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Title

Isocaloric Diets High in Animal or Plant Protein Reduce Liver fat and Inflammation in Individuals with Type 2 Diabetes

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Short title: Liver fat content and high protein diet

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The authors' responsibilities were as follows – SH and AFHP were responsible for concept and design of the study. MM, SH, SS, and JH, conducted the study. MM, OP, SS, KW, JM, KJP, RL, CH, and

MCK performed experiments and data acquisition. MM, OP, SS, and TF performed statistical data analysis. MM, OP, and AFHP were responsible for interpretation of the data and drafting of the manuscript. MM, OP, SH, SS, TF, KW, JM, KP, JH, CH, MCK, MR, NR, SK, RT, RS, SR, and AFHP contributed to the critical revision of the manuscript for important intellectual content. AFHP 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.

Abbreviations

ANOVA	Analysis of variance
AP	Animal protein
AT	Adipose tissue
BCA	Bicinchoninic acid
BCAA	Branched chain amino acids
BSA	Bovine serum albumin
ChREBP	Carbohydrate response element binding protein
CID	Clinical investigation day
DNL_{index}	*De novo* lipogenesis index
ELISA	Enzyme-linked immunosorbent assay
ELF	Enhanced liver fibrosis
FAA	Free amino acid(s)
FGF21	Fibroblast growth factor 21
GC	Gas chromatography
γ-GT	Gamma-glutamyl transpeptidase
HOMA-IR	Homeostatic model assessment insulin resistance
IHL	Intrahepatic lipids
IL	Interleukin
LC-MS	Liquid chromatography mass spectrometry
MCP-1	Monocyte chemotactic protein-1
MRI	Magnetic resonance imaging

MRS	Magnetic resonance spectroscopy
mTORC1	Mammalian target of rapamycin complex 1
MTT	Meal tolerance test
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NVAT	Non-visceral adipose tissue
PIIINP	Amino-terminal propeptide of type III procollagen
PP	Plant protein
PUFA	Polyunsaturated fatty acids
REE	Resting energy expenditure
SAT	Subcutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase-1
SFA	Saturated fatty acids
SREBP1c	Sterol regulatory element-binding transcription factor 1c
TNFα	Tumor necrosis factor alpha
T2D	Type 2 diabetes
VAT	Visceral adipose tissue
qRT-PCR	Quantitative real-time polymerase chain reaction

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is associated with increased risk of hepatic, cardiovascular, and metabolic diseases. High-protein diets, rich in methionine and branched chain amino acids (BCAAs), apparently reduce liver fat but may induce insulin resistance. We investigated the effects of diets high in animal protein vs plant protein, which differ in levels of methionine and BCAA, in subjects with type 2 diabetes and NAFLD. We examined levels of liver fat, lipogenic indices, markers of inflammation, serum levels of fibroblast growth factor 21 (FGF21), and activation of signaling pathways in adipose tissue.

Methods: We performed a prospective study of individuals with type 2 diabetes and NAFLD at a tertiary medical center in Germany, from June 2013 through March 2015. We analyzed data from 37 subjects placed on a diet high in animal protein (AP, rich in meat and dairy foods; n=18) or plant protein (PP, mainly legume protein; n=19) without calorie restriction for 6 weeks. The diets were isocaloric with the same macronutrient composition (30% protein, 40% carbohydrates, and 30% fat). Participants were examined at the start of the study and after the 6-week diet period for body mass index, body composition, hip circumference, resting energy expenditure, and respiratory quotient. Body fat and intrahepatic fat were detected by magnetic resonance imaging and spectroscopy, respectively. Levels of glucose, insulin, liver enzymes, and inflammation markers as well as individual free fatty acids and free amino acids were measured in collected blood samples. Hyperinsulinemic euglycemic clamps were performed to determine whole-body insulin sensitivity. Subcutaneous adipose tissue samples were collected and analyzed for gene expression patterns and phosphorylation of signaling proteins.

Results: Post-prandial levels of BCAAs and methionine were significantly higher in subjects on the AP vs the PP diet. The AP and PP diets each reduced liver fat by 36%–48% within 6 weeks (*P* for AP diet=.0002, *P* for PP diet=.001). These reductions were unrelated to change in body weight but correlated with downregulation of lipolysis and lipogenic indices. Serum level of FGF21 decreased by 50% in each group (*P* for AP diet<.0002, *P* for PP diet<.0002); decrease in FGF21 correlated with loss of hepatic fat. In gene expression analyses of adipose tissue, expression of the FGF21 receptor cofactor klotho beta was associated with reduced expression of genes encoding lipolytic and lipogenic proteins. In subjects on each diet, levels of hepatic enzymes and markers of inflammation decreased, insulin sensitivity increased, and serum level of keratin 18 decreased.

Conclusions: In a prospective study of patients with type 2 diabetes, we found diets high in protein (either animal or plant) to significantly reduce liver fat, independently of body weight, and reduce markers of insulin resistance and hepatic necroinflammation. The diets appear to

mediate these changes via lipolytic and lipogenic pathways in adipose tissue. Negative effects of BCAA or methionine were not detectable. FGF21 level appears to be a marker of metabolic improvement. ClincialTrials.gov no: NCT02402985.

KEY WORDS: KLB, FFA, NAFLD, NASH

Introduction

The ectopic accumulation of excessive fat in the liver, non-alcoholic fatty liver disease (NAFLD) is closely linked to obesity and insulin resistance and may progress to non-alcoholic steatohepatitis (NASH) which provides a high risk of progression to advanced liver diseases. Incidence of NAFLD is rapidly increasing in industrialized countries and is associated with cardiovascular disease and type 2 diabetes (T2D).[1, 2] Liver fat content is highly depending on nutritional intake and energy metabolism. Hypercaloric Western style diets with high content of animal protein, carbohydrates, and saturated fats promote liver fat accumulation.[3] Carbohydrates are known to induce lipogenesis by activating the carbohydrate responsive transcription factor *carbohydrate response element binding protein* (ChREBP).[4] Hepatic lipogenesis additionally requires the activation of *mammalian target of rapamycin complex 1* (mTORC1) to induce *sterol regulatory element-binding transcription factor 1c* (SREBP1c).[5, 6] Branched chain amino acids (BCAA) recruit mTOR and assemble mTORC1 in cooperation with insulin and thereby support tissue hypertrophy and proliferation, inhibit autophagy and proteolysis and impair insulin sensitivity.[7] The mTOR substrate p70S6 kinase was shown to induce insulin resistance in muscle upon activation by infusion of amino acids in humans.[8] Thus, activation of mTORC1 by high intake of protein would be expected to deteriorate metabolic control and to increase hepatic triglycerides. However, high-protein diets have shown variable[9] and sometimes even favorable effects on glucose metabolism and insulin sensitivity in people with T2D[10] and it is unclear which metabolic pathways are involved.[9, 11] Remarkably, diets restricted in methionine were shown to prevent the development of insulin resistance and of the metabolic syndrome in animal models by activating the protective fibroblast growth factor 21 (FGF21) pathway.[12, 13] Moreover, BCAA were proposed to induce insulin resistance by clogging mitochondrial ATP-production.[14]

Therefore, the type of protein may elicit different metabolic responses depending on the amino acid composition. Notably, plant proteins contain much lower levels of methionine and BCAA than proteins of animal origin, which was proposed to mediate some advantages of vegetarian diets.[15] It is therefore hypothesized that high plant-protein diets exert favorable effects on hepatic fat content and metabolic responses as compared to high intake of animal protein rich in BCAA and methionine.

The metabolic hormone FGF21 was shown to be elevated in, and to predict, fatty liver disease in humans.[16, 17] Treatment with FGF21, however, ameliorated fatty liver disease by modifying lipogenic pathways and fat oxidation in animal models.[18] Remarkably, fructose and sugar intake acutely increased circulating FGF21 in humans and mice[19] being partly mediated by ChREBP.[20] Another potent stimulus elevating FGF21 is protein deficiency, which acts via the stress induced transcription factor ATF4.[12] A moderate increase of free fatty acids (FFA) increased FGF21 in humans involving *peroxisome proliferator-activated receptor alpha* (PPARα) associated signaling, as well.[21] Thus, FGF21 is strongly regulated by macronutrient intakes in humans which is closely related to liver fat content. It is further hypothesized that changes of FGF21 might be involved in the effects of protein enriched diets on liver fat and should be particularly effective in plant diets with low content of methionine.

