Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke (DIfE)
Abteilung Klinische Ernährung

Metabolic and molecular effects of two different isocaloric high protein diets in subjects with type 2 diabetes

Dissertation
zur Erlangung des akademischen Grades
"doctor rerum naturalium"
(Dr. rer. nat.)
in der Wissenschaftsdisziplin
„Physiologie und Pathophysiologie“

eingereicht an der
Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Potsdam

von
Mariya Markova

Potsdam, August 2016

Meiner Familie gewidmet.

Благодаря ВИ за всичко!

Index of contents

I. Index of tables

II. Index of figures

III. Abbreviations

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
ACC1	Acetyl-CoA carboxylase
ACOX1	Acyl-CoA oxidase 1
ADA	American Diabetes Association
AdipoIR	Adipose tissue insulin resistance
ADIPOQ	Adiponectin
AGEs	Advanced glycation end products
AKT	Protein kinase B
ALT	Alanine transaminase
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AP	Animal protein
AST	Aspartate transaminase
ATF4	Activating transcription factor 4
AT_{femur}	Adipose tissue on the level of femoral head
ATG5	Autophagy-related protein 5
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
Bad	BCL2 associated agonist of cell death
BCA	Bicinchoninic acid
BCAA	Branched chain amino acids
BCAT2	Branched chain amino acid transaminase 2
BCKDHA	Branched chain keto acid dehydrogenase E1, α polypeptide
BMI	Body mass index
BSA	Bovine serum albumin
CD163	Cluster of differentiation 163
cDNA	complementary DNA
ChREBP	Carbohydrate regulatory element-binding protein
CID	Clinical investigation day
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT2	Diacylglycerol O-acyltransferase 2

DGE	German Nutrition Society (Deutsche Gesellschaft für Ernährung)
DI	Disposition index
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl-peptidase IV
EASD	European Association for the Study of Diabetes
EN%	Percentage of energy intake
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMR1	EGF-like module receptor 1
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAA	Free amino acids
FASN	Fatty acid synthase
FFA	Free fatty acids
FFM	Fat free mass
FGF21	Fibroblast growth factor 21
FM	Fat mass
GC	Gas chromatography
GFR	Glomerular filtration rate
γGT	γ-Glutamyl transpeptidase
GI	Glycemic index
GIP	Glucose-dependent insulinotropic peptide
GIR	Glucose infusion rate
GL	Glycemic load
GLP-1	Glucagon-like peptide 1
GLUT4	Glucose transporter 4
GSK-3α	Glycogen synthase kinase 3 alpha
GSK-3β	Glycogen synthase kinase 3 beta
GUSB	Beta-glucuronidase
HbA_{1c}	Glycated hemoglobin
HDL	High-density lipoprotein
HIC	Hepatic insulin clearance
HOMA-IR	Homeostasis model assessment for insulin resistance
HSL	Hormone sensitive lipase
iAUC	Incremental area under the curve

IDF	International Diabetes Federation
IFNγ	Interferon γ
IFG	Impaired fasting glycaemia
IGT	Impaired glucose tolerance
IHL	Intrahepatic lipids
IL	Interleukin
IQR	Interquartile range
IRS1	Insulin receptor substrate 1
ITGAX	Integrin α X (synonym: Cluster of differentiation 11c; CD11c)
LC3A	Microtubule-associated protein 1A-light chain 3
LC3B	Microtubule-associated protein 1B-light chain 3
LC-MS/MS	Liquid chromatography mass spectrometry
LDL	Low-density lipoprotein
LEP	Leptin
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MRC1	Mannose receptor, C type 1
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MRS	Magnetic resonance spectroscopy
mTOR	Mammalian target of rapamycin
MTT	Meal tolerance test
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NAMPT	Nicotinamide phosphoribosyltransferase
NC	Nutritional counselling
ns	Not significant
NVAT	Non-visceral adipose tissue
OGIS	Oral glucose insulin sensitivity
OGTT	Oral glucose tolerance test
P62	Sequestosome-1
p70S6K	Ribosomal protein S6 kinase B1
PAL	Physical activity level
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PDK1	Pyruvate dehydrogenase kinase 1
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1A	Peroxisome proliferator-activated receptor γ, coactivator 1 α
PPARG	Peroxisome proliferator activated receptor γ
PRAS40	Proline-rich Akt substrate of 40 kDa
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cells
REE	Resting energy expenditure
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RQ	Respiratory quotient
RSK1	Ribosomal protein S6 kinase A1
SAT	Subcutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase-1
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFA	Saturated fatty acids
SGLT	Sodium-dependent glucose cotransporter
SIRT1	Sirtuin 1
SREBP1c	Sterol regulatory element-binding protein 1c
T2D	Type 2 diabetes
TG	Triglycerides
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
UACR	Urine albumin-to-creatinine ratio
VAT	Visceral adipose tissue
WBC	White blood cells
WHO	World Health Organization
WHR	Waist-to-hip-ratio
XBP1	X-box binding protein 1
XBP1s	Spliced X-box binding protein 1

1. Introduction

1.1. Type 2 diabetes

Type 2 diabetes (T2D) represents a complicated network of metabolic disorders including insulin resistance, impaired glucose uptake, impaired β-cell function, and chronic hyperglycemia and hyperinsulinemia [1, 2]. Several risk factors contribute to the development of T2D (Figure 1), the most prominent of which being age, overweight, and genetic predisposition.

Large epidemiological studies have shown an independent influence of age on T2D development [3, 4]. Numerous investigations in twins revealed the genetic susceptibility to T2D [5-9]. Genome-wide association studies verified more than 175 genetic loci related to increased risk of T2D [10], explaining however only 10% of the heritability [11]. Remarkably, the growing epigenetic data suggest additional mechanisms for the relationship between environment and genotype and their contribution to T2D [12].

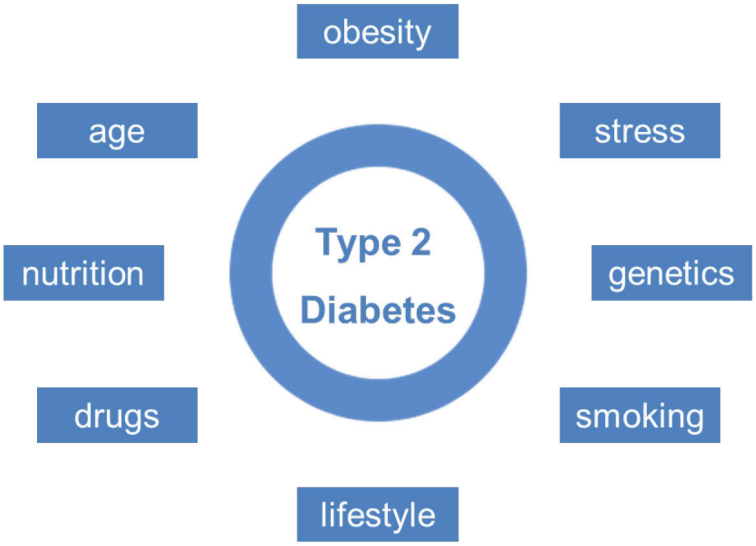

Figure 1. Risk factors for developing type 2 diabetes.

Being overweight or obese is a well-established risk factor for chronic metabolic diseases, including T2D [13-15]. Specifically, a high body mass index (BMI), waist circumference, waist-to-hip-ratio (WHR), and body fat distribution are important predictors of the disease [16, 17]. Overnutrition and poor diet represent other main factors contributing to the increasing incidence of T2D. Carbohydrates and especially consumption of simple sugars lead to a significantly higher risk [18, 19]. In addition, saturated and trans fatty acids are associated

with T2D, while mono- and polyunsaturated fatty acids play a more beneficial role [20]. Lack of physical activity was demonstrated to cause around 7% of the burden of T2D [21], whereas exercise training improves glycemic control and insulin sensitivity of diabetic patients [22].

Epidemiological data indicates psychosocial stress as an independent predictor of T2D [23, 24]. Studies have shown that the intake of pharmaceuticals, such as antihypertensive medications or antidepressants, can increase the risk for T2D [25-27]. Smoking is another important factor that contributes to T2D onset [28-30]. Interestingly, loss of sleep is also associated with increased risk for T2D development [31].

Other contributing factors for the growing evidence of T2D are population growth and increased life expectancy, urbanization and change of lifestyle. According to the 7th edition of the International Diabetes Federation (IDF) Atlas from 2015 around 415 million people worldwide have diabetes and this number will rise to 642 million in the year of 2040, whereby the highest increases are expected in less developed countries in Africa and South Asia.

1.1.1. Definition

T2D has been known as a disease of elevated blood and urine glucose levels associated with excessive weight around 600 years BCE. At that time, an Indian physician described the disease by the passage of large amounts of “honey-tasting” urine and its relationship with a sedentary lifestyle and obesity. As a primary treatment he recommended physical activity [1].

T2D is a chronic disease characterized by insulin resistance accompanied by hyperglycemia and impaired β-cell function. Prediabetes – the precursor stage of T2D – includes condition of impaired fasting glycaemia (IFG) and impaired glucose tolerance (IGT). The progress of prediabetes to T2D can be prevented by lifestyle changes and diet interventions [32]. The criteria of the World Health Organization (WHO) and the American Diabetes Association (ADA) for the diagnosis of IFG, IGT and diabetes are summarized in Table 1. The parameters used for the diagnosis of diabetes include elevated plasma glucose levels in the fasting state and plasma glucose levels 2 hours after the ingestion of 75 g glucose (oral glucose tolerance test (OGTT)). IGT is defined for fasting plasma glucose between 6.1 and 6.9 mmol/l and 2-h plasma glucose from OGTT below 7.8 mmol/l [33]. Of note, the ADA recommends a lower range for impaired fasting glucose of 5.6 to 6.9 mmol/l [34]. An IGT is diagnosed for 2-h plasma glucose between 7.8 and 11.1 mmol/l. For a diabetes diagnosis, a fasting plasma glucose above 7.0 mmol/l and/or 2-h plasma glucose more than 11.1 mmol/l are required.

Table 1. Diagnostic and classification criteria of diabetes.

Metabolic condition	Fasting glucose mmol/l (mg/dl)	2-h glucose mmol/l (mg/dl)	HbA_{1c} % (mmol/mol)
Normal glycaemia	< 6.1 (< 110)	< 7.8 (< 140)	< 6.0 (< 42)
Impaired fasting glycaemia	6.1 – 6.9 (110 – 125)*	< 7.8 (< 140)	6.0 – 6.4 (42 – 46)
Impaired glucose tolerance	< 7.0 (< 126)	7.8 – 11.1 (140 – 200)	6.0 – 6.4 (42 – 46)
Diabetes (type 1 and 2)	≥ 7.0 (≥ 126)	≥ 11.1 (≥ 200)	≥ 6.5 (≥ 48)

*ADA recommendation: 5.6 – 6.9 mmol/l (100 – 125 mg/dl)

An International Expert Committee (ADA, IDF, and European Association for the Study of Diabetes (EASD)) recommend a threshold for glycated hemoglobin (HbA_{1c}) of 6.5%. Patients with levels between 6.0 and 6.4% are at high risk and might be considered for prevention interventions [35].

1.1.2. Pathophysiology

The pathogenesis of T2D is a complicated interplay between raised glucose levels, insulin resistance in different organs, and chronic subclinical inflammation (Figure 2). Although the exact mechanisms of insulin resistance and further T2D development need further investigation, already a lot is known about the pathophysiological aspects.

It is hypothesized that insulin resistance occurs initially in the adipose tissue. In the context of overnutrition and lack of physical activity excess energy is stored in adipose tissue in the form of triglycerides (TG). Adipose tissue has very high capacity for lipid storage and can expand almost infinitely involving cell growth and proliferation of adipocytes. Nevertheless, these processes lead to the impairment of cell function, cytokine secretion, apoptosis and cell death. Moreover, the release of inflammatory markers facilitates the formation of M1 macrophages, which in contrast to the resident M2 macrophages produce pro-inflammatory cytokines (e.g. IL-1β, IL-6, and TNFα) [36]. These cytokines activate signaling pathways in adipocytes resulting in inhibitory phosphorylation of the insulin receptor substrate 2 (IRS2) and thus in impairment of insulin sensitivity. Insulin induces the expression of lipoprotein lipase (LPL) and thereby the lipid supply on the one hand, and suppresses adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) and thus lipolysis and free fatty acid (FFA) release on the other [37] (Figure 2).

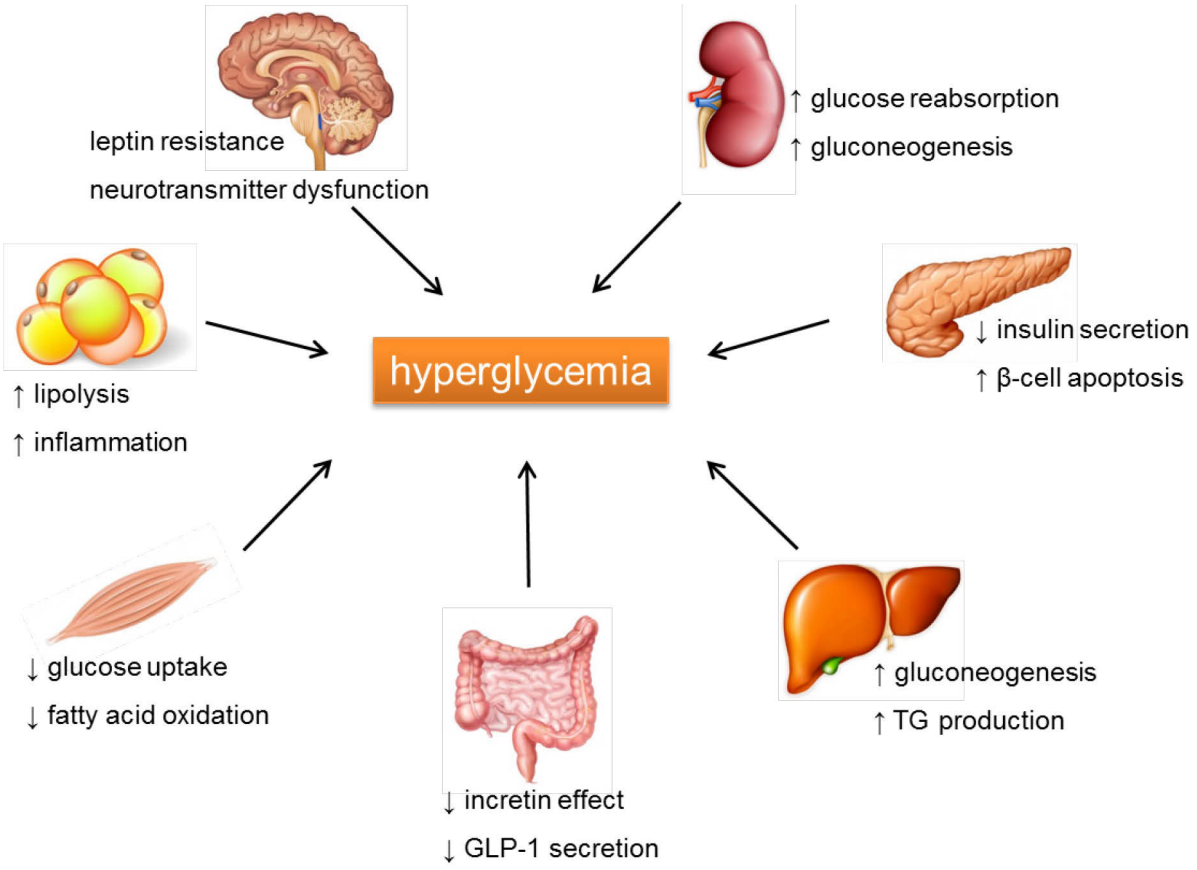

Figure 2. Pathophysiology of type 2 diabetes. Impaired insulin signaling in different organs contributes to chronic elevated blood glucose levels. Adipose tissue insulin resistance leads to increased lipolysis and fatty acids release as well as macrophage formation and cytokine secretion. Glucose uptake and oxidation of fatty acids are decreased in insulin resistant muscle cells. Insulin is not able to suppress gluconeogenesis in the liver, *de novo* lipogenesis and triglyceride storage are induced. Initially, the increased need of insulin for glucose utilization elicits β-cells proliferation, which further results in β-cell loss and decreased insulin synthesis. Response to incretins and GLP-1 secretion in the gut is impaired. Due to the high blood glucose levels increased glucose reabsorption and gluconeogenesis occur in the kidneys. Furthermore, leptin resistance and neurotransmitter dysfunction in the brain aggravate glucose intolerance. Abbreviations: GLP-1: glucagon-like peptide 1; TG: triglycerides. Adapted from Cornell, 2015 [38].

If overnutrition continues, the flux of FFA to the muscle rises. Part of the FFA are oxidized for the energy needs of the body and the excess of FFA are converted to TG [36, 37]. As a consequence, the formation of diacylglycerols (DAG) and ceramides is elevated. Both DAG and ceramides regulate signal chains leading to inhibition of IRS1 followed by enhanced translocation of glucose transporter 4 (GLUT4) and diminished glucose uptake and utilization in the muscle (Figure 2).

In the liver, insulin induces glycolysis and glycogenesis and suppresses gluconeogenesis. On the other hand, it stimulates *de novo* lipogenesis from glucose and fatty acids (originating from lipoprotein remnants and FFA from adipose tissue), triggers their conversion to TG and inhibits very low density lipoprotein (VLDL) release. In obesity and insulin resistant states,

the increased glucose and fatty acids flux into the liver accompanied by systemic hyperinsulinemia promote *de novo* lipogenesis and TG storage [36]. Similar to muscle, DAG and ceramide levels in the hepatocytes increase thereby triggering the development of insulin resistance in the hepatocyte [39]. Thus, gluconeogenesis is no more inhibited by insulin that in turn contributes to postprandial hyperglycemia. *De novo* lipogenesis and TG conversion processes, controlled by carbohydrate- and sterol regulatory element-binding proteins (ChREBP and SREBP), are further increased [39]. Since VLDL secretion is diminished, ectopic TG accumulate leading to development of non-alcoholic fatty liver disease (NAFLD) [37]. Remarkably, because of the high FFA flux into the liver, the β-oxidation is upregulated [40] and the mitochondrial capacity is elevated [41]. This in turn accounts for the enhanced generation of reactive oxygen species (ROS) leading to oxidative and endoplasmic reticulum (ER) stress, inflammation and apoptosis [39]. Moreover, the functional antagonist of insulin, glucagon, plays a major role in T2D pathogenesis. Increased hepatic sensitivity to glucagon and elevated glucagon in the circulation promote hepatic glucose production [42].

Because of the raised glucose levels in the blood, the β-cells of the pancreas produce more insulin. Firstly, the β-cell adaptation to increased demand for insulin results in enhanced proliferation and hyperplasia [43]. Thus, the β-cells can compensate with increased insulin production, which often occurs over a period of several years. Nevertheless, due to insulin resistance of other organs, the glucose and fatty acids supply to the pancreas is increased resulting in glucolipotoxicity [44]. As consequence of increased oxidative and ER-stress, cell apoptosis is induced leading to β-cell loss and transition to T2D [44, 45]. The impairment of IRS2 signaling and the islet amyloid formation were identified as important factors that contribute to β-cell failure [46, 47]. Moreover, the lack of suppression of the glucagon secretion from the α-cells further facilitates gluconeogenesis in the liver [38].

Blood glucose is filtered by the kidneys, whereby 99% of it is reabsorbed in the proximal tubule [38]. Around 90% is reabsorbed by sodium-dependent glucose cotransporter 2 (SGLT2) in concert with glucose transporter GLUT2, and the rest by SGLT1 and GLUT1 [48]. Glucose is excreted in urine when blood concentration rises above the renal absorption threshold (~ 180 mg/dl for healthy people) [38]. Nevertheless, kidneys of patients with T2D have higher capacity for glucose reabsorption which in turn worsens hyperglycemia [42]. On the other hand, besides the liver, the kidney is the other organ that synthesizes glucose and releases it into the circulation. In the normal state, ~ 20% of newly synthetized glucose is derived from the kidney. However, renal gluconeogenesis is increased in T2D [49] and is therefore further conducive to elevated blood glucose levels (Figure 2).

In response to food, the gastrointestinal tract secretes two hormones (incretins), glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which induce insulin release from the β-cells. GLP-1 suppresses also glucagon secretion and promotes satiety [50]. T2D patients have impaired response to GIP and lower GLP-1 secretion [51]. This leads to a decrease of glucose-dependent insulin stimulation and an increase of glucagon secretion, aggravating chronic hyperglycemia (Figure 2).

Insulin is able to cross the blood-brain barrier and to suppress the appetite by regulating neuropeptide expression in the brain [38]. Due to neurotransmitter dysfunction, central insulin resistance may occur in T2D patients so that the effect of insulin on appetite is impaired [48]. Moreover, leptin – a satiety hormone secreted by adipose tissue – is not able to fulfill its function because of leptin resistance in the brain leading to decreased satiety [38] (Figure 2).

High blood glucose and tissue insulin resistance result in a vicious cycle that leads to the development of T2D. This pathophysiological state represents a huge burden that further leads to late comorbidities and shorter life expectancy. T2D strongly correlates with macrovascular (e.g. ischemic heart disease, peripheral vascular disease, cerebrovascular disease) and microvascular (e.g. nephropathy, retinopathy, neuropathy) complications [52]. Vascular alterations include anatomic and functional changes resulting in tissue damage and multiorgan dysfunction. Four major mechanisms were proposed to explain how diabetic hyperglycemia causes late complications: increased flux through the polyol pathway; elevated formation of advanced glycation end products (AGEs); activation of the protein kinase C, involved in numerous metabolic pathways; and upregulated hexosamine pathway activity [53].

1.1.3. Dietary recommendations for type 2 diabetes

Changes in lifestyle like a healthy diet, physical activity, weight loss and self-management are essential for the therapy and prevention of T2D. Large epidemiologic studies and meta-analyses showed metabolic improvements in diabetic patients and a reduced incidence of T2D in people at high risk following lifestyle changes [32, 54-58]. Numerous clinical and epidemiological studies have investigated dietary patterns for the prevention and treatment of T2D. In 2004, the Diabetes and Nutrition Study Group (DNSG) of the EASD published their evidence-based nutritional approaches for diabetes management [59]. The dietary statements of the ADA were updated in 2014 [60].

According to the DNSG recommendations, carbohydrate intake should be between 45 and 65 EN%, with emphasis on fiber-rich vegetables, fruits, legumes, and wholegrain cereals with low glycemic index (GI). Moreover, a minimum of 40 g of fibers per day and ≤ 10 EN% total free sugars are recommended [59, 60].

The maximal intake of total fat is 35 EN%, of which a maximum of 10 EN% trans- and saturated fatty acids (SFA) (ADA: ≤ 7 EN%), 10-20 EN% monounsaturated fatty acids (MUFA), ≤ 10 EN% polyunsaturated fatty acids (PUFA), and ≤ 300 mg/day cholesterol (ADA: ≤ 200 mg/day). Foods rich in ω-3 PUFAs like fatty fish and plant oils should be consumed 2-3 times per week [59, 60].

Protein intake for T2D patients without kidney disease may provide 10 – 20% of total energy intake (ADA: 15 – 20 EN%). Due to insufficient evidence, there are no recommendations for people with impaired renal function. No statements on the preferred type of protein were made [59, 60].

Consumption of foods rich in antioxidants, vitamins, minerals and trace elements is encouraged. Maximal salt intake is 6 g/day (ADA: ≤ 2.3 mg/day sodium). Alcohol consumption should be limited to 10 g/day for women and 20 g/day for men (ADA: 1 drink/day for women and 2 drinks/day for men) [59, 60].

Finally, ADA concludes that there are no universal nutrition patterns, and dietary therapies should be individualized according to the patient's health goals, culture, habits, and preferences for an optimal diet that could be maintained over the long term.

1.2. Dietary protein

Proteins are macromolecules that consist of long amino acid chains and play a major role for the metabolic functions. They perform various functions as structure proteins, enzymes catalyzing metabolic reactions, hormones and signal transducers, and transporters of molecules. There are 20 amino acids, nine of which (valine, leucine, isoleucine, methionine, lysine, histidine, phenylalanine, threonine, and tryptophan) cannot be synthesized by the human body (essential) and need to be taken up with the food [61].

The recommended daily protein intake depends on age, sex and body weight. The German Nutrition Society (DGE) recommends a protein intake of 0.8 g/kg/day for middle-aged and elderly people, which is approximately 9-11 EN%.

1.2.1. Protein metabolism

Dietary proteins are first denatured in the stomach due to the low level of pH, and are then degraded by pepsin, an unspecific protease. In the small intestine, proteins are further degraded to amino acids, di- and tripeptides by proteolytic enzymes, secreted by the pancreas. From the lumen they are transported through the enterocytes, enter the blood flow and are transported to the liver and peripheral tissues [61].

In the cell, damaged proteins are first conjugated with ubiquitin and then cleaved by the proteasome, a big protein complex, involving depletion of ATP. In contrast to glucose and fatty acids, amino acids cannot be stored, so that excess amino acids are used as an energy source. Amino acid turnover takes place mostly in the liver. The first step is the transfer of α-amino group (NH_4^+) from an amino acid to α-ketoglutarate and their conversion to α-ketoacid and glutamate, respectively. This reaction is catalyzed by transaminases; important enzymes are alanine transaminase (ALT) and aspartate transaminase (AST). Further, α-ketoacids are metabolized to pyruvate, acetyl-CoA, oxaloacetate or other intermediates of the citrate cycle, and then either converted to ketone bodies, fatty acids or glucose by gluconeogenesis or oxidized for ATP generation. Most of the amino acids are glucogenic (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, methionine, proline, serine, valine), two are ketogenic (leucine and lysine), and five are both glucogenic and ketogenic (isoleucine, phenylalanine, threonine, tryptophan, tyrosine) [61].
Branched chain amino acids (BCAA) (valine, leucine, isoleucine) are catabolized in the mitochondria, including transamination by the branched chain amino acid transaminase (BCAT), oxidative decarboxylation by the rate-limiting branched chain keto acid dehydrogenase (BCKDH) and formation of acetyl-CoA and succinyl-CoA [61, 62].
Excess NH_4^+ is converted to urea over the urea cycle, a process that takes place in the liver and partly in the kidneys. Urea is transported to the kidneys and excreted with the urine [61].

1.2.2. Protein-rich diets

In recent years, high protein diets have become popular due to their beneficial effect on obesity and metabolic risk factors [63]. In people with T2D, a high protein diet was proven to lower hyperglycemia and hyperinsulinemia [64, 65]. However, adverse effects were also observed [66, 67].

1.2.2.1. Effects on body weight and composition

Higher protein intake facilitates body weight loss and its maintenance thereafter [68, 69]. This is thought to be due to the regulation of appetite by the amino acid induced stimulation of GLP-1, cholecystokinin, and peptide YY and thereby to the resulting satiety [70, 71]. Moreover, high protein intake may increase energy expenditure by triggering postprandial thermogenesis due to increased protein turnover and upregulation of uncoupling proteins [72]. Furthermore, high protein diets induce fat mass loss while preserving fat-free mass, even following body weight loss [73-75].

1.2.2.2. Effects on glycemic control

Protein ingestion does not result in a net increase of blood glucose in people with diabetes even though amino acids are substrates for gluconeogenesis [76, 77]. Ingestion of proteins as well as individual amino acids induce insulin and glucagon secretion, whereby the responses to various proteins and amino acids are different and the metabolic response cannot be predicted based on their functional groups [77]. Stimulating effects on incretin secretion were reported in healthy and diabetic subjects [78, 79], which could partly explain the insulinotropic properties of proteins. Interestingly, bioactive peptides contribute to glycemic control due to their inhibitory effect on carbohydrate degrading digestive enzymes (α-glucosidases) and on dipeptidyl peptidase IV (DPP-IV), which inactivates GLP-1 and GIP [80]. Clinical studies with T2D patients showed reduced blood glucose, HbA_{1c} and insulin levels after high protein diet interventions [65, 81-83].

1.2.2.3. Effects on liver fat and blood lipids

It was observed that increased protein intake/supplementation reduced or blunted diet-induced liver fat content in lean and obese non-diabetic patients [84-87]. A recent meta-analysis of high dietary protein interventions reported no influence on lipid profiles but lower systolic and diastolic blood pressure [64].

1.2.2.4. Effects on renal function

High protein diets increase the formation of urea that should be eliminated by the kidneys. In 1982, Brenner hypothesized that a high protein intake impacts renal function by increasing glomerular pressure and hyperfiltration. He further hypothesized that a higher protein load leads to proliferation of the mesangial cells and thus to increased risk for development or progression of kidney disease [88]. Since T2D patients are at a higher risk for development of diabetic nephropathy, diabetology experts are very critical concerning long-term high protein diets.

Nevertheless, clinical data and meta-analyses that study whether lower protein intake slows down the progression of diabetic nephropathy are inconsistent and controversial [89-91]. Two studies observed no adverse effects of high protein diet on the glomerular filtration rate in T2D patients with microalbuminuria or nephropathy [92, 93]. The absence of a universal definition of high protein intake, bad compliance, and lack of long-term interventions hinder the establishment of dietary recommendations. Hence, this topic remains debatable and further research is warranted.

1.2.2.5. Adverse effects

Some studies have reported adverse effects of high protein intake in rodents and humans. A large epidemiologic study postulated that high protein intake is associated with increased cancer incidence and overall mortality in people up to 65 years old, but not in older populations [94]. In a clinical study, insulin sensitivity was shown to decrease following a high protein diet in overweight humans [95]. Methionine restriction in animals improves body composition and insulin resistance [96-98]. Two metabolomic studies showed associations of BCAA and aromatic amino acids with insulin resistance in obese subjects and with future diabetes development in nondiabetic subjects [66, 67].

A possible explanation for this phenomenon is the activation of the mammalian target of rapamycin (mTOR) pathway. mTOR senses and responds to a variety of environmental factors and regulates organismal growth and homeostasis. mTOR interacts with several proteins to form two distinct complexes mTORC1 and mTORC2. Rapamycin as well as cellular stress are two inhibitors of the mTORC1 action. In contrast, growth factors, amino acids, energy levels and nutrients lead to its activation. mTORC1 induces growth and cell cycle progression, metabolism and synthesis of macromolecules, and inhibits autophagy. mTORC2 is also activated by growth factors and stimulates the cell survival and cytoskeletal organization [99, 100].

In response to insulin, Akt activates mTORC1 and its downstream target p70S6 Kinase (Figure 3). In a negative feedback loop, mTORC1 and p70S6K inhibit insulin receptor substrate 1 (IRS1), thus interrupting the insulin signal cascade. Furthermore, p70S6K inhibits mTORC2, which leads to an attenuation of Akt activation [101, 102]. Essential amino acids (in particular leucine and arginine) activate mTORC1 through another pathway [103]. Thus, an excessive stimulation of mTORC1 by amino acids can decrease the insulin sensitivity of the cell. In humans, infusion of amino acids or ingestion of leucine-enriched essential amino acids, and ingestion of protein rich meals induced activation of the mTOR pathway in skeletal muscle [104-107], which was accompanied by impairment of insulin sensitivity.

