocaloric diets, BMI decreased significantly and similarly in all groups (LP: -1.7 kg/m^2 , $P_{LP} = .001$; HP: -1.5 kg/

m^2 , $P_{HP} = .001$; RP: -1.9 kg/m^2 , $P_{RP} = 3.5 \times 10^{-5}$) as did weight (LP: -5.3 kg , $P_{LP} = 4.43 \times 10^{-4}$; HP: -4.7 kg , $P_{HP} = 2.36 \times 10^{-4}$; RP: -5.3 kg , $P_{RP} = 3.6 \times 10^{-5}$). The changes in BMI among the three groups showed no significant differences ($P_{LPvs,HPvs,RP} = .324$) (Table 1). At the day of surgery, body weight remained decreased in comparison with baseline (LP: -4.2 kg , $P_{LP} = .004$; HP: -4.4 kg , $P_{HP} = .001$).

After 3 weeks of diets, serum γ -glutamyl transferase (GGT) decreased significantly in the LP and RP group, but increased significantly in the HP group (Table 1). The levels of serum urea, as a marker for dietary protein intake, decreased dramatically after the intervention in the LP group ($P_{LP} = 1.63 \times 10^{-4}$), remained unchanged in the RP group and increased significantly in the HP group ($P_{HP} = 0.031$), indicating a good compliance to the dietary intervention (Table 1).

After interventions, serum levels of total, HDL-, LDL-cholesterol and triglyceride decreased in the RP group as well as total and LDL-cholesterol in the HP group ($P_{HP} = .040$ and $P_{HP} = .052$ respectively) (Table 1). Fasting glucose decreased significantly only in the LP group ($P_{LP} = .002$), while the mean fasting serum insulin and HOMA-IR, as a marker of insulin sensitivity, decreased by about 50% in all groups (insulin: $P_{LP} = 4.25 \times 10^{-4}$; $P_{HP} = .042$; $P_{RP} = .010$; HOMA-IR: $P_{LP} = .004$; $P_{HP} = .081$; $P_{RP} = .028$) (Table 1).

3.2 | Intrahepatic lipid and hepatic triglyceride content

In order to determine IHL levels at the beginning of the intervention and 3 weeks later, $^1\text{H-MRS}$ analyses were performed. 21 subjects (6 in the LP group, 5 in the HP group, and 10 in the RP group) completed $^1\text{H-MRS}$ at baseline and after 3 weeks. IHL levels of all these 21 participants before the intervention were above the threshold for NAFLD definition of 5.56% by Szczepaniak et al.²⁹ Baseline levels of IHL in the LP group were higher than in the HP group ($P_{LPvs,HP} = .041$), although both groups had similar baseline BMI ($P_{LPvs,HP} = .387$).

There were strong reductions in IHL by 42.6% in the HP group ($P_{HP} = .014$) and by 36.7% in the RP group ($P_{RP} = 2.45 \times 10^{-4}$). By contrast, no significant reduction in IHL in the LP group ($P_{LP} = .144$) was observed. The differences in the changes in IHL levels between the LP and HP groups, and between the LP and RP groups were significant ($P_{LPvs,HP} = .001$; $P_{LPvs,RP} = .001$) (Figure 1A). However, these 21 participants similarly and significantly reduced their BMI (LP: -4.4% , $P_{LP} = .028$; HP: -4.6% , $P_{HP} = .001$; RP: -4.9% , $P_{RP} = 3.5 \times 10^{-5}$), without difference between the three groups ($P_{LPvs,HPvs,RP} = .895$) (Figure 1B). In addition, there was no correlation between changes of IHL and changes of BMI ($r = .212$, $P = .356$), fat mass ($r = .273$, $P = .417$), fat-free mass ($r = -.173$, $P = .612$) and HOMA-IR ($r = .033$, $P = .900$). Figure 1C moreover shows the impressively greater reductions of IHL in virtually all patients in the HP group as compared to the LP group.

The hepatic triglyceride levels in liver biopsies, which are shown in Figure 1D, were approximately twofold higher in the LP than in the HP group ($P_{LPvs,HP} = 1.09 \times 10^{-4}$) and highly correlated with the