Human interventions studied the effect of high-protein intake on liver fat content.[22-25] In lean and healthy individuals, high protein blunted the increase of intrahepatic lipids (IHL) induced by fructose[22] or high fat diet.[24, 25] In 11 obese women, IHL decreased by 20% after whey supplementation for 4 weeks, only 5 subjects however, exhibited IHL over 5%.[23] High-protein interventions in patients with NAFLD are lacking. Moreover, there is no data whether the origin of dietary protein plays a role in liver fat accumulation in humans with NAFLD or not.

Thus, the aim of the present study was to compare the effects of two isocaloric high-protein diets, containing either animal or plant protein on liver fat content in subjects with T2D accompanied by NAFLD. To additionally explore possible mechanisms of liver fat alteration, lipogenic indices, inflammation markers, serum levels of FGF21 and activation of signaling pathways in adipose tissue and their association with liver fat reduction were assessed.

Materials and Methods

This randomized clinical trial with an open-label, parallel-arm study design was approved by the Ethics Committee of the University of Potsdam, conducted in accordance with the Declaration of Helsinki, and registered at www.clinicaltrials.gov (NCT02402985). All participants provided written informed consent before starting the study. The study took place at the Department of Clinical Nutrition of the German Institute of Human Nutrition Potsdam-Rehbruecke (Nuthetal, Germany) from June 2013 to March 2015. All authors had access to the study data and had reviewed and approved the final version of the manuscript.

Participants and study design

In this 6-week intervention study 44 individuals with T2D were assigned by group matching for age, sex, BMI, HbA_{1c} and glucose lowering drugs using random number generator either to a group consuming an animal protein (AP) or plant protein (PP) rich diet. A total of 37 subjects (n_{AP}=18, n_{PP}=19) aged between 49-78 years completed the study. Both diets were isocaloric and had the same macronutrient composition (30 E% protein, 40 E% carbohydrates, 30 E% fat). Macronutrient intake of individuals prior to enrollment was 17 E% protein, 42 E% carbohydrates, 41 E% fat. The animal-protein diet (AP) was rich in meat and dairy foods, the plant-protein diet (PP) consisted mainly of legume protein (see Supplementary Material for detailed information). On the clinical investigation days (CID) at beginning and end of interventions, hyperinsulinemic euglycemic clamps and meal tolerance tests (MTT) were performed; blood, urine and subcutaneous adipose tissue (SAT) samples were collected (see Supplementary Material).

Individuals had glycated hemoglobin A_{1c} (HbA_{1c}) levels of 5.8-8.8% at recruitment. None of the patients had impaired renal (glomerular filtration rate < 60 ml/min/1.73 m^2), liver, or thyroid function;

none had glucocorticoid therapy, stroke or heart attack in the last 6 months, or cancer in the last 2 years; and none had acute or chronic inflammatory disease, mental disorders and depression, food intolerance and weight change greater than 3 kg within the previous 3 months. Participants were asked to maintain their physical activity patterns for the duration of the study. At each visit they arrived in a fasted state.

Body composition, magnetic resonance imaging (MRI) and spectroscopy (^{1}H-MRS)

Body composition was determined by Air Displacement Plethysmography (BOD POD®, COSMED, Italy). MRI and ^{1}H-MRS were performed on a 1.5 T whole body scanner (Magnetom Avanto, Siemens Healthcare, Germany) for quantification of abdominal fat depots and intrahepatic lipids, respectively[26] (see Supplementary Material for detailed description). Indirect calorimetry (Vmax® Encore metabolic cart, CareFusion, Yorba Linda, USA) was performed in the fasting state to estimate the resting energy expenditure and the respiratory quotient. The substrate oxidation was calculated according following equations: carbohydrate oxidation [g/min] = 4.55 x V_{CO2} − 3.21 x V_{O2}; fat oxidation [g/min] = 1.67 x V_{O2} − 1.67 x V_{CO2}. Protein oxidation was estimated from the urinary urea nitrogen excretion from 24-h urine.[27]

Biomarkers in serum and plasma

Fasting blood was collected at week 0 and 6 of the intervention. Routine markers were measured in serum using ABX Pentra 400 (Horiba, Japan). Biomarkers in serum samples were measured by ELISA: Insulin (Mercodia, Sweden), IL-18, MCP-1, adiponectin, and FGF21 (R&D Systems, USA), caspase-cleaved Keratin 18 fragment by M30-Apoptosense ELISA (Peviva, Sweden); IL-8 and TNFα using Luminex magnetic bead technology (Luminex Performance Human High Sensitivity Cytokine Magnetic Panel, R&D Systems, USA); IL-6 using sandwich immunoassays (MSD, USA). Fasting

plasma levels of FFA were determined by gas chromatography (GC), analysis of free amino acids (FAA) was performed using LC-MS, and the levels of amino-terminal propeptide of type III procollagen (PIIINP) and enhanced liver fibrosis (ELF) score on ADVIA Centaur CP immunochemical analyzer according to manufacturer's instructions (Siemens Healthcare Diagnostics, Germany). Index of whole-body insulin resistance (HOMA-IR) was calculated as: fasting insulin [µU/ml] x fasting glucose in [mM] / 22.5; index of adipose tissue insulin resistance (AdipoIR) as: fasting insulin [µU/ml] x fasting total FFA [µg/ml]. Detailed description of protein and gene analysis in adipose tissue is provided in Supplementary Material.

Statistical analysis

The study enrolled 44 subjects, and 7 of them dropped out. The results are expressed as mean ± SEM. Statistical significance was defined as $P < 0.05$. Data where examined for normality by the Shapiro-Wilk test. Non-normally distributed variables were transformed with the natural logarithm and re-assessed for normality. Comparisons between two groups were tested by Student's *t* test (paired and unpaired) or non-parametric tests (Wilcoxon and Mann-Whitney-U-Test). Multiple comparisons were analyzed by one-way or two-way ANOVA. Depending on data distribution, Pearson's coefficient or Spearman's rank correlation coefficient was used for correlation analysis. All statistical analyses were performed with SPSS 20.0 (Chicago, USA). The network was calculated in R (version 3.1.2, The R Project for Statistical Computing, Vienna, Austria) using RStudio (version 0.98.1091, RStudio Inc., Boston, USA).

Results

Baseline characteristics of the participants

A total of 37 participants concluded the clinical study (animal-protein group (AP): 6 females, 12 males; plant-protein group (PP): 7 females, 12 males). Subjects were 65.0 ± 1.4 (AP) and 63.7 ± 1.5 (PP) years old, moderately obese with BMI of 31.0 ± 0.8 (AP) and 29.4 ± 1.0 (PP).

Intrahepatic lipids and anthropometry

Content of intrahepatic fat was determined at baseline and after 6 weeks of intervention by ^{1}H-MRS. A highly significant reduction of IHL in both groups, by 48.0% and 35.7% in AP and PP, respectively was observed (P_{AP}=0.0002, P_{PP}=0.001) (Figure 1A). Moreover, in 9 subjects (n_{AP}=3, n_{PP}=6) IHL levels fell below 5.56% und thus below the threshold defining NAFLD.[28] Liver enzymes in serum decreased during the intervention in both groups, particularly γ-GT and AST, without significant differences between groups. Additionally, the AST/ALT ratio was below 1 in both groups and not influenced by the diets. Keratin 18 fragment, a marker of necro-apoptotic processes in liver,[29] declined significantly in all participants (P=0.002), and significantly in the PP but not AP subgroup (Table 1). Levels of PIIINP and ELF score were within the reference intervals[30] and no changes were observed in either of the groups (Table 1). However, there was a trend to improvement of the ELF score (P=0.071) in 5 subjects with a score above 9.8 at baseline who were likely to display some degree of fibrosis.[30] Notably, the relative decrease of liver fat correlated with the relative reductions of fasting glucose (Figure 2A) and fasting insulin (Figure 2B). Despite consumption of an isocaloric diet and the attempt to keep body weight constant by increasing food intake, participants in both groups showed a moderate but significant reduction of BMI (AP: -0.8 kg/m^2 ± 0.1 kg; PP: -0.5 ± 0.1 kg/m^2), which was not significantly different between the groups (Table 1). Fat mass decreased and fat-free mass detected by whole-body plethysmography increased in both groups with minor differences. Visceral (VAT) and

non-visceral (NVAT) adipose tissue contents measured by MRI were reduced in both groups being highly significant in the AP group (VAT: -9.7%, $P<0.001$; NVAT: -7.4%, $P<0.0001$) (Figure 1B). Furthermore, hip circumference decreased in both groups, as well as the adipose tissue content, measured at the level of the femoral head (AT_{femur}) (Table 1). None of the changes of body fat correlated with changes of IHL (data not shown). Noteworthy, whole-body insulin sensitivity, expressed as M-value, improved in both groups and correlated significantly with reductions of IHL (Figure 2C).