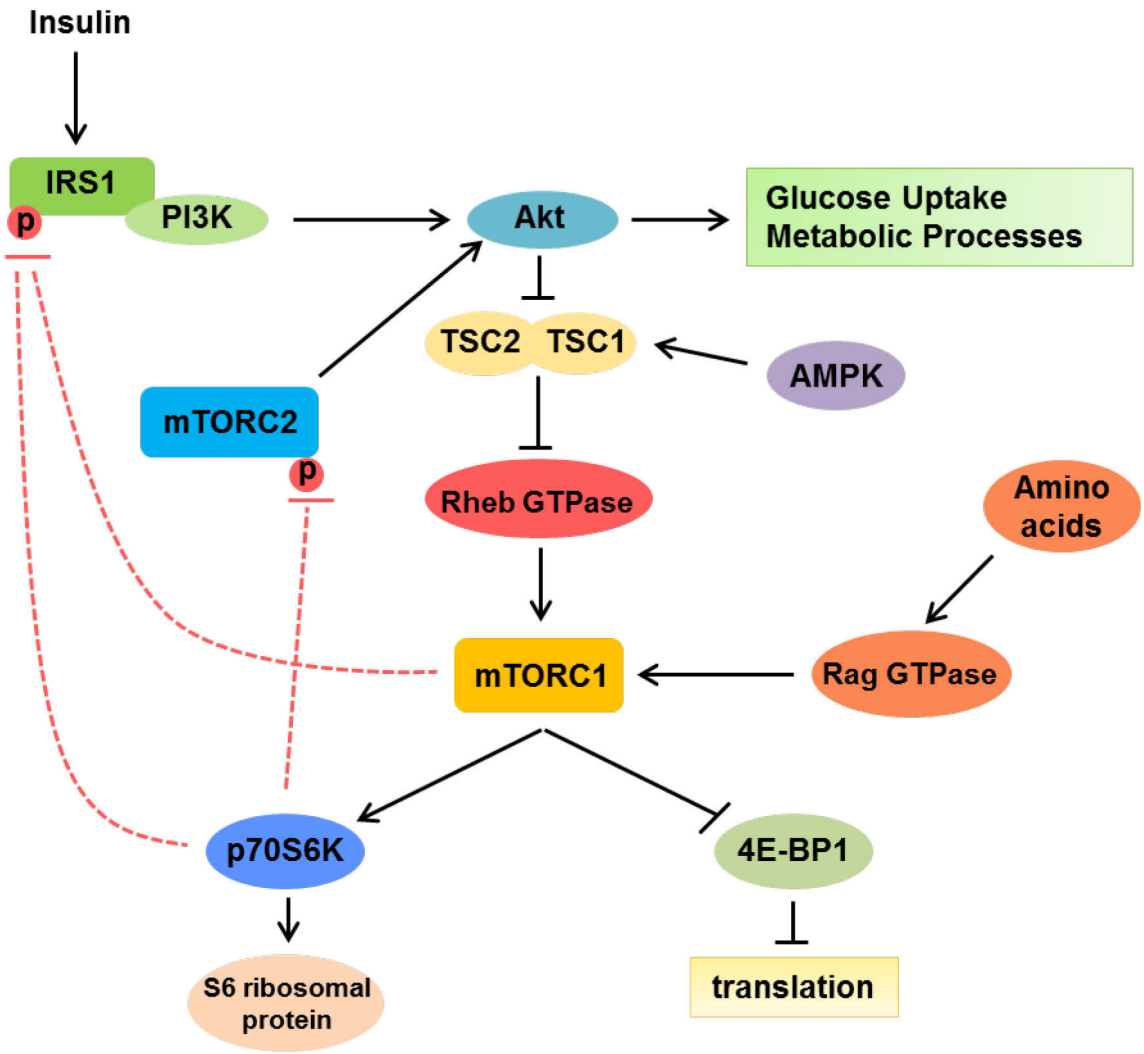

Figure 3. Signaling pathway of the mammalian target of rapamycin complex. Growth factors, nutrients, insulin activate IRS1 and PI3K, followed by induction of Akt pathway, glucose uptake and further metabolic processes. Akt induces mTORC1 (by inhibiting the TSC1-TSC2 complex, thus allowing Rheb GTPase to accumulate and activate mTORC1) which elicits upregulation of protein translation, proliferation and inhibition of autophagy. mTORC2, in response to growth factors, enhances Akt activation. Low energy levels and exercise upregulate AMPK, which inhibits mTORC1 by stimulation of TSC1-TSC2. On the other hand, amino acids (especially essential amino acids) increase mTOR in an Akt-independent manner, by inducing Rag GTPase and thereby promoting translocation of mTORC1 to lysosomal surface and its activation. A negative feedback loop, involving inhibitory phosphorylation of IRS1 by mTORC1 and p70S6K, and of mTORC2 by p70S6K, downregulates the cell response to insulin. Abbreviations: 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; GTP, guanine triphosphate; IRS1, insulin receptor substrate 1; mTOR, mammalian target of rapamycin; p70S6K, ribosomal protein S6 kinase B1; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; Rheb, Ras homolog enriched in brain; TSC, tuberous sclerosis complex.

Amino acids play a further role in the pathogenesis of insulin resistance by acting as gluconeogenic precursors and stimulating hexosamine biosynthesis [108].

1.2.2.6. Role of protein type

Proteins of different origins (animal: meat, dairy, fish; plant: legumes, soy, tree nuts) might provoke different effects on metabolism and diabetes. Epidemiologic studies, prospective cohorts, and meta-analyses postulated red meat as a high risk factor for T2D development [109-112]. Consumption of low-fat dairy products, yoghurt and cheese was found to correlate inversely with T2D risk [113-115]. Vegetarian diets were associated with improved glycemic control [116] and with lower T2D incidence [117, 118] and all-cause mortality [119]. Clinical trials observed reduction of cardiovascular risk factors and insulin resistance in T2D subjects after substituting animal with soy protein [120-123]. Potential mechanisms might involve secondary plant compounds, e.g. phytoestrogens and isoflavones. Fish protein showed beneficial effects on blood lipids and insulin secretion compared to other protein sources [124, 125]. Moreover, non-dairy animal protein may aggravate renal function in people with or without kidney disease in contrast to dairy and vegetable protein [126, 127]. Regarding dietary pulses, studies indicated that higher legume intake leads to improvement of glucose metabolism and lipid profile in T2D [128, 129]. Furthermore, meta-analyses showed beneficial effects of diets high in legumes or other plant proteins on glycemic control in people with T2D [130, 131]. However, the low glycemic index (GI) and high fiber content of the diets could have contributed to these positive effects, so that further studies are needed to explore this in detail. In contrast, Wheeler et al. studied two diets containing moderate protein amount (17 EN%) from either primarily animal (meat, fish, milk) or primarily plant (soy, legumes, vegetables) sources in patients with T2D and microalbuminuria. They found no advantages of the plant protein diet on renal function, cholesterol level, or glucose homeostasis compared to the animal protein diet [132]. Another important aspect is the quality of dietary fats (SFA, MUFA, PUFA), as they are differentially distributed in animal and plant foods. Many of the observed adverse effects of meat and high-fat dairy products are mainly due to their high SFA content. Thus there is no clear recommendation from existing studies regarding the comparison of protein type.

2. Hypothesis and aims of the study

The observed beneficial and adverse effects of high protein diets on the metabolism raise the question whether the origin of the protein (animal or plant) plays a role in those effects. Plant and animal proteins differ in their amino acid composition, which suggests different effects on metabolism. Negative effects on glucose homeostasis have been observed for methionine and branched chain amino acids [66, 97], while arginine ameliorates insulin metabolism [133]. Legumes, in contrast to animal food products, contain lower amounts of methionine and branched chain amino acids and higher levels of arginine [134]. Thus, intake of legumes should improve the metabolism, which is of particular importance for people with impaired glycemic control, especially T2D.

A human clinical intervention study within the framework of the LeguAN project ("Innovative und ganzheitliche Wertschöpfungskonzepte für funktionelle Lebens- und Futtermittel aus heimischen Körner**legu**minosen vom **A**nbau bis zur **N**utzung) was performed in order to investigate the metabolic effects of high protein diets of different origin (animal or plant) in patients with type 2 diabetes. The hypothesis of the LeguAN study was that plant protein from legumes has more beneficial effects in patients with type 2 diabetes than animal protein does. Nevertheless, it is unclear whether the favorable impacts of legumes are due to their amino acid composition or to the lower GI and fat content.

The aim of the intervention study was to compare the effects of the two high protein diets differing only in their amino acid composition. In order to eliminate the impact of other macronutrients, contents of carbohydrates, fats (SFA, MUFA, PUFA), fibers and GI were the same in both diets. The following aspects were investigated in detail:

- glycemic control and cardiovascular risk factors
- liver fat and body composition
- kidney function
- regulation of molecular pathways (e.g. mTOR) in blood and adipose tissue.

3. Materials and Methods

3.1. Materials

3.1.1. Chemical compounds

Chemicals	Company
Antibiotic Antimycotic Solution	Sigma Aldrich Chemie (Steinheim, Germany)
Bovine serum albumin (BSA), fatty acid free, low endotoxin	Sigma Aldrich Chemie (Steinheim, Germany)
Complete Mini EDTA-free Protease	Roche Diagnostics (Mannheim, Germany)
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich Chemie (Steinheim, Germany)
DPP-IV inhibitor	EMD Millipore (St. Charles, USA)
Paraformaldehyde	Carl Roth (Karlsruhe, Germany)
Lipopolysaccharides from Escherichia coli (LPS)	Applied Biosystems (Forster City, USA)
Phosphostop	Roche Diagnostics (Mannheim, Germany)
Sodium dodecyl sulfate (SDS)	Sigma Aldrich Chemie (Steinheim, Germany)
Triton X-100	Sigma Aldrich Chemie (Steinheim, Germany)
Water For Injection for Cell Culture	Life technologies (Gaithersburg, USA)
10x Lysis Buffer	Cell Signaling (Danvers, USA)
10x Dulbecco's Phosphate-Buffered Saline (DPBS)	Life technologies (Gaithersburg, USA)
RPMI 1640 Medium	Life technologies (Gaithersburg, USA)
RNase/DNase-free water	MP Biomedicals Inc. (Solon, USA)

3.1.2. Assays

Kits, ELISA	Company
ABX Cholesterol 100	Horiba ABX (Montpellier, France)
ABX Pentra Albumin	Horiba ABX (Montpellier, France)

ABX Pentra ALT CP	Horiba ABX (Montpellier, France)
ABX Pentra AST CP	Horiba ABX (Montpellier, France)
ABX Pentra CREA	Horiba ABX (Montpellier, France)
ABX Pentra CRP	Horiba ABX (Montpellier, France)
ABX Pentra GGT CP	Horiba ABX (Montpellier, France)
ABX Pentra Glucose HK CP	Horiba ABX (Montpellier, France)
ABX Pentra HbA_{1c}	Horiba ABX (Montpellier, France)
ABX Pentra HDL Direct CP	Horiba ABX (Montpellier, France)
ABX Pentra NEFA	Wako Chemicals GmbH (Neuss, Germany)
ABX Pentra Triglycerides CP	Horiba ABX (Montpellier, France)
ABX Pentra URAC	Horiba ABX (Montpellier, France)
ABX Pentra UREA	Horiba ABX (Montpellier, France)
Agilent RNA 6000 Nano LabChip® Kit	Agilent Technologies (Santa Clara, USA)
Agilent small RNA Nano LabChip® Kit	Agilent Technologies (Santa Clara, USA)
BCA Protein Assay Kit	Thermo Fisher Scientific (Rockford, USA)
Elisa Serum Adiponectin	R&D Systems (Minneapolis, USA)
Elisa Serum C-peptide	Mercodia (Uppsala, Sweden)
Elisa Serum Cystatin C	BioVendor (Brünn, Czech Republik)
Elisa Serum FGF21	R&D Systems (Minneapolis, USA)
Elisa Serum GIP	Meso Scale Diagnostics (Rockville, USA)
Elisa Serum GLP-1	Meso Scale Diagnostics (Rockville, USA)
Elisa Serum Glucagon	Mercodia (Uppsala, Sweden)
Elisa Serum IL-8	R&D Systems (Minneapolis, USA)
Elisa Serum Insulin	Mercodia (Uppsala, Sweden)
Elisa Serum MCP-1	R&D Systems (Minneapolis, USA)
Illumina TruSeq® RNA	Illumina (San Diego, USA)
Luminex Performance Human High Sensitivity Cytokine Magnetic Panel	R&D Systems (Minneapolis, USA)
miRNeasy Mini Kit	Qiagen (Hilden, Germany)
miScript II RT Kit	Qiagen (Hilden, Germany)
PathScan® Akt Signaling Antibody Array Kit	Cell Signaling (Danvers, USA)

PAXgene Blood miRNA Kit	PreAnalytiX / Qiagen (Hilden, Germany)
Power SYBR®Green PCR Master Mix	Applied Biosystems (Forster City, USA)
ProcartaPlex™ Multiplex Immunoassay	Affymetrix eBiosciences (Vienna, Austria)
RNase-free DNase Set	Qiagen (Hilden, Germany)
V-Plex Proinflammatory Panel Human IL-1β, IL-6, TNF-α	Meso Scale Diagnostics (Rockville, USA)

3.1.3. Primers

Gene	Forward primer	Reverse primer
Lipid metabolism		
ACC1	TCGCTTTGGGGGAAATAAAGTC	ACCACCTACGGATAGACCGC
ACOX1	CCAGTCTGAAATCAAGCCAGGT	AAACTGGAAGGCATAGGCAGTG
ATGL	AGCTCATCCAGGCCAATGTCT	GGTTGTCTGAAATGCCACCAT
DGAT2	AAGGGCTTTGTGAAACTGGC	CCTCCTCGAAGATCACCTGC
FASN	AGACACTCGTGGGCTACAGCAT	ATGGCCTGGTAGGCGTTCT
HSL	GCGCCCTCCTTGGCTCAACA	ATGCCATCTGGCACCCGCAC
LPL	TGCAGGAAGTCTGACCAATAAG	CCCTCTGGTGAATGTGTGTAAG
PGC1A	CAATGGAAGAGCGCCGTGT	GTGCACTCCTCAATTTCACCAA
PPARG	CGAGGGCGATCTTGACAG	TCTTTGCTCTGCTCCTG
SCD1	CATAACAGCAGGAGCTCATCGT	ACGAGCCCATTCATAGACATCA
SREBP1	CCACCGTTTCTTCGTGGAT	TGCTCGCTCTAAGAGATGTTCC
Adipokines		
ADIPOQ	GCAGTCTGTGGTTCTGATTCCA	GCAGTAGAACAGCTCCCAGCAA
LEP	TTTCTATGTCCAAGCTGTGCCC	GGAGACTGACTGCGTGTGTGAA
NAMPT	GCCAGCAGGGAATTTTGTTA	TGATGTGCTGCTTCCAGTTC
Glucose metabolism		
GLUT4	GCCGGACGTTTGACCAGAT	GGTGTTTCACCTCCTGCTCTA
PDK4	GCTGTCCATGAAGCAGCTACTG	AATGTTGGCGAGTCTCACAGG

PEPCK	AAGTATGACAACTGCTGGTTGGC	NICHT LESBAR
BCAA metabolism		
BCAT2	GGGCAGATCTGGGCACG	GCAGCCTTGAAACTGGAGGA
BCKDHA	CGATGGCATTGCAGCACGAG	GCCTCCTTTGTGGCGTTGTA
Energy metabolism		
SIRT1	ATGCTGGCCTAATAGAGTGGCA	CCTCAGCGCCATGGAAAAT
Autophagy		
ATG5	GGCCATCAATCGGAAACTCA	AGCCACAGGACGAAACAGCTT
LC3A	CCAGCAAAATCCCGGTGAT	CCGGATGATCTTGACCAACTC
LC3B	ATTCGAGAGCAGCATCCAACC	AGCATTGAGCTGTAAGCGCCT
P62	AATCAGCTTCTGGTCCATCGG	CCCTGCTCCACATCGATATCA
ER-stress		
ATF4	CAGCGACAAGGCTAAGGCGGG	CCGGAGAAGGCATCCTCCTTGCT
XBP1	GTGAGCTGGAACAGCAAGTGGT	CCAAGCGCTGTCTTAACTCCTG
XBP1s	CCGCAGCAGGTGCAGG	GAGTCAATACCGCCAGAATCCA
FGF21 pathway		
FGFR1	GAATTGGAGGCTACAAGGTCCG	TGCTGCCGTACTCATTCTCCAC
FGFR2	CCCGTGGAGGAACTTTTTAAGC	TGCCAACAGTCCCTCATCATC
KLB	CCAGGTGCTTCAAGCAATAAGG	AATAATCCTCGGCGGATGGT
mTOR pathway		
4EBP1	ACCCGATGACGCACAATTTG	TTTGGATGCCCCAGGAAGA
AKT	GCTTCTATGGCGCTGAGATTGT	TGATCTTAATGTGCCCGTCCTT
MTOR	TCATTGGAGACGGTTTGGTGA	CTTGCGTTGGAACATCCAAAGT
P70S6K	TGGCATGGAACATTGTGAGA	TAGCCCCCTTTACCAAGTACCC
TLR pathway		
TLR2	AGCACTGGACAATGCCACATAC	CATTGCGGTCACAAGACAGAGA
TLR4	TCTACAAAATCCCCGACAAC	TGGATTTCACACCTGGATA
Cytokines		
IL1B	GCAATGAGGATGACTTGTTCTTTG	CAGAGGTCCAGGTCCTGGAA
MCP1	CATAGCAGCCACCTTCATTCC	TCTGCAGTGAGATCTTCCTATTGG

TNFA	GGACCTCTCTCTAATCAGCCCTC	TCGAGAAGATGATCTGACTGCC
Macrophage marker		
CD163	TTGCCAGCAGTTAAATGTG	AGGACAGTGTTTGGGACTGG
ITGAX	TCGTGAGAGCTGTGATAAGCCA	GCGCCTGAATTCCTCGAAA
MRC1	CAGCGGTTGGCAGTGGA	CAGCTGATGGACTTCCTGGTAAC
Housekeeping gene		
GUSB	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA

3.1.4. Laboratory equipment

Instrument	Company
ABI Prism ViiA7	Applied Biosystems (Forster City, USA)
ABX Pentra 4000	Horiba ABX SAS (Montpellier, France)
Agilent 2100 Bioanalyzer	Agilent Technologies (Santa Clara, USA)
Bio–Plex™ 200 System	Bio-Rad Laboratories GmbH (Munich, Germany)
BOD POD®	COSMED (Rome, Italy)
Centrifuge 4k15C	Qiagen (Hilden, Germany)
Centrifuge 5417R	Eppendorf (Hamburg, Germany)
Digital scale	Soehnle Professional (Nassau, Germany)
Mastercycler ®	Eppendorf (Hamburg, Germany)
Metabolic Cart MV_{max} Series 29	SensorMedics (Bilthoven, The Netherlands)
NanoDrop® ND-1000	Thermo Scientific (Wilmington, USA)
ODYSSEY Infrared Imaging System	LI-COR Biosciences (Lincoln, USA)
SpeedMill P12	Analytik Jena (Jena, Germany)
Vmax® Encore metabolic cart	CareFusion (Yorba Linda, USA)
Wallac Viktor2 1420 Multilabel Counter	PerkinElmer Life and Analyt. Sciences (Waltham, USA)

3.1.5. Software

Software	Company
2100 Expert Software Version 2.5	Agilent Technologies (Santa Clara, USA)
Bio-Plex Manager™ 6.0	Bio-Rad Laboratories GmbH (Munich, Germany)
GraphPad Prism Version 5.0	GraphPad Prism Inc. (La Jolla, USA)
ODYSSEY Software 3.0.30	LI-COR Biosciences (Lincoln, USA)
SPSS Statistics Version 20.0	IBM (Armonk, USA)
ViiA™ 7 Software Version 1.2.2	Applied Biosystems Inc (Forster City, USA)
WallacWorkout Version 1.5	PerkinElmer Life and Analyt. Sciences (Waltham, USA)

3.2. Clinical intervention study – the LeguAN study

The study was performed in the framework of the LeguAN project ("Innovative und ganzheitliche Wertschöpfungskonzepte für funktionelle Lebens- und Futtermittel aus heimischen Körner**legu**minosen vom **A**nbau bis zur **N**utzung) aimed at the efficient cultivation of legumes and the development of innovative food products, which was financed by the German Ministry of Food and Agriculture (Federal Ministry of Food and Agriculture, BMEL).

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. It is registered at www.clinicaltrials.gov with NTC Identifier NCT02402985. All participants were studied in the clinical research center, at the German Institute of Human Nutrition (DIfE) in the Department of Clinical Nutrition. The subjects provided a written informed consent before starting the study. They were informed about the aim, intent, and risks of the intervention and of their right to drop out at any stage of the trial. The study duration was approximately 2 years: from June 2013 to March 2015.

3.2.1. Inclusion criteria

Subjects were recruited from newspaper, poster, flyer, and internet announcements. The inclusion criteria were a diagnosed T2D, treated with diet and/or oral therapy, HbA_{1c} level between 6 and 11%, and no other severe diseases. People, who volunteered, were first

interviewed on the phone. They were asked about their medical history and health status. Exclusion criteria were:

- glucocorticoid therapy
- immunosuppression
- anemia
- endocrine disease
- severe liver disease
- kidney dysfunction
- GFR < 60 ml/min/1.73m^2
- malignant disease / cancer in the last 2 years
- apoplexy / myocardial infarction in the last 6 months
- coagulopathy
- mental or psychiatric disorder, addiction
- pregnancy
- relevant change of body weight (+/- 5 kg) in the last 8 weeks
- food intolerance against any animal or plant protein
- participation in another study

The oral medications metformin, DPP-IV inhibitors, and sulfonyl urea derivatives were accepted, while insulin or long acting insulin analogues and glucocorticoid drugs were not.
Individuals who met the inclusion criteria were invited for screening at the clinical unit. On the screening day the following investigations were performed: medical examination, family anamnesis, anthropometric measurements, fasting blood samples, blood pressure, fasting energy expenditure. If HbA_{1c} level as well as all other parameters were fulfilled, the subject was enrolled in the study.

3.2.2. Study design

The study included Caucasian subjects with diagnosed T2D on diet or medication therapy. Individuals, who met the inclusion criteria, were enrolled in the study 2-3 weeks after the screening day (Figure 4). The participants were put on either a high animal protein diet (AP) or a high plant protein diet (PP) for a period of 6 weeks. The macronutrient composition of both diets was 30 EN% proteins, 40 EN% carbohydrates, and 30 EN% fats. Moreover, consumed dietary fats were composed from 1/3 saturated fatty acids (SFA), 1/3 monounsaturated fatty acids (MUFA), and 1/3 polyunsaturated fatty acids (PUFA). The fiber content (~ 35 g/day) and the glycemic index (~ 55) of the two diets were identical.

A total of 44 participants were randomized in one of the two groups. After 7 dropped out, 37 individuals completed the study: 18 in the AP and 19 in the PP group. Following parameters were matched: age, sex, BMI, HbA_{1c}, and oral medication.

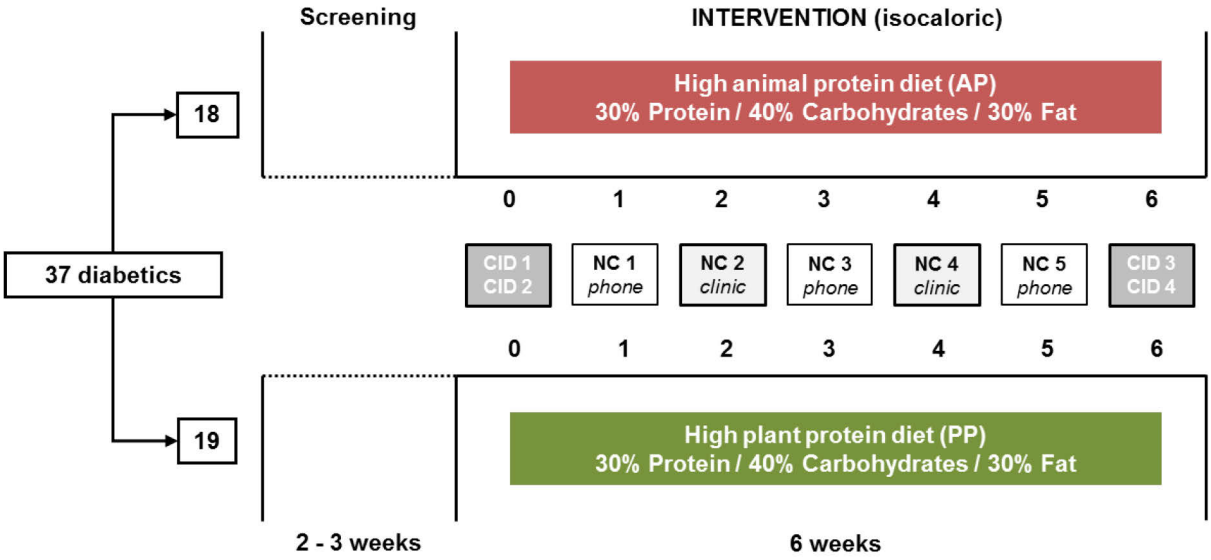

Figure 4. Design of the LeguAN-Study. Abbreviations: AP animal protein, CID clinical investigation day, NC nutritional counselling, PP plant protein.

At the beginning and at the end of the study (week 0 and week 6) the clinical investigation days (CID) took place. All medical investigations were divided on two consecutive days, CID 1 and 2 in week 0, and CID 3 and 4 in week 6 (Figure 4). During the study, at week 2 and 4 the subjects visited the institute for nutritional counselling (NC), blood sampling, and anthropometric measurements. Additionally, in week 1, 3 and 5 the participants were counselled about their diet on the phone and their body weight was documented.

3.2.3. Dietary intervention

The planning and preparation of dietary plans, supervision of participants and nutritional counselling, evaluation of dietary protocols and calculation of compliance (including macronutrients, single amino acids, fibers, and glycemic index) were conducted by Frau ***Stephanie Sucher****, a supervisor and nutritionist of the LeguAN study. Here I would like to acknowledge her huge contribution to the study.*

Participants in the study were asked to follow one of two different isocaloric high protein diets for a period of 6 weeks. In order to design individual isocaloric food plans, the energy expenditure of every participant was calculated before entering the study. Therefore, the total energy expenditure was estimated using the self-reported physical activity and the basal metabolic rate determined by indirect calorimetry on the screening day (chapter 3.3.2.). In

addition, the mean daily calorie intake was obtained via a 5-day dietary record which the participants completed prior to the intervention. Based on this data, individual isocaloric food plans were created to maintain the body weight constant throughout the study. Participants were asked not to change their physical activity habits for the duration of the study.

Both diets had the same macronutrient composition but differed in the origin of the proteins. The AP diet was rich in dairy and meat products, while the subjects in the PP group consumed high amounts of peas and other legumes. The Institute for Cereal Processing (IGV, Potsdam, Germany) had developed diverse food products enriched with pea proteins (e.g. bread, noodles, pancake mix, mashed potato, instant drink) especially for the PP diet. Nevertheless, the total exclusion of animal protein such as dairy products in the PP group was not advised, in order to achieve a good compliance. Therefore, the maximal amount of animal protein in the PP diet was around 30% of total protein intake, while the plant protein content in the AP diet was limited to 30% of total protein intake. Approximately 50% of the food was provided: pea protein enriched foods for the PP group (IGV, Potsdam, Germany), protein shakes for the AP group (Rossmann GmbH, Burgwedel, Germany), and ready-to-eat meals for all participants (FRoSTA GmbH, Hamburg, Germany).

Detailed daily meal plans were provided. In order to increase the compliance, the food plans were adapted to fit individual preferences and the participants received detailed substitution lists for food groups to allow more flexibility. Body weight was recorded every week. If there was a steady change of the body weight, the amount of energy intake was adapted respectively. Participants were asked to fill out dietary protocols every day and to weigh and note each consumed food item throughout the day. These food protocols were analyzed with PRODI® 6.2 software (Nutri-Science GmbH, Hausauch, Germany).

In addition, the glycemic index (GI), the glycemic load (GL), and dietary fiber content were set at similar levels for both diets to ensure the eventual influence of these factors on the results (GI ≈ 55, GL ≈ 130, fiber ≈ 35 g/day). The GI of a particular type of food is defined as the rise of blood glucose following consumption of this food in relation to the rise of blood glucose after consuming the same amount of glucose [135]. The incremental area under the two-hour blood glucose response curve (AUC) after consumption of the food (usually 50 g) is estimated and then divided by the 2h-AUC after intake of 50 g glucose: $GI = (AUC_{food} / AUC_{glucose}) \times 100$ [135, 136]. According to the American Diabetes Association (ADA), foods with GI of 55 or less have a low GI. Medium GI is between 56 and 69, while levels above 70 are foods with a high GI. GL estimates the impact of food intake according to the GI and the amount of carbohydrate that is consumed: $GL = (GI_{food} \times Amount_{food}) / 100$ [137].

The GI was estimated from the dietary protocols of every individual using values for each food item from GI tables [137-139] and then calculating the sum of GIs of consumed foods according to their proportion of total dietary carbohydrates [136, 139]. The following equation was used:

meal GI = $\sum$ [GI_{food} x (amount of available carbohydrates provided by food) / total dietary available carbohydrates)] [136, 139].

Dietary fibers – the indigestible components of plant derived foods – are associated with a lower risk for T2D [140-142]. The German Nutrition Society (DGE) recommends a daily intake of 30 g/day of fibers for adults and 40 g/day for type 2 diabetic patients.

Exemplary food plans are shown in the appendix (Table 20).

3.2.4. Clinical investigation days (CID)

Clinical investigation days took place on two consecutive days before the beginning and at the end of the dietary intervention, when all medical examinations were performed. The participants arrived at the institute ambulance early in the morning after overnight fasting (minimum of 10 hours). Furthermore, they were asked not to consume alcohol during the previous day. During the tests, the intake of medication was omitted in order to avoid the possible effect of the diabetic drugs on the examinations and the results. After consultation with the study doctor, the patients took their oral medicine at the end of the CID/NC.

On CID 1 and 3, the participants brought stool as well as 24-h urine samples, collected on the previous day. The intervention days started with anthropometric measurements (body weight, height, waist and hip circumference, body composition), blood pressure assessment and blood collection (Figure 5). Subsequently, an adipose tissue biopsy was performed, followed by a hyperinsulinemic euglycemic clamp (chapters 3.3.9. and 3.3.3.).

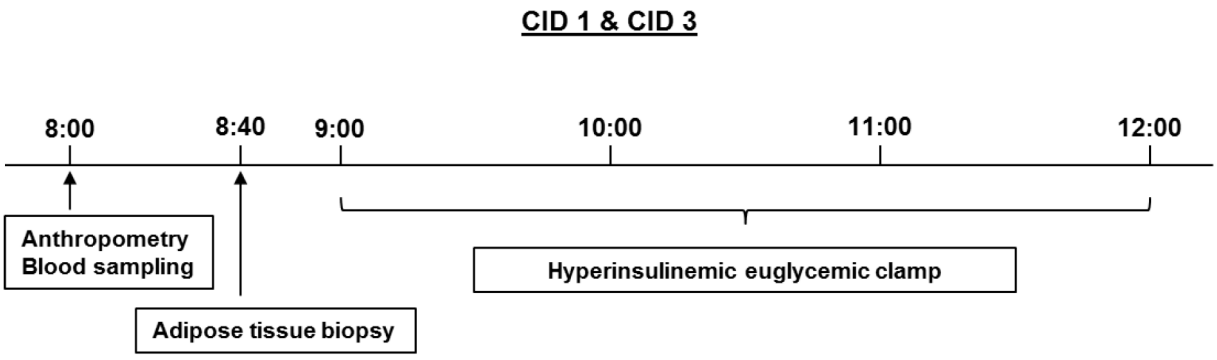

Figure 5. Day workflow on clinical investigation days (CID) 1 and 3.

CIDs 2 and 4 started with indirect calorimetry to determine the resting energy expenditure of the participants (Figure 6). After blood sampling, the meal tolerance test (MTT) took place, for which they received a standardized meal according to their dietary intervention (around 8:00). The subjects were asked to consume the meal within approximately 15 minutes. Blood samples were taken right before (0 min) and then 30, 60, 90, 120, 180, and 240 min after the time the meal was finished. At the end of the CID, magnetic resonance imaging (MRI) for quantification of abdominal fat depots and magnetic resonance spectroscopy (MRS) for determination of hepatic lipid content were performed (chapter 3.3.8.).

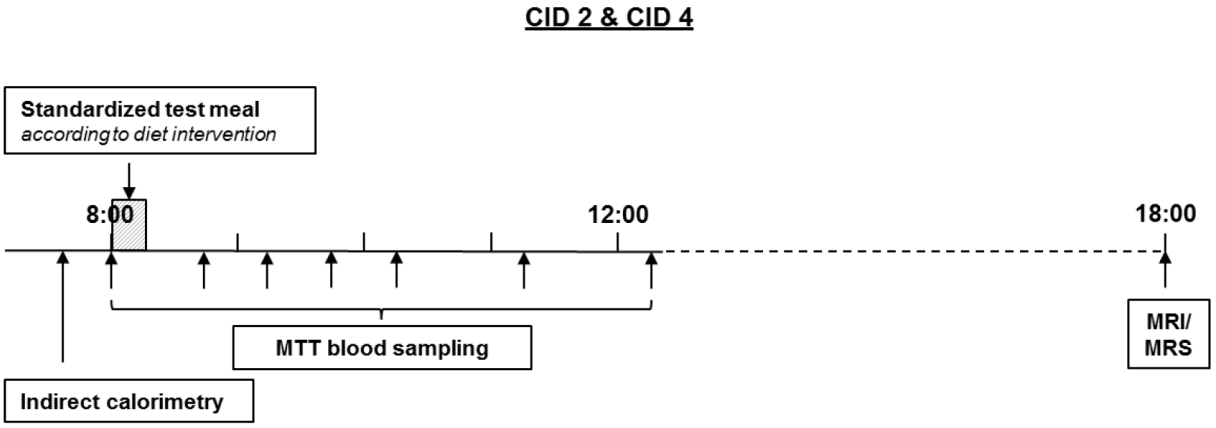

Figure 6. Day workflow on clinical investigation days (CID) 2 and 4. Abbreviations: MRI magnetic resonance imaging, MRS magnetic resonance spectroscopy, MTT meal tolerance test.