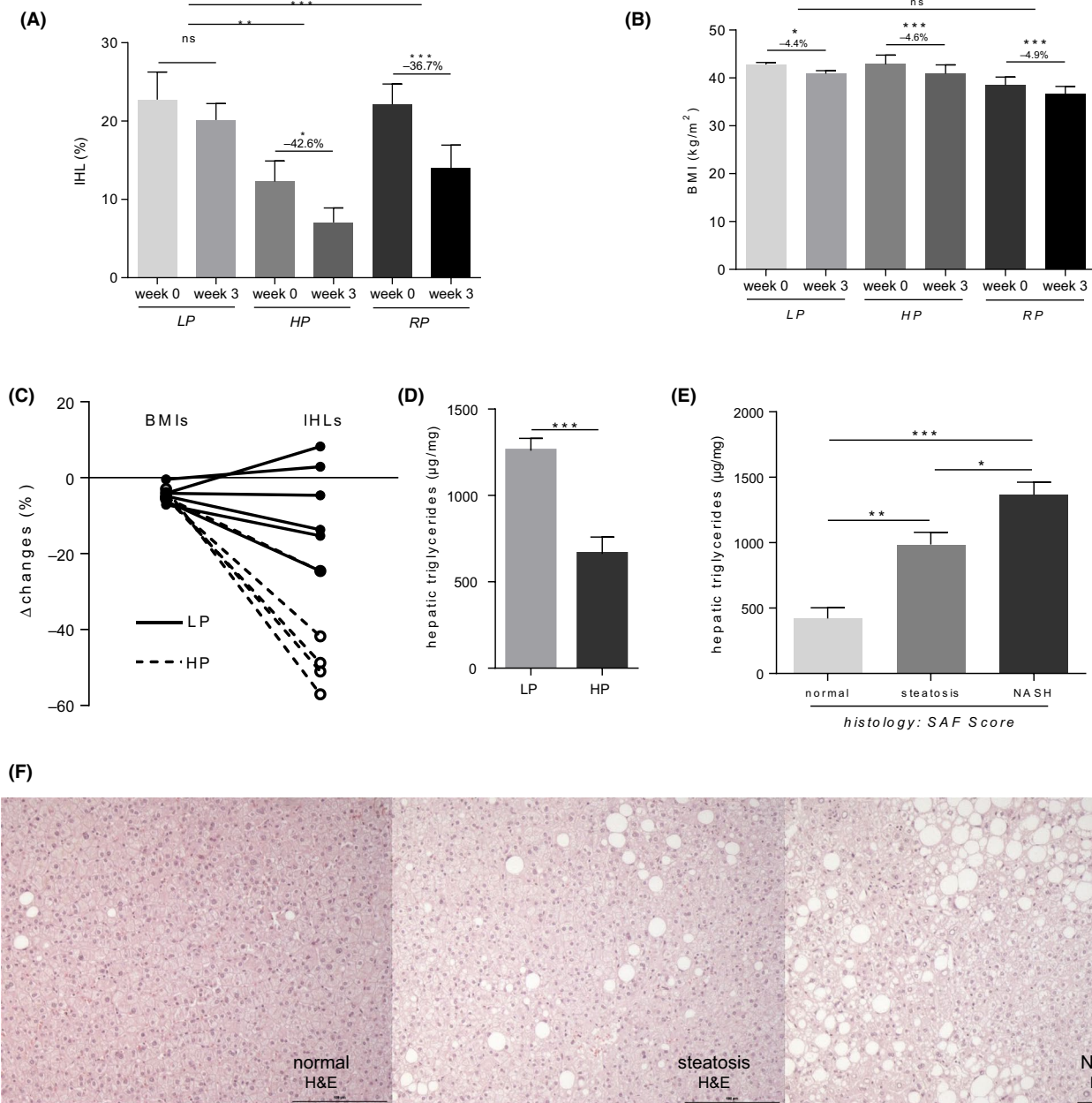


FIGURE 1 IHL reduced effectively in the HP group after the dietary intervention; high correlation between hepatic triglycerides and histology. (A) IHL at the baseline and after 3-week dietary intervention in the LP, HP and RP groups by ¹H-MRS. $n_{LP} = 6$, $n_{HP} = 5$, $n_{RP} = 10$. (B) Body mass index at the baseline and after 3-week dietary intervention in the LP, HP and RP groups. $n_{LP} = 6$, $n_{HP} = 5$, $n_{RP} = 10$. (C) Δ BMI change and Δ IHL change. $n_{LP} = 6$, $n_{HP} = 5$. (D) Liver triglyceride levels in the LP and HP groups (quantified in liver samples and normalized to protein concentration). $n_{LP} = 10$, $n_{HP} = 9$. (E) Hepatic triglyceride content in different stages of NAFLD by histology (scored by SAF). $n_{LP} = 10$, $n_{HP} = 9$. (F) Microscopy images of liver biopsies of three patients as examples in different stages of NAFLD or hepatic triglyceride content after H&E stain (scored by SAF). Abbreviations: BMI, body mass index; HP, high-protein diet; IHL, intrahepatic lipid; LP, low-protein diet; NASH, non-alcoholic steatohepatitis; SAF, steatosis-activity-fibrosis; TG, triglyceride. * $P < .05$, ** $P < .01$, *** $P < .001$. ns, not significant ($P > .05$). Data are presented as mean \pm SEM

IHL contents detected via ¹H-MRS after the 3-week intervention ($r = .790$, $P = .004$), indicating that both methods for determining liver fat content should be considered as correct and reliable (Table 2). Hepatic triglyceride levels were significantly different between "normal", "steatosis" and "NASH" groups divided according to the SAF score ($P_{\text{normal vs. steatosis vs. NASH}} = .002$) being the lowest in the "normal" group, and highest in the "NASH" group (Figure 1F-E).

3.3 | LC3B II protein levels in autophagy flux

As the autophagy was shown to essentially contribute to the regulation of hepatic lipid metabolism and intracellular lipid stores,^{13,14} we investigated dynamic indicators of autophagy (LC3B II flux) upon HP and LP diets. For this, fresh liver tissue was obtained during bariatric surgery and incubated with or without lysosomal proteolytic inhibitors

TABLE 2 Post-diet correlation analysis of autophagy, liver fat content, citrate synthase (CS) activity and fibroblast growth factor 21 (FGF21)

	Liver			Serum	Hepatic gene
	Autophagy flux	Triglycerides	IHL	CS activity	FGF21
Triglycerides					
<i>r</i>	.764				
<i>P</i> value	.002				
IHL					
<i>r</i>	.786	.790			
<i>P</i> value	.036	.004			
CS activity					
<i>r</i>	.357	.411	.650		
<i>P</i> value	.231	.080	.030		
Serum FGF21					
<i>r</i>	.615	.606	.919	.106	
<i>P</i> value	.025	.006	6.5×10^{-5}	.667	
Hepatic FGF21					
<i>r</i>	.555	.511	.828	-.270	.709
<i>P</i> value	.049	.025	.002	.263	.001

Bold font shows significant *P*-values ($P < .05$).

Abbreviations: CS, citrate synthase; FGF21, fibroblast growth factor 21; IHL, intrahepatic lipid.

(bafilomycin and leupeptin) in ex vivo experiments. Subsequently, LC3B II protein levels were assessed via Western blotting.

Ratios of LC3B II levels with/without proteolytic inhibitors were used for the assessment of autophagy flux. Incubation with lysosomal proteolytic inhibitors induced a significant increase in LC3B II (the lipidated form of LC3) protein levels by 66.7% in the LP group compared to the incubation without the inhibitors ($P_{LP} = .038$). The average ratio with/without inhibitors was 1.83 for the LP group and 0.97 for the HP group. However, in the HP group, incubation with lysosomal proteolytic inhibitors had no effect on LC3B II (-2.8% , $P_{HP} = .322$), resulting in a marked difference between the LP and HP groups ($P_{LPvs,HP} = .029$; $P_{adj} = .056$ after the adjustment for the baseline IHL) (Figure 2 A, B).

In order to gain further insight into the metabolic mechanism associated with autophagy, bivariate correlation analyses were performed. Autophagy flux was significantly correlated with IHL ($r = .786$, $P = .036$), hepatic triglycerides ($r = .764$, $P = .002$), serum FGF21 ($r = .615$, $P = .025$), hepatic gene expression of FGF21 ($r = .555$, $P = .049$) (Table 2) and ATF4 ($r = .621$, $P = .024$). However, there was no significant correlation between autophagy flux and expression of autophagy related genes (LC3A, LC3B, and ATF5).

3.4 | Serum FGF21 and expression levels of hepatic FGF21 pathway related genes

FGF21 is a metabolic hormone which is strongly regulated by protein restriction and autophagy and also involved in endoplasmic

reticulum (ER) stress upon liver fat accumulation.^{15,16} To investigate the role of FGF21 in the effects of dietary protein on the liver fat, gene expression levels of FGF21, fibroblast growth factor receptor 1 (FGFR1), fibroblast growth factor receptor 2 (FGFR2) and klotho beta (KLB) were measured after the dietary intervention in the liver samples.

During the dietary intervention, serum FGF21 levels were not significantly changed in the HP group ($P_{HP} = .220$) and increased in the LP group by 42.2% ($P_{LP} = .047$). The difference in the FGF21 changes between the LP and HP groups was significant ($P_{LPvs,HP} = .005$) (Figure 2C). Post-intervention, hepatic gene expression levels of FGF21, FGFR1, FGFR2 and KLB were higher in the LP group compared to the HP group (FGF21: $P_{LPvs,HP} = .008$; FGFR1: $P_{LPvs,HP} = .007$; FGFR2: $P_{LPvs,HP} = .005$; KLB: $P_{LPvs,HP} = .049$) (Figure 2D). In addition, there was a positive correlation between serum FGF21 and hepatic FGF21 gene expression levels ($r = .709$, $P = .001$) (Table 2).

3.5 | Hepatic gene expression levels

We further investigated expression levels of genes involved in autophagy, ER-stress, fat uptake, fatty acid β -oxidation, de novo lipogenesis and lipid storage – processes involved in the regulation of liver fat accumulation – in the LP and HP groups. Expression levels of LC3A, ATF5, BiP, XBP1s, XBP1, DDIT3, LPL, ChREBP, FASN, SREBP1c, ACC1, ACC2, AMPK γ 1 and SCD1 were higher in the LP group than those in