Resting energy expenditure (REE) and respiratory quotient did not change during the intervention. As expected, protein oxidation, calculated from the urinary urea nitrogen excretion, increased significantly in all participants without differences between groups (Table 1).

FFA and FAA blood levels

In the fasting state FFA in the circulation are largely derived from SAT lipolysis[31] and provide an index of insulin sensitivity and processes occurring in adipose tissue. To assess changes of lipid metabolism in adipose tissue upon the high-protein diet and its possible contribution to the IHL reduction, FFA levels before and after the intervention were measured. A significant decrease of all saturated FFA (SFA) from C14:0 to C24:0 and of monounsaturated FFAs (MUFA) was observed in both groups with the exception of odd-chain fatty acid levels (Table 2). With regard to polyunsaturated fatty acids (PUFA), levels of linoleic acid (C18:2n6) did not change while arachidonic acid (C20:4n6) decreased significantly in both groups. Remarkably, the levels of γ-linolenic acid (C18:3n6) decreased, while levels of α-linolenic acid (C18:3n3) increased in both groups with the changes being more pronounced in the AP group. This was accompanied by a significant decrease of the AdipoIR index in both groups (Table 1).

Furthermore, the index of *de novo* lipogenesis (DNL_{index} = 16:0/18:2n6) decreased significantly in both groups and a significant decrease of the elongase activity index (C18:0/C16:0), but no change of the stearoyl-CoA desaturase-1 (SCD1) index (C16:1n7/C16:0) was observed. Importantly, Δ5 desaturase activity index (C20:4n6/C20:3n6) decreased significantly in PP, while Δ6 desaturase activity decreased (C18:3n6/C18:2n6) in AP group (Table 2). Collectively, these results show a very specific pattern of changes regarding preserved ω-6 FFA which were associated with reduced diabetes risk.[32]

As expected, at baseline we observed a significant correlation of circulating free fatty acids with IHL. Strong correlations were observed for saturated FFA including C14:0, C16:0 and C18:0 as well as for palmitoleic acid (C16:1n7) with IHL (Supplementary Figure 1A). These correlations remained significant after intervention (Supplementary Figure 1B). The reduction of γ-linoleic acid (Figure 2E) and the reduction of the DNL_{index} correlated with the change in IHL (Figure 2F).

To investigate whether alterations of circulating amino acids contributed to IHL reduction, fasting plasma FAA levels before and after intervention were measured. Analysis showed no significant changes in the levels of 14 amino acids. Only taurine decreased in both groups (Supplementary Figure 2). Interestingly, changes of plasma asparagine correlated with the reduction of IHL in the PP group (r= -0.568, *P*=0.027).

As amino acids are rapidly taken up by the cells, postprandial levels of FAA after a test meal with the typical composition of the AP and PP diets were also determined. BCAA (leucine, isoleucine, and valine) and methionine contents were higher after the AP meal in comparison to the PP meal, while arginine and asparagine were higher after the PP meal in comparison to the AP meal (Supplementary Table 1). The PP group showed larger increases of asparagine and arginine (Figure 3A-B) while the

AP meal resulted in much larger increases in methionine and BCAA (Figure 3C-F), corresponding with the amino acid content of the different protein types.

FGF21

FGF21 correlates with anthropometric indices in humans including liver fat as we could confirm in our cohort (Supplementary Table 2), and the increase was proposed to reflect ER-stress in human NAFLD.[33] FGF21 might therefore play a role in the protein induced reduction of liver fat. Unexpectedly, FGF21 levels were reduced by 42.6% and 51.1% in the AP and PP groups, respectively (P_{AP}<0.0002, P_{PP}<0.0002) (Figure 1C). There was also a positive association between the reduction of FGF21 and IHL in the entire cohort (r=0.363, P=0.049).

FGF21 stimulates the release of adiponectin and requires adiponectin to reduce liver fat and improve insulin sensitivity in mice.[34] Indeed, a modest reduction of circulating adiponectin which was most pronounced in the PP group (Table 1) and a correlation with FGF21 after the intervention was observed (Supplementary Table 2).

Inflammatory markers

The metabolic syndrome and NAFLD are linked to subclinical inflammation.[1, 3] Therefore, changes of important inflammatory cytokines were assessed. IL-4, IL-6, IL-8 and MCP-1 did not change during the intervention, while the inflammasome processed cytokine IL-18 decreased in the AP group, and TNFα decreased in the PP group (Supplementary Figure 3). This indicated a modest reduction of inflammatory markers with the high-protein diets.

Gene expression and protein modification in adipose tissue

In view of the extensive changes in lipid metabolism and of FGF21 associated with IHL reduction upon high-protein diet, we further investigated its molecular mechanisms using available SAT samples

collected before and after the intervention (n_{AP}=12, n_{PP}=15). We analyzed diet-induced changes of signaling pathways in SAT and measured expression of genes playing a key role in *de novo* lipogenesis, lipid storage, lipolysis and lipid uptake, as well as main enzymes in BCAA metabolism, ER-stress and FGF21 pathways using real-time PCR (Figure 4). Overall, the gene expression of lipolytic and lipogenic enzymes was decreased which was significant for *ACC1*, involved in *de novo* lipogenesis, in the AP group (Figure 4A). Furthermore, expressions of enzymes of lipid storage were decreased in both groups (*PGC1A* and *PPARG* in PP, *SCD1* in AP). Expression of the lipolytic enzyme *HSL* and *ADIPOQ* were also lower in the PP group (Figure 4A). ER-stress related genes remained unchanged (Figure 4B). *FGFR1* expression was significantly reduced in the PP group (Figure 4C).

We further tested the hypothesis that high intake of BCAA from animal-protein rich diet activates the mTOR pathway in humans. The expression of the main enzymes in BCAA-metabolism, *BCAT2* and *BCKDH*, was unchanged in adipose tissue of either group (Figure 4D). In addition, we measured the mRNA expression and phosphorylation state of enzymes in the Akt-mTOR pathway in SAT. No differences were observed in gene expression of kinases in the Akt-mTOR pathway (*AKT, MTOR, P70S6K, 4EBP1*) (Figure 5A). Furthermore, the phosphorylation state of several key enzymes of this pathway (assessed in a subgroup: n_{AP}=8, n_{PP}=8) did not change, except for a significant decrease of the phosphorylation of p70S6K (Thr421/Ser424) in the AP group (Figure 5B).

Although expression of particular genes was significant only in one of dietary groups studied, similar trends were found in both groups, and there were no statistical differences between groups. We then used a bioinformatics approach to understand the interaction of pathways involved and calculated correlation networks. The changes of gene expression showed high correlations of β-Klotho (*KLB*) with the major lipid metabolic enzymes *FASN*, *ACC1*, *SCD1*, *ATGL*, *HSL*, but also *PPARG* and

SREBP1c indicating a critical role of the FGF21 pathway (Figure 4E). Remarkably *MTOR* and its pathway including *P70S6K*, *4EBP1*, as well as components of autophagy *ATG5* and *LC3A/B* were highly correlated confirming their central regulatory role in human metabolic adaptation to high-protein diet (Figure 4E).

Discussion

The most important finding of our study is that provision of a 6-week high-protein diet that did not restrict caloric intake, very effectively reduced IHL by 36 - 48% in subjects with T2D and NAFLD. This strategy was accompanied by an improvement of metabolic and inflammatory parameters and therefore provides a powerful and safe treatment approach. The loss of liver fat was highly related to changes of lipid metabolism as shown by the decreases of saturated and other FFA and indices of lipogenesis. Despite the elevated intake and postprandial uptake of methionine and BCAA in the AP group, there was no indication of negative effects of these components. The origin of protein – animal or plant – did not play a major role. Both high-protein diets unexpectedly induced strong reductions of FGF21 which was associated with metabolic improvements and the decrease of IHL.

Hepatic fat is regulated by a complex array of metabolic phenomena which reflect metabolic balance in numerous organs.[2, 35] Imbalance between lipid supply (*de novo* lipogenesis and FFA flux) and lipid disposal (VLDL secretion and lipid oxidation) leads to increased fat accumulation in liver. In the post-absorptive state fatty acid uptake by the liver is directly related to the concentration of FFA in the circulation.[31, 36] NAFLD is associated with insulin resistance of adipose tissue and increased rates of lipolysis,[37, 38] which increase further depends on the extent of obesity.[39] Indeed, the reduction of IHL in our study was accompanied by the improvement of whole-body insulin sensitivity and adipose tissue insulin resistance. As expected, at baseline and after the intervention we observed significant correlations of circulating FFA with IHL. Strong associations were seen for saturated FFA including C14:0, C16:0 and C18:0 as well as for palmitoleic acid (C16:1n7) confirming previous observations.[40] The decrease of these FFA therefore was associated with the reduction of liver fat. This conclusion is supported by the correlated reduction of hepatic fat and the saturated stearic acid in both groups.