At the two additional visits in weeks 2 and 4 (NC 2 and 4), fasting blood samples and anthropometric data were collected. The participants were counselled in detail about the dietary plans and any problems associated with them. In weeks 1, 3 and 5, the subjects were contacted on the phone for additional nutritional consultations and asked for their health status, including body weight.

3.3. Methods

3.3.1. Anthropometric measurements

The anthropometric data of the participants were collected at each visit. The height was measured with a wall-mounted stadiometer, the body weight was taken with a calibrated digital scale (Soehnle Professional, Nassau, Germany), and the body mass index (BMI) was calculated as BMI [kg/m^2] = body weight [kg] / body height^2 [m^2]. The waist and hip circumferences were recorded, and the waist-to-hip-ratio (WHR) was subsequently

calculated. Blood pressure was measured in the resting state, and the mean of three measurements was recorded.

In order to determine the body composition of the participants, an Air Displacement Plethysmography was carried out using the BOD POD® (COSMED, Rome, Italy). On the basis of whole-body densitometry this technique enables the determination of the fat and the fat-free mass. The body volume is determined as the displaced air when the subject is seated, based on the change of pressure in the chamber and the physical relationship between volume and pressure. After calculating the body density as the ratio of mass to volume, the relative proportions of fat and lean mass are determined using mathematical equations.

3.3.2. Estimation of energy expenditure

The energy expenditure of the participants was determined for the design of the isocaloric dietary plans. For this purpose, the resting energy expenditure was measured by indirect calorimetry, the physical activity level (PAL) was evaluated according to a questionnaire, and 5-day dietary reports of the participants were used to estimate their regular calorie intake.

3.3.2.1. Indirect calorimetry

On the screening day the resting energy expenditure of the participants was estimated by indirect calorimetry (Vmax® Encore metabolic cart, CareFusion, Yorba Linda, USA). The principle of this technique is based on the oxygen (O_2) consumption and carbon dioxide (CO_2) production. In general, the inhaled O_2 is used for the oxidative catabolism of nutrients, which is then exhaled as CO_2. By the indirect calorimetry the volume of O_2 consumption and the volume of CO_2 production are measured while the subject is lying down and breathing spontaneously under a canopy hood. The measurement lasts 30 minutes. The estimated volumes of O_2 and CO_2 serve for the calculation of the resting energy expenditure (REE) and the respiratory quotient (RQ), as well as the oxidation rate of glucose (Gox), lipids (Lox), and protein (Pox). The formulas are [143]:

$$REE\ [kcal/day] = 3.91 \times V_{O2} + 1.1 \times V_{CO2} - 3.34 \times N_{const}$$

$$RQ = V_{CO2} / V_{O2}$$

$$Gox\ [g/min] = 4.55 \times V_{CO2} - 3.21 \times V_{O2}$$

$$Lox\ [g/min] = 1.67 \times V_{O2} - 1.67 \times V_{CO2}$$

$$Pox\ [g/min] = 6.25 \times N_{24\text{-h urine}}$$

The amount of nitrogen (N_2) in the urine was not estimated before the measurement, but a standard rate of 15 g/d was used instead (N_{const}). Protein oxidation was estimated from the urinary nitrogen excretion measured in 24-h urine samples ($N_{24\text{-h urine}}$).

3.3.2.2. Physical activity level (PAL)

For the estimation of physical activity each of the participants was asked to fill out a questionnaire about their activities throughout the day. This included the time spent sleeping, exclusively sitting, predominantly sitting, predominantly standing/walking, physical exercising, and the duration and frequency of sport activity per week. Table 2 lists some exemplary PAL values from the German Nutrition Society (DGE):

Table 2. Physical activity levels (PAL) according to the German Nutrition Society (DGE).

PAL value	Activity	Example
1.4	exclusively sitting with little or no physical activity	clerical worker, precision mechanic
1.6	sitting, sporadic standing and walking activity	laboratory assistant, driver, assembly-line worker, student
1.8	predominantly standing and walking activity	waiter, salesman, mechanic

The product of REE x PAL gives the energy requirement of the individual. Thus, the calorie intake for each participant was set according to the energy requirements and considering the energy intake from the 5-day dietary self-report. The questionnaires are included in the appendix.

3.3.3. Hyperinsulinemic euglycemic clamp

For estimating the influence of the dietary interventions on insulin sensitivity hyperinsulinemic euglycemic clamps were performed at the beginning and at the end of the study. The participants were asked to arrive fasted at the institute ambulance, and after blood sampling, two peripheral venous catheters were inserted in both arms. First, the plasma insulin concentration was raised by infusing an insulin solution of 800 mU/ml (Insuman®Rapid, Sanofi Aventis, Frankfurt, Germany) into a peripheral vein, whereby infusion rate was adjusted individually according to participant's body surface area. Then variable infusion of a 10% glucose (B. Braun, Melsungen, Germany) solution was started 10 minutes later, insulin infusion rate was kept constant at 40 mU/kg/min. The starting glucose infusion rate (GIR) is

calculated from individual body weight. The blood sugar glucose was measured every 5 minutes and the glucose infusion was adjusted to keep the plasma glucose concentration between 90 and 110 mg/dl. The steady state (ss) was achieved after approximately two hours, when the glucose infusion rate equaled the glucose uptake by the tissues for over 30 minutes. The insulin sensitivity or whole-body glucose disposal (expressed as insulin-mediated glucose uptake – M-value) was assessed from the GIR during the steady state, divided by body weight. Blood samples were collected at 0, 60, and 120 minutes and at steady state (ss1, ss2, ss3) to measure blood parameters and hormones.

3.3.4. Meal tolerance test (MTT)

The meal tolerance test (MTT) gives information about the hormone response after food intake. In contrast to an intravenous glucose tolerance test, in the MTT the food reaches the gastrointestinal tract and can affect the metabolic response and the secretion of gastrointestinal hormones. The MTTs were performed before and after the diet intervention. The participants received standardized meals according to their dietary intervention, nutrient compositions are shown in Table 3.

Each test lasted 4 hours; blood samples were taken in the fasting state (0 min) and throughout the test (at 30, 60, 90, 120, 180, and 240 min). The participants were asked to finish the meals within approximately 15 minutes and then the time points for the blood sampling were adjusted according to the time the meal was finished.

Food composition of the meals is shown in the appendix (Table 21).

Table 3. Nutrient composition of the meals in the MTTs.

Variables		Animal Protein MTT1	Plant Protein MTT1
Macronutrients			
Energy	[kcal]	486.6	457.0
Protein	**[EN%]**	**30.0**	**28.8**
	[g]	35.8	32.3
Animal protein	[%]	86.8	8.4
Plant protein	[%]	13.2	91.6
Carbohydrates	[**EN%**]	**40.3**	**39.7**
	[g]	48.2	44.5
Fat	[**EN%**]	**29.7**	**31.5**
	[g]	16.0	15.9
Fatty acids			
SFA	[g]	9.35	8.63
MUFA	[g]	4.66	4.25
PUFA	[g]	1.14	1.41
Glycemic Index			
Meal GI		65.2	65.2
Amino Acids			
Arginine	[mg]	1539.9	2825.6
Asparagine + Aspartic Acid	[mg]	3151.9	3932.2
Glutamine + Glutamic Acid	[mg]	8870.6	7384.2
Isoleucine	[mg]	2277.8	1707.9
Leucine	[mg]	3946.6	3065.5
Methionine	[mg]	1078.8	472.5
Tryptophan	[mg]	526.1	391.3
Valine	[mg]	2821.6	1883.5

Values are presented as means ± SEM.

3.3.5. Measurement of routine parameters in blood

Blood samples were collected throughout the clinical investigation days (CID 1-4, NC 2 and 4); 24-h urine samples were provided from the participants at CID1 and CID3. Tubes containing EDTA, lithium-heparin or citrate were used for plasma (S-Monovette®, Sarstedt AG & Co, Nümbrecht, Germany), depending on the further analysis. Immediately after blood sampling, the tubes were centrifuged (10 min, 3,000 x rpm, 4°C), whereas the serum samples were allowed to clot 10 min before centrifugation. Serum, plasma, and urine samples were aliquoted and stored at -80°C.

Glucose

The glucose concentration was estimated by the Hexokinase method of Slein [144], spectrophotometrically measured at 340 nm. Capillary blood glucose was determined via the glucose oxidase method at the Super GL glucose analyzer (Dr. Müller Gerätebau, Freital, Germany).

Insulin, C-peptide, glucagon

Enzyme-linked immunosorbent assays (ELISA) were used for the evaluation of insulin, c-peptide, and glucagon (Mercodia, Uppsala, Sweden).

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1)

GIP and active GLP-1 were determined with sandwich immunoassay (MSD, Rockville, USA), based on electrochemiluminescence. DPP-IV inhibitors (EMD Millipore, St. Charles, USA) were added to EDTA-plasma to avoid degradation of the incretins. The samples were added to a plate pre-coated with capture antibodies and then incubated with detection antibodies conjugated with electrochemiluminescent labels. Subsequently, a buffer was added to create an appropriate chemical environment for electrochemiluminescence and the plate was loaded into an MSD instrument. Voltage was applied to the plate electrodes causing the captured labels to emit light, whose intensity is proportional to the concentration of the analytes.

Indices of insulin resistance, degradation, and secretion

To estimate insulin resistance, homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated by the formula: HOMA-IR = (fasting insulin in mU/l x fasting glucose in mmol/l) / 22.5. Additionally, index of adipose tissue insulin resistance (AdipoIR) was calculated as fasting insulin in mU/l x fasting free fatty acids in mmol/l.

The index of hepatic insulin clearance (HIC = C-peptide$_{iAUC0-240}$ / Insulin$_{iAUC0-240}$) defines the postprandial insulin increase. Reduced HIC is associated with markers of the metabolic syndrome [145]. Oral glucose insulin sensitivity (OGIS) index, estimated from 2-h and 3-h OGTT [146], was assessed in the MTT using serum glucose (at 0, 120, 180 min) and insulin (at 0 and 120 min) levels, weight, height, and glucose dose. Matsuda index, developed from OGTT as an index of whole-body insulin sensitivity [147], was calculated in the MTT using fasting (0 min) and mean levels (over the time course 0-240 min) of glucose and insulin. The insulinogenic index (= Insulin$_{iAUC0-30}$ / Glucose$_{iAUC0-30}$) was used for estimation of first phase insulin secretion [148]. The disposition index (DI = Matsuda$_{0-240}$ x Insulin$_{iAUC0-240}$ / Glucose$_{iAUC0-240}$) was calculated as estimate of β-cell function after meal ingestion [149].

Blood lipids

The levels of the blood lipids (triglycerides (TG), total cholesterol, high-density lipoprotein (HDL cholesterol), low-density lipoprotein (LDL cholesterol), and free fatty acids (FFA)) were measured on the spectrophotometry based ABX Pentra 4000 analyzer (HORIBA ABX SAS, Montpellier, France) using colorimetric enzymatic kits (see chapter 3.1.2.).

C-reactive protein (CRP)

For the determination of CRP an immunoturbidimetric assay (Horiba ABX SAS, Montpellier, France) was used. It contains latex microbeads agglutinating with the CRP molecules and thus increasing the turbidity of the suspension due to the scattering effect of the particles. The concentration was measured by turbidimetry at 850 nm.

Other biomarkers

Established routine laboratory methods were carried out for the quantification of glycated hemoglobin (HbA_{1c}), urea, uric acid, creatinine, albumin, and the liver enzymes: aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), gamma-glutamyl transferase (γGT).

ELISA assays were used for the determination of cystatin C (BioVendor, Brünn, Czech Republic), as well as of FGF19 and FGF21 (R&D, Minneapolis, USA).

The glomerular filtration rate (GFR) was estimated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation. Table 4 is shows the CKD-EPI creatinine equation [150] and Table 5 the CKD-EPI creatinine – cystatin C equation [151].

Table 4. Chronic Kidney Disease Epidemiology Collaboration creatinine equation.

Sex	Scr [mg/dL]	Equation for GFR [ml/min/1.73m^2]
female	≤ 0.7	$144 \times (Scr/0.7)^{-0.329} \times 0.993^{Age}$
female	> 0.7	$144 \times (Scr/0.7)^{-1.209} \times 0.993^{Age}$
male	≤ 0.9	$141 \times (Scr/0.7)^{-0.441} \times 0.993^{Age}$
male	> 0.9	$141 \times (Scr/0.7)^{-1.209} \times 0.993^{Age}$

Abbreviations: Scr, serum creatinine level.

Table 5. Chronic Kidney Disease Epidemiology Collaboration creatinine – cystatin C equation.

Sex	Scr [mg/dL]	Scys [mg/L]	Equation for GFR [ml/min/1.73m^2]
female	≤ 0.7	≤ 0.8	$130 \times (Scr/0.7)^{-0.248} \times (Scys/0.8)^{-0.375} \times 0.995^{Age}$
		> 0.8	$130 \times (Scr/0.7)^{-0.248} \times (Scys/0.8)^{-0.711} \times 0.995^{Age}$
female	> 0.7	≤ 0.8	$130 \times (Scr/0.7)^{-0.601} \times (Scys/0.8)^{-0.375} \times 0.995^{Age}$
		> 0.8	$130 \times (Scr/0.7)^{-0.601} \times (Scys/0.8)^{-0.711} \times 0.995^{Age}$
male	≤ 0.9	≤ 0.8	$130 \times (Scr/0.7)^{-0.207} \times (Scys/0.8)^{-0.375} \times 0.995^{Age}$
		> 0.8	$130 \times (Scr/0.7)^{-0.207} \times (Scys/0.8)^{-0.711} \times 0.995^{Age}$
male	> 0.9	≤ 0.8	$130 \times (Scr/0.7)^{-0.601} \times (Scys/0.8)^{-0.375} \times 0.995^{Age}$
		> 0.8	$130 \times (Scr/0.7)^{-0.601} \times (Scys/0.8)^{-0.711} \times 0.995^{Age}$

Abbreviations: Scr, serum creatinine level; Scys, serum cystatin C level.

Cytokines in blood

The levels of multiple inflammatory markers were measured by the cooperating partner Dr. Christian Herder (Institute for Clinical Diabetology, German Diabetes Center (DDZ), Düsseldorf, Germany). IL-1RA, IL-18, adiponectin and MCP-1 were detected by ELISA (R&D Systems, Minneapolis, USA). IL-4, IL-6, IL-8, IL-12p70, and TNF-α were determined using the Luminex magnetic bead technology according to the manufacturer's manual (Luminex Performance Human High Sensitivity Cytokine Magnetic Panel, R&D Systems, Minneapolis, USA).

3.3.6. Free fatty acids (FFA) analysis

The detection of FFA species was carried out by Dr. Klaus-Jürgen Petzke (Research Group Physiology of Energy Metabolism, German Institute of Human Nutrition (DIfE), Potsdam, Germany). FFA were measured with a modified method using extraction with isooctane, methylation with 10% H_2SO_4/CH_3OH, and subsequent analysis by gas chromatography (GC)

with flame ionization detection [152, 153]. Individual fatty acids were calibrated against an internal standard (IS, heneicosanoic acid 21:0) and were presented in µg/ml.
Briefly, 50 µl of plasma was mixed with 25 µl IS (prepared as 1 mg in 10 ml toluene), 500 µl methanol, 200 µl i-propanol, and 25 µl 1N HCl. The extraction of FFA was performed twice using 1.5 ml isooctane. After vortexing (10 min, 720 x rpm) and centrifugation (10 min, 1,000 x g, 15°C) the upper isooctane layer was evaporated to dryness under a stream of N_2 at room temperature. For methylation the dried FFA extract was re-dissolved in 2 ml 10% H_2SO_4/CH_3OH, vortexed (120 min, 750 x rpm, 60°C) and after cooling 2 ml saturated NaCl and 1.8 ml hexane were added and vortexed again (10 min, 720 x rpm). After centrifugation (5 min, 1,000 x g, 15°C) the upper hexane phase was evaporated under N_2 at room temperature, and re-dissolved in 200 µl toluene. Analysis of FFA was performed on an Agilent GC system 7890A equipped with Agilent 7000 GC/MS Triple Quad (Agilent Technologies, Waldbronn, Germany) and a flame ionization detector.

3.3.7. Free amino acids (FAA) analysis

The determination of free amino acids in the plasma was performed by Prof. Dr. Sascha Rohn and Katrin Wagner (Institute for Food Chemistry, University of Hamburg, Germany) via liquid chromatography mass spectrometry (LC-MS/MS). Plasma samples from the MTTs at time points 0, 60, 120, and 240 min were taken for the measurements. 245 µl acetonitrile/water (8/2, v/v) and 5 µl internal standard (d3-methionine) were added to 50 µl plasma sample. These were vortexed twice, shaken continuously over a period of 10 min and centrifuged (10 min, 12,000 x g, 10°C). After centrifugation, 200 µl of the supernatants were evaporated until dryness. The dried samples were reconstituted with 100 µl acetonitrile/water (1/1, v/v) and used for HPLC-ESI-QqQ-MS/MS analysis. Amino acids were analyzed on Agilent 1260 Infinity Quaternary LC System (Agilent Technologies, San Diego, USA) coupled to a QqQ-MS/MS API 4000 QTRAP® (AB Sciex, Darmstadt, Germany) equipped with a turbo ion spray source, operating in positive ion mode. Liquid chromatography was performed on a Kinetex® HILIC LC column (2.6 µm, 150 mm x 2.1 mm i.d.), equipped with a Kinetex® HILIC security guard column (Phenomenex, Torrance, USA).

3.3.8. Magnetic resonance imaging (MRI) and spectroscopy (^{1}H-MRS)

At the end of CID2 and CID4 the participants underwent an MRI scan at the Department of Diagnostic and Interventional Radiology, Ernst von Bergmann Hospital (Potsdam, Germany). The evaluation was performed by Dr. Jürgen Machann (Institute for Diabetes Research and Metabolic Diseases (IDM), Helmholtz Center Munich, Germany). MRI and MRS were

performed on a 1.5 T whole body scanner (Magnetom Avanto, Siemens Healthcare, Erlangen, Germany). For quantification of abdominal fat depots, an axial T1-weighted fast spin echo technique was applied as described previously [154]. Visceral adipose tissue (VAT) from femoral head to thoracic diaphragm and non-visceral abdominal adipose tissue (NVAT) integrating subcutaneous, intermuscular and intrathoracic fat from femur to humerus were quantified in liters by an automatic segmentation algorithm [155]. Additionally, adipose tissue on the level of femoral head was (AT_{femur}), which is representative for adipose tissue of the lower extremities, was determined [156]. Intrahepatic lipids (IHL) were quantified by single voxel ^{1}H-MRS in the posterior part of segment 7 [157] and IHL are given as ratio of fat (methylene+methyl resonances) divided by water+fat.

3.3.9. Adipose tissue biopsy

Subcutaneous adipose tissue samples were obtained periumbilically (in the region of the belly button) by fine-needle biopsy. Firstly, the skin area (2x2 cm) was numbed with local anesthetic (lidocaine). Then the fine-needle (2.1 mm), connected to a vacuum syringe with sterile NaCl solution, was inserted in a 3 mm cut in the skin. The vacuum enabled the suction of small tissue pieces (approx. 2 g), which were immediately washed with NaCl solution and finally flash-frozen in liquid nitrogen and stored at -80°C until analysis.

3.3.10. Blood cells isolation

The main problem of the protein analysis of the blood samples is that many of the phospho-epitopes of interest are part of signaling networks that respond to the environment and turn over rapidly. Therefore, the interval and manipulations used to eliminate red blood cells (RBC) from samples have the potential to introduce artifacts. For this study, a procedure was established including fixation of whole blood with subsequent removing of erythrocytes by lysis, based on a protocol published previously [158]. First, 2 ml of heparinized blood were fixed with 1.3 ml 10% formaldehyde (4% final concentration) for 10 min, followed by RBC lysis with 20 ml 0.15% Triton X-100 for 30 min at 37°C. Then the cell pellet was washed twice with 1x phosphate buffered saline (PBS; Life technologies Inc., Gaithersburg, USA) and centrifuged (10 min, 1,000 x g, room temperature). Finally, the WBC pellet was lysed with Cell Lysis Buffer (Cell Signaling, Danvers, USA), containing proteinase and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). To increase the solving of fixed proteins, 2% sodium dodecyl sulfate (SDS) was added to the protein lysis buffer followed by heating at 100°C for 20 min and incubation at 60°C for 2 hours [159]. After

sonication of the samples followed by centrifugation (10 min, 14,000 x g, 4°C), the protein lysates were stored at -80°C until further analysis.

3.3.11. *Ex vivo* stimulation of whole blood

Before and after the intervention, postprandial blood (4 h after MTT) was taken in order to perform an *ex vivo* stimulation to measure the effect of the diet on inflammatory responses. Heparinized blood was diluted 1:5 with cell culture medium (Gibco® RPMI 1640, Thermo Fisher Scientific Life Technologies, Gaithersburg, USA) containing 1% antibiotic antimycotic solution (Life technologies, Gaithersburg, USA; Sigma Aldrich Chemie, Steinheim, Germany). It was added to 6-well-plates and directly stimulated with 100 ng/mL LPS (Applied Biosystems, Forster City, USA) for 20 hours at 37°C and 5% CO_2. After centrifugation (5 min, 500 x g, room temperature) the supernatants were collected and stored at -80°C.
Further, the levels of several inflammation markers (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-18, IFNγ, MCP-1, and TNFα) were determined in the supernatants using ProcartaPlex™ Multiplex Immunoassay (Affymetrix eBioscience, Vienna, Austria) according to the manufacturer's manual. The prepared samples were diluted 1:2 with Universal Assay Buffer and added to the analyte-specific capture magnetic fluorescent-dye microspheres (beads), which are coated with target-specific capture antibodies. The bead-analyte was further incubated with a biotinylated analyte-specific detection antibody with subsequent incubation with a fluorescent detection label (streptavidin-conjugated R-phycoerythrin). The quantification was carried out using the Luminex technology on a Bio-Plex™ 200 System (Bio-Rad Laboratories, Munich, Germany).

3.3.12. Protein analysis

The lysates of the white blood cells (see chapter 3.3.10) and the adipose tissue samples were investigated on the proteomic level.

Protein isolation from adipose tissue

The adipose tissue samples were stored at -80°C before proceeding with the analysis. The samples were first washed with cold 1x PBS (Life technologies, Gaithersburg, USA), containing proteinase and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany), in order to remove the blood remains. Afterwards the samples were lysed with Cell Lysis Buffer (Cell Signaling, Danvers, USA), containing proteinase and phosphatase inhibitors, followed by bead mill homogenization (Speed Mill P12 Analytik Jena, Jena, Germany) and two-step centrifugation (15 min, 21,000 x g, 4°C).

Protein concentration

First, the samples (white blood cells and adipose tissue) were sonicated and centrifuged (10 min, 14,000 x g, 4°C). The quantity of total protein was detected spectrophotometrically using a commercially available kit Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA). The method is based on the reduction of Cu^{2+} to Cu^{+} in an alkaline protein solution (biuret reaction) and the chelation of the cuprous cation with two molecules of bicinchoninic acid (BCA). This complex exhibits an absorbance at 562 nm which was measured at Wallac Victor2 plate reader (PerkinElmer Life and Analyt. Sciences, Waltham, USA). Bovine serum albumin (BSA) was used for the preparation of the standard curve.

Protein phosphorylation

The phosphorylation status of the proteins was estimated using the PathScan® Akt Signaling Antibody Array Kit with fluorescent readout (Cell Signaling, Danvers, USA) for simultaneous detection of 16 phosphorylated proteins predominantly belonging to the Akt signaling network: Akt (Thr308), Akt (Ser473), Ribosomal protein S6 (Ser235/236), AMPKα (Thr172), PRAS40 (Thr246), mTOR (Ser2481), GSK-3α (Ser21), GSK-3β (Ser9), p70S6K (Thr389), p70S6K (Thr421/Ser424), Bad (Ser112), RSK1 (Ser380), PTEN (Ser380), PDK1 (Ser241, ERK1/2 (Thr202/Tyr204), 4EBP1 (Thr37/46). Based on the sandwich immunoassay principle, the array uses a nitrocellulose-coated glass slide spotted with target-specific capture antibodies. Protein lysates were diluted to a total protein concentration of 0.4 mg/ml, and 75 µl were incubated on the slide. The assay was performed according to the manufacturer's protocol with subsequent incubation with a biotinylated detection antibody cocktail. Finally, a streptavidin-conjugated dye was added and a fluorescent signal was detected at the ODYSSEY Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). The spot intensities were quantified with ODYSSEY software.

3.3.13. Gene expression

RNA isolation from whole blood

Blood samples (fasting and 4h postprandial) were collected in PAXgene Blood RNA Tubes (PreAnalytiX/Qiagen, Hilden, Germany) for immediate stabilization of intracellular RNA after collection. First, the tubes were stored for 2 hours at room temperature, then frozen at -20°C for 24 hours and finally stored at -80°C until further processing. The purification of total RNA, including miRNA, was carried out by PAXgene Blood miRNA Kit (PreAnalytiX/Qiagen, Hilden, Germany) according to the manufacturer's manual.

RNA isolation from adipose tissue

Total RNA, including miRNA, was extracted from adipose tissue samples (ca. 400 mg) using miRNeasy Kit (Qiagen, Hilden, Germany). The homogenization step was performed with bead mill (Speed Mill P12 Analytik Jena, Jena, Germany) and further purification according to the kit instructions.

Quality and quantity assessment of RNA

The purity of the RNA samples affects the results of the further analysis. Therefore, the quality as well as the quantity of the isolated RNA was estimated before the gene expression analysis to ensure reliable results. The RNA quality was assessed using NanoDrop and Bioanalyzer.

The spectrophotometric analysis of the RNA samples was performed on NanoDrop® ND-1000 (Thermo Scientific, Wilmington, USA). All nucleotides (RNA, DNA) absorb at 260 nm, while protein, phenolic compounds or other contaminants absorb at 280 nm. So, the ratio of absorbance at these two wavelengths (260/280) is used to assess the purity of the sample. A ratio above 1.8 was considered “pure” for RNA. Additionally, a 260/230 ratio is used as a secondary measure of RNA purity, as carbohydrates and phenols have absorbance near 230 nm. The ranges are commonly between 2.0 and 2.2.

A further criterion for RNA quality is the rate of degradation of the RNA by nucleases during the purification process. Therefore, a capillary electrophoresis was performed using Agilent RNA 6000 Nano LabChip® Kit on a Bioanalyzer (Agilent Technologies, Santa Clara, USA). The RNA samples were first denatured for 2 min at 70°C and further processed according to the instructions. The 18S and 28S subunits of ribosomal RNA were separated in an electrical field, and visualized in an electropherogram and a gel-like image. The ratio of the peak areas 18S/28S gives information about RNA degradation, with the optimal value being 2. The RNA Integrity Number (RIN) describes the rate of degradation in a range of 1 (completely degraded, fragmented RNA) to 10 (not degraded, intact RNA). A RIN value > 7 was assessed as suitable for high-throughput RNA analysis by next-generation sequencing.

Next-generation sequencing

A next-generation sequencing (RNAseq) of whole blood RNA (n = 10) was performed by the cooperation partners Dr. Holger Prokisch and Robert Kopajtich (Institute for Human Genetics, Helmholtz Center Munich, Germany) with Illumina TruSeq® RNA (Illumina, San Diego, USA). Briefly, the poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads. Then the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into

first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNase H. After that, the cDNA fragments went through an end repair process, the addition of a single 'A' base, and ligation of the adapters. The products are then purified and enriched with PCR to create the final cDNA library.

cDNA synthesis

Synthesis of cDNA was performed using miScript II Reverse Transcription Kit (Qiagen, Hilden, Germany), which enabled the transcription of total RNA, including miRNA. An amount of 1 µg total RNA was used for the reaction. The components for a single reaction are listed in Table 6.

Table 6. Reverse-transcription reaction components.

Component	Volume
5x miScript HiFlex Buffer	4 µl
10x Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA (1 µg)	Variable
Total volume	**20 µl**

All steps were performed on ice. Subsequently, the cDNA synthesis occurred in a Mastercycler® (Eppendorf, Hamburg, Germany):

1. Incubation for 60 minutes at 37°C for reverse transcription reaction
2. Incubation for 5 minutes at 95°C for inactivation of reverse transcriptase
3. Cooling at 4°C und storage at -20°C

Quantitative real-time PCR

The gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). The polymerase reaction was performed using Power SYBR®Green PCR Master Mix (Applied Biosystems, Forster City, USA), containing DNA polymerase, deoxynucleotides, and SYBR Green I dye, which intercalates with double-stranded DNA and emits a fluorescent signal.

The cDNA templates were diluted 1:50 in RNase/DNase-free water. A standard stock solution was prepared as a pool from all cDNA samples. The standard dilution series was a set of standards prepared by serially diluting standard stock (1:4, 1:16, 1:64, 1:256, 1:1024,

1:4096, 1:16384) for which the arbitrary value range was set up in the ViiA 7 software. Water was used as a no-template control to check for extraneous nucleic acid contamination, and a no-reverse transcriptase control was used to assess DNA contamination. The reactions were run in triplicates; each reaction contained the following components (Table 7):

Table 7. Quantitative real-time PCR components.

Component	Volume
Power SYBR®Green PCR Master Mix	2 µl
Forward primer	0.35 µl
Reverse primer	0.35 µl
cDNA template	1 µl
RNase/DNase-free water	1.3 µl
Total volume	**5 µl**

Primer Express 2.0 (Applied Biosystems, Carlsberg, USA) was used to design the primers (primer sequences are summarized in chapter 3.1.3); all primers were purchased from Invitrogen (Carlsbad, USA). β-Glucuronidase (GUSB) was used as housekeeping gene for adipose tissue [160]. The qRT-PCRs were run on ViiA 7 Real-Time PCR System (Life Technologies, USA) for a total of 45 two-step cycles followed by melt curve analysis.

Table 8. Quantitative real-time PCR cycling conditions.

Reaction step	Time	Temperature
Taq-polymerase activation	10 min	95°C
PCR stage (45 cycles):		
• Denaturation	15 s	95°C
• Annealing and Elongation	1 min	60°C
Melt curve analysis		
	15 s	95°C
	1 min	60°C
	15 s	95°C

After the run (Table 8), the software ViiA™ 7 1.2.2 interpolated the relative gene quantity using the standard curve. The analysis of the melt curve enabled the examination of possible unspecific PCR products. For analysis, the relative expression of the target gene was normalized to the expression of the corresponding housekeeping gene.

3.3.14. Statistical analysis

Before the data was released for analysis, an evaluation of plausibility was performed for each parameter and time point. Values that were outside of the 3-fold interquartile range (IQR) with IQR = $\text{quartile}_{0.75} - \text{quartile}_{0.25}$ were declared as extreme outliers and were not considered for further analysis.

After separating the data set by the diet group, each variable was tested for its normal distribution using the Shapiro-Wilk test. For small samples sizes ($n < 50$) Shapiro-Wilk test has greater statistical power than the usually used Kolmogorov-Smirnov test. Since the null-hypothesis of the Shapiro-Wilk test is that the population is normally distributed, a normal distribution was considered for significance values greater than 0.05. If a normal distribution could not be detected even after a natural logarithm (ln)-transformation, or if other requirements were violated, a non-parametric statistic test was used as an alternative.

For comparisons at two time points within groups, either a paired Student's *t*-test or a non-parametric Wilcoxon test was used. Comparisons at more than two time points were tested using one-way repeated-measures analysis of variance (ANOVA) with the different time points set up as within-subject contrasts and a Bonferroni adjustment. According to Mauchly's test of sphericity, significance was considered from a within-subjects effects test, either using not corrected values or applying a Greenhouse-Geisser and Huynh-Feldt correction. A non-parametric Wilcoxon test was used for non-normally distributed variables for pairwise comparisons.

An unpaired *t*-test or non-parametric Mann-Whitney-U test was used for comparisons between groups for two time points. For measurements at more than two time points a two-way repeated-measures ANOVA was used with the different time points as within-subject variables and the intervention group as a between-subject factor with a Bonferroni adjustment. Non-normally distributed variables were compared using a non-parametric Kruskal-Wallis test.

Bivariate correlation was performed to investigate possible relations between changes of the parameters. In accordance with the data distribution, either Pearson or Spearman correlation coefficient was used, and r (ρ, respectively) greater than 0.3 (with $p < 0.05$) were considered as strong correlations.

Data, if not indicated otherwise, are presented as mean ± standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$. The incremental area under the curve (iAUC) was estimated by the trapezoid rule.