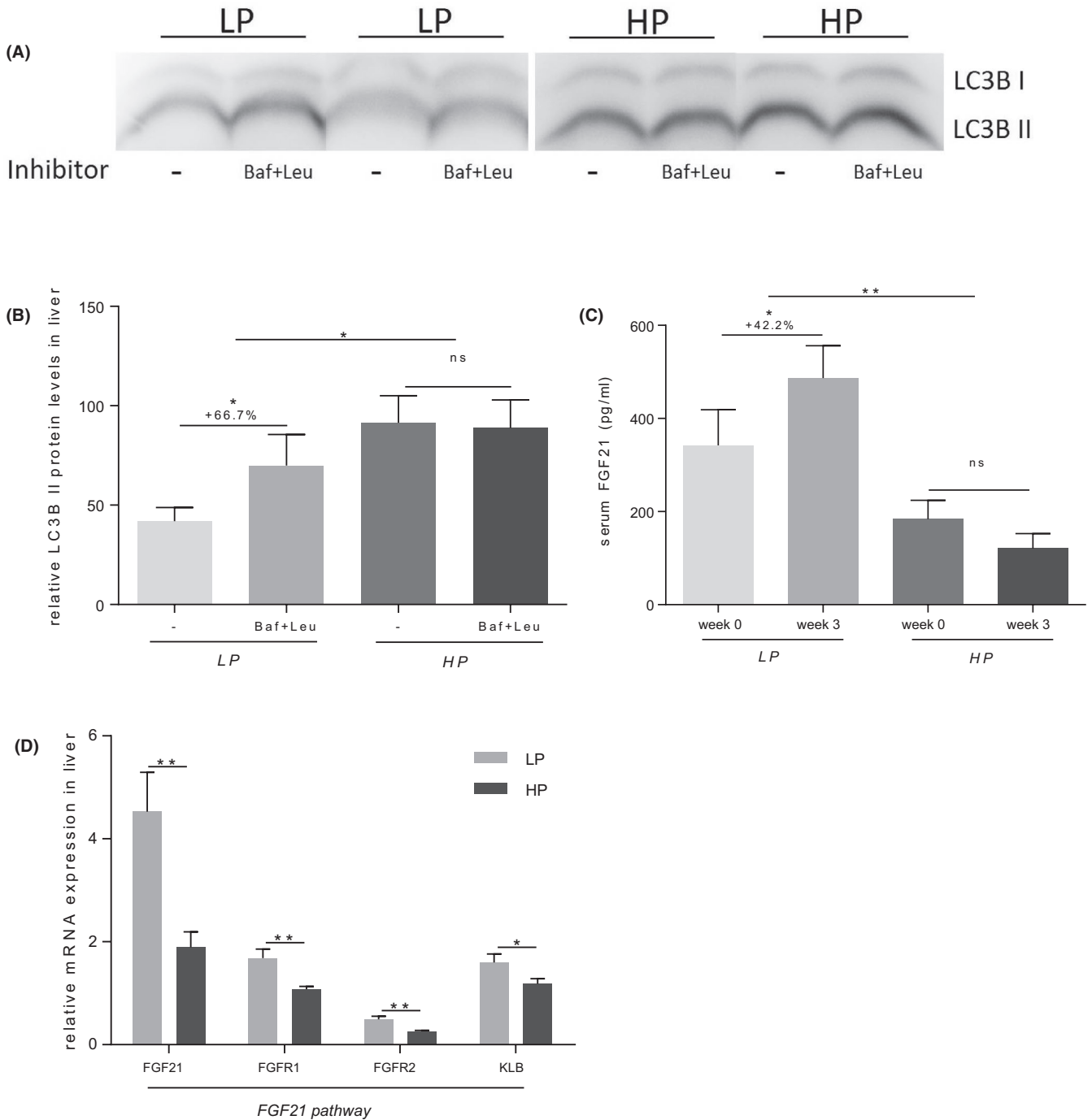


FIGURE 2 Autophagy flux and FGF21 increased significantly in the LP group after the dietary intervention. (A-B) Fold changes of LC3 II levels (in the absence and the presence of autophagy inhibitor) for dynamic autophagy flux. $n_{LP} = 7$, $n_{HP} = 6$. (C) Serum FGF21 at the baseline and 3-week dietary intervention in the LP and HP groups by ELISA. $n_{LP} = 10$, $n_{HP} = 7$. (D) Expression levels of FGF21 pathway (FGF21, FGFR1, FGFR2 and KLB) related genes via qRT-PCR (normalized to the geometric mean of HPRT and RPLP0). $n_{LP} = 10$, $n_{HP} = 9$. Abbreviations: Baf, bafilomycin A1; FGF21, fibroblast growth factor 21; FGFR1, fibroblast growth factor receptor 1; FGFR2, fibroblast growth factor receptor 2; HP, high-protein diet; KLB, klotho beta; Leu, leupeptin; LC3B, microtubule-associated protein 1B-light chain 3; LP, low-protein diet. * $P < .05$, ** $P < .01$, *** $P < .001$. ns, not significant ($P > .05$). Data are presented as mean \pm SEM

the HP group (Figure 3 A, B, Table S5). Expression of genes involved in fatty acid β -oxidation was not different between groups (Figure 3 A, B).

The expression of autophagy-related genes was positively correlated with that of ER-stress-related genes (Table 3).

Furthermore, *ATF4* gene expression was positively correlated with autophagy flux ($r = .621$, $P = .024$). Moreover, hepatic triglyceride content was significantly correlated with the gene expression levels of *LC3A*, *ATG5*, *BiP*, *XBP1s*, *XBP1*, *ATF4*, *DDIT3* and *FGF21* (Table 3).

3.6 | RNA-seq-based pathway analysis

To gain further insight into the molecular mechanisms involved in dietary protein induced changes in liver, RNA-seq was performed ($n = 10$ in the LP group and $n = 9$ in the HP group). Differential expression analyses showed that 66 genes were higher and 70 genes were lower expressed in the HP group compared with the LP group from a total of 22 273 detected transcripts. GO analyses revealed a range of changed pathways (Table S6) including pathways linked to hepatic amino acid biosynthesis and amine metabolism which were enriched in the HP group (Table 4). Moreover, transcript coding argininosuccinate synthetase (ASS1), an important enzyme in the urea cycle, was expectedly up-regulated upon HP diet (Table S6). Furthermore, pathways involved in glucose import and glycogen biosynthesis in the liver were enriched in the HP group compared with the LP group (Table 4). In contrast, in the LP group, genes involved in triglyceride hydrolysis (*LPL*, *FABP4*, *FABP5*) were up-regulated compared with HP group (Table 4). Moreover, in the LP group, pathways involved in inflammation, apoptosis, chemotaxis, I- κ B/NF- κ B signalling, ERK1/ERK2 cascade showed higher activity and several markers of liver fibrosis (*COL1A2*, *COL1A1*) were expressed higher (Table 4). PCR analysis confirmed that the proinflammatory cytokine *MCP1* and the M1 macrophage marker *ITGAX* (*CD11c*) were expressed higher upon LP diet (*MCP1*: $P_{LPvs,HP} = .015$; *ITGAX*: $P_{LPvs,HP} = .043$) (Figure 3C). Expression of fibrosis markers α SMA, *COL1A1*, *COL3A1*, *COL6A1* and *TGF β 1* showed no significant between-group differences (data not shown).

3.7 | Hepatic mitochondrial and citrate synthase activity

We further hypothesized that HP diet reduced liver fat by triggering mitochondrial activity. In order to investigate the influence of dietary protein on the mitochondria of the liver, mitochondrial respiration analyses in fresh liver samples and citrate synthase (CS) activities were determined ($n = 5$ in the LP group and $n = 5$ in the HP group). Hepatic mitochondrial activity was normalized by CS activity as a marker of mitochondrial mass.³⁰

There were significant differences of CS activity between "normal", "steatosis" and "NASH" groups ($P_{\text{"normal"vs."steatosis"vs."NASH"}} = .030$). "NASH" group had the highest CS activity and was significantly different from the "steatosis" and the "normal" group ($P_{\text{"normal"vs."NASH"}} = .045$; $P_{\text{"steatosis"vs."NASH"}} = .014$). Additionally, the OXPHOS respiration and the maximal uncoupled respiration (ETS_{max}) were significantly lower in the "NASH" compared to the "normal" group (OXPHOS: $P_{\text{"normal"vs."NASH"}} = .050$; ETS_{max}: $P_{\text{"normal"vs."NASH"}} = .040$) (Figure 4 A-C).

The CS activity, however, was not influenced by protein intake ($P_{LPvs,HP} = .735$). Mitochondrial activity in the HP group was not significantly different from that in the LP group although it showed a tendency to increase in all the respiration processes

in the HP group (Figure 4 D, E). However, there were strong correlations between hepatic CS activity and IHL content ($r = .963$, $P = .002$) (Table 2), and between ETS_{max} respiration and IHL content ($r = -.906$, $P = .013$).

4 | DISCUSSION

The most important finding of our study is that, compared to LP intake, the 3-week HP diet more effectively reduced IHL in morbidly obese patients which was the primary outcome in this study. Both strategies were differentially accompanied by metabolic improvements of secondary outcomes - serum transaminases, lipid, uric acid, glucose and insulin levels. The HP strategy additionally showed a decrease in oxidative stress conditions. Furthermore, our study showed that different mechanisms are involved in metabolic improvements induced by HP and LP diets. Indeed, FGF21 and autophagy, which have been associated with metabolic improvements, increased only in the LP but not in the HP diets. Contrary with expectations, the mitochondrial activity and expression levels of genes involved in fatty acid β -oxidation were not increased in the HP compared to LP diets. Interestingly, however, genes related to lipid biosynthesis displayed lower expression levels in the condition of HP diets.