Importantly, FFA were recently shown to directly induce hepatic glucose production in addition to their contribution to liver fat.[41, 42]

Plasma FFA and enzyme activity indices reflect mostly fatty acid metabolism in SAT since they are predominantly secreted from SAT.[31] Reductions of fasting FFA point to improved insulin sensitivity in adipose tissue supported by the increased whole-body insulin sensitivity in AP and decreased *HSL* expression in PP group as well as the diminished adipose tissue insulin resistance index in the entire cohort. Both, elongase activity index and DNL_{index} were significantly decreased in AP and PP groups, the latter of which correlated with the IHL decrease. The data also agree with the reductions of total fat mass, VAT, NVAT, and IHL. Expression of genes involved in lipogenesis (defined as both the process of fatty acid synthesis and triglyceride synthesis) were decreased in SAT (*ACC1* and *SCD1* in AP group and *PGC1A* and *PPARG* in PP group). As we did not use isotope tracers, we cannot distinguish exactly between changes in adipose tissue and liver but rather observe an overall decrease in lipogenesis and lipolysis as reflected by the reduction of FFA.

Recently, long term correlations of FFA and Δ5 and Δ6 desaturase activities with diabetes risk were published from a 19 year follow up of the Kuopio ischemic heart disease study including 2189 men.[32] Diabetes risk was inversely related to linoleic acid and directly correlated with γ-linolenic acid as well as with Δ6 desaturase activity. This mirrors the changes of FFA observed in response to the high-protein diets which showed a correlation of γ-linolenic acid with liver fat, while linoleic acid remained at a high level and Δ6 desaturase decreased. A positive association of γ-linolenic acid with liver fat has been observed in plasma lipidomic studies of NAFLD and NASH in agreement with our data.[43] This not only confirms the diabetes preventive effect of lowering γ-linolenic acid but also links it to the reduction of hepatic fat content which is closely related to diabetes risk.[32] The high level of

linoleic acid is known to inhibit ChREBP and was shown to reduce liver fat in a human study.[44] Recent analyses linked prostanoid metabolites of arachidonic acid and ω-6 PUFAs to NAFLD and to its progression to NASH[45] supporting the positive impact of the high-protein diets which decreased arachidonic acid. The high-protein diets thus exert important parts of their activities through the changes of lipid and adipose tissue metabolism.

Since we essentially exchanged fat versus protein (carbohydrate intake was 40 E% before and during the study), the lower fat intake might have also contributed to diminished *de novo* lipogenesis in liver. However, most studies report a positive association of carbohydrate intake with lipogenesis,[46] while the role of fat appears to depend on the type of fat and extend of NAFLD.[39, 44]

Remarkably, the improvement of IHL was not related to changes in body weight and fat mass, neither to VAT or NVAT content. We observed no increase in whole-body REE and no change of fat oxidation rates in the fasting state as calculated from indirect calorimetry. Expectedly, protein oxidation was highly induced in all participants.

As the AP diet induced much larger postprandial increases in BCAA and also methionine we expected a more favorable response to the PP diet than to AP. Levels of BCAA were not increased in the fasting state but showed large differences between the diets postprandially. Therefore, their degradation must have been increased which may involve the liver, adipose tissue, and muscle.[11] This may be reflected by changes of gene expression of the degrading pathway,[11] but in adipose tissue the expression of *BCAT* and *BCKDH* did not change. Still, the BCAA catabolism in human occurs mostly in muscle and liver,[47] suggesting the activation of the BCAA degrading pathway in those tissues.

In contrast to our expectations, we found no evidence for an activation of the mTOR pathway in adipose tissue. Activation of mTOR requires leucine which was higher in the AP group. Nevertheless,

phosphorylation of p70S6 kinase decreased significantly in the AP group suggesting decreased activation of the mTOR pathway in adipose tissue. No activation was observed at gene expression level. This may also explain the preferential loss of adipose since mTOR mediates trophic stimuli. We did not determine a possible activation of the mTOR pathway in muscle. Gran et al. showed a postprandial induction of the mTOR pathway in skeletal muscle after ingestion of protein rich meal[48] which may be expected in our cohort since lean tissue mass increased. However, the significant improvement of insulin sensitivity which we observed in hyperinsulinemic euglycemic clamps excludes a clinically relevant activation of the p70S6 kinase pathway in skeletal muscle.[8] Therefore, the detrimental effects of BCAA or their α-ketoacid degradation products described in some settings do not apply to a high animal-protein intake in the presence of an isocaloric diet without restriction of energy intake.[14] This is in line with other studies demonstrating metabolic improvements with high-protein diets[24, 49] and strongly argues against an important role of an activation of the mTOR pathway upon increased BCAA intake in humans. Clearly, BCAA are related to insulin resistance but they do not seem to respond to changes in protein intake.

The extensive reduction of FGF21 with the high-protein diet was unexpected. The reduction might reflect an improvement of hepatic ER-stress and improved mitochondrial function due to the reduction of liver fat. Markers of ER-stress such as XBP-1 and IRE1 were shown to correlate highly with elevated levels of FGF21.[33, 50] Although we had no access to liver biopsy samples, it appears likely that the IHL reduction was accompanied by decrease of hepatic ER-stress and thus FGF21 release. We did not observe changes in the expression of ER-stress markers in human adipose tissue, which is a primary target of FGF21 where it acts on FGFR1c and FGFR2c in combination with β-Klotho.[34, 51, 52] Resistance to FGF21 has been proposed and represents a possible cause of elevated FGF21 in obesity

and T2D.[33] However, expression of β-Klotho (*KLB*) was not altered and FGFR1 decreased in our study, which does not support changes of peripheral FGF21-sensititvity and is similar to data in mouse models.[51]

The reduction of FGF21 was accompanied by an improvement of markers of hepatic metabolism and of FFA. Changes in lipid metabolic enzyme transcripts were highly associated with the changes in FGF21 signaling pathways which implies that FGF21 plays some role in the metabolic effects of high-protein diets or is highly co-regulated. The decrease in FGF21 adds a novel aspect to the puzzle of FGF21. Although application of FGF21 rapidly improves metabolism in all models of obese mice,[51] increased levels of FGF21 are induced by obesity and associated with increased risks of metabolic and cardiovascular diseases.[16-18, 33, 50] The reduction of IHL might therefore reduce FGF21 which is supported by the correlation of FGF21 with IHL and by the highly correlated gene expression network in adipose tissue. FGF21 thus appears to regulate lipid biosynthetic pathways closely linked to the loss of IHL induced by the high-protein intake. Since increased levels of FGF21 were invariably linked to unfavorable outcomes the decrease may be interpreted as a positive signal reflecting the metabolic improvement.

Nevertheless it is difficult to envision how a decrease of FGF21 should be instrumental in mediating the improvements of metabolism. The increased protein intake therefore is likely to involve other gene regulatory pathways, such as the NO-arginine cycle which has a major impact on glucose and lipid metabolism.[53, 54]

Notably, FGF21 was associated to higher carbohydrate and lower fat and protein consumption[55, 56] and thus might act as a nutrient sensor to regulate macronutrient intake. It was shown that exogenous as well as fructose induced FGF21 suppresses sweet preference in a negative feedback loop.[20, 57] On the

other hand, protein/amino acids restriction stimulates FGF21,[12, 13] but decreased with high protein intake as shown in this study. Collectively is could be assumed that FGF21 is tightly coupled with macronutrient intake and choice.

Longitudinal observations showed a strong association of keratin 18 decrease with improvement in liver histology in children and adults with NAFLD.[58] The diminished keratin 18 levels indicated a beneficial role of high-protein diets on hepatic necroinflammation. The absence of changes in the ELF score may reflect a low degree of fibrosis in our cohort. Despite the extensive reduction of IHL, levels of aminotransferases showed only minor reductions by 10 – 15% which may be explained by the low baseline levels and low AST/ALT ratio. Blood levels of γ-GT declined in both groups with 24-30% suggesting a reduced oxidative challenge.