Statistical analysis was performed with IBM SPSS Statistics Version 20.0 (IBM, Armonk, USA).

4. Results

4.1. Baseline characteristics of the participants

The participant flow diagram is depicted in Figure 7. Almost 250 people responded to the advertisements. They were first interviewed on the telephone for eligibility according to the inclusion criteria (chapter 3.2.1.). Two thirds of those volunteers were either not eligible because of exclusion criteria or withdrew participation before the screening. Thus, 85 type 2 diabetics were examined for their suitability for enrollment in the clinical study. Almost half of them (n = 41) failed the screening. 22 of them did not meet the inclusion criteria based on an HbA_{1c} level below 6% or other medical reasons; the rest withdrew because of job-related reasons or because they considered participation in the study to be too burdensome. Thus, 44 type 2 diabetics were randomly assigned by group matching to one of the diet interventions, either animal (AP) or plant (PP) protein. The matching parameters were age, BMI, sex, HbA_{1c} level, and diabetes treatment. Throughout the intervention, 4 subjects of the AP group and 3 subjects of the PP group dropped out due to medical reasons (n = 2) or loss of interest (n = 4); in one case the vein access was not possible. This resulted in 37 individuals who successfully completed the 6-week clinical intervention – 18 in the AP diet group, and 19 in the PP diet group.

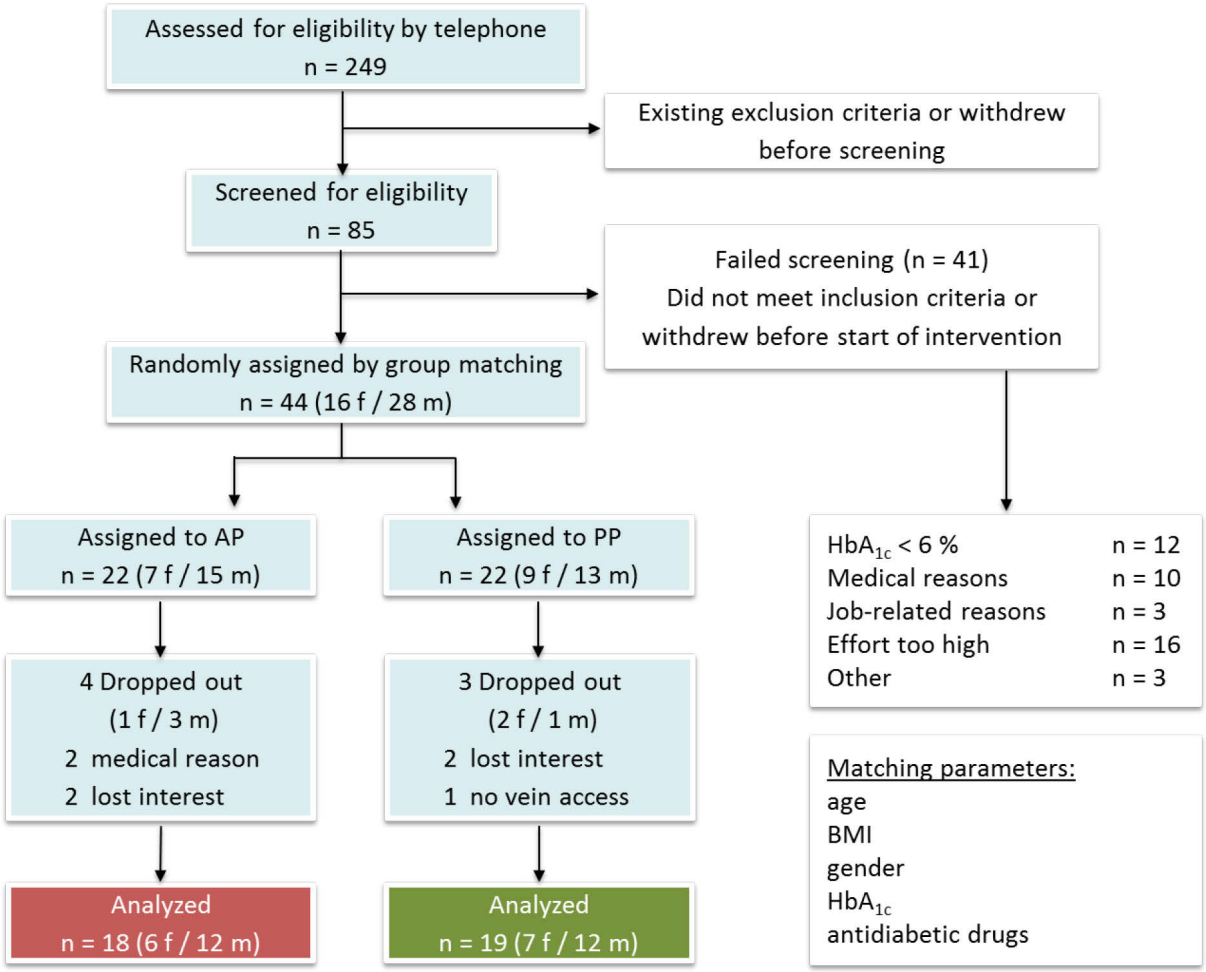

Figure 7. Participant flow throughout the study. Abbreviations: AP, animal protein; BMI, body mass index; f, female; m, male; PP, plant protein.

The randomizing parameters and baseline characteristics for each group are summarized in Table 9. The average age was 65 (57 - 78) years in the AP, and 64 years (49 - 75) in the PP group. Females and males were not equally distributed, approximately two thirds were men (n = 24) and one third women (n = 13). Nevertheless, the number of females and males within the two diet groups were the same. BMI averaged out at 31.0 kg/m^2 in the AP, and 29.4 kg/m^2 in the PP group. Hence, the individuals were overweight to moderately obese. The baseline glycated hemoglobin (HbA_{1c}) level in both groups was 7.0% (≙ 53 mmol/mol). With respect to diabetes treatment, the participants were randomized as follows: 4 per group did not receive any drugs, 6 per group received metformin, 2 in the PP group received DPP-IV inhibitors, 6 in AP and 5 in PP – metformin and DPP-IV inhibitor, 1 in AP and 2 in PP – metformin and sulfonyl urea derivative, 1 in AP metformin, DPP-IV inhibitor and insulin detemir, and 1 in the AP group received sulfonyl urea derivative and a DPP-IV inhibitor (Table 9).

Table 9. Matching parameters and baseline characteristics.

Parameter	Animal protein (AP)	Plant protein (PP)	p_{APvsPP}
Matching parameters			
Age [years]	65 ± 1	64 ± 2	0.512
Sex [female/male]	6 / 12	7 / 12	
BMI [kg/m²]	31.0 ± 0.8	29.4 ± 1.0	0.222
HbA_{1c} [%]	7.0 ± 0.2	7.0 ± 0.1	0.893
Antidiabetic drugs			
No drugs	4	4	
Metformin	6	6	
DPP-IV inhibitor	0	2	
Metformin + DPP-IV inhibitor	6	5	
Metformin + Sulfonyl urea derivative	1	2	
Metformin + DPP-IV inhibitor + Insulin detemir	1	0	
Sulfonyl urea derivative + DPP-IV inhibitor	1	0	
Baseline characteristics			
Body weight [kg]	92.6 ± 2.9	86.0 ± 3.2	0.137
Glucose [mmol/l]	9.64 ± 0.43	9.48 ± 0.35	0.776
Insulin [mU/l]	10.07 ± 1.69	8.74 ± 1.32	0.577
C-peptide [µg/l]	2.33 ± 0.27	2.05 ± 0.21	0.411
HOMA-IR	4.45 ± 0.87	3.82 ± 0.53	0.688
Glucagon [pmol/l]	8.75 ± 1.01	8.15 ± 0.78	0.642
GLP-1 [ng/l] *(active)*	3.01 ± 0.54	3.83 ± 0.83	0.580
GIP [ng/l] *(total)*	53.17 ± 7.22	40.58 ± 3.38	0.254
FFA [mmol/l]	0.66 ± 0.04	0.73 ± 0.04	0.259
Triglycerides [mmol/l]	1.72 ± 0.13	1.64 ± 0.14	0.702
Cholesterol [mmol/l]	5.16 ± 0.27	5.24 ± 0.16	0.789
LDL cholesterol [mmol/l]	3.25 ± 0.23	3.40 ± 0.17	0.590
HDL cholesterol [mmol/l]	1.13 ± 0.07	1.09 ± 0.05	0.683
systolic BP [mmHg]	128 ± 4	135 ± 4	0.204
diastolic BP [mmHg]	75 ± 2	84 ± 2	0.006
CRP [mg/l]	2.17 ± 0.56	2.09 ± 0.50	0.976
Uric acid [µmol/l]	349.00 ± 17.35	387.26 ± 18.52	0.142

Values are presented as means ± SEM. Abbreviations: BMI, body mass index; DPP-IV, dipeptidyl-peptidase IV; BP, blood pressure.

Furthermore, body weight as well as baseline levels of routine parameters (glucose, insulin, glucagon, incretins, blood lipids, CRP, uric acid) did not differ significantly between AP and PP group. Additionally, systolic blood pressure was not significantly different between the groups, but diastolic blood pressure in the PP group was significantly higher than in the AP group (Table 9).

4.2. Compliance to dietary intervention

*All results presented in this chapter were estimated and calculated by Frau **Stephanie Sucher**, a supervisor and nutritionist of the LeguAN study. Here I would like to acknowledge again her huge contribution to the study. Since these data give essential information about the conducted study and are further essential for the interpretation of the outcome results, I included them in the present work.*

The provided dietary protocols from every participant for every day of the 6-week-long intervention and 5-day-long habitual food intake prior to entering the study were gathered in order to analyze the compliance with the prescribed diet. Caloric intake and the amount of macronutrients, minerals and trace elements were calculated using PRODI® 6.2 software (Nutri-Science GmbH, Hausauch, Germany). Furthermore, the glycemic indexes (GI), fibers, and the percentage of animal and plant protein were estimated in both diet groups. The average energy intake was 10467 ± 1563 kJ/day (2500 ± 90 kcal/day) in the AP group and 9794 ± 1911 kJ/day (2337 ± 111 kcal/day) in the PP group.

The pie charts in Figure 8 illustrate consumption of proteins, carbohydrates, and fats in percentage of energy intake (EN%) before and during the intervention. They show that all subjects in the study used to consume a typical western diet containing a high amount of fat of more than 40 EN% and relatively low amounts of carbohydrates. The experimental diets showed a change compared to the previous nutrient intake, with an increase in the protein intake (from ~ 17 EN% to ~ 30 EN%) and a decrease in the dietary fat intake (from ~ 40 EN% to ~ 30 EN%). Carbohydrate consumption remained close to the levels of the habitual diet (~ 40 EN%).

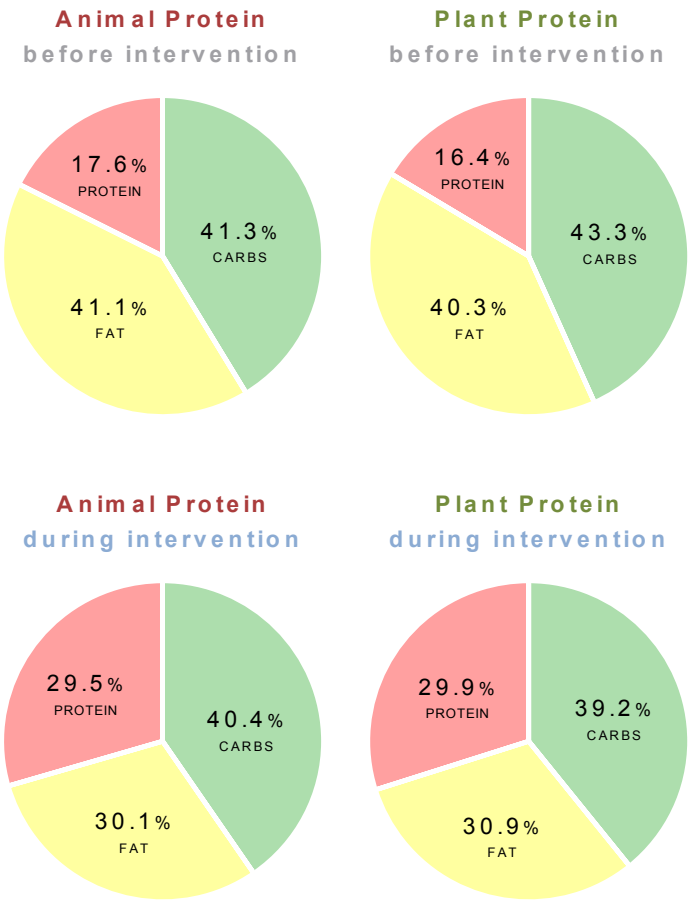

Figure 8. Comparison of the macronutrient composition for both groups. Upper panel: before intervention; lower panel: during the intervention. Values are presented as means in energy % (EN%).

Thus, fat intake was substituted with protein, since both groups consumed around 40 EN% from carbohydrates before and during the intervention. The adherence to the dietary protocols was excellent with 30 EN% protein, 40 EN% carbohydrates, and 30 EN% fat in both groups (Figure 8).

Figure 9 portrays the habitual consumption of fats before enrollment and the intake throughout the 6 weeks of the study. Prior to the diet intervention, the fat intake represented around 40 EN%: ~ 17 EN% SFA, ~ 14.5 EN% MUFA, and ~ 6.3 EN% PUFA. By contract, the fat intake throughout the study was set at 30 EN% and the ratio of SFA : MUFA : PUFA at 1:1:1 (≙ 10 EN% SFA, 10 EN% MUFA, and 10 EN% PUFA).
Thus, the compliance of the participants with regard to the fat intake was very good.

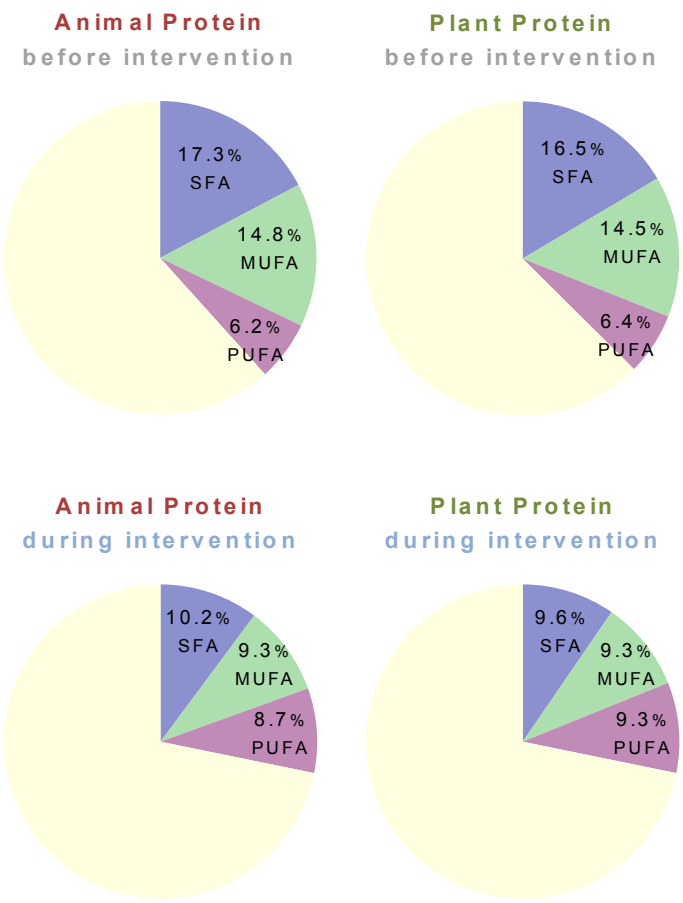

Figure 9. Comparison of the fat composition for both groups. Upper panel: before intervention; lower panel: during the intervention. Values are presented as means in energy % (EN%).

Table 10 shows the values for GI, GL, and dietary fiber consumption during the intervention. The mean meal GI of the diets averaged in the low range, 54.48 and 55.72 for the AP and PP group, respectively. Correspondingly, the GL in the AP group was 133.65 and in PP group 123.18. Moreover, the dietary fiber intake was above 30 g in accordance with the DGE recommendations for adults. There were no differences between the diets regarding these parameters.

Table 10. Glycemic index, glycemic load, and fiber content of both diets.

	Animal Protein (AP)	Plant Protein (PP)
Glycemic index (GI)	54.48 ± 0.39	55.72 ± 1.25
Glycemic load (GL)	133.65 ± 5.00	123.18 ± 6.00
Fiber [g]	35.36 ± 1.42	33.27 ± 1.78

Values are presented as means ± SEM.

By equalized fatty acid composition, GI, GL, and fiber intake, it could be ascertained that the dietary groups only differed in their amino acid compositions.

The respective parts of animal and plant proteins in the two diets were as follows: AP diet – 80% animal and 20% plant protein, PP diet – 28% animal and 72% plant protein. The amounts of amino acids in the diets were calculated with PRODI® 6.2 software and are summarized in Table 11. As expected, the two protein types differed in their amino acid compositions. For instance, the amounts of BCAA (leucine, isoleucine und valine) as well as methionine were higher in the animal proteins, while arginine was higher in the plant foods.

Table 11. Amounts of amino acids in both diets.

Amino acid [mg]	Animal Protein (AP)	Plant Protein (PP)
Alanine	7918.6 ± 300.5	6683.1 ± 340.2
Arginine	8933.7 ± 331.0	9980.0 ± 490.1
Asparagine + Aspartic Acid	15507.5 ± 532.3	14734.4 ± 719.5
Cysteine	1821.1 ± 71.8	2042.9 ± 102.7
Glutamine + Glutamic Acid	36870.9 ± 1199.6	33911.8 ± 1781.3
Glycine	6211.0 ± 262.0	6094.7 ± 314.4
Histidine	5493.2 ± 197.1	4019.9 ± 220.8
Isoleucine	9415.5 ± 296.2	7302.0 ± 373.7
Leucine	15868.2 ± 491.1	14374.7 ± 710.0
Lysine	13887.9 ± 458.0	9617.5 ± 512.5
Methionine	4483.5 ± 137.1	2995.9 ± 156.7
Phenylalanine	8446.1 ± 263.7	7853.5 ± 404.4
Proline	14930.2 ± 474.3	11135.0 ± 612.0
Serine	9356.8 ± 287.3	8080.9 ± 407.9
Threonine	7827.4 ± 250.4	5978.6 ± 310.7
Tryptophan	2205.6 ± 65.9	1803.9 ± 87.3
Tyrosine	7631.7 ± 235.2	5849.4 ± 305.7
Valine	10886.1 ± 336.8	8218.9 ± 424.1

Values are presented as means ± SEM.

4.3. Anthropometry and body composition

Body weight and BMI were determined every two weeks over the intervention period (at week 0, 2, 4, and 6). Figure 10 illustrates the changes in body weight (left panel) and BMI (right panel) for the AP and PP group, depicted in red and green, respectively.

The body weight of the subjects in both groups (AP: 92.6 kg; PP: 86.0 kg) was not different at baseline. Despite consumption of an isocaloric diet accompanied by intensive nutritional consultations, after two weeks of intervention body weight decreased significantly in both groups (AP: - 0.9 kg; PP: - 0.8 kg). Therefore, the dietary plans were corrected for the caloric intake in order to avoid further body weight loss. Nevertheless, at the end of the study the moderate loss of body weight (AP: - 2.5 kg (- 2.7%); PP: - 1.3 kg (- 1.5%)) was significant in both groups; the difference between the two diet groups was significant (p_{APvsPP} = 0.033).

BMI was not different between the groups at baseline (AP: 31.0 kg/m^2; PP: 29.4 kg/m^2). Appropriate to body weight loss, BMI was significantly lower after 2 weeks in both AP and PP groups. At the end of the study, a moderate significant reduction of BMI was observed in both groups (AP: - 0.8 kg/m^2; PP: - 0.5 kg/m^2), but this change was not significantly different between the interventions (p_{APvsPP} = 0.089).

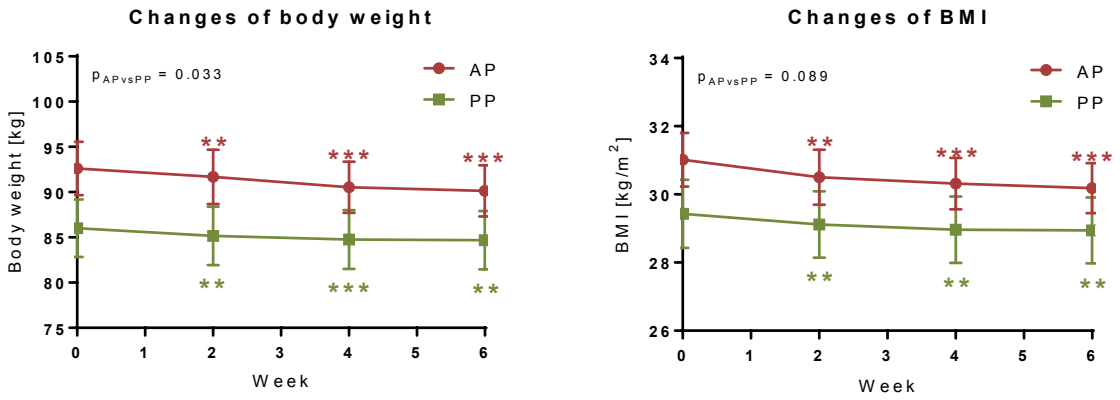

Figure 10. Changes of body weight and BMI. Parameters were determined every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. Height measurements were performed only on CID 1. Abbreviations: BMI, body mass index; AP, animal protein; PP, plant protein. Values are presented as mean ± SEM. **p < 0.01, ***p < 0.001 different from week 0 within group.

In order to distinguish a potential effect of change in body weight regarding different parameters, linear regression analyses were performed. A significant correlation was found for systolic blood pressure (r = 0.349, p = 0.034), total free fatty acids (r = -0.447, p = 0.006), total cholesterol (r = 0.392, p = 0.016), and LDL cholesterol (r = 0.362, p = 0.028). Thus, for comparisons of these four parameters between the diet groups, the change of body weight was added as covariate in a two-way ANOVA.

There were no changes of the waist circumference and the waist-to-hip ratio (WHR) (Table 12). The hip circumference was reduced only in the PP group (p_{PP} = 0.034), no differences between diets were observed. Moreover, the adipose tissue on the level of the femoral head (AT_{femur}) was measured at week 0 and 6 by MRI. AT_{femur} was reduced in the AP group, and tended to decrease in the PP group (p_{AP} = 0.016; p_{PP} = 0.084).

Individuals were not hypertensive at baseline (blood pressure (BP) < 140/90). After the intervention systolic BP decreased only in the PP group (p_{PP} = 0.022) resulting in a significant difference between the two diets (p_{APvsPP} = 0.029; with change of body weight as covariate). Moreover, initial diastolic BP in the PP group was significantly higher than in the AP group (p_{APvsPP} = 0.006 (Table 9)). Similar to systolic BP, diastolic BP was reduced only in the PP group after 6 weeks of intervention (p_{PP} = 0.008), the difference between AP und PP was significant as well (p_{APvsPP} = 0.009).

Table 12. Dietary effects on waist and hip circumferences, waist-to-hip ratio, AT_{femur}, and blood pressure.

Parameter	Animal protein (AP), n = 18			Plant protein (PP), n = 19			AP vs PP
	week 0	week 6	p_{AP}	week 0	week 6	p_{PP}	p_{APvsPP}
Anthropometry							
Waist [cm]	104.2 ± 2.6	102.2 ± 2.0	1.000	100.7 ± 3.0	99.4 ± 2.9	0.830	0.875
Hip [cm]	107.8 ± 1.8	106.3 ± 1.6	0.434	105.3 ± 2.0	103.2 ± 1.9	0.034	0.110
WHR	0.97 ± 0.02	0.96 ± 0.02	1.000	0.96 ± 0.02	0.96 ± 0.03	1.000	0.657
AT_{femur} [ml]	394.25 ± 17.51	372.15 ± 19.66	0.016	372.73 ± 26.18	348.05 ± 17.56	0.084	0.490
Blood pressure (BP) [mmHg]							
systolic BP	128 ± 4	126 ± 3	0.635	135 ± 4	126 ± 3	0.022	0.029
diastolic BP	75 ± 2	76 ± 2	0.478	84 ± 2	77 ± 2	0.008	0.009

Waist and hip circumferences as well as WHR were measured every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. AT_{femur} was determined by MRI at week 0 and 6. Systolic and diastolic blood pressures were determined at week 0 and 6, average value of three measurements in the resting state. Abbreviations: AT_{femur}, adipose tissue on the level of femoral head; BP, blood pressure; MRI: magnetic resonance imaging; WHR, waist-to-hip ratio. Values are presented as means ± SEM.

Total fat and fat free mass were determined using whole-body densitometry technique (chapter 3.3.1.) at week 0 and 6; data are shown in Figure 11 (upper panel). Fat mass (FM) decreased and fat free mass (FFM) increased in the AP group by ~ 1.9% (p_{AP} = 0.023), while body composition of PP participants did not change significantly (p_{PP} = 0.107). Comparing the two groups there were no significant differences (p_{APvsPP} = 0.188).

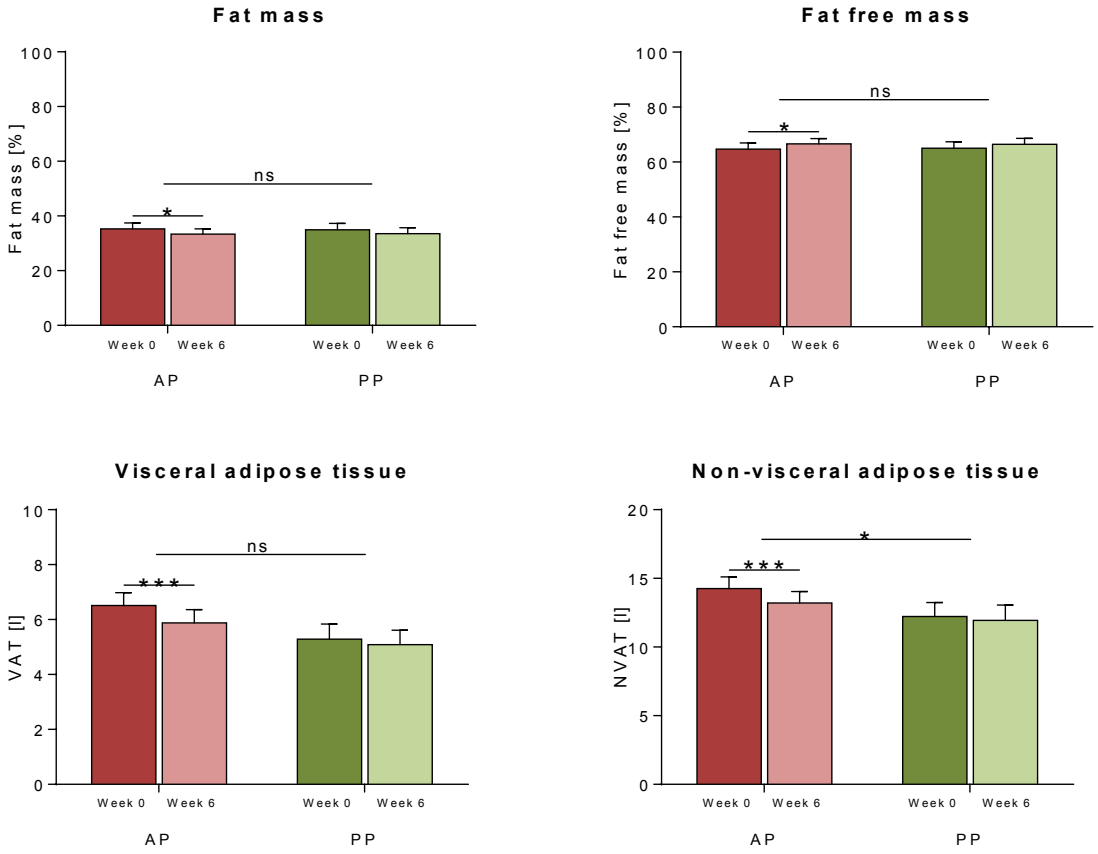

Figure 11. Body composition and adipose tissue distribution. Fat and fat free mass were measured at week 0 and 6 by whole-body densitometry in the fasting state (n_{AP} = 14, n_{PP} = 15). VAT and NVAT were determined by MRI at week 0 and 6 (n_{AP} = 15, n_{PP} = 17). Values are presented as mean ± SEM. *p < 0.05, ***p < 0.001. ns, not significant (p > 0.05).

Additionally, MRI was used to evaluate the visceral (VAT) and non-visceral (NVAT) abdominal adipose tissue (Figure 11, lower panel). In agreement to the FM results, only in the AP group the values of VAT and NVAT were reduced ($p_{AP} < 3x10^{-4}$ and $p_{AP} < 8.5x10^{-5}$, respectively). The decrease of NVAT in the AP group was significantly higher compared to the PP group ($p_{APvsPP} = 0.042$).

Taken together, 6 weeks of isocaloric high protein diet led to a moderate body weight loss and decrease of BMI, which was more pronounced after the AP diet. Further, fat depots (FM, VAT and NVAT) were reduced significantly only in the AP group. Notably, despite undesired weight loss the FFM was increased by the high protein diet. Moreover, blood pressure was improved by the PP diet, pointing eventual benefit of the higher arginine content in the plant diet [161].

4.4. Energy metabolism

Resting energy expenditure (REE) was determined by indirect calorimetry and substrate oxidation was calculated using correspondent equations (chapter 3.3.2.1.). Respiratory quotient (RQ) values range between 1.0 for pure carbohydrate oxidation, 0.8 – 0.9 for pure protein oxidation, and 0.7 for pure fat oxidation. Here, mean REE was around 1600 kcal/day, RQ ~ 0.85. These parameters did not change after the intervention (Table 13).

The oxidation of carbohydrates and fats remained unchanged as well. As expected, the protein oxidation, calculated from the 24-h urinary nitrogen excretion, was significantly increased in all participants without differences between the groups (Table 13).

Table 13. Dietary effects on energy metabolism of study participants.

Parameter	Animal protein (AP), n = 18			Plant protein (PP), n = 19			APvsPP
	week 0	week 6	p_{AP}	week 0	week 6	p_{PP}	p_{APvsPP}
Resting energy expenditure [kcal/day]	1682.1 ± 68.8	1628.3 ± 67.2	0.184	1604.6 ± 61.5	1594.4 ± 75.6	0.563	0.203
Respiratory quotient	0.86 ± 0.02	0.84 ± 0.02	0.623	0.84 ± 0.02	0.85 ± 0.02	0.791	0.548
Carbohydrate oxidation [g/min]	0.158 ± 0.017	0.144 ± 0.023	0.367	0.139 ± 0.017	0.138 ± 0.014	0.651	0.316
Fat oxidation [g/min]	0.060 ± 0.008	0.064 ± 0.008	0.769	0.066 ± 0.007	0.067 ± 0.008	0.486	0.846
24-h urinary nitrogen [g/day]	11.477 ± 0.876	17.240 ± 1.403	$7x10^{-6}$	11.245 ± 1.012	16.027 ± 1.337	0.005	0.532
Protein oxidation [g/min]	0.050 ± 0.004	0.075 ± 0.006	$7x10^{-6}$	0.049 ± 0.004	0.070 ± 0.006	0.005	0.532

All parameters were determined by indirect calorimetry in the fasting state at week 0 and week 6. Values are presented as means ± SEM.

4.5. Biomarkers in blood

Important parameters of glucose and insulin metabolism, blood lipids and markers of kidney function were determined. Further, diet effects on liver enzymes and gut hormones were verified. The biomarkers were measured in fasting serum samples at every visit (week 0, 2, 4, 6). Compositions of free fatty acids (FFA) and free amino acids (FAA) were detected (at week 0 and 6) as attempt to elucidate possible mechanisms of dietary effects.

4.5.1. Glucose metabolism

Initial fasting plasma glucose levels were 9.64 and 9.48 mmol/l in the AP and PP group, respectively. Concentrations decreased after 2 and 4 weeks of intervention in both groups. However, after 6 weeks the reductions were not significant. Fasting insulin was reduced after 6 weeks of AP diet (- 17.43%), while the PP intervention did not induce a significant change of insulin levels. C-peptide concentrations did not change throughout the study in both diet groups. Correspondingly to the changes of glucose and insulin, the HOMA-IR index was reduced significantly only in the AP group by 29.2%, indicating favorable effect on whole body insulin sensitivity (Figure 12).

Total FFA in the AP group remained unchanged, while in the PP group the concentration was diminished already after two weeks of intervention. The difference between the groups tended to significance ($p_{APvsPP} = 0.056$). The calculated AdipoIR index improved significantly only in the PP group (Figure 12).