To our knowledge, this is the first human study investigating whether HP or LP diets are more effective for liver fat reduction in patients with morbid obesity and NAFLD indication undergoing bariatric surgery. Although all groups, LP, HP and RP, reduced weight and BMI because of the moderate caloric restriction, the HP group was more effective in eliminating the intrahepatocellular lipids than the LP group. Two participants in the LP group even slightly gained liver fat despite weight loss. Remarkably, there was no correlation between weight (or BMI) loss and reduction of liver fat. Moreover, although the LP and RP groups had similar baseline liver fat, the liver fat reduction was more effective in the RP group, suggesting that even a moderate increase of the protein intake up to 20-22 EN% has a stronger effect on liver fat reduction than LP intake. Similar results were published in other groups of subjects. Martens et al reported IHL reduction after isocaloric HP diet compared to LP diet.³¹ However, the study was conducted in healthy subjects with BMI of 24 kg/m² and IHL below 0.4% at baseline. Similarly, Drummen et al observed a significant decrease in IHL in obese participants after 8 weeks of hypocaloric HP diet (35-40 E% protein) and 20 weeks of hypocaloric HP diet.³² Important result of our study is that we demonstrated the strong reduction in liver fat already after a short-term (3-weeks) HP diet. Taken together, we concluded that HP diet reduced liver fat more effectively than LP diet in the presence of moderate weight loss, regardless of the baseline liver fat content.

Hence, the effective reduction of liver fat by HP diets seems to be accomplished by pathways independent of overall fat mass reduction. We hypothesize that HP diet reduced liver fat by triggering mitochondrial activity and fatty acid β -oxidation. It was previously demonstrated that obesity, NAFLD, and insulin resistance are associated with mitochondrial dysfunction.³³⁻³⁶ The lower mitochondrial

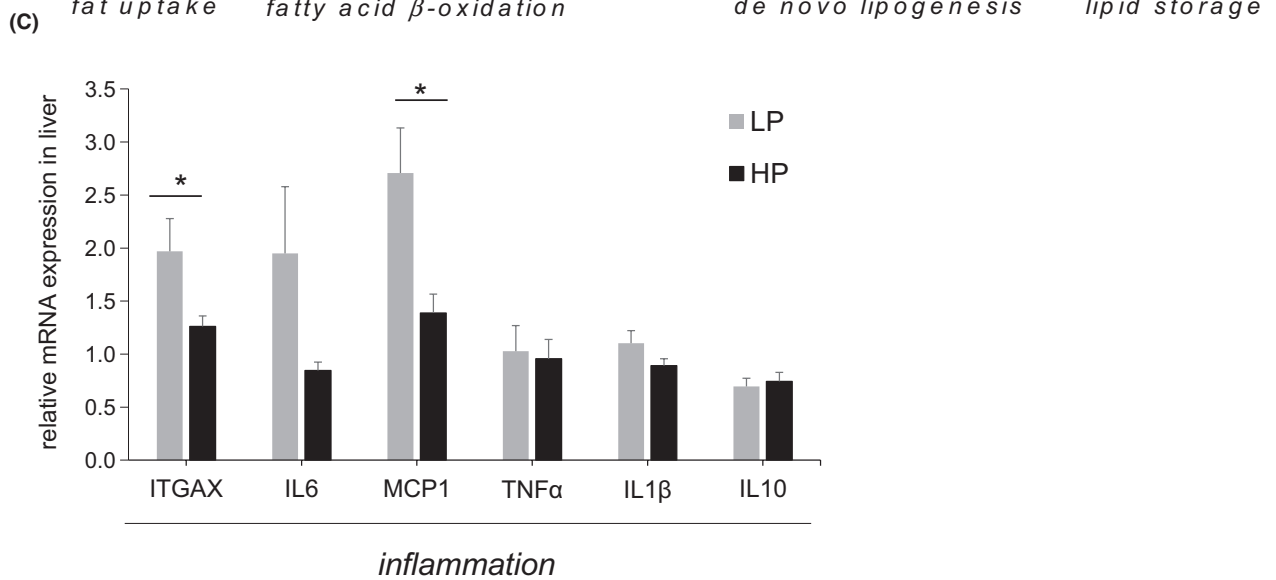
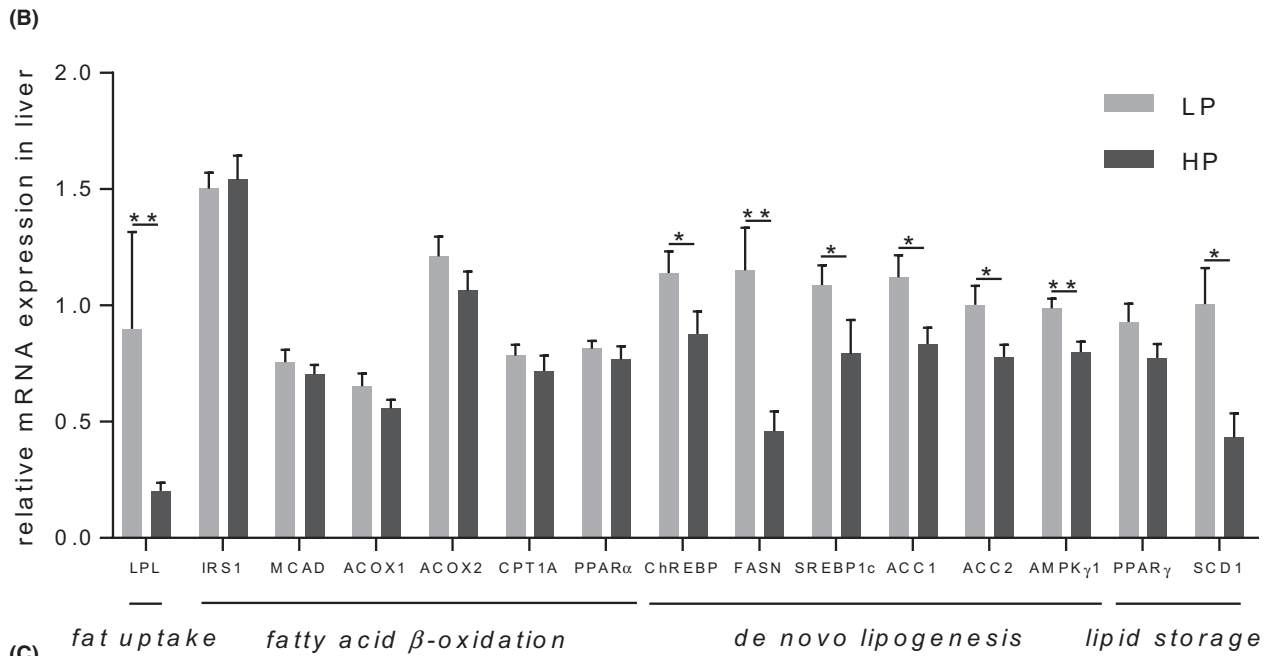
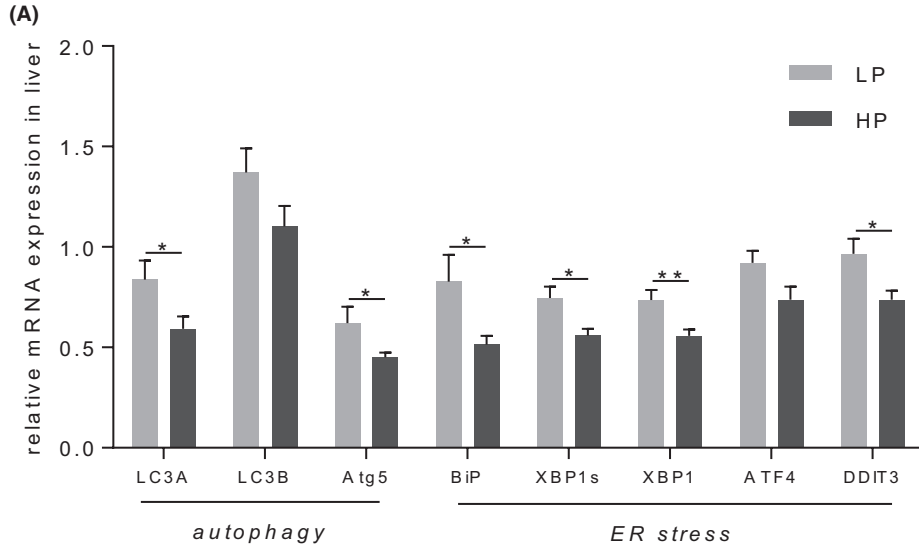