In conclusion, we demonstrated the effective reduction of IHL in patients with T2D and NAFLD consuming isocaloric high-protein diets. Unexpectedly, both AP and PP groups showed this beneficial effect associated with decrease of hepatic markers, fasting FFA and FGF21 as well as increase of insulin sensitivity. Moreover, molecular analysis of the signaling pathways in SAT revealed no activation of mTOR pathway upon the AP or PP diet. Nevertheless, larger and longer dietary intervention studies are needed to show the durability of the responses and eventual adverse effects of the diets. Moreover, the beneficial responses may be age related since our cohort was over 60 years old and age related effects may play a particular role in protein intake.

Figure Legends

Figure 1. High-protein diet reduced IHL, VAT, SAT, and serum FGF21 in T2DM. Boxplots and individual changes in intrahepatic lipid content *(A)* and serum FGF21 *(C)* after 6 weeks of

intervention; *B:* changes in adipose tissue depots after 6 week of intervention. $15 \leq n_{AP} \leq 18$, $17 \leq n_{PP} \leq 19$, results are shown as mean±SEM (B). **P*<.05, ***P*<.01, ****P*<.001. NS, not significant.

Figure 2. Relative reduction of IHL correlates with relative changes in metabolic parameters. Correlation between the relative change in IHL from week 0 to week 6 with the relative change in fasting plasma glucose *(A)*, fasting serum insulin *(B)*, whole-body insulin sensitivity (M-value) *(C)*, fasting plasma stearic acid C18:0 *(D)*, fasting plasma γ-linoleic acid C18:3n6 *(E)*, and lipogenic index C16:0/C18:2n6 *(F)*. $n_{AP}=15$, $n_{PP}=17$.

Figure 3. Differences in postprandial FAA levels after AP and PP meals. Plasma levels of arginine *(A)*, asparagine *(B)*, methionine *(C)*, valine *(D)*, leucine *(E)*, and isoleucine *(F)* over 240 minutes after test meal ingestion and corresponding iAUCs. AP week 0: black line, black bar; AP week 6: grey line, grey bar; PP week 0: dotted black line, dashed black bar; PP week 6: dotted grey line, dashed grey bar. $n_{AP}=18$, $n_{PP}=19$, results are shown as mean±SEM. *significant difference between AP and PP at week 0, #significant difference between AP and PP at week 6. **P*<.05, ***P*<.01, ****P*<.001.

Figure 4. High-protein diet regulates gene expression in subcutaneous adipose tissue. Changes in relative mRNA expression of genes involved in fat metabolism *(A)*, ER-stress *(B)*, FGF21 pathway *(C)*, and BCAA degradation *(D)* after 6 weeks of intervention. Results are shown as mean±SEM. *E*: correlation network of changes in gene expression. The color of the edges represents the quantity of the correlation coefficients (blue: $0.75>\tau\geq0.5$; black: $0.5>\tau\geq0.3$). All correlation coefficients were positive and highly significant. $n_{AP}=12$, $n_{PP}=15$. **P*<.05, ***P*<.01, ****P*<.001.

Figure 5. High-protein diet does not induce activation of the mTOR pathway in SAT. A: changes in relative the mRNA expression of genes involved in mTOR pathway after 6 weeks of intervention,

n_{AP}=12, n_{PP}=15. B: phosphorylation state of key enzymes of Akt-mTOR pathway at baseline and week 6, n_{AP}=8, n_{PP}=8. Results are shown as mean±SEM. *P<.05, **P<.01, ***P<.001.

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Table 1. Changes in anthropometric measurements, routine blood parameters and substrate oxidation.

Parameter	Animal protein (AP), n = 18			Plant protein (PP), n = 19			AP versus PP
	week 0	week 6	P_{AP}	week 0	week 6	P_{PP}	P_{APvsPP}
BMI [kg/m^2]	31.0 ± 0.8	30.2 ± 0.7	0.003	29.4 ± 1.0	28.9± 1.0	0.001	NS
Waist [cm]	104.2 ± 2.6	102.2 ± 2.0	NS	100.7 ± 3.0	99.4 ± 2.9	NS	NS
Hip [cm]	107.8 ± 1.8	106.3 ± 1.6	NS	105.3 ± 2.0	103.2 ± 1.9	0.034	NS
WHR	0.967 ± 0.018	0.962 ± 0.015	NS	0.957 ± 0.024	0.964 ± 0.025	NS	NS
Fat mass [%]	35.26 ± 2.19	33.36 ± 1.94	0.023	34.95 ± 2.30	33.55 ± 2.20	NS	NS
Fat-free mass [%]	64.74 ± 2.19	66.64 ± 1.94	0.023	65.05 ± 2.30	66.45 ± 2.20	NS	NS
AT_{femur} [ml]	394.25 ± 17.51	372.15 ± 19.66	0.016	372.73 ± 26.18	348.05 ± 17.56	NS	NS
AST [U/l]	26.64 ± 1.85	22.36 ± 1.44	NS	23.88 ± 2.13	20.37 ± 1.23	0.020	NS
ALT [U/l]	30.44 ± 2.47	27.09 ± 1.93	NS	29.59 ± 2.97	26.52 ± 2.01	NS	NS
AST/ALT ratio	0.88 ± 0.06	0.84 ± 0.05	NS	0.80 ± 0.04	0.80 ± 0.04	NS	NS
γ-GT [U/l]	44.31 ± 6.82	31.51 ± 4.23	0.017	41.76 ± 5.25	31.94 ± 3.61	NS	NS
Keratin 18 [U/l]	184.9 ± 28.9	159.7 ± 20.8	NS	197.4 ± 26.2	151.2 ± 13.9	0.021	NS
ELF score	9.19 ± 0.15	9.01 ± 0.16	NS	9.02 ± 0.17	9.11 ± 0.15	NS	NS
PIIINP [ng/ml]	7.73 ± 0.53	7.05 ± 0.26	NS	8.07 ± 0.49	7.98 ± 0.41	NS	NS
Adiponectin [ng/ml]	4063.6 ± 836.1	3661.4 ± 727.0	NS	4239.1 ± 395.6	3653.5 ± 317.1	0.003	NS
AdipoIR	11.99 ± 2.32	8.61 ±1.34	0.019	10.24 ± 1.71	9.87 ± 2.20	0.026	NS
Substrate oxidation							
Resting energy expenditure [kcal/day]	1682.1 ± 68.8	1628.3 ± 67.2	NS	1604.6 ± 61.5	1594.4 ± 75.6	NS	NS
Respiratory quotient	0.86 ± 0.02	0.84 ± 0.02	NS	0.84 ± 0.02	0.85 ± 0.02	NS	NS
Carbohydrate oxidation [g/min]	0.158 ± 0.017	0.144 ± 0.023	NS	0.139 ± 0.017	0.138 ± 0.014	NS	NS
Fat oxidation [g/min]	0.060 ± 0.008	0.064 ± 0.008	NS	0.066 ± 0.007	0.067 ± 0.008	NS	NS
24-h urinary nitrogen [g/day]	11.477 ± 0.876	17.240 ± 1.403	<0.001	11.245 ± 1.012	16.027 ± 1.337	0.005	NS
Protein oxidation [g/min]	0.050 ± 0.004	0.075 ± 0.006	<0.001	0.049 ± 0.004	0.070 ± 0.006	0.005	NS

Values are shown as mean±SEM. AdipoIR (adipose tissue insulin resistance) = fasting insulin [µU/ml] x fasting total FFA [µg/ml]. NS, not significant (*P*>.05).

Table 2. Changes in plasma free fatty acids and enzyme activity indices in the fasting state.