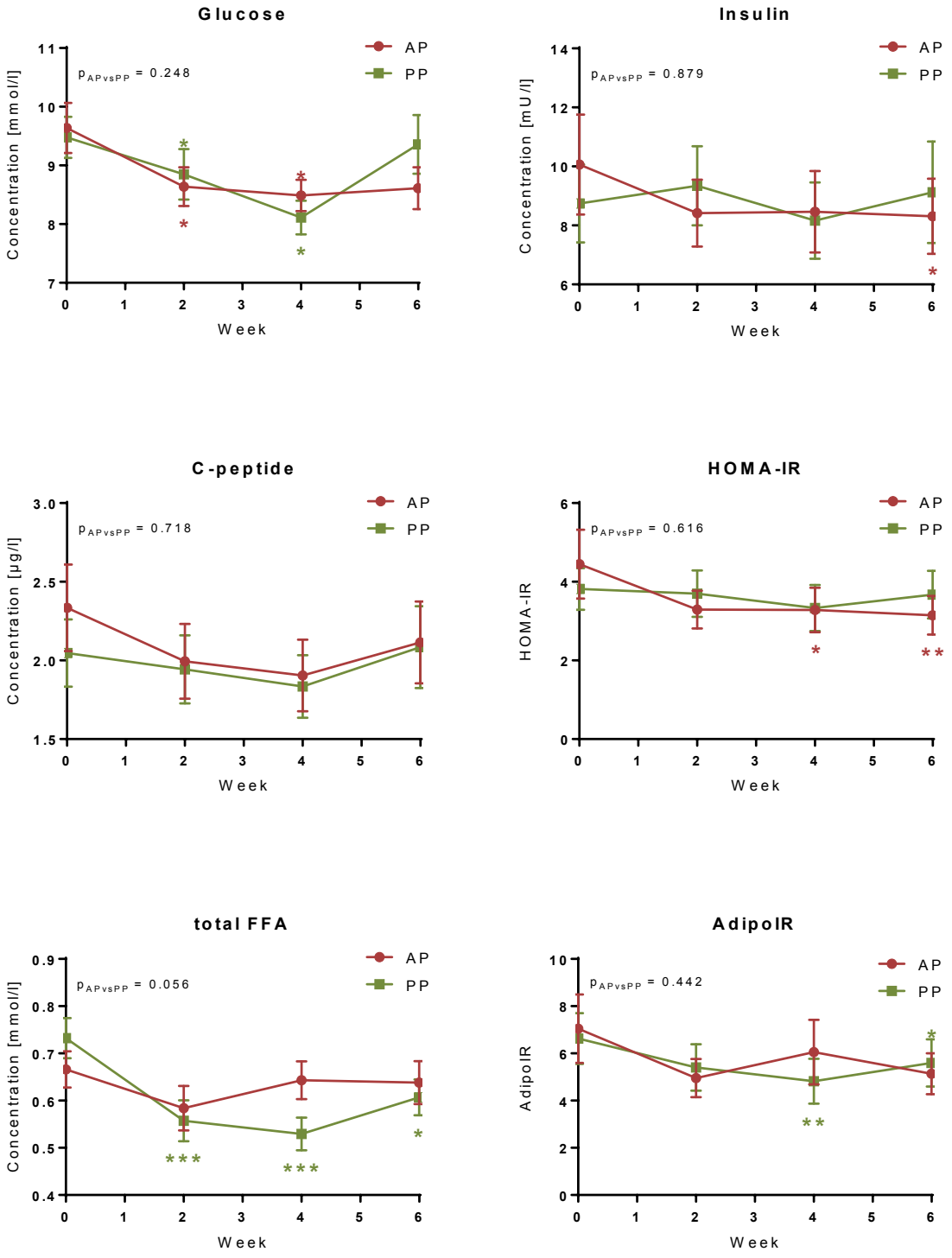

Figure 12. Fasting concentrations of glucose, insulin, and C-peptide. HOMA-IR, free fatty acids (FFA), and adipose tissue insulin resistance (AdipoIR). All parameters were determined in blood serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. HOMA-IR = (fasting insulin in mU/l x fasting glucose in mmol/l) / 22.5. AdipoIR = fasting insulin in mU/l x fasting free fatty acids in mmol/l. Abbreviations: AdipoIR, index of adipose tissue insulin resistance; AP, animal protein; FFA: free fatty acids; HOMA-IR: homeostasis model assessment for insulin resistance; PP, plant protein. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 different from week 0 within group.

At baseline, patients were randomized also for their HbA_{1c} value, as one of the most important markers of type 2 diabetes. Both groups had an initial HbA_{1c} level of approximately 7% (Figure 13, left panel). It decreased significantly in the PP group (- 0.55%, $p_{PP} < 1x10^{-6}$). In the AP group, interestingly, only the reduction after 4 weeks was significant but not after 6 weeks (week 4: - 0.47%, $p_{APweek4} = 0.010$, week 6: - 0.34%, $p_{APweek6} = 0.353$). Nevertheless, there were no significant differences between the two protein diets ($p_{APvsPP} = 0.703$).

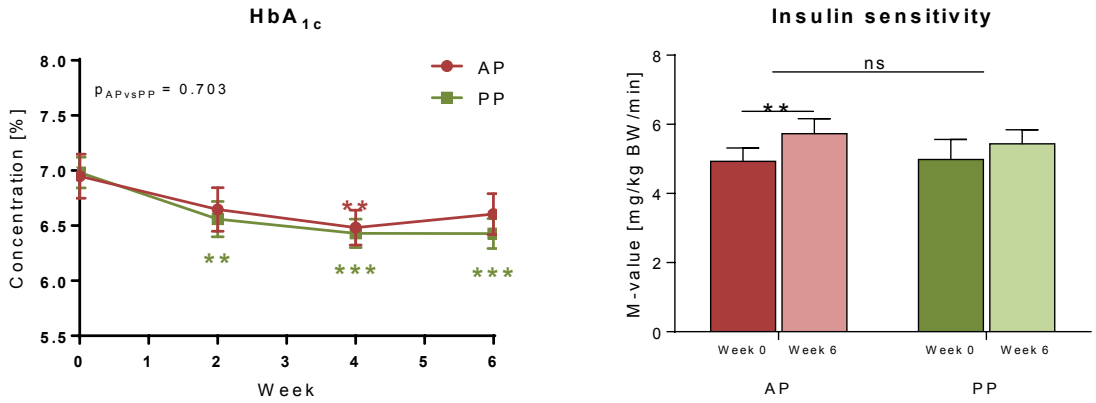

Figure 13. Dietary effects on HbA_{1c} and whole-body insulin sensitivity. HbA_{1c} was measured in blood serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. Whole-body insulin sensitivity (expressed as M-value) was determined using hyperinsulinemic euglycemic clamps performed at week 0 and 6. Abbreviations: AP, animal protein; HbA_{1c}: glycated hemoglobin A_{1c}; PP, plant protein. $n_{AP} = 18$, $n_{PP} = 19$. Values are presented as mean ± SEM. **p < 0.01, ***p < 0.001 different from week 0 within group. ns, not significant (p > 0.05).

Whole-body insulin sensitivity was determined by a hyperinsulinemic euglycemic clamp, performed before and after the diet intervention (Figure 13, right panel). The M-value indicates the glucose uptake in the tissues, thus the higher the M-value the higher the insulin sensitivity. The improvement was statistically significant only in the AP group (AP: + 16.3%, $p_{AP} = 0.005$; PP: + 9.1%, $p_{PP} = 0.224$), nevertheless without difference between AP and PP ($p_{APvsPP} = 0.438$) (Figure 13, right panel).

4.5.2. Meal tolerance test

During the meal tolerance test (MTT) blood samples were collected at 7 time points (0, 30, 60, 90, 120, 180, and 240 min) and levels of glucose, hormones and FFA were measured. Figures 20 and 21 illustrate the meal-induced response and incremental areas under the curve ($iAUC_{0-240}$) of insulin, C-peptide, glucose, and FFA and for glucagon, active GLP-1 and total GIP, respectively. Table 14 summarizes peaks and $iAUCs_{0-30}$ for these biomarkers, as well as indices of insulin secretion, sensitivity and degradation.

Concentrations of all parameters did not differ at baseline. Insulin and C-peptide increased after meal ingestion and peaked at 90 and 120 min, respectively (Figure 14). Meal-induced responses and single time points, as well as iAUCs did not differ between measurements (week 0 and 6) or diet groups (AP and PP). After 6 weeks of AP diet, postprandial glucose levels at time points 0, 30, 60, and 90 min were lower compared to week 0; in the PP group only fasting blood glucose was diminished. However, iAUCs values remained unchanged. Further, in the AP group the glucose peak at week 6 was lower than at baseline ($p_{AP} = 0.017$) (Table 14). For FFA, there were no differences for time courses and individual time points, but in the AP group the $iAUC_{0-240}$ at week 6 was smaller than at week 0 ($p_{AP} = 0.033$) (Figure 14, Table 14).

Glucagon peak and $iAUC_{0-30}$ increased after PP diet ($p_{PP} = 0.001$ and 0.079, respectively), resulting in significant difference between the two interventions ($p_{APvsPP} = 0.012$ and 0.015, respectively). Nevertheless, $iAUCs_{0-240}$ did not differ between weeks of intervention or between groups (Figure 15, Table 14). Active GLP-1 $iAUC_{0-240}$ was reduced by more than half in the AP group ($p_{AP} = 0.008$), but without further differences for the other outcomes (Figure 15, Table 14). Meal-induced response of total GIP differed between groups: secretion in the PP group was higher at 60 min at week 0 and at 60, 90, and 120 min at week 6, resulting in a significant higher $iAUC_{0-240}$. Moreover, the $iAUC_{0-30}$ increased only in the AP group (Figure 15, Table 14).

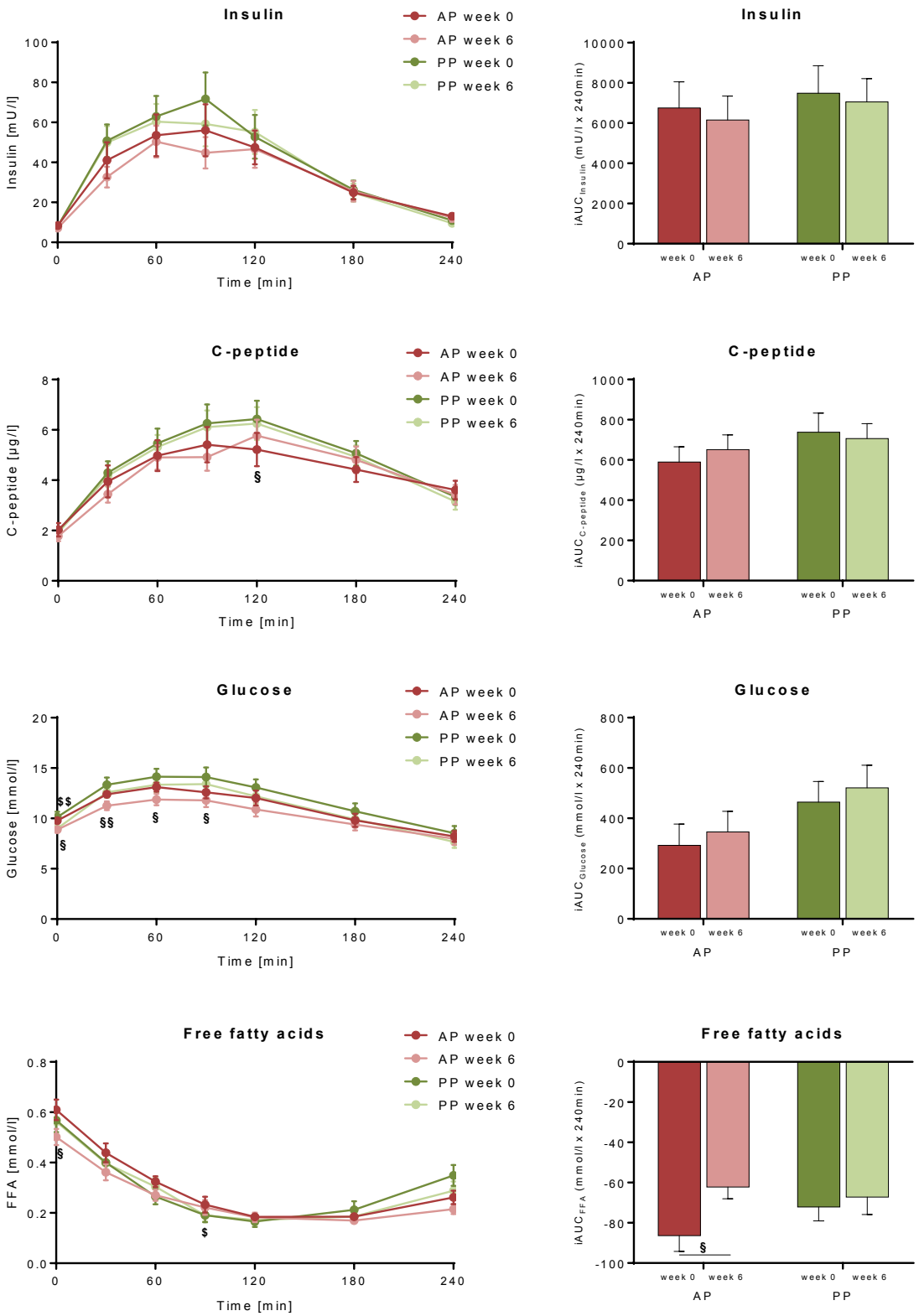

Figure 14. Postprandial meal-induced responses of insulin, C-peptide, glucose, and FFA. Postprandial meal-induced response over 240 min and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; FFA: free fatty acids; iAUC: incremental area under the curve; PP,

plant protein. Values are presented as means ± SEM. $^{§}p < 0.05$, $^{§}p < 0.01$ different from week 0 within AP group; $^{$}p < 0.05$, $^{$$}p < 0.01$ different from week 6 within PP group.

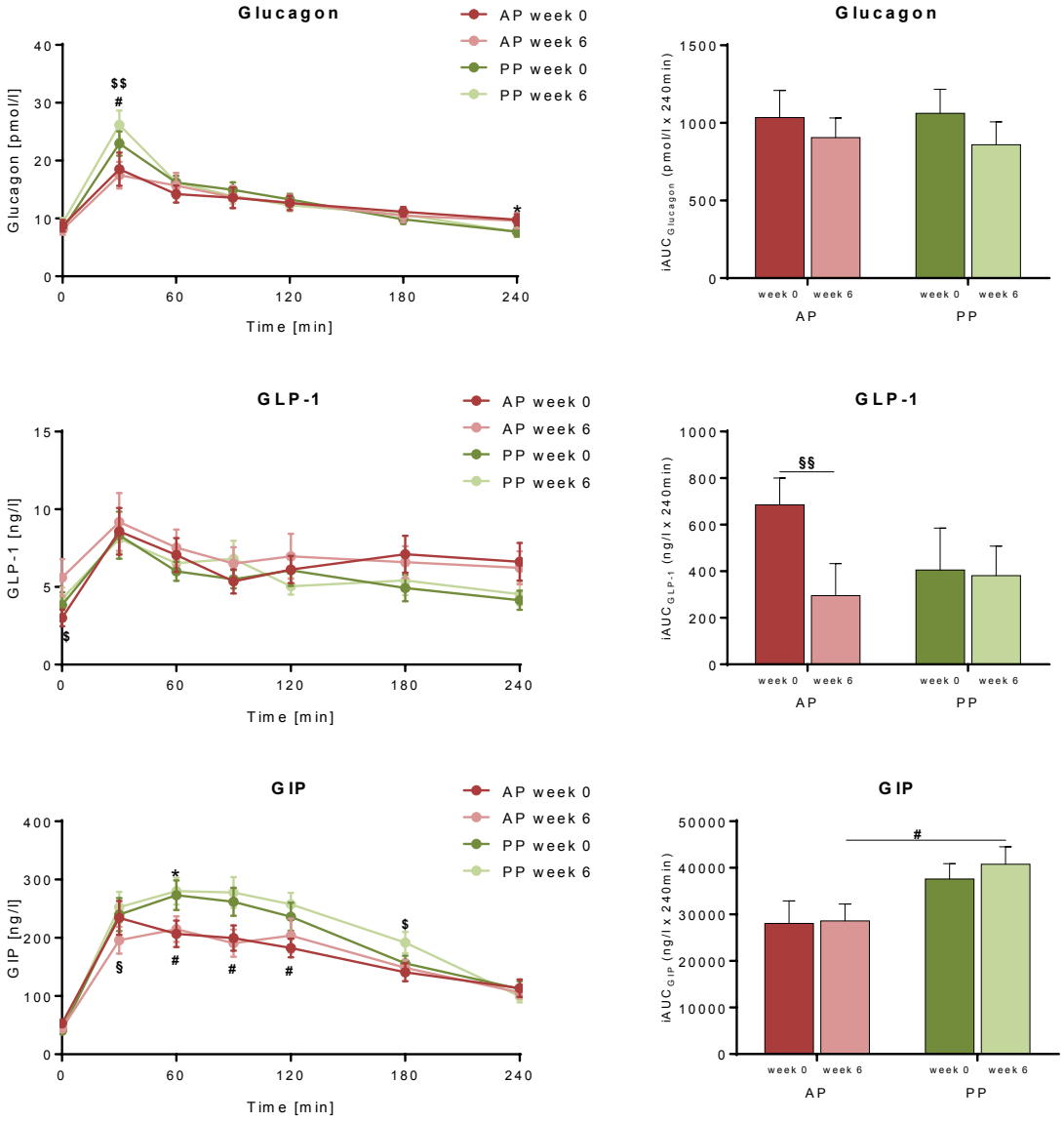

Figure 15. Postprandial meal-induced responses of glucagon, active GLP-1, and total GIP. Postprandial meal-induced responses over 240 min and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; GIP: glucose-dependent insulinotropic peptide; GLP-1: glucagon-like peptide 1; iAUC: incremental area under the curve; PP, plant protein. Values are presented as means ± SEM. *p < 0.05 different between AP and PP group at week 0; $^{#}p < 0.05$ different between AP and PP group at week 6; $^{§}p < 0.05$; $^{§§}p < 0.01$ different from week 0 within AP group; $^{$}p < 0.05$, $^{$$}p < 0.01$ different from week 0 within PP group.

The HIC index tended to improve in the AP group ($p_{AP} = 0.055$) and the OGIS and Matsuda indices in the PP group ($p_{PP} = 0.050$ and 0.063, respectively). The insulinogenic index did not change, while the DI increased only in the AP group ($p_{AP} = 0.035$) (Table 14).

Table 14. Peak, iAUCs at different intervals, and indices of insulin secretion, sensitivity and degradation.

	Animal Protein (AP), n = 18			Plant Protein (PP), n = 19			APvsPP
	week 0	week 6	p_{AP}	week 0	week 6	p_{PP}	p_{APvsPP}
Insulin							
Peak [mU/l]	62.8 ± 12.2	54.4 ± 9.2	0.932	74.9 ± 12.9	73.6 ± 12.7	0.319	0.773
$iAUC_{0-30}$ [mU/l x 30 min]	488.9 ± 116.4	382.0 ± 63.3	0.300	637.2 ± 109.9	626.4 ± 108.5	0.918	0.487
C-peptide							
Peak [µg/l]	6.1 ± 0.6	5.9 ± 0.6	0.748	6.7 ± 0.7	6.6 ± 0.7	0.528	0.522
$iAUC_{0-30}$ [µg/l x 30 min]	29.2 ± 5.3	25.3 ± 2.8	0.551	34.3 ± 5.0	34.1 ± 4.4	0.796	0.696
Glucose							
Peak [mmol/l]	13.4 ± 0.5	12.5 ± 0.5	0.017	14.9 ± 0.8	13.9 ± 0.7	0.161	0.553
$iAUC_{0-30}$ [mmol/l x 30 min]	38.9 ± 4.8	34.6 ± 4.7	0.307	49.1 ± 4.9	54.0 ± 5.8$^{\#}$	0.714	0.323
FFA							
Nadir [mmol/l]	0.16 ± 0.01	0.15 ± 0.01	0.793	0.15 ± 0.02	0.14 ± 0.01	0.378	0.419
$iAUC_{0-30}$ [mmol/l x 30 min]	-3.10 ± 0.67	-2.11 ± 0.32	0.375	-2.59 ± 0.48	-2.03 ± 0.52	0.109	0.940
$iAUC_{0-240}$ [mmol/l x 240 min]	-86.4 ± 7.9	-62.2 ± 5.9	0.033	-72.1 ± 7.0	-67.2 ± 8.7	0.329	0.283
Glucagon							
Peak [pmol/l]	18.8 ± 18.8	18.7 ± 2.3	0.631	22.1 ± 2.0	26.2 ± 2.5$^{\#}$	0.001	0.012
$iAUC_{0-30}$ [pmol/l x 30 min]	154.2 ± 30.3	128.5 ± 21.5	0.133	195.1 ± 14.6	227.0 ± 20.5$^{\#\#}$	0.079	0.015
active GLP-1							
Peak [µg/l]	10.08 ± 1.35	10.44 ± 1.78	0.795	9.05 ± 1.41	8.92 ± 1.10	0.302	0.357
$iAUC_{0-30}$ [µg/l x 30 min]	69.1 ± 14.5	57.8 ± 17.2	0.279	63.7 ± 25.3	58.2 ± 13.0	0.570	0.925
total GIP							
Peak [µg/l]	246.0 ± 22.3	243.5 ± 27.9	0.554	327.0 ± 25.8*	330.6 ± 24.0$^{\#\#}$	0.647	0.466
$iAUC_{0-30}$ [µg/l x 30 min]	2718.7 ± 465.1	2254.1 ± 319.5	0.048	3093.6 ± 422.0	3141.1 ± 420.4	0.613	0.438

Indices of insulin secretion, sensitivity and degradation							
HIC_{0-240}	0.11 ± 0.01	0.13 ± 0.01	0.055	0.11 ± 0.01	0.12 ± 0.01	0.219	0.282
OGIS [ml/min x m^2]	225.1 ± 8.9	228.4 ± 8.1	0.828	213.4 ± 8.9	231.8 ± 8.2	0.050	0.189
$Matsuda_{0-240}$	4.92 ± 0.82	5.41 ± 0.63	0.184	4.30 ± 0.65	4.92 ± 0.80	0.063	0.948
Insulinogenic index	17.6 ± 5.2	29.1 ± 18.0	0.925	14.8 ± 2.8	18.1 ± 5.4	0.833	0.483
DI_{0-30}	68.6 ± 29.7	91.0 ± 36.7	0.035	42.6 ± 5.8	63.6 ± 19.6	0.571	0.424

Abbreviations: DI, disposition index; FFA, free fatty acids; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; HIC, hepatic insulin clearance; iAUC, incremental area under the curve; OGIS, oral glucose insulin sensitivity. Values are presented as means ± SEM. *$p < 0.05$ different between AP and PP group at week 0; $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ different between AP and PP group at week 6.

Summarizing the data, only minor differences were found for metabolic and hormonal responses to meal tests. Changes of glucose, insulin, and C-peptide were not significantly different from baseline or between the diets. Glucagon peaked higher after PP meal ingestion but did not result in higher iAUC. Secretion of GIP induced by the PP meal, however, was significantly higher than by the AP meal. On the other hand, 6 weeks of AP diet lowered total postprandial levels of FFA and GLP-1. The indices of insulin secretion, sensitivity and degradation showed slight improvements without variation between the protein types.

The animal and plant protein test meals differed in their AA composition (chapter 3.3.4., Table 3). The BCAA (valine, leucine and isoleucine) and methionine were higher in the AP, while arginine and asparagine acid in PP meal. The postprandial plasma levels of FAA were determined at time points 60, 120, and 240 min after meal ingestion. Figure 16 shows meal-induced changes over time and $iAUCs_{0-240}$ of methionine and BCAA. Plasma concentrations peaked at 60 min and then fell down. As expected, plasma methionine was much higher in the AP group. The same was observed for valine and leucine. Remarkably, valine levels in the AP group remained nearly unchanged after the initial increase at 60 min pointing to a slower or shifted degradation. In contrast, isoleucine decreased below baseline resulting in negative iAUCs in both groups (Figure 16). Nevertheless, there were no significant differences within the two interventions.

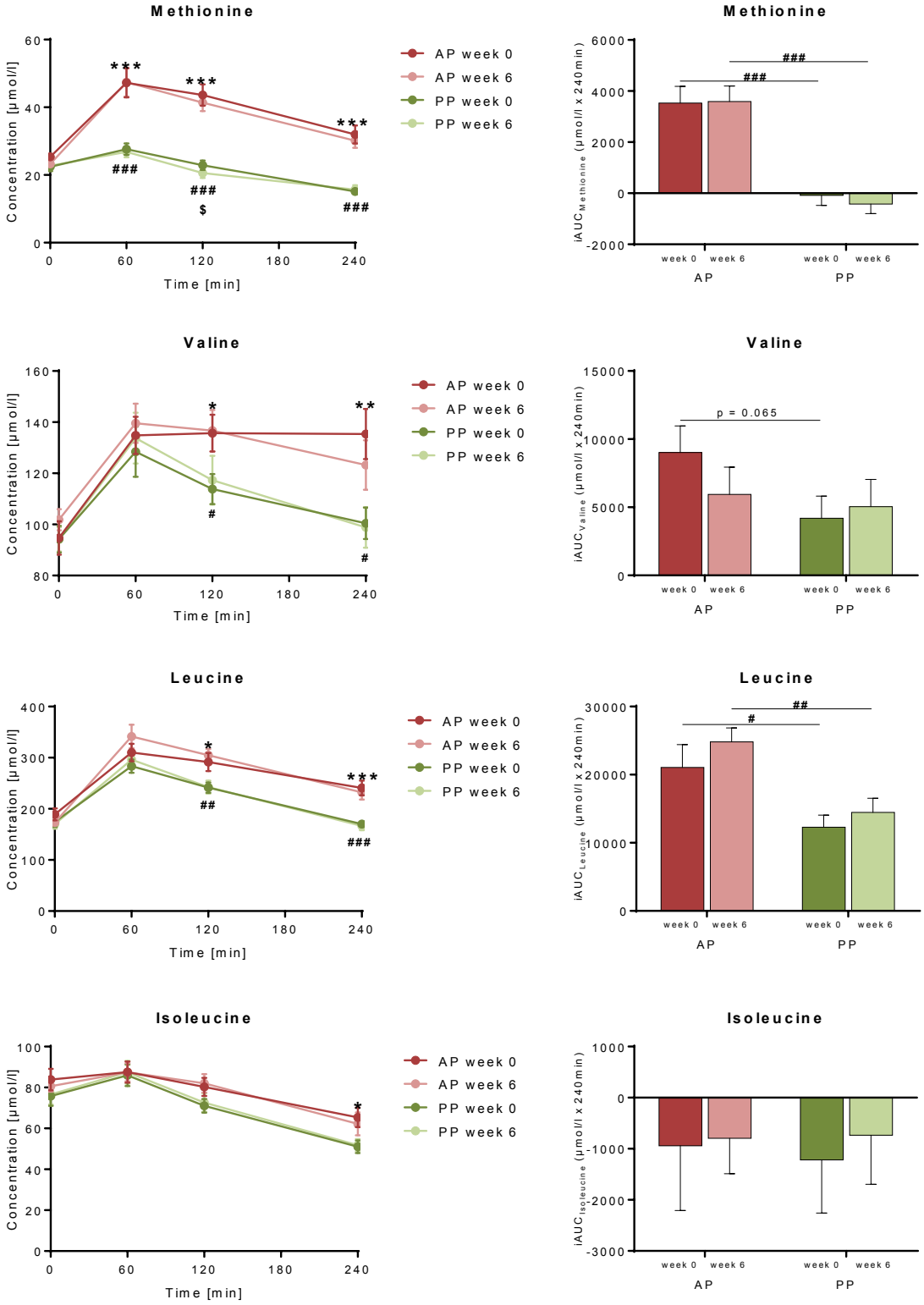

Figure 16. Postprandial levels of methionine, valine, leucine, and isoleucine. Postprandial changes over 240 min and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; iAUC: incremental area under the curve; PP, plant protein. Values are presented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 different between AP and PP group at week 0; #p < 0.05, ##p < 0.01, ###p < 0.001 different between AP and PP group at week 6; $p < 0.05, different from week 0 within PP group.

Results for arginine and asparagine are depicted in Figure 17. After AP meal arginine levels almost did not change from baseline, resulting in significant difference at 60 min from the PP meal. iAUCs, however, were not significantly different between groups. For asparagine there was a similar pattern without differences between AP and PP meals (Figure 17).

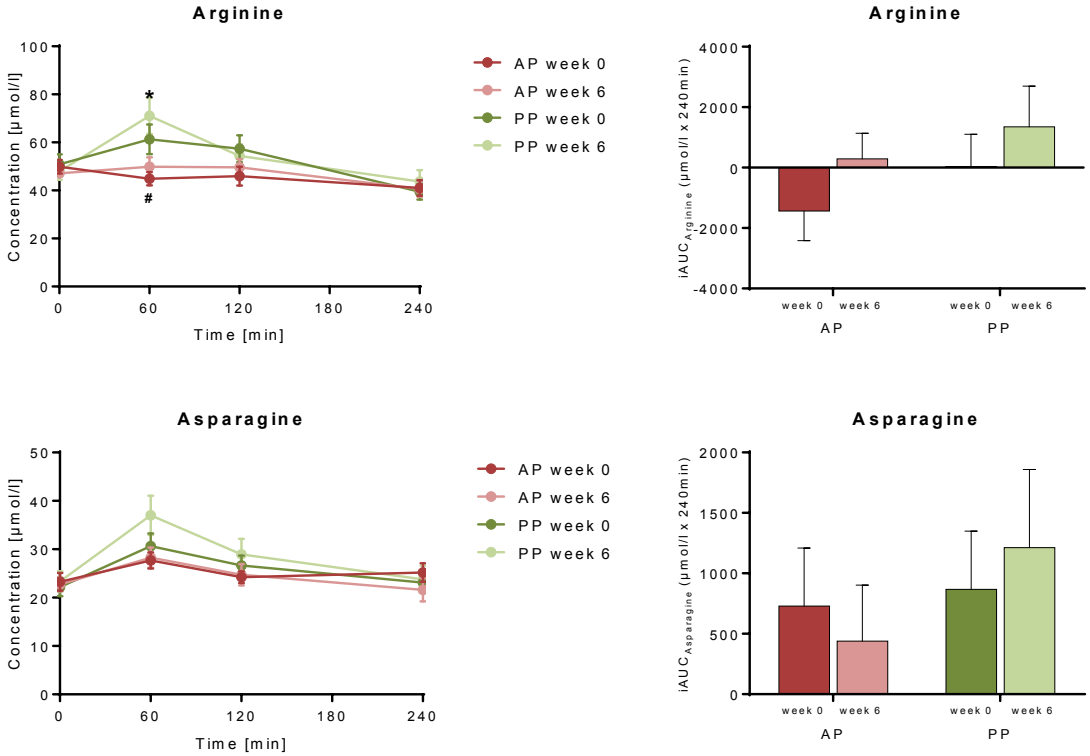

Figure 17. Postprandial levels of arginine and asparagine. Postprandial changes over 240 min and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; iAUC: incremental area under the curve; PP, plant protein. Values are presented as means ± SEM. *p < 0.05 different between AP and PP group at week 0; #p < 0.05 different between AP and PP group at week 6.

Although there were no significant group differences in fasting FAA, postprandial levels of methionine, BCAA and arginine differed between the two groups, mirroring the amino acid compositions of the AP and PP test meals. No differences were observed in plasma levels and iAUCs of other FAA. Graphs for lysine, histidine, glutamine, serine, alanine, phenylalanine, tyrosine, and tryptophan are shown in Figures 35 and 36 in the appendix.

4.5.3. Cardiovascular parameters

The blood lipids (triglycerides, LDL, HDL, and total cholesterol) are shown in Figure 18. The initial levels of triglycerides, total and LDL cholesterol were in the normal range; only HDL cholesterol was lower than the recommended values of 1.4 and 1.7 mmol/l for males and females, respectively. Triglycerides decreased after the first 2 weeks in all subjects, but were not significantly lower at the end of the study. Total cholesterol levels were lowered after 2 weeks, the reduction at week 6 was highly significant in both groups (AP: - 15.7%, $p_{AP} = 2x10^{-6}$, PP: - 11.4%, $p_{PP} = 2x10^{-5}$). LDL cholesterol declined with 16.3% in the AP and 11.7% in the PP group. HDL cholesterol decreased as well with 15.5% and 14.5% in AP and PP, respectively.