FIGURE 3 Expression of autophagy, ER stress, lipid biosynthesis and inflammatory genes were significantly higher in the LP group. (A) Expression levels of autophagy (LC3A, LC3B and Atg5) and ER stress (BiP, XBP1s, XBP1, ATF4 and DDIT3) related genes via qRT-PCR (normalized to HPRT). (B) Expression levels of fat uptake (LPL), fatty acid β -oxidation (IRS-1, MCAD, ACOX1, ACOX2, CPT1A and PPAR α), de novo lipogenesis (ChREBP, FASN, SREBP1c, ACC1, ACC2 and AMPK γ 1), and lipid storage (PPAR γ and SCD1) related genes via qRT-PCR (normalized to the geometric mean of HPRT and RPLP0). (C) Expression of genes involved in inflammatory pathways (ITGAX, IL6, MCP1, TNF α , IL1B, IL10). $n_{LP} = 10$, $n_{HP} = 9$. Abbreviations: ACC1, acetyl-CoA carboxylase 1; ACC2, acetyl-CoA carboxylase2; ACOX1, acyl-CoA oxidase 1; ACOX2, acyl-CoA oxidase 2; AMPK γ 1, AMP-activated protein kinase γ 1; ATF4, activating transcription factor 4; Atg5, autophagy-related protein 5; BiP, binding of immunoglobulin protein; ChREBP, carbohydrate-responsive element-binding protein; CPT1A, carnitine palmitoyltransferase 1A; DDIT3, DNA damage-inducible transcript protein 3; ER, endoplasmic reticulum; FASN, fatty acid synthase; HP, high-protein diet; IL, interleukin; IRS1, insulin receptor substrate 1; ITGAX, Integrin subunit alpha x; LC3A, microtubule-associated protein 1A-light chain 3; LC3B, microtubule-associated protein 1B-light chain 3; LP, low-protein diet; LPL, lipoprotein lipase; MCAD, medium-chain specific acyl-CoA dehydrogenase; MCP1, monocyte chemoattractant protein 1; PPAR α , peroxisome proliferator activated receptor α ; PPAR γ , peroxisome proliferator activated receptor γ ; SCD1, stearoyl-CoA desaturase 1; SREBP1c, sterol regulatory element-binding protein 1c; TNF α , tumor necrosis factor alpha; XBP1, X-box binding protein 1; XBP1s, spliced X-box binding protein 1. * $P < .05$, ** $P < .01$, *** $P < .001$. ns, not significant ($P > .05$). Data are presented as mean \pm SEM

activity and higher CS activity in NASH patients compared to subjects with normal liver or simple steatosis showed a similar pattern as previously reported by Koliaki et al.³⁷ In addition, CS activity was positively correlated with IHL via ¹H-MRS and NAFLD stages diagnosed by SAF score indicating that CS activity is up-regulated by hepatic fat accumulation and may represent an attempt to counterbalance the increased lipids by increased oxidation. Interestingly, methionine-restriction was shown to increase mitochondrial mass,

size and capacity in liver, muscle and adipose tissue in some rodent studies.³⁸⁻⁴⁰ However, this was not the case in the LP group where methionine intake was very low because of the vegetarian protein source. On the other hand, Garcia-Caraballo et al.⁴¹ found an increased mitochondrial oxidative capacity, but no changes in CS activity by a HP diet, which corresponds to our findings.

Importantly, the expression levels of genes regulating fat uptake, de novo lipogenesis and lipid storage were down-regulated

TABLE 3 Post-diet correlations between autophagy-related and endoplasmic reticulum (ER)-stress-related genes in liver

	Autophagy			ER-stress				
	LC3A	LC3B	Atg5	BiP	XBP1s	XBP1	ATF4	DDIT3
LC3B								
<i>r</i>	.632							
<i>P</i> value	.004							
Atg5								
<i>r</i>	.837	.486						
<i>P</i> value	7.9×10^{-6}	.035						
BiP								
<i>r</i>	.704	.413	.598					
<i>P</i> value	.001	.079	.007					
XBP1s								
<i>r</i>	.822	.545	.575	.606				
<i>P</i> value	1.6×10^{-5}	0.016	.010	.006				
XBP1								
<i>r</i>	.821	.624	.595	.561	.976			
<i>P</i> value	1.7×10^{-5}	.004	.007	.012	1.3×10^{-12}			
ATF4								
<i>r</i>	.638	.652	.533	.475	.814	.771		
<i>P</i> value	.003	.002	.019	.040	2.3×10^{-5}	1.1×10^{-4}		
DDIT3								
<i>r</i>	.717	.464	.470	.679	.882	.840	.801	
<i>P</i> value	.001	.045	.042	.001	5.8×10^{-7}	6.8×10^{-6}	3.8×10^{-5}	

Abbreviations: ATF4: activating transcription factor 4; Atg5: autophagy-related protein 5; BiP: binding of immunoglobulin protein; DDIT3: DNA damage-inducible transcript protein 3; ER: endoplasmic reticulum; HP: high-protein diet; LC3A: microtubule-associated protein 1A-light chain 3; LC3B: microtubule-associated protein 1B-light chain 3; LP: low-protein diet; XBP1: X-box binding protein 1; XBP1s: spliced X-box binding protein 1. Bold font shows significant *p*-values ($P < .05$).

TABLE 4 Affected GO pathways related to the metabolic regulation

Category	Term	P value	Count
Higher in the HP vs. LP group			
GO:0008652	Cellular amino acid biosynthetic process	2.7×10^{-5}	4
GO:0046326	Positive regulation of glucose import	0.002	3
GO:0009308	Amine metabolic process	0.018	2
GO:0045725	Positive regulation of glycogen biosynthetic process	0.033	2
Lower in the HP vs. LP group			
GO:0030199	Collagen fibril organization	2.8×10^{-4}	4
GO:0070374	Positive regulation of ERK1 and ERK2 cascade	0.003	5
GO:0019433	Triglyceride catabolic process	0.003	3
GO:0050900	Leukocyte migration	0.007	4
GO:0043123	Positive regulation of I- κ B kinase/NF- κ B signaling	0.016	4
GO:0050776	Regulation of immune response	0.021	4
GO:0030198	Extracellular matrix organization	0.026	4
GO:2000406	Positive regulation of T cell migration	0.032	2
GO:0006954	Inflammatory response	0.035	5
GO:0050921	Positive regulation of chemotaxis	0.039	2
GO:0097284	Hepatocyte apoptotic process	0.042	2

Note: $n_{LP} = 10$, $n_{HP} = 9$.

by the HP diet as confirmed by RNA-seq data for LPL, FABP4 and FABP5. LPL, which was down-regulated upon HP intake, can hydrolyse chylomicron triglycerides to NEFAs and promote hepatocellular uptake of chylomicron remnants and NEFAs.⁴² FABP4 and FABP5 can bind and promote uptake of fatty acids as lipid transport proteins. Zhu et al⁴³ found that NASH patients had much higher expression of lipid transport proteins such as CD36 and FABP1 and concluded that an increase in fatty acid uptake contributes more to fat deposition than changes in de novo lipogenesis or fatty acid β -oxidation.