Free fatty acid		Animal Protein Diet (AP), n = 18			Plant Protein Diet (PP), n = 19			AP versus PP
[µg/ml]		week 0	week 6	P_{AP}	week 0	week 6	P_{PP}	P_{APvsPP}
Myristic acid	C14:0	27.04 ± 1.56	24.46 ± 1.69	0.045	27.28 ± 1.95	23.47 ± 1.87	0.013	NS
Pentadecylic acid	C15:0	4.51 ± 0.26	4.57 ± 0.25	NS	4.25 ± 0.26	4.05 ± 0.24	NS	NS
Palmitic acid	C16:0	216.84 ± 13.33	189.32 ± 13.35	0.005	217.56 ± 14.00	183.58 ± 11.51	0.007	NS
Palmitoleic acid	C16:1n7	45.92 ± 3.56	41.12 ± 3.80	0.005	47.35 ± 3.98	40.38 ± 3.57	0.030	NS
Margaric acid	C17:0	4.01 ± 0.21	3.90 ± 0.21	NS	3.78 ± 0.18	3.68 ± 0.23	NS	NS
Stearic acid	C18:0	45.77 ± 2.10	36.63 ± 2.13	0.000	44.82 ± 2.09	35.61 ± 1.85	0.000	NS
Oleic acid	C18:1n9	297.08 ± 15.99	263.83 ± 14.32	0.012	302.99 ± 18.94	249.24 ± 15.48	0.002	NS
Vaccenic acid	C18:1n7	30.48 ± 1.73	28.11 ± 1.52	0.018	30.35 ± 2.02	26.27 ± 1.58	0.025	NS
Linoleic acid	C18:2n6	277.69 ± 11.41	270.79 ± 8.69	NS	282.38 ± 8.61	278.31 ± 10.98	NS	NS
γ-Linolenic acid	C18:3n6	13.60 ± 0.77	11.92 ± 0.74	0.005	14.13 ± 0.81	13.00 ± 1.03	NS	NS
α-Linolenic acid	C18:3n3	16.42 ± 0.84	22.28 ± 1.40	0.001	17.40 ± 0.90	19.03 ± 1.71	NS	0.024
Dihomo-γ-linolenic acid	C20:3n6	12.68 ± 0.58	11.75 ± 0.64	NS	12.51 ± 0.75	12.24 ± 0.72	NS	NS
Arachidonic acid	C20:4n6	63.54 ± 2.76	57.70 ± 3.00	0.006	65.09 ± 4.08	51.19 ± 3.55	0.001	NS
Eicosapentaenoic acid	C20:5n3	19.82 ± 1.27	19.18 ± 1.30	NS	20.22 ± 1.70	16.45 ± 1.18	0.019	NS
Lignoceric acid	C24:0	2.64 ± 0.24	2.39 ± 0.19	0.046	2.82 ± 0.20	2.55 ± 0.17	0.036	NS
Nervonic acid	C24:1n9	2.61 ± 0.15	2.62 ± 0.14	NS	2.60 ± 0.14	2.53 ± 0.10	NS	NS
Docosapentaenoic acid n6	C22:5n6	16.35 ± 1.28	11.23 ± 0.64	0.000	17.97 ± 1.44	11.63 ± 0.75	0.000	NS
Docosapentaenoic acid n3	C22:5n3	9.08 ± 0.27	8.31 ± 0.30	0.023	8.99 ± 0.27	8.05 ± 0.38	0.019	NS
Docosahexaenoic acid	C22:6n3	22.56 ± 0.60	20.89 ± 1.09	NS	21.66 ± 1.74	17.06 ± 0.99	0.003	NS
Enzyme activity index								
SCD1 activity index	C16:1 / C16:0	0.21 ± 0.01	0.21 ± 0.01	NS	0.22 ± 0.01	0.22 ± 0.00	NS	NS
Elongase activity index	C18:0 / C16:0	0.22 ± 0.01	0.20 ± 0.01	0.014	0.21 ± 0.01	0.20 ± 0.00	0.027	NS
Lipogenic index	C16:0 / C18:2n6	0.78 ± 0.04	0.70 ± 0.04	0.003	0.77 ± 0.05	0.67 ± 0.04	0.006	NS

Δ5 desaturase activity index	C20:4n6 / C20:3n6	5.13 ± 0.27	5.05 ± 0.27	NS	5.43 ± 0.36	4.93 ± 0.42	0.037	NS
Δ6 desaturase activity index	C18:3n6 / C18:2n6	0.050 ± 0.004	0.045 ± 0.003	0.045	0.050 ± 0.002	0.047 ± 0.004	NS	NS

Values are shown as mean±SEM. NS, not significant (*P*>.05).

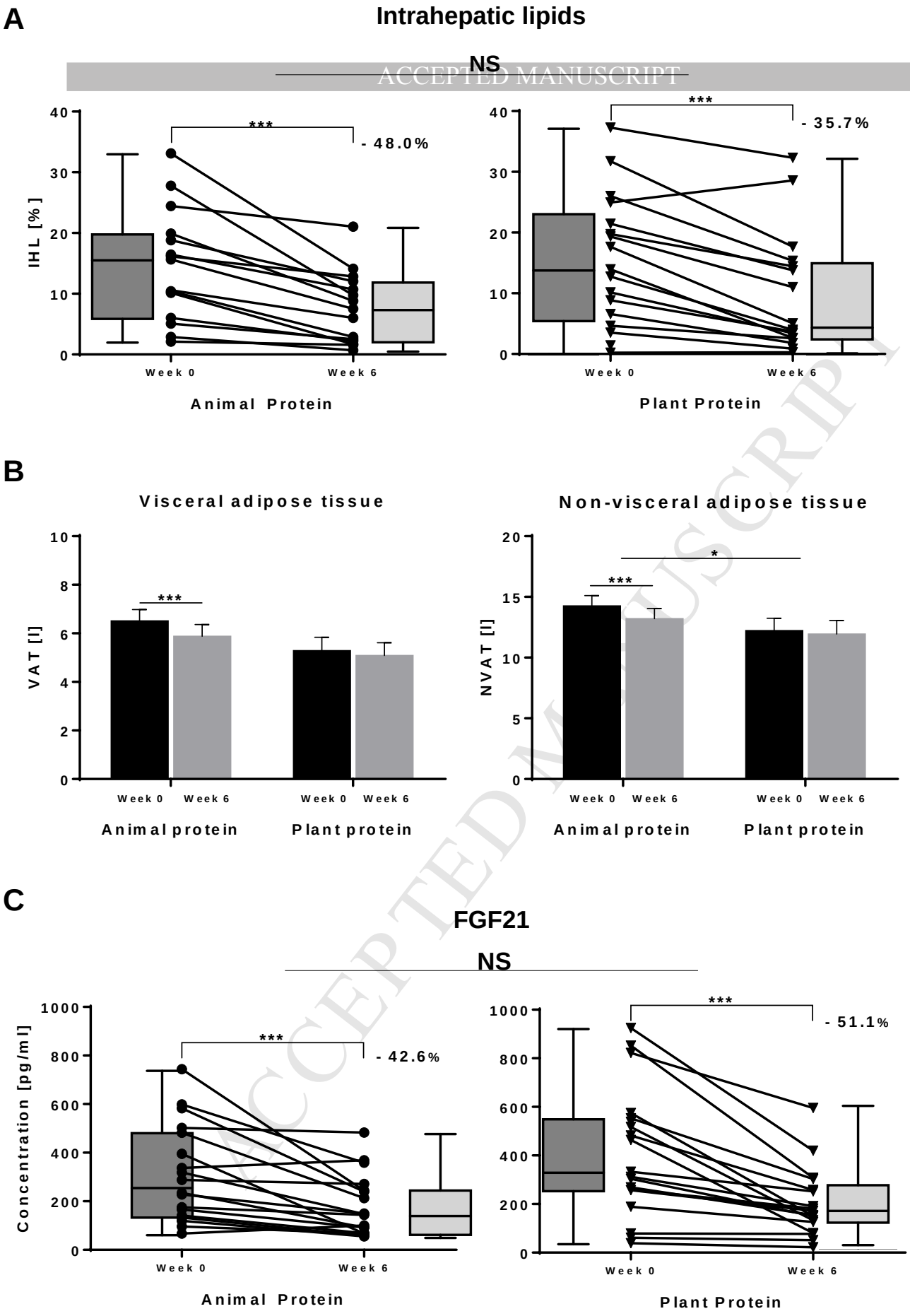

Figure 1. High-protein diet reduced IHL, VAT, SAT, and serum FGF21 in T2DM. Boxplots and individual changes in intrahepatic lipid content *(A)* and serum FGF21 *(C)* after 6 weeks of intervention; *B:* changes in adipose tissue depots after 6 week of intervention. $15 \leq n_{AP} \leq 18$, $17 \leq n_{PP} \leq 19$, results are shown as mean±SEM (B). **P*<.05, ***P*<.01, ****P*<.001. NS, not significant.