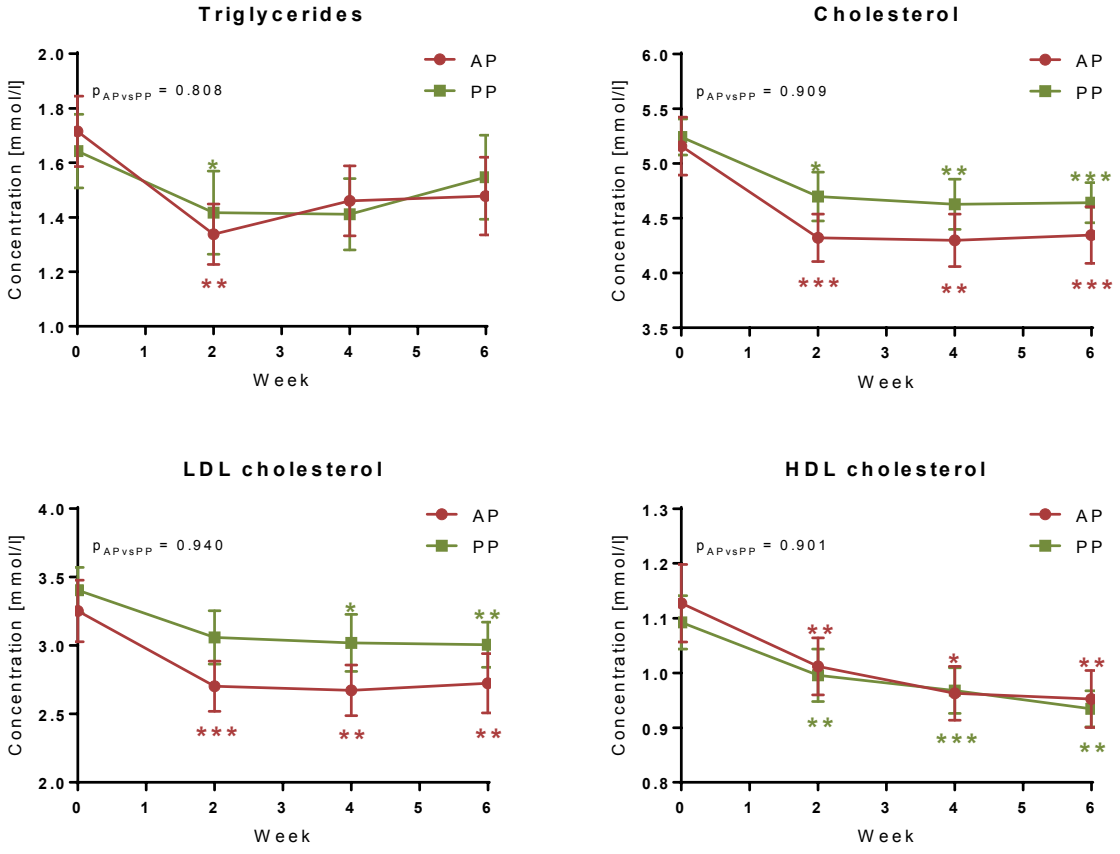

Figure 18. Dietary effects on blood lipids. All parameters were determined in blood serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. Abbreviations: AP, animal protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; PP, plant protein. $n_{AP} = 18$, $n_{PP} = 19$. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 different from week 0 within group.

Changes in these biomarkers did not vary between groups. Presumably, the improvements in the blood lipid profile mirror the lower fat content of the dietary interventions (Figure 8).

4.5.4. Renal function

High protein intake is presumed to worsen kidney function, especially in individuals with impaired kidney function, i. a. diabetics [88]. To evaluate effects of the high protein diets on kidney function, corresponding biomarkers were measured in fasting blood serum and 24-h urine samples.

Table 15 summarizes the levels of urea, creatinine, albumin and the urine albumin-to-creatinine ratio (UACR). Urea levels in all participants increased both in serum (week 2: $urea_{AP}$ = 8.1 ± 0.4 mmol/l, $p_{AP} = 3x10^{-5}$; $urea_{PP}$ = 7.6 ± 0.4 mmol/l, p_{PP} = 0.003) and in urine samples. This indicates the rise in the dietary protein intake and the high compliance to the food plans. Further, serum creatinine decreased in the PP group (Table 15, Figure 19), but was not changed in the AP group. Similar results were determined in the 24-h urine samples. These diverse tendencies suggest specific metabolic effects probably based on creatine intake or the different amino acid composition of the two diets.

Microalbuminuria is defined as albumin excretion between 30 and 300 mg/24h. At onset, 4 subjects from the AP and 2 from the PP group had excretion rates above 30 mg/24h. Despite the small sample size, levels in this subgroup decreased significantly. Urinary albumin excretion did not change in individuals without microalbuminuria (Table 15). UACR is an another diagnostic marker for microalbuminuria, that is defined as UACR values between 30 and 300 mg/g. UACR improved in subjects with microalbuminuria (considering baseline albumin levels) and remained unchanged in those with normal albumin excretion (Table 15).

Table 15. Dietary effects on urea, creatinine, and albumin.

Parameter	Animal protein (AP), n = 18			Plant protein (PP), n = 19			AP vs PP
	week 0	week 6	p_{AP}	week 0	week 6	p_{PP}	p_{APvsPP}
Urea							
in serum [mmol/l]	6.0 ± 0.3	8.0 ± 0.6	$<1x10^{-6}$	6.0 ± 0.3	7.9 ± 0.3	$1x10^{-6}$	0.538
in urine [mmol/24h]	409.9 ± 31.3	615.7 ± 50.1	$7x10^{-6}$	401.6 ± 36.1	572.4 ± 47.7	0.005	0.532
Creatinine							
in serum [µmol/l]	82.1 ± 4.2	80.6 ± 4.4	0.446	80.2 ± 3.1	74.0 ± 2.7	0.007	0.475
in urine [g/24h]	1.6 ± 0.1	1.7 ± 0.1	0.442	1.5 ± 0.1	1.3 ± 0.1	0.067	0.053
Albumin in urine							
albumin < 30 [mg/24h] at baseline	8.5 ± 2.1	6.6 ± 2.4 (n = 13)	0.695	9.7 ± 2.1	22.1 ± 10.7 (n = 14)	0.534	0.254
albumin > 30 [mg/24h] at baseline	65.1 ± 17.3	22.4 ± 12.3 (n = 4)	0.007	107.4 ± 4.8	24.2 ± 16.5 (n = 2)	0.089	0.028
Urine Albumin-to-Creatinine Ratio (UACR) [mg/g]							
albumin < 30 [mg/24h] at baseline	4.63 ± 0.86	4.33 ± 1.59 (n = 13)	0.754	6.73 ± 1.37	13.34 ± 5.53 (n = 13)	0.074	0.144
albumin > 30 [mg/24h] at baseline	46.6 ± 23.8	11.2 ± 5.7 (n = 4)	0.012	70.0 ± 15.0	27.0 ± 22.6 (n = 2)	0.112	0.739

Fasting serum concentrations of urea and creatinine were determined every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively), n_{AP} = 18, n_{PP} = 19. 24-h urine samples were collected by the participants on the days prior CID 1 and CID 3 for detection of urea, creatinine and albumin, n_{AP} = 17, n_{PP} = 16. Abbreviations: UARC, urine albumin-to-creatinine ratio. Values are presented as means ± SEM.

The glomerular filtration rate (GFR) describes the flow rate of filtered fluid through the kidney and is used in laboratory diagnostics to estimate renal function. Values under 60 ml/min/1.73m^2, together with increased albumin excretion rate and UACR, indicate 3rd degree impaired kidney function. The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation was developed in 2009 [150] for estimation of GFR. Dependent variables are serum creatinine values, age and sex (chapter 3.3.5., Table 4).

Serum creatinine can be influenced by several factors, e.g. age, sex, BMI, muscle mass, protein intake. Cystatin C, a proteinase inhibitor, represents a more eligible marker for glomerular filtration, since it is absorbed in the glomerulus, reabsorbed in the proximal tubule and completely catabolized. Unlike creatinine, cystatin C does not depend on age, sex,

protein intake etc. In 2012, the same work group developed an equation for GFR estimation using both serum creatinine and cystatin C [151]. This combined equation is more accurate than equations based on either of the two parameters alone. Equations in accordance to sex, creatinine and cystatin C are listed in chapter 3.3.5., Table 5.

Figure 15 illustrates the levels of serum creatinine and cystatin C as well as the GFR values calculated with the two CKD-EPI equations. Creatinine decreased only in the PP group, while cystatin C remained constant in all participants throughout the study period. Notably, due to the reduced creatinine concentration, GFR calculated with the creatinine equation improved significantly in the PP group. On the other hand, GFR values estimated with the combined equation did not change in either of the groups (Figure 19).

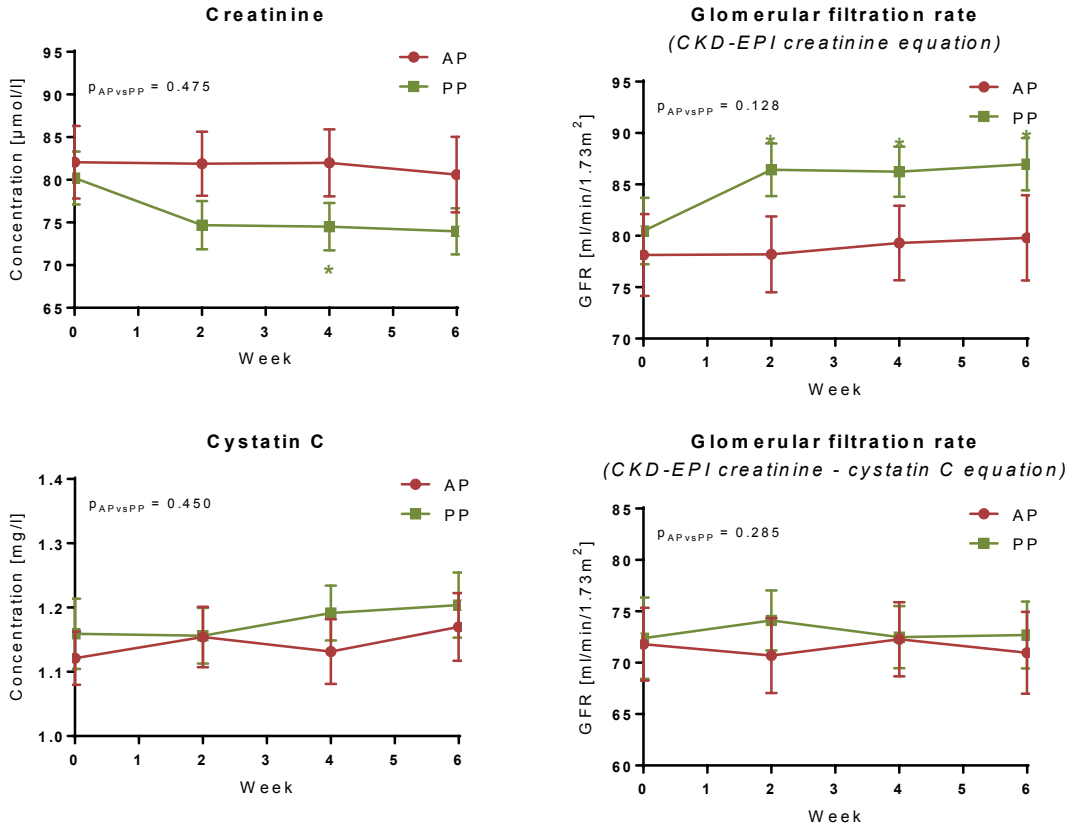

Figure 19. Dietary effects on creatinine, cystatin C, and glomerular filtration rate (GFR). Fasting serum concentrations of creatinine and cystatin C were determined every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively). GFR was calculated with CKD-EPI equations using creatinine concentrations or a combination of creatinine and cystatin C concentrations (chapter 3.3.5., Tables 4 and 5). Abbreviations: AP, animal protein; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; PP, plant protein. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. *p < 0.05 different from week 0 within group.

The diverse results of the two equations show that creatinine alone is not a suitable marker for GFR estimation. The high plant protein intake induced a decrease of serum creatinine, which could lead to a misleading interpretation of GFR improvement. Thus, GFR values

estimated with creatinine in combination with cystatin C should be considered correct and reliable.

It could be concluded that neither high animal nor high plant protein intake for a period of 6 weeks impaired the kidney function in type 2 diabetic individuals.

4.5.5. Liver enzymes

Liver enzymes aspartate transaminase (AST), alanine transaminase (ALT), and γ-glutamyl transpeptidase (γ-GT) were detected in fasting plasma samples as clinical biomarkers for liver health. Baseline levels of the three enzymes were in the normal range and the AST/ALT ratio was < 1, suggesting no severe liver damage (Figure 20).

The PP diet reduced the AST, while the levels in the AP group remained unchanged. Both protein diets did not affect the ALT values, as well as the AST/ALT ratio throughout the period of intervention (Figure 20). The decrease of γ-GT was significant only in the AP group (AP: - 28.9%, p_{AP} = 0.017; PP: - 23.5%, p_{PP} = 0.240).

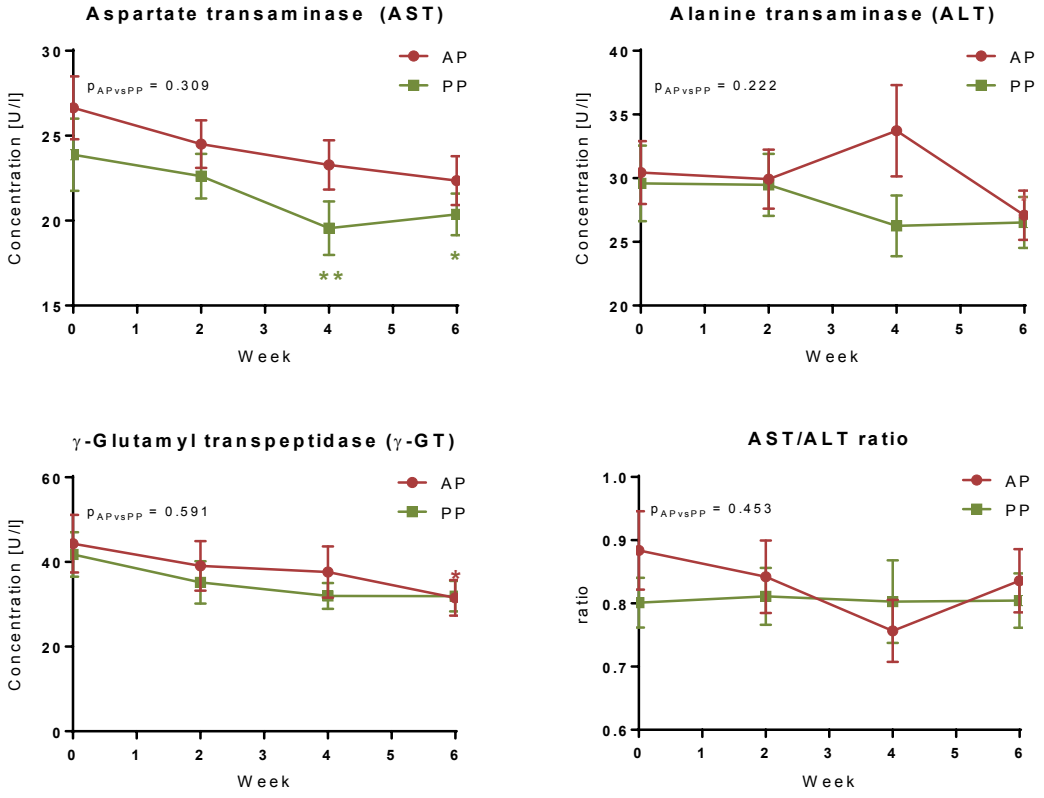

Figure 20. Dietary effects on liver enzymes. All parameters were determined in blood serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. Abbreviations: ALT: alanine transaminase; AP, animal protein; AST: aspartate transaminase; γ-GT: γ-glutamyl transpeptidase; PP, plant protein. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01 different from week 0 within group.

The high protein diets had a beneficial effect on the liver enzymes, although participants had no elevated levels at baseline. Protein origin showed not to play a role.

4.5.6. Uric acid, adiponectin, FGF21

Uric acid is a breakdown product of the purine metabolism and higher blood concentrations can cause gout [162]. It is also associated with metabolic syndrome, cardiovascular diseases and type 2 diabetes [163, 164]. Participants in the study had uric acid levels in the normal range at onset (Figure 21, left panel). Significant decreases of ~ 10% were observed in both groups.

Adiponectin, an adipokine secreted exclusively by adipose tissue, increases fat oxidation, glucose utilization in muscle, and reduces inflammation [165]. Low adiponectin levels are associated with metabolic syndrome, insulin resistance, and T2D in humans [166]. Adiponectin levels were measured in fasting serum in a subcohort of 20 participants (n_{AP} = 10, n_{PP} = 10). The levels of adiponectin were significantly reduced in the PP group, and tended to significance in the AP group (Figure 21, right panel).

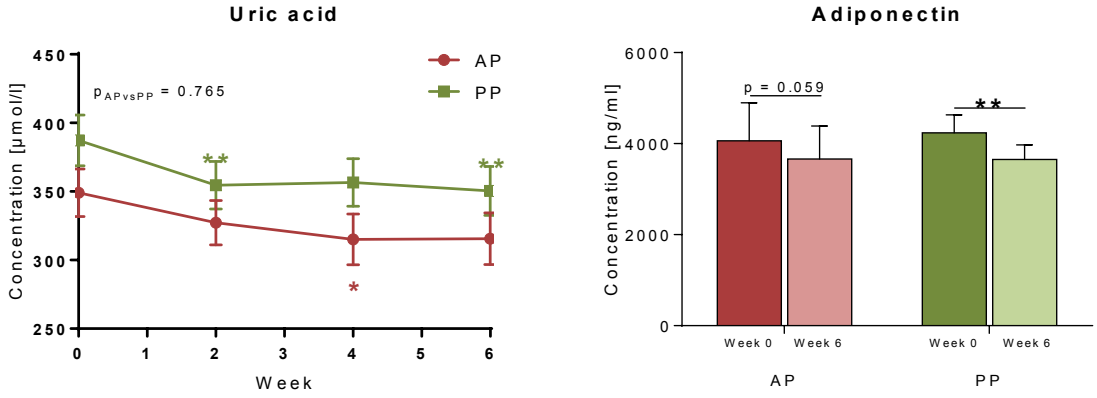

Figure 21. Dietary effects on uric acid and adiponectin. Fasting serum concentrations of uric acid (n_{AP} = 18, n_{PP} = 19) were determined every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) and of adiponectin (n_{AP} = 10, n_{PP} = 10) at week o and 6. Abbreviations: AP, animal protein; PP, plant protein. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01 different from week 0 within group.

Fibroblast growth factor 21 (FGF21) is a hepatokine associated with improvements in insulin sensitivity and lipid metabolism [167]. FGF21 concentration was measured in fasting serum samples (Figure 22). At baseline, participants showed elevated levels above 300 pg/ml confirming data from patients with metabolic syndrome and type 2 diabetes [168, 169]. After 6 weeks of intervention, a remarkable decrease was observed in all participants, 42.6% and 51.1% in the AP and PP group, respectively (Figure 22, left panel). In addition, levels after 2

and 4 weeks of intervention were assessed in a subgroup of 10 subjects (n_{AP} = 5, n_{PP} = 5). The analysis showed that FGF21 was significantly decreased after 2 weeks in both groups by approximately 50% (Figure 22, right panel). No differences between the diets were observed.

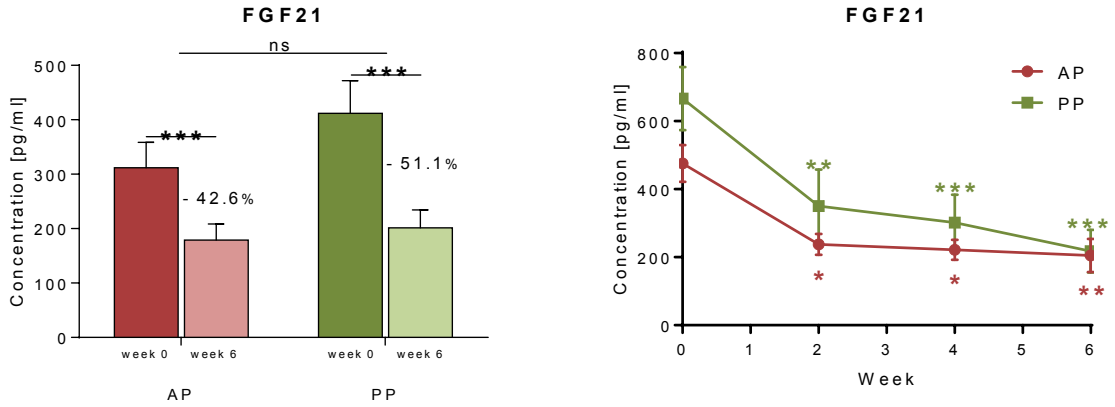

Figure 22. Decrease of serum fibroblast growth factor 21 (FGF21) after high protein diet. Left panel: FGF21 concentrations were determined in fasting serum samples taken at week 0 and 6 (n_{AP} = 18, n_{PP} = 19). Right panel: FGF21 concentrations were determined in fasting serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in a subcohort (n_{AP} = 5, n_{PP} = 5). Abbreviations: AP, animal protein; FGF21: fibroblast growth factor 21; PP, plant protein. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 different from week 0 within group. ns, not significant (p > 0.05).

4.5.7. Free fatty acid species and enzyme activity indices

The concentrations of different FFA in fasting blood plasma samples were determined using gas chromatography. The data in Table 16 summarizes the single fatty acids with names, abbreviations and chemical formulas, and the values for each group and time point together with the respective statistics. Calculated activity indices for enzymes involved in lipid metabolism are listed as well.

All measured SFAs (C14:0, C16:0, C18:0, C24:0) and MUFAs (C16:1n7, C18:1n9, C18:1n7) decreased significantly in both groups. However, odd-chain fatty acids (C15:0, C17:0) were not changed by the diets (Table 16). The main PUFA, linoleic acid (C18:2), did not change in either group. Interestingly, γ-linolenic acid (C18:3n6) decreased and α-linolenic acid (C18:3n3) increased in the AP group while there were no changes in the PP group resulting in a significant group difference for α-linolenic acid (p_{APvsPP} = 0.024). Arachidonic acid (C20:4) was reduced in both groups, dihomo-γ-linolenic acid (C20:3) in AP, and eicosapentaenoic acid (C20:5) in PP without group differences. Regarding the long-chain PUFAs, there was a significant decrease of docosapentaenoic acids (C22:5n3 and C22:5n6) in both groups, while docosahexaenoic acid (C22:6) was diminished only in the PP group.

Table 16. Fasting plasma free fatty acids concentrations and enzyme activities.

Free fatty acid		Animal Protein (AP), n = 18			Plant Protein (PP), n = 19			APvsPP
[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	0.717
Pentadecylic acid	C15:0	4.51 ± 0.26	4.57 ± 0.25	0.902	4.25 ± 0.26	4.05 ± 0.24	0.277	0.559
Palmitic acid	C16:0	216.84 ± 13.33	189.32 ± 13.35	0.005	217.56 ± 14.00	183.58 ± 11.51	0.007	0.931
Palmitoleic acid	C16:1n7	45.92 ± 3.56	41.12 ± 3.80	0.005	47.35 ± 3.98	40.38 ± 3.57	0.030	0.944
Margaric acid	C17:0	4.01 ± 0.21	3.90 ± 0.21	0.140	3.78 ± 0.18	3.68 ± 0.23	0.598	0.793
Stearic acid	C18:0	45.77 ± 2.10	36.63 ± 2.13	$1x10^{-5}$	44.82 ± 2.09	35.61 ± 1.85	$8x10^{-5}$	0.796
Oleic acid	C18:1n9	297.08 ± 15.99	263.83 ± 14.32	0.012	302.99 ± 18.94	249.24 ± 15.48	0.002	0.465
Vaccenic acid	C18:1n7	30.48 ± 1.73	28.11 ± 1.52	0.018	30.35 ± 2.02	26.27 ± 1.58	0.025	0.624
Linoleic acid	C18:2n6	277.69 ± 11.41	270.79 ± 8.69	0.490	282.38 ± 8.61	278.31 ± 10.98	0.713	0.822
γ-Linolenic acid	C18:3n6	13.60 ± 0.77	11.92 ± 0.74	0.005	14.13 ± 0.81	13.00 ± 1.03	0.109	0.352
α-Linolenic acid	C18:3n3	16.42 ± 0.84	22.28 ± 1.40	0.001	17.40 ± 0.90	19.03 ± 1.71	0.341	0.024
Dihomo-γ-linolenic acid	C20:3n6	12.68 ± 0.58	11.75 ± 0.64	0.112	12.51 ± 0.75	12.24 ± 0.72	0.543	0.303
Arachidonic acid	C20:4n6	63.54 ± 2.76	57.70 ± 3.00	0.006	65.09 ± 4.08	51.19 ± 3.55	0.001	0.749
Eicosapentaenoic acid	C20:5n3	19.82 ± 1.27	19.18 ± 1.30	0.458	20.22 ± 1.70	16.45 ± 1.18	0.019	0.181
Lignoceric acid	C24:0	2.64 ± 0.24	2.39 ± 0.19	0.046	2.82 ± 0.20	2.55 ± 0.17	0.036	0.839
Nervonic acid	C24:1n9	2.61 ± 0.15	2.62 ± 0.14	0.889	2.60 ± 0.14	2.53 ± 0.10	0.536	0.759
Docosapentaenoic acid n6	C22:5n6	16.35 ± 1.28	11.23 ± 0.64	$4x10^{-4}$	17.97 ± 1.44	11.63 ± 0.75	$4x10^{-8}$	0.589
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	0.858
Docosahexaenoic acid	C22:6n3	22.56 ± 0.60	20.89 ± 1.09	0.202	21.66 ± 1.74	17.06 ± 0.99	0.003	0.120

Enzyme activity indices								
SCD1 activity index	C16:1 / C16:0	0.21 ± 0.01	0.21 ± 0.01	0.916	0.22 ± 0.01	0.22 ± 0.00	0.994	0.973
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	0.957
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	0.973
Δ5 desaturase activity index	C20:4n6 / C20:3n6	5.13 ± 0.27	5.05 ± 0.27	0.718	5.43 ± 0.36	4.93 ± 0.42	0.037	0.203
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	0.351	0.402

Concentrations of free fatty acids were determined in fasting plasma samples taken at week 0 and 6. Enzyme activities were calculated using free fatty acid concentrations in [µg/ml]. Abbreviations: SCD1: stearoyl-CoA desaturase-1. n_{AP} = 18, n_{PP} = 19. Values are presented as means ± SEM.

The levels of FFA in blood give information about the enzyme activities in mainly SAT, since it is the major FFA contributor to the systemic venous circulation (about 80%) [170]. Elongase activity and lipogenic index diminished in all participants, while SCD1 activity remained unchanged. Noteworthy, Δ5 desaturase activity index was lower in the PP group and Δ6 desaturase activity index in the AP group, no difference between the groups were seen.

Correlation analyses between the relative changes of FFA with the relative changes of fat depots were performed in order to suggest possible mechanisms contributing to the decrease of fat mass. Table 17 summarizes the significant correlations between relative changes of FFAs and enzyme activity indices with the relative changes of VAT, NVAT, total FM (measured with BODPOD®), AdipoIR, and clamp-derived whole-body sensitivity, expressed as M-value. Correlation coefficients (r, ρ) are shown for each group, asterisks (*) indicate significant correlation for the entire cohort.

Of note, in the PP group the change of the odd-chain pentadecylic acid (C15:0) correlated positively with the changes of VAT and NVAT (Table 17). Moreover, the change of FM was negatively correlated with the changes of PUFAs linoleic (C18:2n6) and docosapentaenoic acid (C22:5n3) in the PP group and α-linolenic acid (C18:3n3) in all subjects. On the other hand, there was a positive correlation with the change of lignoceric acid (C24:0) in the AP group and of lipogenic index (C16:0 / C18:2n6) in the whole cohort. Furthermore, the change of AdipoIR correlated positively with changes of stearic (C18:0) and docosapentaenoic acid (C22:5n3) in the PP and Δ5 desaturase activity index (C20:4n6 / C20:3n6) in the AP group (Table 17). The change of M-value correlated negatively with the change of the ω-6 PUFAs

γ-linolenic (C18:3n6) and dihomo-γ-linolenic acid (C20:3n6) and positively with the Δ5 desaturase activity index (C20:4n6 / C20:3n6) in the PP group and in the entire cohort.

Table 17. Correlations between the relative changes of plasma free fatty acids from week 0 to week 6 with the relative changes of fat depots and insulin sensitivity.

Parameter		Animal Protein (AP), n = 15		Plant Protein (PP), n = 17	
		r, ρ	p_{AP}	r, ρ	p_{PP}
VAT					
Pentadecylic acid	C15:0	0.106	0.720	0.493	**0.045**
NVAT					
Pentadecylic acid	C15:0	-0.062	0.833	0.489	**0.046**
FM					
Linoleic acid	C18:2n6	-0.154	0.633	-0.545	**0.044**
α-Linolenic acid *	C18:3n3 *	-0.027	0.937	-0.525	0.065
Lignoceric acid	C24:0	0.601	**0.039**	0.165	0.573
Docosapentaenoic acid n3	C22:5n3	0.287	0.366	-0.553	**0.040**
Lipogenic index *	C16:0 / C18:2n6 *	0.273	0.391	0.120	0.682
AdipoIR					
Stearic acid	C18:0	-0.028	0.916	0.493	**0.032**
Docosapentaenoic acid n3	C22:5n3	0.139	0.595	0.473	**0.041**
Δ5 desaturase activity index	C20:4n6 / C20:3n6	0.636	**0.006**	-0.317	0.186
M-value					
γ-Linolenic acid *	C18:3n6 *	-0.234	0.367	-0.458	**0.049**
Dihomo-γ-linolenic acid *	C20:3n6 *	-0.439	0.078	-0.561	**0.012**
Δ5 desaturase activity index*	C20:4n6 / C20:3n6 *	0.225	0.386	0.482	**0.036**

*significant correlation in the whole cohort (n = 32). *p < 0.05.

These results suggest a role of FFA in the changes of fat depots and insulin sensitivity. While SFAs and lipogenic index showed positive associations with fat depots and AdipoIR, there were negative correlations for PUFAs with FM and M-value. Controversially, Δ5 desaturase activity index correlated positively with AdipoIR in the AP but also with M-value in the PP group.

4.5.8. Free amino acids

Since the intake of dietary amino acids was different in the two high protein diets, the free amino acid (FAA) composition in the circulation was of major interest. LC-MS technique was used to determine the plasma concentrations of 14 FAA and taurine (Figure 23). Fasting levels of the FAA did not reveal any changes for week 0 and week 6. Noteworthy, the analysis showed no significant differences between the groups regarding the fasting levels.