As the main source of de novo lipogenesis, dietary carbohydrates are widely accepted to up-regulate hepatic lipogenesis. Interestingly, previous studies revealed that dietary protein also played a role in the regulation of lipid biosynthesis. Expression levels of SREBP1c,^{44,45} FASN,^{18,46} ACC,^{18,46} SCD1⁴⁵ and PPAR γ ,¹⁸ involved in de novo lipogenesis and lipid storage, were down-regulated by HP intake in several rodent studies. The diminished lipogenesis could be related to HP diet stimulated glucagon secretion but lower glycemic action,⁴⁷ but also to the function of dietary ketogenic essential amino acids.⁴⁵ The relatively lower carbohydrate content in HP diets might have also partly contributed to the larger decrease in de novo lipogenesis and intrahepatic fat. Also, in contrast with lipogenesis genes, dietary protein intake did not change the expression of genes involved in lipid oxidation or the substrate oxidation estimated by indirect calorimetry in several rat studies.^{44,46} In our study, no difference in gene expression levels involved in fatty acid β -oxidation between the LP and HP diets was observed. Stepien et al⁴⁶ attributed the unchanged lipid oxidation to a phenomenon observed by starvation, since the gene expression

of enzymes involved in lipid oxidation changed more slowly than those involved in lipogenesis.⁴⁸ Additionally, Zhu et al⁴³ showed that the increased hepatic fat uptake and de novo lipogenesis might be the most common cause for the formation of steatosis in NASH patients. Then, HP diets might reverse steatosis through the suppression of these two processes.

Previous studies showed that LP diets promote autophagy by a GCN2-mediated stress response to the deficiency of amino acids. Also, 'lipophagy' as a potential player of autophagy in lipid metabolism has been described in several papers.^{13,49} Originally, we hypothesized that autophagy could help to eliminate the intrahepatocellular lipids in LP diets by supporting lipophagy. From the dynamic analyses of autophagy flux after 3 weeks of intervention, we confirmed that the LP group displayed significantly elevated autophagy flux, while autophagy flux in the HP group was unchanged. We conclude that, although LP diets increased autophagy levels, the ability to eliminate liver fat did not increase correspondingly. Some papers reported that autophagy is involved in the formation and growth of lipid droplets.¹⁴ How autophagy keeps a balance between 'lipophagy' or the genesis of lipid droplets and the final effect of autophagy on hepatic lipid metabolism is still not known. In order to gain further insight into the molecular mechanism associated with autophagy, correlation analyses were performed. Expression of autophagy-related genes was positively correlated with ER-stress-related genes, and autophagy flux was positively correlated with ATF4 gene expression, which is in line with the fact that the ATF4 pathway can promote stress-induced autophagy gene expression.⁵⁰⁻⁵² However, we found no significant correlation between autophagy flux and autophagy-related gene expression which might be explained by the fact

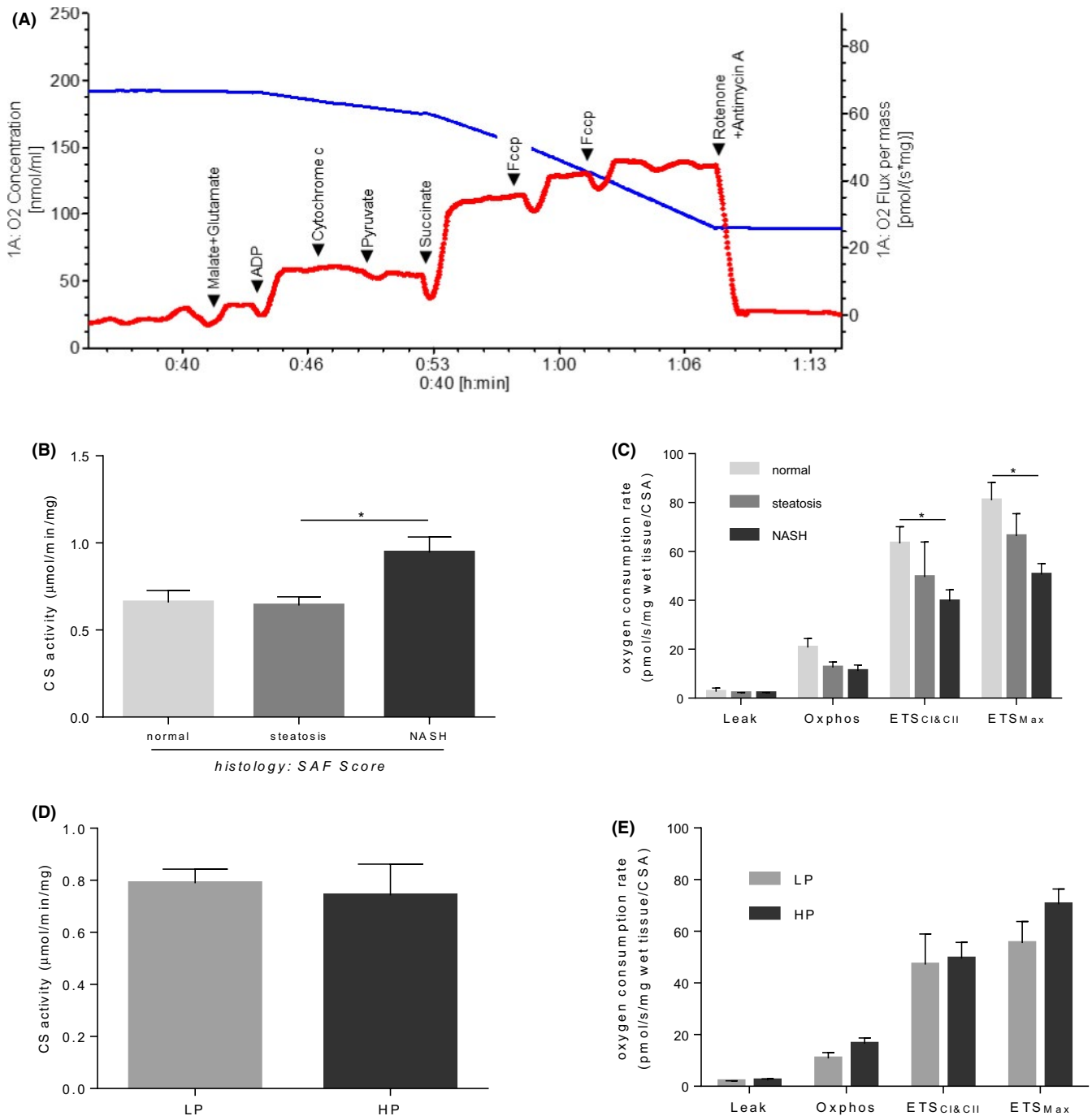


FIGURE 4 Liver mitochondrial and citrate synthase activity. (A) Representative trace of oxygen (O₂) concentration (blue line) and O₂ flux (red line) during substrate-uncoupler-inhibitor-titration (SUIT) protocol for mitochondrial respiratory capacity in human liver biopsies: Malate + Glutamate (Leak respiration), ADP (OXPHOS capacity), Cytochrome c (Integrity of outer mitochondrial-membrane), Pyruvate & Succinate (ETSCI&CII), FCCP (ETS maximum capacity), Rotenone + Antimycin A (less than 2% residual oxygen consumption, ROX). (B) Hepatic CS activity in different stages of NAFLD by histology (histology scored by SAF; normalized to protein concentration). $n_{LP} = 5$, $n_{HP} = 5$. (C) Hepatic mitochondrial activity in different stages of NAFLD by histology (histology scored by SAF; normalized to CS activity). $n_{LP} = 5$, $n_{HP} = 5$. (D) Hepatic CS activity in the LP and HP groups (normalized to protein concentration). $n_{LP} = 5$, $n_{HP} = 5$. (E) Hepatic mitochondrial activity in the LP and HP groups (normalized to protein concentration). $n_{LP} = 5$, $n_{HP} = 5$. Abbreviations: CS, citrate synthase; ETS, mitochondrial electron transport system; HP, high-protein diet; LP, low-protein diet; NASH, non-alcoholic steatohepatitis; Oxphos, oxidative phosphorylation; SAF, steatosis-activity-fibrosis; TG, triglyceride. * $P < .05$, ** $P < .01$, *** $P < .001$. ns, not significant ($P > .05$). Data are presented as mean \pm SEM

that the dynamic levels of autophagy flux (LC3B II) are correlated with the function of autophagosomes rather than the number.⁵³ In addition, hepatic autophagy flux was positively correlated with

circulating FGF21 and hepatic FGF21 gene expression levels. Zhu et al¹¹ proposed that FGF21, which is potently induced by ER-stress, may up-regulate autophagy.