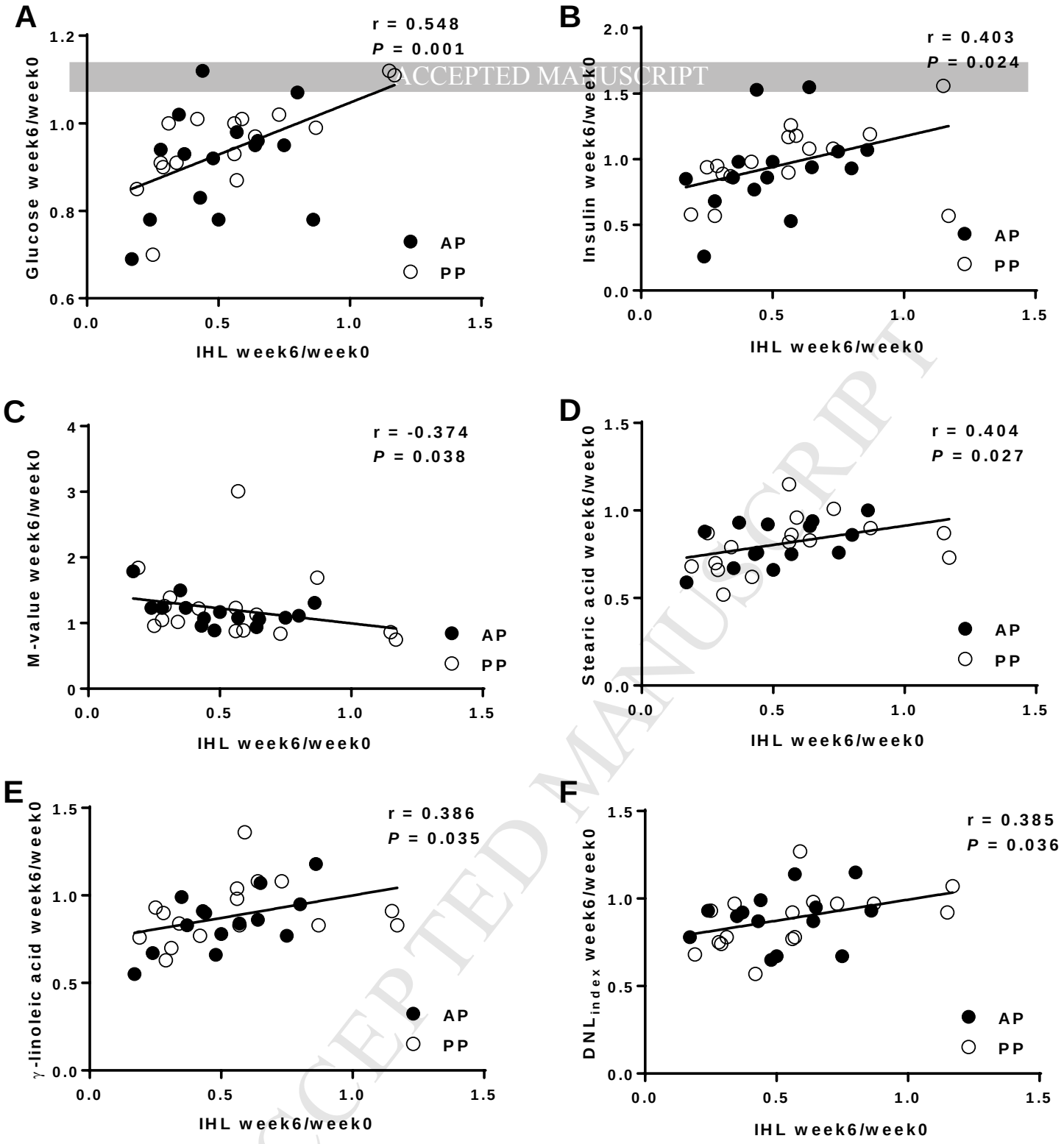

Figure 2. Relative reduction of IHL correlates with relative changes in metabolic parameters. Correlation between the relative change in IHL from week 0 to week 6 with the relative change in fasting plasma glucose *(A)*, fasting serum insulin *(B)*, whole-body insulin sensitivity (M-value) *(C)*, fasting plasma stearic acid C18:0 *(D)*, fasting plasma γ-linoleic acid C18:3n6 *(E)*, and lipogenic index C16:0/C18:2n6 *(F)*. n_{AP}=15, n_{PP}=17.

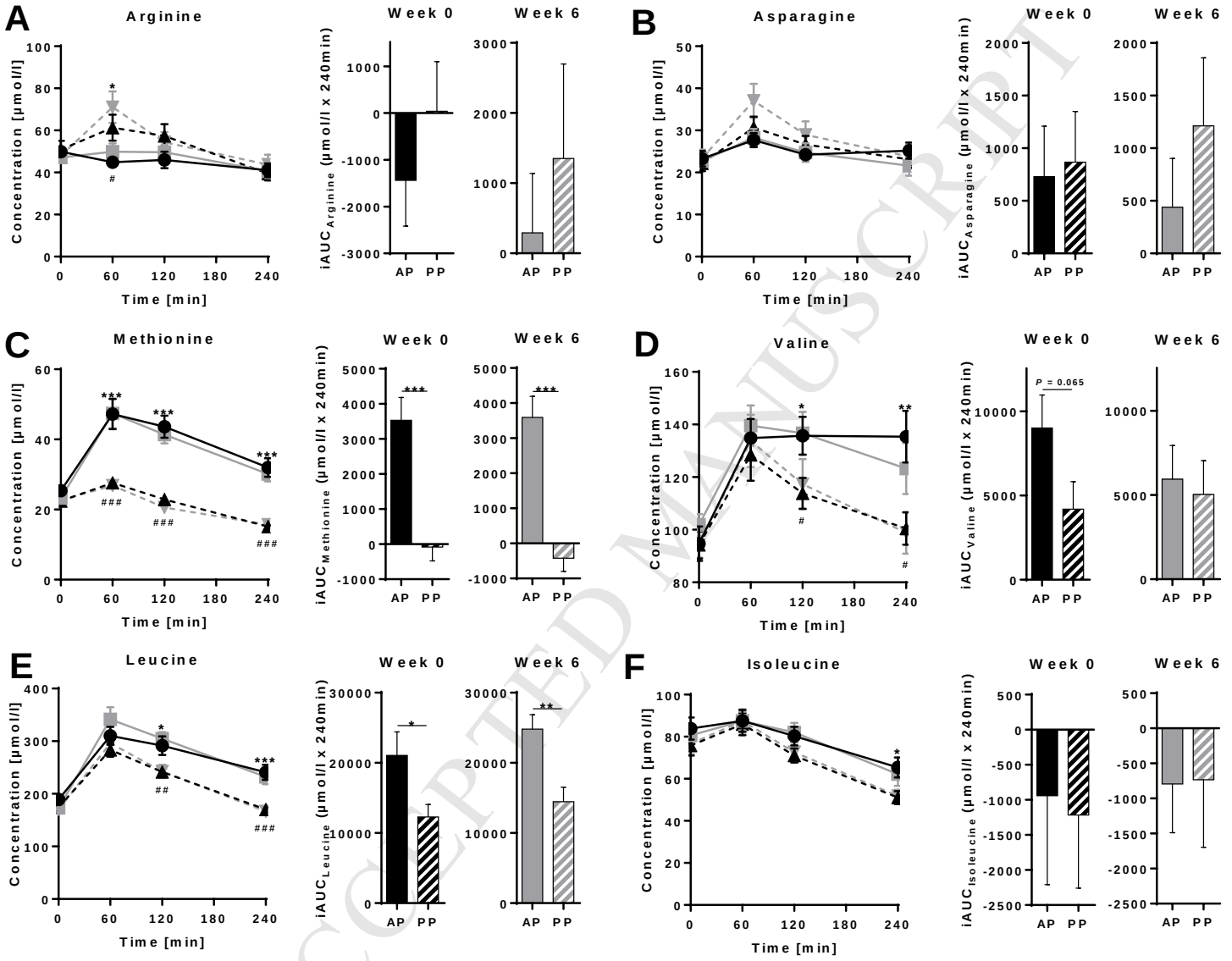

Figure 3. Differences in postprandial FAA levels after AP and PP meals. Plasma levels of arginine *(A)*, asparagine *(B)*, methionine *(C)*, valine *(D)*, leucine *(E)*, and isoleucine *(F)* over 240 minutes after test meal ingestion and corresponding iAUCs. AP week 0: black line, black bar; AP week 6: grey line, grey bar; PP week 0: dotted black line, dashed black bar; PP week 6: dotted grey line, dashed grey bar. n_{AP}=18, n_{PP}=19, results are shown as mean±SEM. *significant difference between AP and PP at week 0, #significant difference between AP and PP at week 6. **P*<.05, ***P*<.01, ****P*<.001.