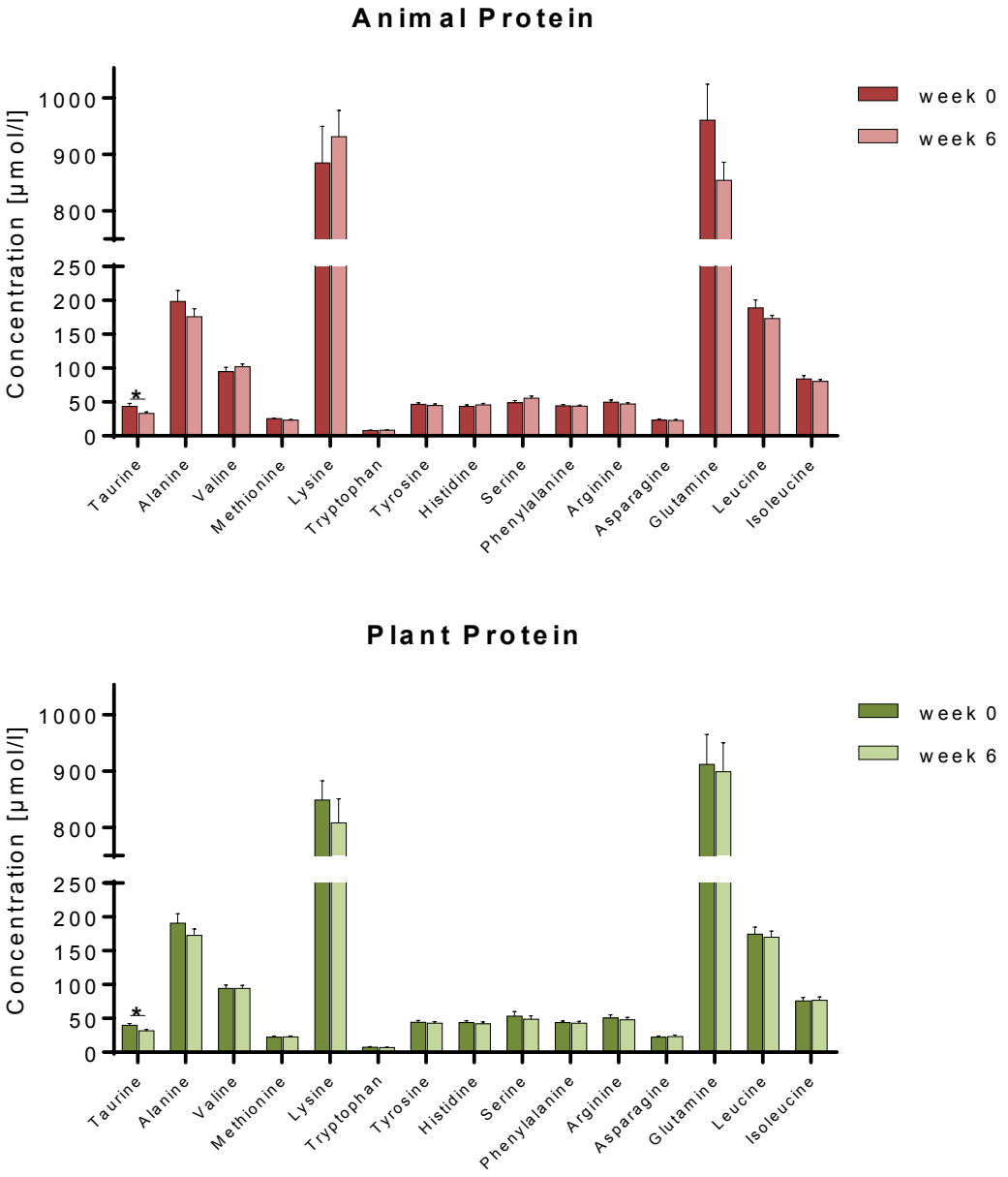

Figure 23. Fasting free amino acid concentrations after high protein diet. Concentrations of free amino acids were determined in fasting plasma samples taken at week 0 and 6. n_{AP} = 18, n_{PP} = 19. Values are presented as means ± SEM. *p < 0.05.

Only taurine, a sulfonic acid containing an amino group and involved in cardiovascular functions and bile acid synthesis, decreased significantly in the entire cohort. Taurine occurs naturally in meat and fish and it was shown to be lower in vegans [171].

Table 18 summarizes the significant correlations between relative changes of fasting FAAs and relative changes of body composition and whole-body insulin sensitivity (M-value). In the AP group the change of tryptophan correlated positively with changes of VAT and NVAT, while in the PP group the change of asparagine correlated negatively with the changes of the fat depots. Furthermore, there were positive correlations between changes of NVAT and glutamine in the AP group and leucine and isoleucine in the entire cohort (Table 18). Moreover, in the PP group the change of lysine correlated positively with the change of FFM and negatively with the change of FM. Notably, there was a positive correlation for change of serine with change of FFM in all subjects and with the change of M-value in the PP group and in the whole cohort.

Table 18. Correlations between the relative changes of fasting plasma free amino acids from week 0 to week 6 with the relative changes of fat depots, fat free mass and whole-body insulin sensitivity.

Parameter	Animal Protein (AP), n = 15		Plant Protein (PP), n = 17	
	r, ρ	p_{AP}	r, ρ	p_{PP}
VAT				
Tryptophan	0.684	**0.007**	-0.294	0.252
Asparagine	0.026	0.929	-0.588	**0.017**
NVAT				
Tryptophan	0.631	**0.016**	-0.111	0.672
Asparagine	0.442	0.113	-0.566	**0.022**
Glutamine	0.612	**0.020**	-0.086	0.742
Leucine *	0.307	0.286	0.312	0.223
Isoleucine *	0.441	0.115	0.267	0.301
FFM				
Lysine	0.035	0.914	0.583	**0.037**
Serine *	0.322	0.308	0.502	0.080
FM				
Lysine	0.042	0.897	-0.609	**0.027**
M-value				
Serine **	0.259	0.299	0.612	**0.007**

*significant correlation in the whole cohort (n = 32). *p < 0.05, **p < 0.01.

These data propose a beneficial effect of asparagine on abdominal fat depots and of lysine and serine on body composition and insulin sensitivity. However, rather negative effects on abdominal fat were indicated for tryptophan and glutamine in the AP group, and the BCAA leucine and isoleucine in the entire cohort.

4.6. Liver fat

The content of intrahepatic lipids (IHL) was estimated in 32 study subjects (n_{AP} = 15, n_{PP} = 17) using magnetic resonance spectroscopy. Per definition, non-alcoholic fatty liver disease (NAFLD) is diagnosed when the hepatic triglyceride content is more than 5.56% [172]. At onset, 25 of the participants had IHL levels above 5.56%.

Figure 24 illustrates the changes of IHL in every individual from week 0 to week 6. After 6 weeks of intervention the IHL content was markedly reduced in both groups, by 48.0% and 35.7% of mean in AP and PP, respectively. Moreover, in 9 of the 25 subjects, who had NAFLD at baseline, the IHL levels fell below 5.56% and thus below the definition limit of NAFLD.

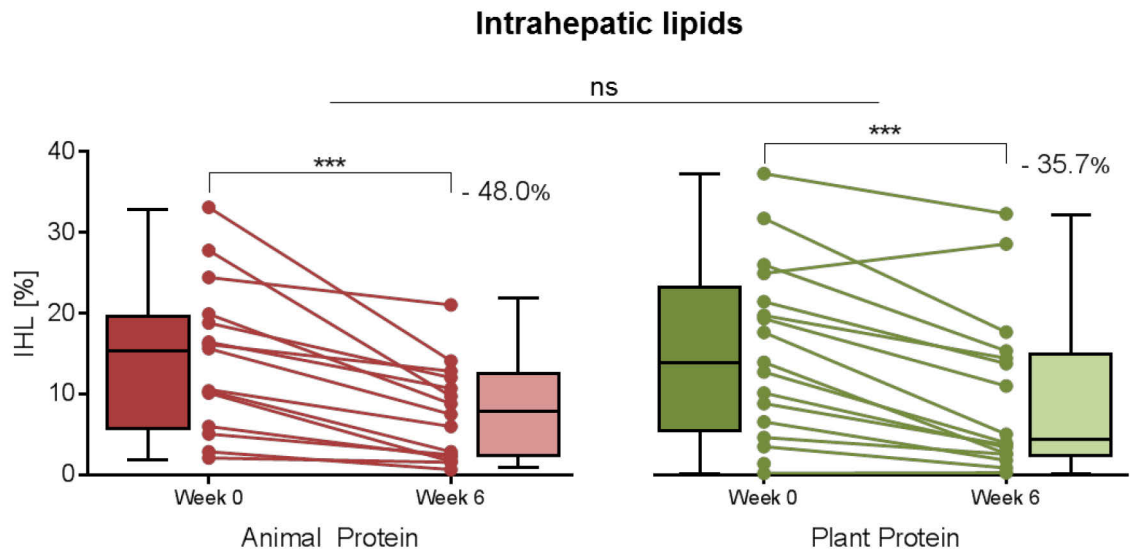

Figure 24. Effects of high protein diets on intrahepatic lipids (IHL). IHL content was determined by MRS at week 0 and 6. Graphs show individual changes from week 0 to week 6 and the respective boxplots (boxplots represent the median values together with the 25th and 75th percentiles; the whiskers show the lowest and highest values). Abbreviations: IHL: intrahepatic lipids; MRS: magnetic resonance spectroscopy. n_{AP} = 15, n_{PP} = 17. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant (p > 0.05).

The reduction of IHL was not correlated with both body weight and BMI decrease, or with the changes of FM, VAT or NVAT. Even after adjusting for body weight change the IHL reduction remained significant in both groups (p_{AP} = 0.006, p_{PP} = 0.039). This indicates that the very pronounced loss of hepatic triglycerides was primarily due to dietary effects rather than

changes in body composition. Furthermore, the decrease of liver fat was not different between the groups (Figure 24), suggesting that the amino acid composition in the diet is not determinant for the IHL diminishing effect.

The IHL reduction correlated positively with changes of glucose and HOMA-IR in the PP group and in all subjects (Table 19). Moreover, there was a positive correlation with the change of insulin and a negative one with M-value in the entire cohort. Positive correlations were observed for total, LDL, and HDL cholesterol in the AP group and in the whole cohort. Furthermore, changes of stearic acid (C18:0), γ-linolenic acid (C18:3n6), lipogenic index (C16:0 / C18:2n6), and Δ6 desaturase activity index (C18:3n6/C18:2n6) correlated positively with IHL change in all participants. On the other hand, the changes of IHL and plasma asparagine correlated negatively in the PP group and in the entire cohort (Table 19).

Table 19. Correlations between the relative change of intrahepatic lipids with the relative changes of blood parameters.

Parameter		Animal Protein (AP), n = 15		Plant Protein (PP), n = 17	
		r, ρ	p_{AP}	r, ρ	p_{PP}
Glucose **		0.293	0.289	0.761	**$6x10^{-4}$**
Insulin *		0.398	0.142	0.424	0.102
HOMA-IR **		0.377	0.166	0.597	**0.015**
M-value *		-0.437	0.104	-0.441	0.087
Cholesterol **		0.548	**0.034**	0.455	0.077
LDL cholesterol *		0.471	0.076	0.395	0.130
HDL cholesterol *		0.588	**0.021**	-0.141	0.602
Stearic acid *	C18:0 *	0.507	0.064	0.361	0.170
γ-Linolenic acid *	C18:3n6 *	0.642	**0.013**	0.241	0.369
Lipogenic index *	C16:0 / C18:2n6 *	0.198	0.497	0.496	0.051
Δ6desaturase activity index*	C18:3n6/C18:2n6 *	0.476	0.085	0.307	0.247
Asparagine *		-0.121	0.669	-0.568	**0.027**

*significant correlation in the whole cohort (n = 32). *p < 0.05, **p < 0.01.

From the data it can be observed that IHL reduction was associated with the improvements of blood markers of glucose and lipid metabolism. Moreover, it indicates a role of the reduced stearic and γ-linolenic acid as well as lipogenic and Δ6 desaturase activity indices in the decrease of IHL. Notably, asparagine might favor fatty liver reduction.

4.7. Gene expression in adipose tissue

Subcutaneous adipose tissue (SAT) biopsies were obtained in the fasting state (n_{AP} = 12, n_{PP} = 15). Gene expression analyses were performed using quantitative real-time polymerase chain reaction (qRT-PCR). Several key genes from lipid metabolic pathways were selected from published studies indicating their role in the pathogenesis of diabetes [87, 173]. Figure 25 illustrates the relative mRNA expression of genes involved in lipid uptake, *de novo* lipogenesis, lipid storage and lipolysis. *ACC1* and *SCD1* decreased in the AP, *PGC1A* and *PPARG* in the PP group. mRNA expression of lipolytic enzyme *HSL* decreased significantly in the PP group and tended to significance in the AP group (p_{AP} = 0.052), while *ATGL* was not altered.

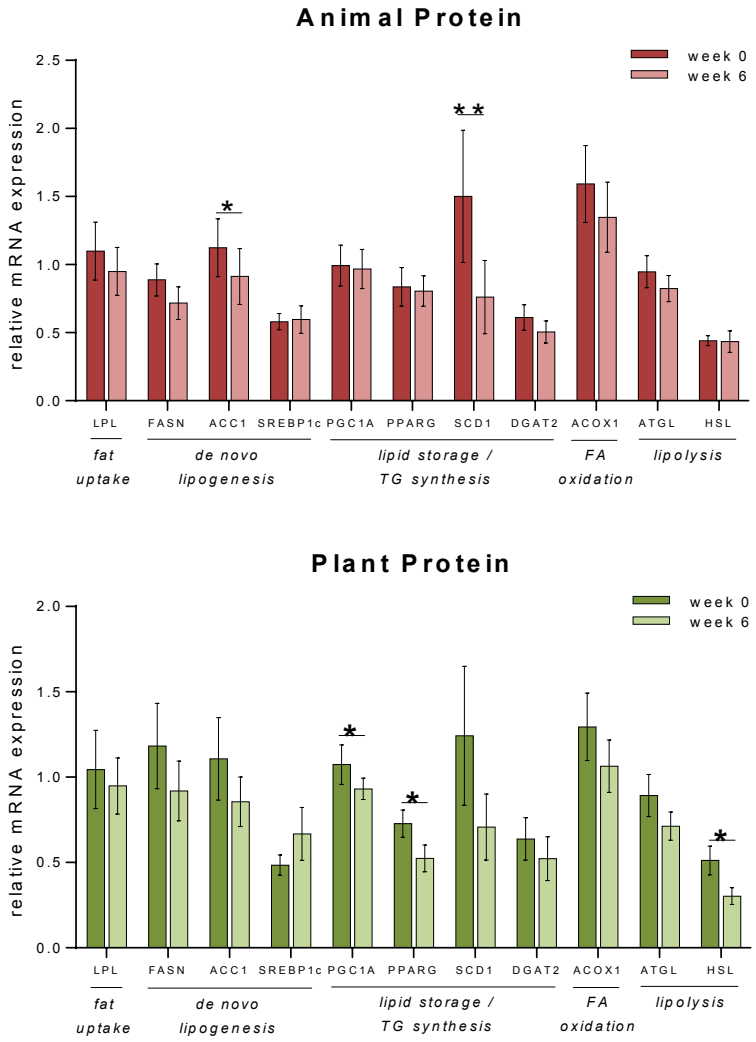

Figure 25. Dietary effects on mRNA expression of genes involved in fat metabolism in adipose tissue. Gene expression analyses were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 12, n_{PP} = 15. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01.

Additionally, adipokines and genes involved in glucose and energy metabolism, altered in the insulin resistant state, were studied [174-176]. Eventual changes in metabolism of BCAA provoked by the increased intake were further investigated [177]. Gene expression of adiponectin (*ADIPOQ*) was reduced in the PP group, whereas leptin (*LEP*) remained unchanged (Figure 26). *PDK4* and *GLUT4* expression did not change, while *PEPCK* decreased in the PP group. No alteration was observed in expression of BCAA-oxidizing enzymes *BCAT2* and *BCKDH* as well as *SIRT1*.

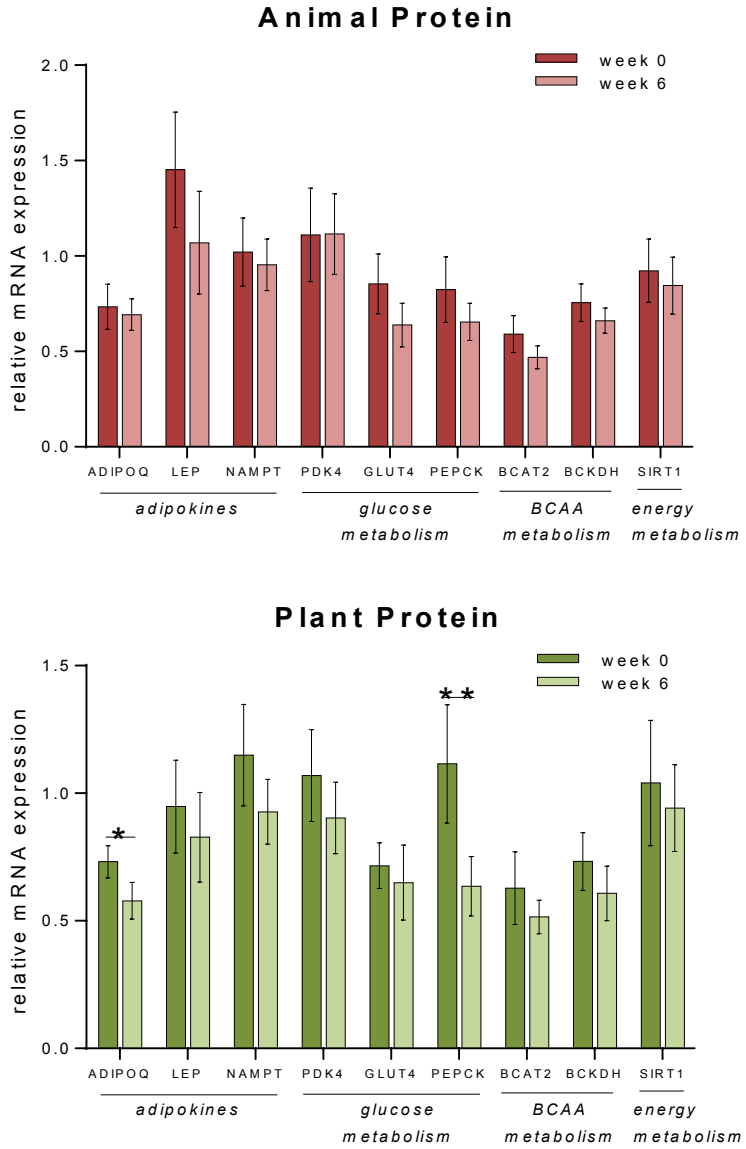

Figure 26. Dietary effects on mRNA expression of adipokines and of genes involved in glucose, branched-chain amino acids (BCAA), and energy metabolism in adipose tissue. Gene expression analyses were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 12, n_{PP} = 15. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01.

Furthermore, since autophagy and ER-stress are elevated in adipose tissue of obese humans [178, 179], influence of the interventions on selected genes was studied. Relative mRNA expression of proteins playing major roles in autophagy (*P62*, *ATG5*, *LC3A*, *and LC3B*) and ER-stress (*ATF4*, *XBP1*, and *XBP1s*) were not influenced by the diet intervention. The observed decrease of circulating FGF21 raised the question whether this was due to FGF21 resistance in adipose tissue of obese and insulin resistant subjects as proposed by Gallego-Escuredo and coworkers [169]. *FGFR1* expression decreased in the PP, but β-Klotho (*KLB*) was unchanged in either of the groups (Figure 27).

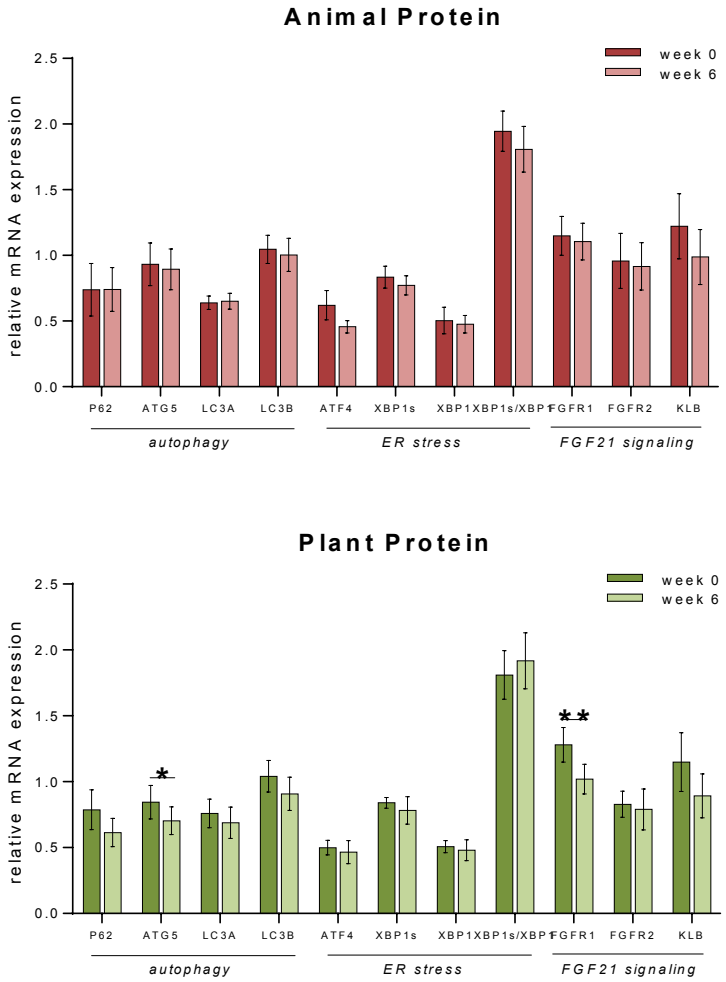

Figure 27. Dietary effects on mRNA expression of genes involved in autophagy, endoplasmic reticulum (ER) stress, and FGF21 signaling in adipose tissue. Gene expression analyses were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 12, n_{PP} = 15. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01.

Apparently, the high protein diets induced decreases in *de novo* lipogenesis, lipid storage and lipolysis in SAT. Nevertheless, gene expression analysis showed no differences between animal and protein diets.

4.8. Inflammation

T2D is characterized by subclinical low-grade inflammation [180]. In order to investigate whether the high protein diets had an effect on inflammatory processes, analyses in blood and adipose tissue were performed. The acute-phase-protein C-reactive protein (CRP), an inflammatory marker elevated in T2D patients [181], decreased significantly throughout the intervention only in the AP group (p_{AP} = 0.001, p_{PP} = 0.723, p_{APvsPP} = 0.198) (Figure 28).

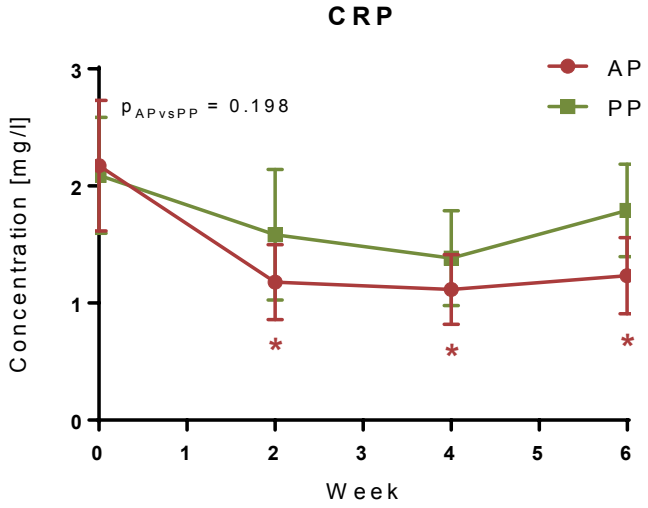

Figure 28. Dietary effects on C-reactive protein (CRP). Concentrations of CRP were determined in blood serum samples taken every two weeks (on CID 1, NC 2, NC 4, and CID 3, respectively) in the fasting state. Abbreviations: AP, animal protein; CRP: C-reactive protein; PP, plant protein. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. *p < 0.05 different from week 0 within group.

Levels of IL-6, an interleukin associated with the risk of developing T2D [182], tended to decrease in the AP and did not change in the PP group (Figure 29).

Moreover, in a subgroup of 20 participants (n_{AP} = 10, n_{PP} = 10), 7 further cytokines, related to T2D, were measured in fasting serum samples [183-185]. IL-18 levels decreased significantly in the AP group and TNFα in the PP group. However, IL-12p70 increased in the PP group resulting also in a significant difference between the groups (p_{APvsPP} = 0.019). MCP-1 tended to be reduced in the PP group. There was no change in the levels of IL-4, IL-8 and IL-1RA (Figure 29).

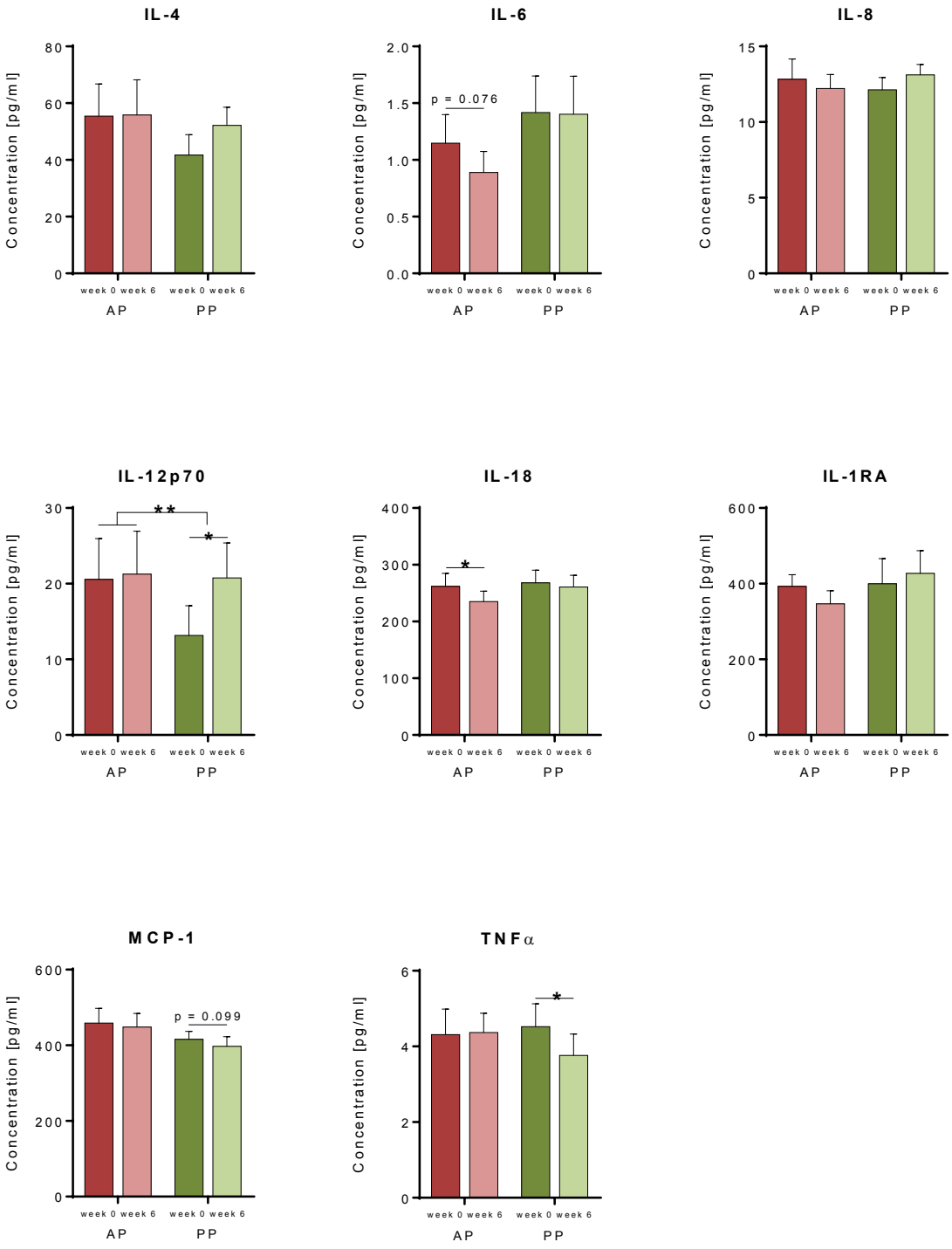

Figure 29. Dietary effects on inflammatory markers in serum. In a subcohort (n_{AP} = 10, n_{PP} = 10), concentrations of several inflammatory markers were detected in fasting serum samples taken at week 0 and 6 (IL-6 levels were determined in the entire cohort, n_{AP} = 18, n_{PP} = 19). Abbreviations: AP, animal protein; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; PP, plant protein; TNFα: tumor necrosis factor α. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. *p < 0.05, **p < 0.01.

Moreover, expression of inflammatory genes was determined in adipose tissue (Figure 30). Genes were selected from previously published studies showing their increased expression in insulin resistance and T2D [186, 187]. No differences were observed for the expression of toll-like receptors *TLR2, TLR4,* cytokines *IL-1B, MCP-1,* and macrophage markers *ITGAX, MRC1,* and *CD163.* Changes between AP and PP were not observed.

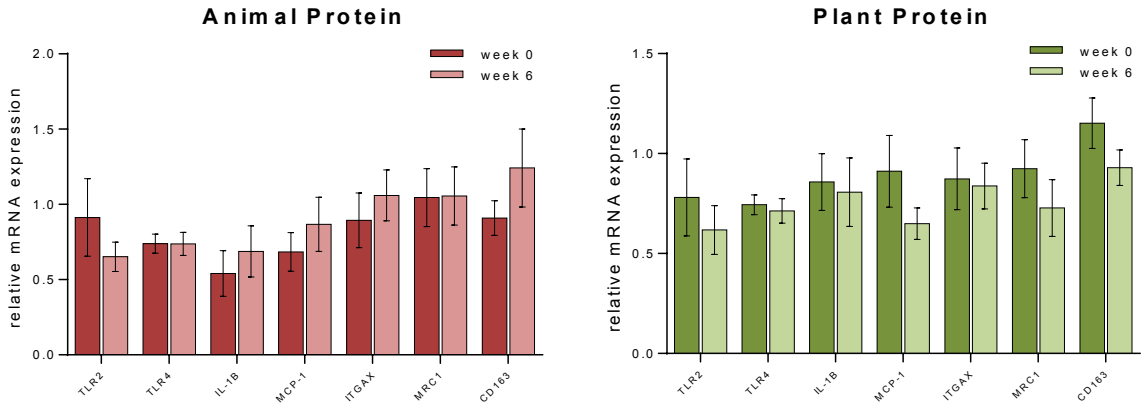

Figure 30. Dietary effects on mRNA expression of inflammatory genes in adipose tissue. Gene expression analyses were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 12, n_{PP} = 15. Values are presented as mean ± SEM.

Whole blood samples were stimulated with LPS followed by detection of secreted cytokines in order to elucidate the effect of the diets on inflammatory responses adapting an experimental setting published previously [188]. Figure 31 illustrates the LPS-induced levels of diverse cytokines at week 0 and week 6. Only IL-18 was significantly lower in the AP group. Trends towards an increase of the anti-inflammatory IL-10 and pro-inflammatory IFNγ were found in the PP (p_{APvsPP} = 0.052) and AP group (p_{APvsPP} = 0.082), respectively. The secretion of the other inflammatory markers (IL-1β, IL-6, IL-12p70, MCP-1, and TNFα) was not altered by either of the diets.

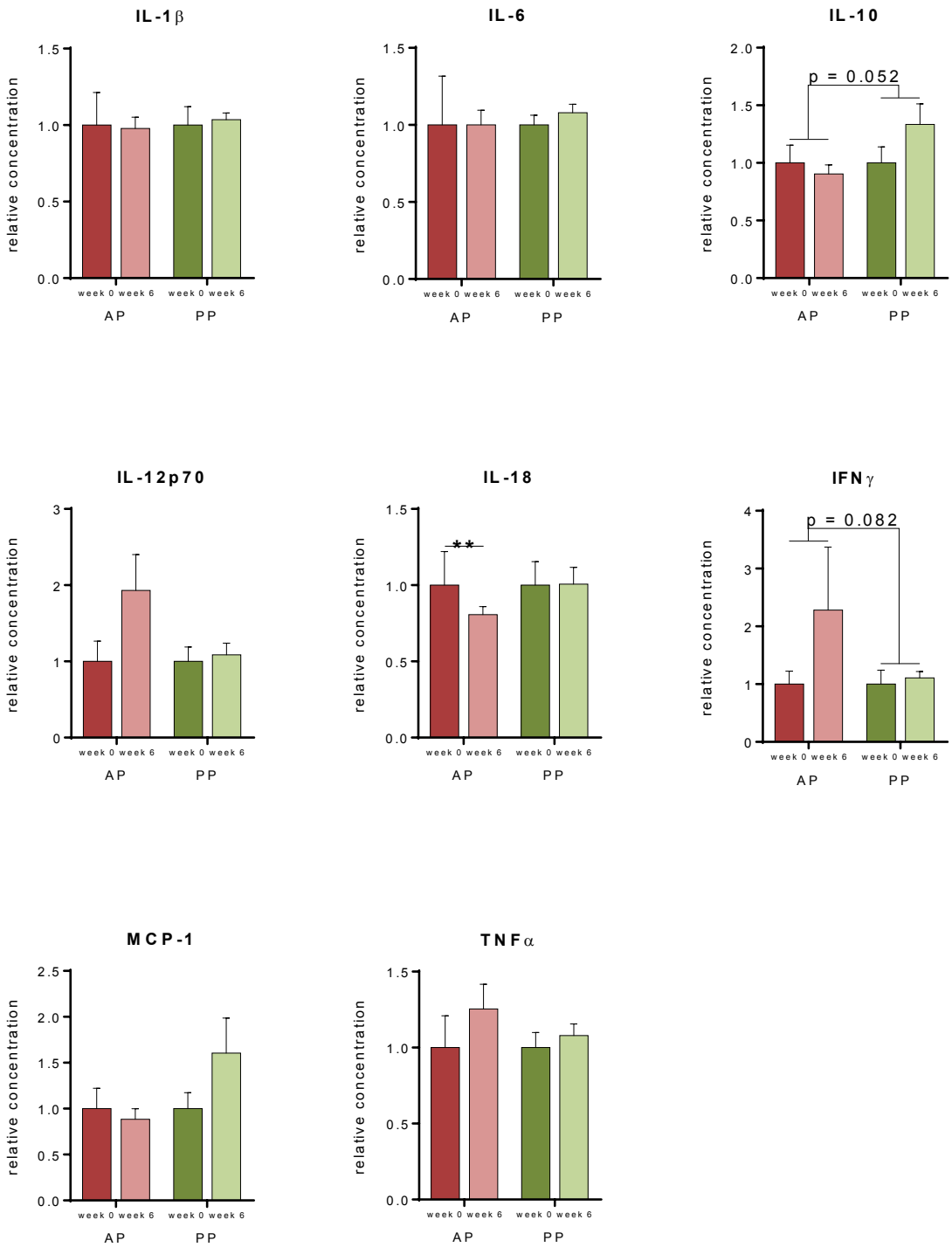

Figure 31. Dietary effects on LPS-induced cytokine secretion. Whole blood samples taken 4 hours after high protein meal consumption were stimulated with 100 ng/ml LPS for 20 h. Experiments were performed at week 0 and week 6. Cytokine concentrations were detected in supernatants. Abbreviations: AP, animal protein; IFNγ: interferon γ; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; PP, plant protein; TNFα: tumor necrosis factor α. n_{AP} = 18, n_{PP} = 19. Values are presented as mean ± SEM. **p < 0.01.