Our data show a lower post-diet level of ER-stress in the HP group which may be related to the stronger reduction of liver fat. However, this is difficult to assess in our study cohort because liver fat in the HP group was lower at the onset of the study. Therefore, this group may have started out with lower levels of ER-stress. However, the correlation of ER-stress markers and hepatic triglycerides persisted in the HP group indicating that the protein induced reduction in liver fat also reduced hepatic ER-stress. This is also supported by the trend towards a reduction in serum FGF21 in the course of the study. Although the FGF21 decrease upon the HP diet did not achieve the statistical significance in the current study, possibly because of the relatively low subject number, it was clearly demonstrated in our previous study in 37 subjects upon diets rich in both animal or plant proteins.⁵⁴ The reduction of protein intake may have added the stress induced by amino acid deficiency on to the preexisting ER-stress induced by obesity and hepatic steatosis and thereby may have reduced the efficiency of liver fat reduction.

The RNA-seq analysis of liver samples revealed a range of pathways which are differently regulated upon HP and LP intake and could affect NAFLD pathogenesis. As expected, GO analyses revealed that pathways linked to hepatic amino acid biosynthesis and amine metabolism were enriched in the HP group. Moreover, transcript coding ASS1, an important enzyme in the urea cycle, was expectedly up-regulated upon HP diet. We also found a range of additional pathways differently regulated upon HP and LP intake. Particularly, we found that inflammatory pathways (involved in leukocyte migration, chemotaxis, I- κ B/NF- κ B signaling, and ERK1/ERK2 cascade) showed lower activity upon HP diet which was confirmed by qPCR data of the expression of proinflammatory markers MCP1 and ITGAX in the liver. These data agree with our previous observation that HP diet moderately reduces proinflammatory cytokine levels in the circulation of subjects with type 2 diabetes.³ Interestingly, inflammasome NLRP6, which expression was also affected by the protein intake, is regulated by microbial-derived metabolites and represents a promising cross-point between innate immunity and metabolic control.⁵⁵ Further studies are required to elucidate detailed molecular mechanisms of the regulation of liver inflammation by dietary protein intake.

There are some limitations of the study that should be mentioned. 19 subjects were divided into two different dietary groups prior to the intervention matched by age, gender and BMI. However, as it was mentioned above, individuals in the LP group had higher liver fat content and higher serum FGF21 and GGT levels at baseline. Considering the correlation of autophagy flux with IHL, serum FGF21, hepatic gene expression of FGF21 and ATF4 levels, the unequal liver fat content might impede the interpretation of the effect of dietary intervention. Furthermore, a higher fat accumulation in the LP group may signal a depot more resistant to mobilization upon the dietary intervention. Nevertheless, adjustment of the data on the autophagy flux and gene expression to the baseline hepatic fat content showed a similar tendency as unadjusted data. Four genes remained significantly lower in the HP

group and many others showed a trend ($0.1 > P > .05$) to be lower expressed in the HP group (Table S5). Thus, our analysis confirmed the effect of the dietary protein intake on various mechanisms of the liver fat reduction independent from the baseline hepatic fat content.

The next limitation is the relatively small number of study subjects because of the inclusion criteria prior to a bariatric surgery. Nevertheless, according to the power calculation, our study was powered sufficiently to detect dietary-induced differences of liver fat content.

The next limitation is that protein intake during the intervention was not checked directly (eg by food protocol), so one cannot be sure about the consumed levels. However, study participants obtained food plans given as 10-d rotating menus which were easy to follow. Furthermore, we measured serum urea levels, a well-established marker of protein intake, and found a significant increase in the HP group and a significant decrease in the LP group, from where we could conclude a good compliance to the diets, although we cannot define the exact protein intake achieved. Although a 3-5 days gap was between the dietary intervention and the surgery, patients were asked to continue following the diets and their body weight remained decreased in comparison with baseline without marked regain. Data from the RP group demonstrate that even a moderate increase in protein intake leads to more effective decrease in liver fat compared to the LP group, supporting our findings and interpretation. Future studies in rodents and cell culture are needed to elucidate molecular mechanisms underlying the observed effects of dietary protein intake and the role of FGF21, inflammatory factors and other biomarkers.

In summary, HP diet had stronger effects on liver fat reduction than LP diet, even though LP diet seemed to increase glucose tolerance at least as effectively as HP diet. Although LP diet could dramatically elevate autophagy flux and FGF21 levels in liver and circulation compared to HP diets, there was no evidence that autophagy could help to eliminate the intrahepatocellular lipids. A triggered mitochondrial activity and fatty acid β -oxidation upon HP diet was also one of our mechanistic hypotheses to explain the effective liver fat reduction. However, hepatic mitochondrial activity and fatty acid β -oxidation gene expression did not change upon HP diet. Diet induced changes of other metabolic pathways, eg suppression of hepatic fat uptake, de novo lipogenesis and lipid storage upon HP diet might be explanatory for the marked liver fat reduction.

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DISCLOSURE

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows - AFHP, MM, OPR, SK and SH designed the research; CX, MM, NS, SH, TG, SK, KH, JL, MO, VC, FK, AR and VL conducted the research; CX, AL and OPR analysed data and performed the statistical analysis; CX, MM, OPR and AFHP wrote the manuscript; CX, MM, OPR, NS, TG, SK, SKI, SH and AFHP contributed to the critical revision of the manuscript for important intellectual content; AFHP had primary responsibility for the final content. All authors read and approved the final manuscript.