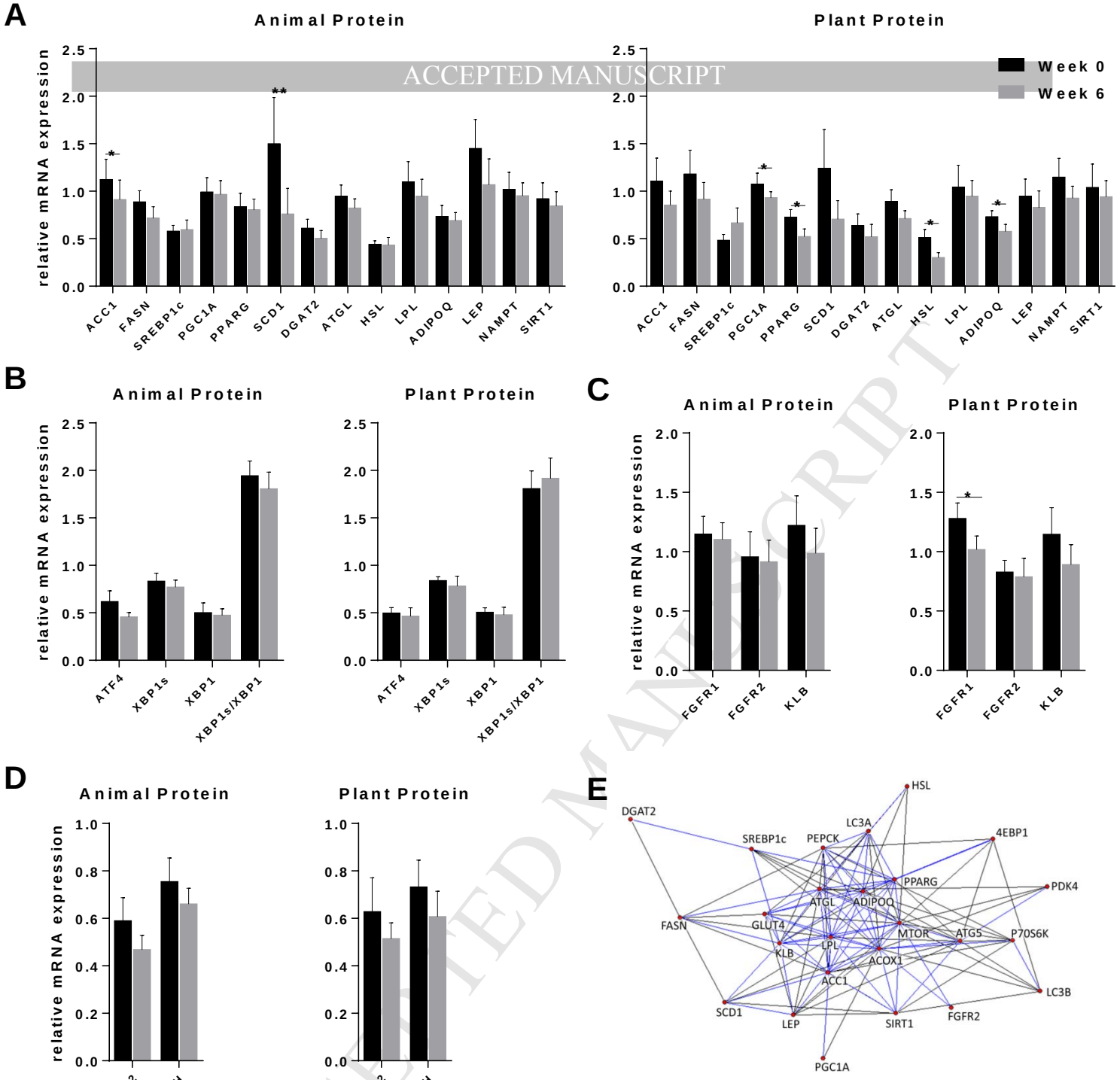

Figure 4. High-protein diet regulates gene expression in subcutaneous adipose tissue. Changes in relative mRNA expression of genes involved in fat metabolism *(A)*, ER-stress *(B)*, FGF21 pathway *(C)*, and BCAA degradation *(D)* after 6 weeks of intervention. Results are shown as mean±SEM. *E*: correlation network of changes in gene expression. The color of the edges represents the quantity of the correlation coefficients (blue: 0.75>r≥0.5; black: 0.5>r≥0.3). All correlation coefficients were positive and highly significant. n_{AP}=12, n_{PP}=15. **P*<.05, ***P*<.01, ****P*<.001.

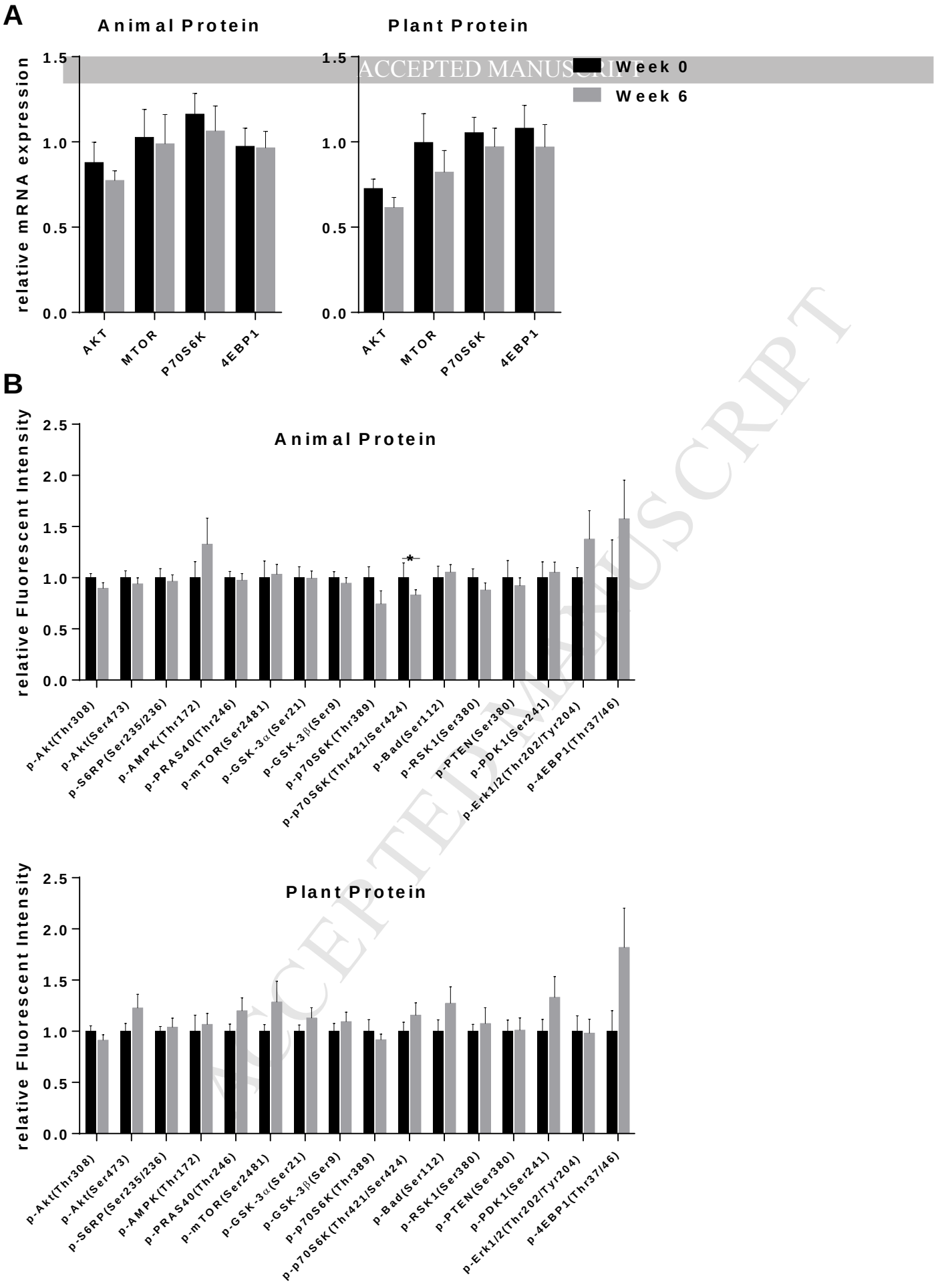

Figure 5. High-protein diet does not induce activation of the mTOR pathway in SAT. A: changes in relative the mRNA expression of genes involved in mTOR pathway after 6 weeks of intervention, n_{AP}=12, n_{PP}=15. B: phosphorylation state of key enzymes of Akt-mTOR pathway at baseline and week 6, n_{AP}=8, n_{PP}=8. Results are shown as mean±SEM. **P*<.05, ***P*<.01, ****P*<.001.