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REFERENCES

- Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther.* 2011;34(3):274-285.
- LaBrecque DR, Abbas Z, Anania F, et al, World Gastroenterology Organisation global guidelines: nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *J Clin Gastroenterol.* 2014;48(6):467-473.
- Markova M, Pivovarova O, Hornemann S, et al, Isocaloric diets high in animal or plant protein reduce liver fat and inflammation in individuals with type 2 diabetes. *Gastroenterology.* 2017; 152(3):571-585.e8.
- Benjaminov O, Beglaibter N, Gindy L, et al, The effect of a low-carbohydrate diet on the nonalcoholic fatty liver in morbidly obese patients before bariatric surgery. *Surg Endosc.* 2007;21(8):1423-1427.
- Bortolotti M, Maiolo E, Corazza M, et al, Effects of a whey protein supplementation on intrahepatocellular lipids in obese female patients. *Clin Nutr.* 2011;30(4):494-498.
- Arslanow A, Teutsch M, Walle H, Grunhage F, Lammert F, Stokes CS. Short-term hypocaloric high-fiber and high-protein diet improves hepatic steatosis assessed by controlled attenuation parameter. *Clinical and translational gastroenterology.* 2016;7(6):e176.
- Maida A, Zota A, Sjøberg KA, et al, A liver stress-endocrine nexus promotes metabolic integrity during dietary protein dilution. *J Clin Invest.* 2016;126(9):3263-3278.
- Laeger T, Henagan TM, Albarado DC, et al, FGF21 is an endocrine signal of protein restriction. *J Clin Invest.* 2014;124(9):3913-3922.
- Solon-Biet S, McMahon A, Ballard J, et al, The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metab.* 2014;19(3):418-430.
- Solon-Biet S, Mitchell S, Coogan S, et al, Dietary protein to carbohydrate ratio and caloric restriction: comparing metabolic outcomes in mice. *Cell Rep.* 2015;11(10):1529-1534.
- Zhu S, Wu Y, Ye X, et al, FGF21 ameliorates nonalcoholic fatty liver disease by inducing autophagy. *Mol Cell Biochem.* 2016;420(1-2):107-119.
- Henagan TM, Laeger T, Navard AM, et al, Hepatic autophagy contributes to the metabolic response to dietary protein restriction. *Metabolism.* 2016;65(6):805-815.
- Singh R, Kaushik S, Wang Y, et al, Autophagy regulates lipid metabolism. *Nature.* 2009;458(7242):1131-1135.
- Shibata M, Yoshimura K, Furuya N, et al, The MAP1-LC3 conjugation system is involved in lipid droplet formation. *Biochem Biophys Res Commun.* 2009;382(2):419-423.
- Kim KH, Jeong YT, Oh H, et al, Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. *Nat Med.* 2013;19(1):83-92.
- Kim KH, Lee MS. FGF21 as a stress hormone: the roles of FGF21 in stress adaptation and the treatment of metabolic diseases. *Diabetes Metab J.* 2014;38(4):245-251.
- Petzke KJ, Freudenberg A, Klaus S. Beyond the role of dietary protein and amino acids in the prevention of diet-induced obesity. *Int J Mol Sci.* 2014;15(1):1374-1391.
- Freudenberg A, Petzke KJ, Klaus S. Comparison of high-protein diets and leucine supplementation in the prevention of metabolic syndrome and related disorders in mice. *J Nutr Biochem.* 2012;23(11):1524-1530.
- Freudenberg A, Petzke KJ, Klaus S. Dietary L-leucine and L-alanine supplementation have similar acute effects in the prevention of high-fat diet-induced obesity. *Amino Acids.* 2013;44(2):519-528.
- de Andrade KQ, Moura FA, dos Santos JM, de Araujo OR, de Farias Santos JC, Goulart MO. Oxidative stress and inflammation in hepatic diseases: therapeutic possibilities of N-acetylcysteine. *Int J Mol Sci.* 2015;16(12):30269-30308.
- Chavin KD, Yang ShiQi, Lin HZ, et al, Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. *The Journal of biological chemistry.* 1999;274(9):5692-5700.
- Bedossa P; Consortium FP. Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. *Hepatology.* 2014;60(2):565-575.
- Weitkunat K, Schumann S, Petzke KJ, Blaut M, Loh G, Klaus S. Effects of dietary inulin on bacterial growth, short-chain fatty acid production and hepatic lipid metabolism in gnotobiotic mice. *J Nutr Biochem.* 2015;26(9):929-937.
- Kovsan J, Blüher M, Tarnovscki T, et al, Altered autophagy in human adipose tissues in obesity. *J Clin Endocr Metab.* 2011;96(2):E268-E277.
- Keipert S, Ost M, Chadt A, et al, Skeletal muscle uncoupling-induced longevity in mice is linked to increased substrate metabolism and induction of the endogenous antioxidant defense system. *Am J Physiol Endocrinol Metab.* 2013;304(5):E495-506.
- Pesta D, Gnaiger E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol Biol.* 2012;810:25-58.
- Ost M, Werner F, Dokas J, Klaus S, Voigt A. Activation of AMPK α 2 is not crucial for mitochondrial uncoupling-induced metabolic effects but required to maintain skeletal muscle integrity. *PLoS One.* 2014;9(4):e94689.
- Coleman V, Sa-Nguanmoo P, Koenig J, et al, Partial involvement of Nrf2 in skeletal muscle mitohormesis as an adaptive response to mitochondrial uncoupling. *Sci Rep.* 2018;8(1):2446.
- Szczepaniak LS, Nurenberg P, Leonard D, et al, Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab.* 2005;288(2):E462-468.
- Wredenberg A, Wibom R, Wilhelmsson H, et al, Increased mitochondrial mass in mitochondrial myopathy mice. *Proc Natl Acad Sci USA.* 2002;99(23):15066-15071.
- Martens EA, Gatta-Cherifi B, Gonnissen HK, Westerterp-Plantenga MS. The potential of a high protein-low carbohydrate diet to preserve intrahepatic triglyceride content in healthy humans. *PLoS One.* 2014;9(10):e109617.
- Drummen M, Dorenbos E, Vreugdenhil ACE, et al, Long-term effects of increased protein intake after weight loss on intrahepatic lipid content and implications for insulin sensitivity: a PREVIEW study. *Am J Physiol Endocrinol Metab.* 2018;315(5):E885-E891.
- Mootha VK, Bunkenborg J, Olsen JV, et al, Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell.* 2003;115(5):629-640.
- Morino K, Petersen KF, Dufour S, et al, Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of

- insulin-resistant offspring of type 2 diabetic parents. *J Clin Investig.* 2005;115(12):3587-3593.
35. Szendroedi J, Schmid AI, Chmelik M, et al, Muscle mitochondrial ATP synthesis and glucose transport/phosphorylation in type 2 diabetes. *PLoS Medicine.* 2007;4(5):e154.
 36. Bournat JC, Brown CW. Mitochondrial dysfunction in obesity. *Curr Opin Endocrinol Diabetes Obes.* 2010;17(5):446-452.
 37. Koliaki C, Szendroedi J, Kaul K, et al, Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab.* 2015;21(5):739-746.
 38. Hasek BE, Boudreau A, Shin J, et al, Remodeling the integration of lipid metabolism between liver and adipose tissue by dietary methionine restriction in rats. *Diabetes.* 2013;62(10):3362-3372.
 39. Patil YN, Dille KN, Burk DH, Cortez CC, Gettys TW. Cellular and molecular remodeling of inguinal adipose tissue mitochondria by dietary methionine restriction. *J Nutr Biochem.* 2015;26(11):1235-1247.
 40. Perrone CE, Mattocks DA, Jarvis-Morar M, Plummer JD, Orentreich N. Methionine restriction effects on mitochondrial biogenesis and aerobic capacity in white adipose tissue, liver, and skeletal muscle of F344 rats. *Metabolism.* 2010;59(7):1000-1011.
 41. Garcia-Caraballo SC, Comhair TM, Verheyen F, et al, Prevention and reversal of hepatic steatosis with a high-protein diet in mice. *Biochim Biophys Acta.* 2013;1832(5):685-695.
 42. Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med.* 2002;80(12):753-769.
 43. Zhu L, Baker SS, Liu W, et al, Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis. *Metabolism.* 2011;60(7):1001-1011.
 44. Chaumontet C, Even PC, Schwarz J, et al, High dietary protein decreases fat deposition induced by high-fat and high-sucrose diet in rats. *Br J Nutr.* 2015;114(8):1132-1142.
 45. Noguchi Y, Nishikata N, Shikata N, et al, Ketogenic essential amino acids modulate lipid synthetic pathways and prevent hepatic steatosis in mice. *PLoS One.* 2010;5(8):e12057.
 46. Stepien M, Gaudichon C, Fromentin G, Even P, Tome D, Azzout-Marniche D. Increasing protein at the expense of carbohydrate in the diet down-regulates glucose utilization as glucose sparing effect in rats. *PLoS One.* 2011;6(2):e14664.
 47. Uebanso T, Taketani Y, Fukaya M, et al, Hypocaloric high-protein diet improves fatty liver and hypertriglyceridemia in sucrose-fed obese rats via two pathways. *Am J Physiol Endocrinol Metab.* 2009;297(1):E76-84.
 48. Palou M, Priego T, Sánchez J, et al, Sequential changes in the expression of genes involved in lipid metabolism in adipose tissue and liver in response to fasting. *Pflugers Arch.* 2008;456(5):825-836.
 49. Debeer LJ, Thomas J, De Schepper PJ, Mannaerts GP. Lysosomal triacylglycerol lipase and lipolysis in isolated rat hepatocytes. *J Biol Chem.* 1979;254(18):8841-8846.
 50. B'Chir W, Maurin AC, Carraro V, et al, The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res.* 2013;41(16):7683-7699.
 51. Carroll B, Korolchuk VI, Sarkar S. Amino acids and autophagy: cross-talk and co-operation to control cellular homeostasis. *Amino Acids.* 2015;47(10):2065-2088.
 52. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem J.* 2013;449(1):1-10.
 53. Czaja MJ. Functions of autophagy in hepatic and pancreatic physiology and disease. *Gastroenterology.* 2011;140(7):1895-1908.
 54. Markova M, Koelman L, Hornemann S, et al, Effects of plant and animal high protein diets on immune-inflammatory biomarkers: a 6-week intervention trial. *Clin Nutr.* 2020;39(3):862-869.
 55. Prochnicki T, Latz E. Inflammasomes on the crossroads of innate immune recognition and metabolic control. *Cell Metab.* 2017;26(1):71-93.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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