Collectively, the two high protein diets did not induce a rise in inflammation. Quite on the contrary, levels of CRP, IL-18 and TNFα were reduced after the intervention, especially in the AP group. The inflammatory response to an *ex vivo* stimulation was not affected either.

4.9. Regulation of the mTOR pathway

Animal and human studies have shown amino acid induced activation of mTOR followed by inhibitory phosphorylation of IRS1 and alteration of insulin signaling in the cell [104-106]. Therefore, the mTOR signaling pathway was investigated in blood cells and adipose tissue samples at the protein and mRNA levels. In theory, the higher amounts of leucine in foods of animal origin might promote induction of the mTOR pathway [103], in contrast to foods of plant origin.

In SAT, no changes of the gene expression of *AKT*, *MTOR*, *p70S6K*, or *4EBP1* were found (Figure 32). There were no differences between the diets.

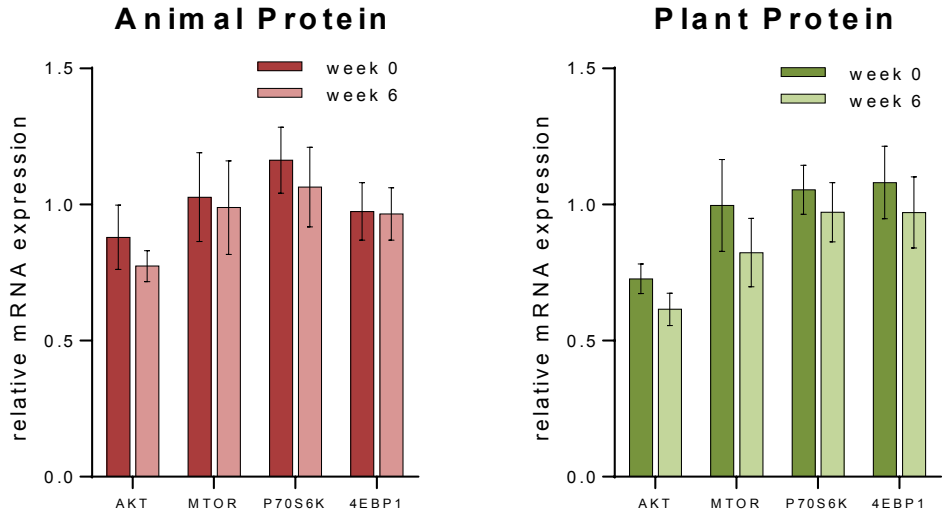

Figure 32. Dietary effects on mRNA expression of genes involved in the mTOR pathway in adipose tissue. Gene expression analyses were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 12, n_{PP} = 15. Values are presented as mean ± SEM.

Further, the phosphorylation status of several key enzymes of the Akt-mTOR signaling was detected with PathScan protein arrays in a subgroup of 8 subjects from each group (Figure 33). There was no activation of the pathway in either of the two groups. In the AP group the phosphorylation of p70S6K (Thr421/Ser424) was even lower after 6 weeks of intervention which was different from the PP group (p_{APvsPP} = 0.041).

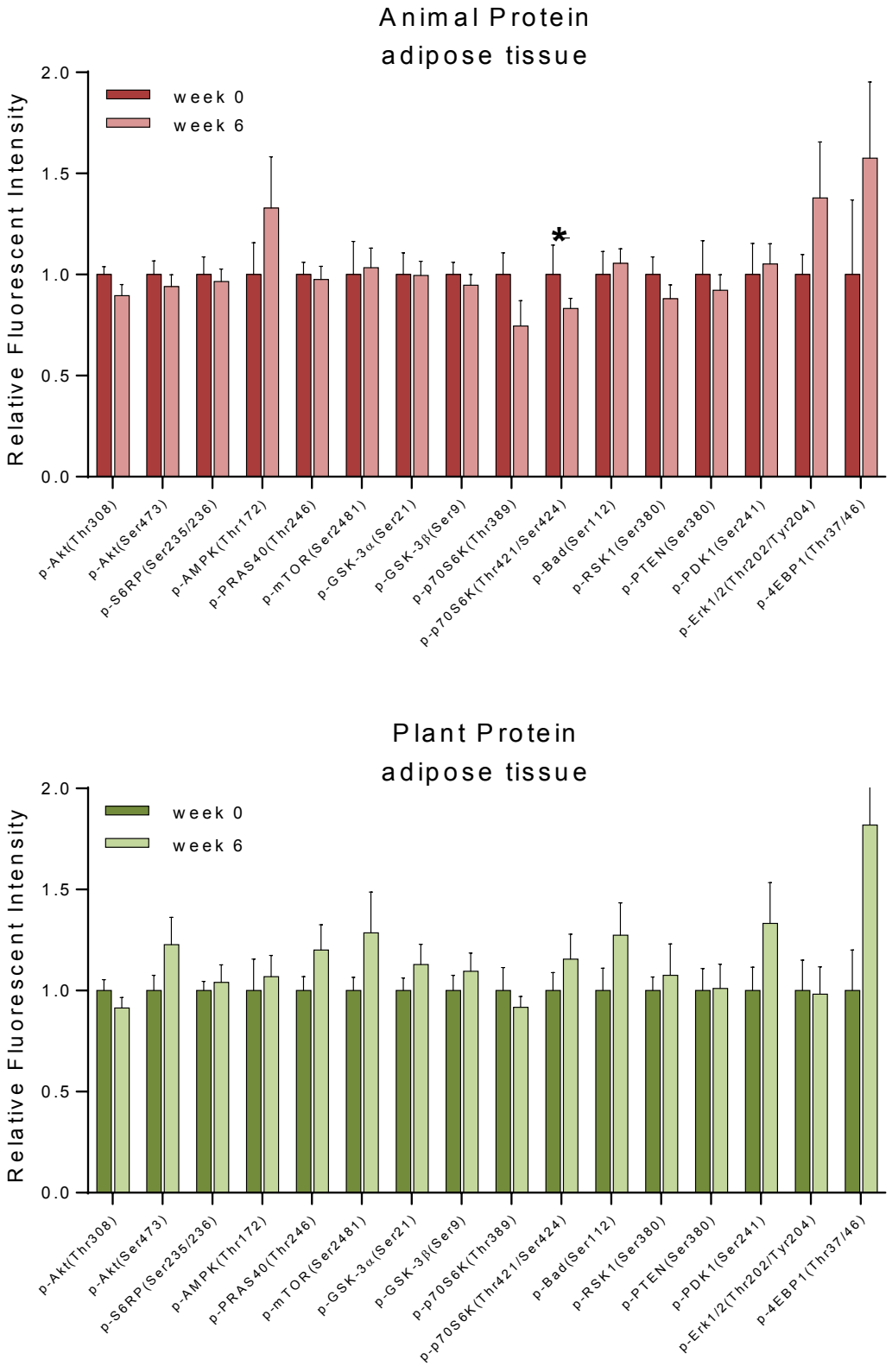

Figure 33. Dietary effects on protein activation in adipose tissue. Analyses of the phosphorylation status of key proteins of the Akt-mTOR pathway were performed in subcutaneous adipose tissue samples obtained at week 0 and 6. n_{AP} = 8, n_{PP} = 8. Values are presented as mean ± SEM. *p < 0.05.

The RNA sequencing analysis, performed in whole blood RNA samples of 10 participants from the AP group, revealed no differences in the gene expression of genes involved in glucose and insulin metabolism, including the Akt-mTOR pathway, neither pre- nor postprandially (4 h).

The PathScan protein array was performed in isolated white blood cells (WBC) from pre- and 4-h postprandial blood samples ($n_{AP} = 4$, $n_{PP} = 4$) (Figure 34). At week 0, there was a decrease of p-p70S6K (Thr421/Ser424), p-RSK1 (Ser380), p-PDK1 (Ser241), p-Erk1/2 (Thr202/Tyr204), and p-4EBP1 (Thr37/46) after AP meal. At week 6 no further changes for pre- and postprandial samples were observed (Figure 34). In the other intervention group, at week 0, only a decrease of p-Erk1/2 (Thr202/Typ204) was detected. At week 6, p-S6RP (Ser235/236), p-mTOR (Ser2481), p-p70S6K (Thr389), p-PTEN (Ser380), and p-Erk1/2 (Thr202/Tyr204) were reduced (Figure 34).
The activation of p-PTEN (Ser380) and p-Erk1/2 (Thr202/Tyr204) in the fasting state was increased after 6 weeks of PP diet, while it was reduced after the AP diet (Figure 34), resulting in significant differences between the groups at the preprandial time point at week 6 ($p_{APvsPP} = 0.014$ and 0.036, respectively).

The detection of anabolic pathways provided a snapshot of the phosphorylation state of proteins in SAT and WBC. Nevertheless, the analyses in SAT were performed solely in fasting samples so that no postprandial effects of high protein meals could be evaluated. Further, only the 4-h postprandial time point was analyzed in WBC. It is reasonable to think that a possible activation of the Akt-mTOR signaling occurred prior this measurement.

In conclusion, 6 weeks of high protein diets – from animal and plant origin – did not induce an activation of the mTOR pathway as shown in fasting adipose tissue samples and in fasting and postprandial WBC samples. These results agree with the observed improvement of whole-body insulin sensitivity (chapter 4.5.1., Figure 13).

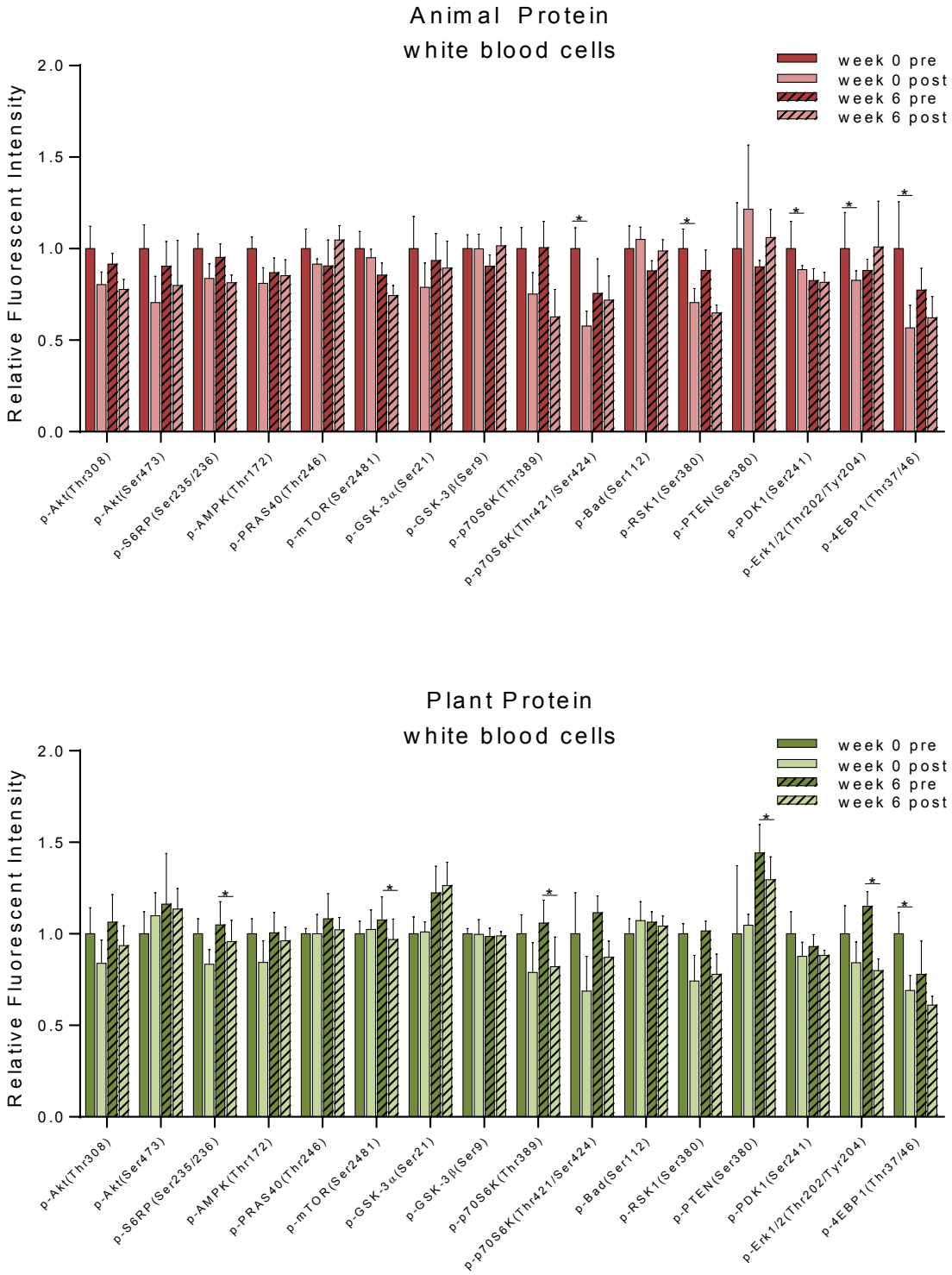

Figure 34. Dietary effects on protein activation in white blood cells. Analyses of the phosphorylation status of key proteins of the Akt-mTOR pathway were performed in white blood cell isolated from blood samples taken in the fasting state (pre) and 4 h after high protein meal (post) at week 0 and 6. $n_{AP} = 4$, $n_{PP} = 4$. Values are presented as mean ± SEM. *$p < 0.05$.

5. Discussion

The LeguAN trial studied effects of two high protein diets in subjects with T2D on the metabolic and the molecular level. Improvements of body composition, glycemic control, liver fat, and markers of the metabolic syndrome were shown in both intervention groups. Moreover, no indications of impairment of renal function were observed. Molecular studies of the mTOR pathway revealed no activation induced by high intake of protein or specific amino acids. Comparisons between diets showed no remarkable differences.

5.1. Diet effects on body composition

5.1.1. Body weight

Over the 6 weeks of intervention, despite the isocaloric diet design, the BMI was modestly but significantly reduced in all participants without group variation. The body weight of the participants decreased by 2.7% in the AP and by 1.5% in the PP group. It proved difficult to achieve the aim of keeping body weight constant throughout the study in spite of weekly nutritional counseling and readjustment of dietary plans. Reduction of body weight was significantly more pronounced in the AP group.

One reason for the body weight loss was most likely that these healthy diets had a greater volume than the typical local Western diet with high amounts of fat and less vegetables and fruits. Moreover, body weight lowering effect was shown for high protein diets, explained by triggered resting energy expenditure [72]. However, whole-body REE in the fasting state was not altered after intervention by either of the diets, which was observed also by Rietman and colleagues [87]. On the contrary, high protein intake induced increase of REE was observed in other human studies [189, 190].

Moreover, the high protein content is known to increase satiety [74]. Amino acids induce secretion of GLP-1 as well as peptide YY and cholecystokinin and thereby stimulate satiety signals in the brain. Indeed, study participants reported to feel satiated and often failed to eat all foods as advised in the dietary plans. These effects may explain the observed body weight loss of the participants.

Further, it was reported that different proteins (whey, casein, soy, etc.) affect satiety differentially [72]. For example, a more rapid gastric emptying and higher postprandial amino acids concentrations followed by increased stimulation of gastrointestinal hormones were observed after whey protein load in comparison to casein load [191]. Subjects in the PP group received products made with purified pea protein which have only a slightly lower ileal digestibility than milk protein [192, 193]. MTTs in the present study were performed with

mixed meals differing only in the amino acid composition. Apart from the two protein drinks, test meals included also bread, yoghurt, cheese and butter. The postprandial levels of amino acids peaked at 60 min after meals ingestion in both groups. Additionally, GLP-1 secretion after the meal tests showed no variation between the two different protein meals pointing to similar rates of digestion. However, meal effects on satiety hormones and gastric emptying need to be investigated. In case the two diets influenced satiety hormone secretion differentially might eventually explain the more pronounced weight loss in the AP group.

5.1.2. Fat-free mass

High protein diets selectively promote body weight and fat mass loss but preserve fat-free mass [73, 75]. This is in parallel with observed changes in both groups. Remarkably, the loss of body weight and fat mass were more pronounced in the AP group and at the same time FFM increased more. This interesting phenomenon might be due to specific effects of amino acids enriched in AP foods (e.g. BCAAs, methionine) as discussed below.

Dietary proteins, especially dietary protein-derived essential amino acids, are main anabolic stimuli of skeletal muscle mass. There were no differences in fasting FAA levels neither after the 6-week intervention period, nor between the two diets. This indicates that amino acids were effectively catabolized even after consumption of high protein diets. As seen, postprandial levels rise substantially, which increases the protein balance and therefore FFM. Amino acids were shown to activate mTOR and subsequent processes of cell growth and proliferation [99]. Indeed, FFM was enhanced in all participants, whereby more pronounced in AP group. Intake of leucine, a potent activator of mTOR, was higher in the AP diet which might explain this observation [103]. Animal studies revealed acute leucine-dependent induction of protein synthesis in muscle, adipose and other tissues associated with activation of mTOR downstream targets p70S6K and 4EBP1, as published by Lynch and coworkers [194]. On the other hand, the same group showed that chronic leucine supplementation (12 days) in rats increased protein synthesis in muscle, adipose tissue and liver without adaptive changes in mTOR signaling and BCAA degrading enzymes (BCAT and BCKDH) [195].

In general, elderly people have diminished muscle protein synthesis than young populations potentially due to resistance to protein intake. This might result from lower sensitivity of mTOR to feeding in older population [196]. Insulin resistance of protein metabolism was further reported in men with T2D [197]. Thus, elderly obese individuals with T2D might have an altered mTOR signaling in muscle in response to protein. Nevertheless, in view of the FFM increase, induction of protein synthesis and proliferative processes must have occurred in muscle, with or without interference of mTOR.

Examination of muscle biopsy samples on molecular level would reveal if mTOR was differentially regulated by animal and plant protein-derived amino acids. A human study observed increased phosphorylation of mTOR and S6 ribosomal protein in muscle after consumption of a dairy rich meal with higher BCAA amounts but not soy rich meal (meals contained 18 EN% protein) [106]. Further human studies have shown lower muscle protein synthesis after soy consumption in comparison to foods of animal protein [198]. However, investigations on other plant protein sources, e.g. legumes, are lacking. Results of the present study suggest higher anabolic properties of the AP than the PP diet.

5.1.3. Fat mass

Total fat mass and fat depots were reduced significantly only in the AP group, whereby the decrease of NVAT was significantly higher compared to the PP group, most likely due to the more pronounced body weight loss in the AP group. Both high protein diets downregulated the mRNA expression of genes involved in lipogenesis and lipid storage in SAT samples (*ACC1* and *SCD1* in AP and of *PGC1A* and *PPARG* in PP group), which might be a possible explanation for the observed decreases of fat depots. Further, stimulation of autophagy processes might also play a role since autophagy is upregulated in adipose tissue of obese humans [179]. Nevertheless, mRNA levels of key genes were not altered in SAT samples. Analysis of protein expression and autophagic rate (flux) will give additional information of possible regulation.

No indications for mTOR pathway activation were detected in SAT; p-p70S6K (Thr421/Ser424) was even lower after AP diet; mRNA expression of *AKT*, *MTOR*, *P70S6K*, *4EBP1* as well as *BCAT* and *BCKDH* were not altered, at least not in the fasting state. The chronic effects of the 6-week high protein diets observed in adipose tissue samples are similar to Lynch and colleagues [195]. And, the BCAA catabolism in humans occurs mostly in muscle and liver [177] suggesting the activation of the BCAA degrading pathways in those tissues. Moreover, mTOR induces adipogenesis and lipogenesis [199] and thus the lack of chronic activation of mTOR in SAT is in agreement with the downregulation of lipogenic genes and the overall decrease of fat mass.

5.1.4. Relationships between body composition, FAA, and FFA

Although levels of fasting amino acids were not altered in both groups, some relationships were shown between amino acids and body composition. Fasting asparagine was associated with beneficial effects on abdominal fat depots. Further, changes of serine and lysine correlated positively with FFM increase. Negative effects on abdominal fat were seen for

tryptophan, glutamine, leucine, and isoleucine. Similarly, Takashina et al. reported associations of visceral obesity with lower asparagine, serine, and glutamine but with higher tryptophan, lysine, leucine, isoleucine, and valine. However, for lysine and glutamine observations were opposite to present study [200].

Further, relationships were seen between fat depots and FFA, which are released mainly by adipose tissue into the circulation [170]. Interestingly, the odd-chain pentadecylic acid (C15:0), found primarily in dairy products, did not decrease after intervention but correlated directly with VAT and NVAT in the PP group. This was not expected since pentadecylic (C15:0) and margaric (C17:0) acids are linked to lower CVD risk [201]. Moreover, FM was negatively associated with the essential linoleic acid (C18:2n6) and α-linolenic acid (C18:3n3) and directly with lignoceric acid (C24:0) and lipogenic index (C16:0 / C18:2n6). In accordance, high levels of linoleic acid (C18:2n6) were inversely associated with prevalence of the metabolic syndrome in the PREDIMED study [202].

The observed correlations suggest possible mechanisms for changes in body composition, which require further investigation.

5.2. Diet effects on glucose metabolism

5.2.1. Fasting parameters

The results of this study confirmed previous observations of lowering hyperglycemia and hyperinsulinemia after high protein diet [64, 65, 81-83]. Both high protein diets induced improvements in glucose homeostasis in subjects with type 2 diabetes after 6 weeks of intervention. Unlike the initial hypothesis, intake of foods rich in animal proteins did not induce adverse effects on insulin sensitivity. In fact, animal and plant proteins had similar effects without significant differences between the interventions.

Fasting glucose levels decreased in both groups throughout the study, but this was not significant at the end of the trial. This was certainly unexpected. Possible explanation might be decline in the compliance towards the end of the intervention. However, this is not seen for the rest of the data, which supposes other reasons more likely. Nevertheless, both animal and plant protein diets elicited the same effect, as previously seen by Wheeler and coworkers [132].

Fasting insulin and respectively HOMA-IR levels were significantly reduced only in the AP group. This agrees also with the significant improvement of whole-body insulin sensitivity in the AP group. Still, the lack of significant difference between AP and PP groups excludes a possible advantage of animal over plant proteins.

On the other hand, total FFA and AdipoIR index were lower only in the PP diet group. This indicates an improvement of insulin sensitivity in adipose tissue and thus a diminished lipolysis in the fasting state. The decreased expression of the lipolytic gene *HSL* in adipose tissue in this group further confirmed that. Noteworthy, the chromatographic analysis of single FFA in plasma revealed significant reduction of FFA species in both groups. Since differences between the dietary interventions were not significant for FFA and AdipoIR, it cannot be concluded that proteins from plant origin were more advantageous.

Main parameter used for monitoring glycemic control in diabetic patients is $HbA_{1c.}$ It reflects the 2-3 month average plasma glucose concentration, since the lifespan of red blood cells is around 110 days, which can vary between 70 and 140 days [203]. Over the 6 weeks of intervention, the HbA_{1c} levels decreased by 0.5% on average in both groups. Similarly, Gannon and Nuttall observed an HbA_{1c} reduction of 2.2% in diabetic individuals after 5 weeks of high protein diet [65]. In contrast, next to 30% protein content, their diet contained only 20% carbohydrates, which could explain the greater effect compared to the finding in the LeguAN study. Importantly, iron intake and changes in hemoglobin levels might bias HbA_{1c} levels. In the present study, iron intake in the PP group did not differ from previous intake, while it was a bit higher in the AP diet. Also no significant changes of hemoglobin levels in blood were observed (data not shown), so that eventual bias caused by lower iron intake is rather unlikely.

5.2.2. Insulin sensitivity

Whole-body insulin sensitivity (M-value) was increased significantly only in the AP group. This is in contrast to a previously reported decrease of M-value in overweight humans after 6 weeks of high protein diet containing 28% protein [95]. The authors discussed a potential role of p70S6K that displayed elevated total protein levels in subcutaneous adipose tissue after the high protein diet. However, this was not seen in the present study. In SAT samples taken in the fasting state, no alteration of p70S6K gene expression was detected. Further, the phosphorylated state of p70S6K was unchanged after PP diet; p-p70S6K (Thr421/Ser424) levels were even reduced after the AP diet.

Yet, the hyperinsulinemic euglycemic clamp estimates primarily muscle insulin sensitivity. Tremblay and colleagues detected increased activation of p70S6K and inhibitory phosphorylation of IRS1 in muscle biopsies of healthy men after amino acid infusion. This led to an impairment of insulin's ability to inhibit endogenous glucose production and to stimulate glucose disposal in muscle [104, 105]. The same effects were observed in elderly women after ingestion of whey protein but not after leucine intake [107]. Hence, one could hypothesize that in the present study insulin resistance in muscle was enhanced after high

protein meal ingestion via mTOR activation. However, the 6-week long high protein intake improved insulin sensitivity together with glycemic control.

Acute effects of protein ingestion on the mTOR pathway were investigated only in white blood cells 4 hours after test meals. No indications of amino acid induced stimulation of the mTOR signaling were found, on protein and gene expression levels. Nevertheless, it is possible that activation of the Akt-mTOR signaling occurred prior this measurement. Also the low sample number (n = 4 per group) limits the validity of data.

Some investigators assume a chronic elevation of mTOR in diabetic patients [108] since circulating amino acids are increased in obesity and T2D [204] thereby promoting tissue insulin resistance as well as tumor growth. However, in the current study, the high protein diets did not further affect whole-body insulin sensitivity after 6 weeks of intervention. The initial hypothesis, that high animal protein diet, rich in leucine, will impair insulin sensitivity could not be proven. Certainly unexpected, the improvement of insulin sensitivity was more pronounced in the AP group despite the higher postprandial BCAA levels. This raises the possibility that more complex metabolic adaptations occur upon prolonged consumption of BCAA.

In addition, methionine is thought to exert adverse effects on metabolism. Moderate methionine restriction ameliorates body composition and insulin sensitivity in young and old rodents [96-98]. One hypothesis in the LeguAN study was that low methionine content in pea protein [134] will lead to pronounced improvements of insulin sensitivity in the PP group in opposition to animal foods rich in methionine. Indeed, foods consumed in the PP group had lower methionine levels than in the AP group, which was concordant with the postprandial blood levels after test meal ingestion. However, insulin sensitivity increased significantly only in AP group. Moreover, there was no significant difference in insulin sensitivity between the diets and thus the effect cannot be ascribed to methionine restriction. Although methionine restriction stimulates FGF21 secretion in mice [98, 205, 206], this was not observed here. Yet, despite lower methionine amounts, the PP diet cannot be defined as a methionine restricted diet since the methionine intake was still higher than before owing to the high protein consumption. Long-term studies are needed to evaluate possible benefits of low methionine diets in humans.

5.2.3. Relationships between insulin sensitivity, FFA, and FAA

Metabolomic studies search for new biomarkers predicting development of T2D and its comorbidities. Recent data from a 19 year follow-up study reported an inverse correlation between γ-linolenic acid (C18:3n6) and dihomo-γ-linolenic acid (C20:3n6) and a positive correlation of Δ5 desaturase activity index (C20:4n6 / C20:3n6) with T2D risk [207]. In

agreement, the improvement of insulin sensitivity (M-value) in the present study correlated with decreases of γ-linolenic acid and dihomo-γ-linolenic acid levels and elevated Δ5 desaturase activity index. Inconsistently, Δ5 desaturase activity index and the polyunsaturated docosapentaenoic acid (C22:5n3) were linked to insulin resistance of adipose tissue (AdipoIR) in the AP and PP group, respectively. Further, stearic acid (C18:0) was additionally associated with AdipoIR in the PP group. This was in line with metabolome analyses in patients of the German Diabetes Study that identified inverse association of stearic acid with insulin sensitivity in recent-onset T2D [208].

As for fasting amino acid levels, increased serine concentrations correlated with improved insulin sensitivity and increased FFM pointing to a role in muscle insulin signaling. Surprisingly, elevated BCAA levels were not related to insulin resistance in contrast to data from several metabolomic studies [66, 67, 200, 208, 209].

Thus, changes in FFA and FAA induced by the diets might have played a role in insulin metabolism and needs further analyses.

5.2.4. Postprandial metabolism

Meal tolerance tests allowed direct comparisons of the metabolic and hormonal responses to the AP and PP diets before and after intervention. Amount of energy, carbohydrates, fats, proteins, and fiber as well as fatty acid composition and GI of the two test meals were similar (Table 3). Solely amino acids amounts differed between the meals, as methionine and BCAA were higher in the AP and arginine and asparagine in the PP group. Proteins and single amino acids are known for their stimulatory effects on insulin and incretin secretion [77-79]. Moreover, rate of digestibility, which depends mainly on the form of ingested food (solid or liquid), affects the hormonal response. Also protein type plays a role in the speed of digestion, for example whey is absorbed faster than casein, both milk protein fractions [210]. AP meals contained milk (mostly casein), whey, and egg proteins, while subjects in the PP group received high amounts of purified pea protein with small amounts of casein (Table 21). It was previously shown, that purified pea protein exhibits only a slightly lower ileal digestibility than milk protein [192, 193]. Postprandial amino acid profiles and hormone secretion rates in blood peaked at same time points in both groups. This suggests that meals had similar rates of gastric emptying and amino acid bioavailability. Unfortunately, gastric emptying was not estimated during the MTTs.

Further, the postprandial plasma amino acids reflected the meal composition – methionine, valine, and leucine levels were higher in the AP, arginine in the PP group. Secretion of insulin and glucagon stimulated by individual amino acids is very diverse and depends on form of ingestion – alone, as mixture, hydrolysate, or in the presence of glucose [77, 211].

Here, despite differences in amino acid composition of meals and subsequently in circulation, metabolic responses during the MTT were similar in both groups. Glucose profiles were not different, which may be mainly due to the same GI and carbohydrate amount. Concordantly, C-peptide and insulin release did not vary between the meals. Similarly, van Loon et al. did not observe differences in insulin secretion in healthy humans after ingestion of casein or pea protein hydrolysates [212]. Glucagon secretion was enhanced after 6 weeks of PP diet in contrast to the AP diet suggesting a role of specific amino acids. For instance arginine, the intake of which was higher in the PP group, is known to induce glucagon secretion [213] and that might explain the present observations. On the other hand, circulating FFA during MTT decreased significantly after 6 weeks of AP diet which points to enhancement of adipose tissue insulin sensitivity. In parallel, indices of insulin secretion, degradation and sensitivity demonstrated trends of improvement without group differences.

Noticeably, total GIP levels were significantly higher after PP than after AP meals. After secretion GIP is rapidly cleaved by DPP-IV so total GIP levels (sum of intact peptide and inactive form) indicate the overall GIP secretion from the K cells of the proximal small intestine [214]. Bioactive dietary peptides can inhibit DPP-IV and thus increase the half-life of active GIP and GLP-1 [80]. It would be interesting to determine the amount of bioactive peptides in the meals as well as the DPP-IV activity in order to compare diets and verify if this was the cause. Amino acid administration induces GIP secretion [79], but there is no data whether one or more specific amino acids are responsible. It can be assumed that the higher plasma arginine and/or the lower methionine and BCAA levels in the PP meal might have contributed to this effect. Further studies should investigate this in detail. Nevertheless, insulinotropic actions of GIP are largely reduced in T2D likely due to lower sensitivity or receptor expression in pancreatic β-cells [214].

AP diet led to significantly lower active GLP-1 after 6 weeks, while the levels in the PP group were not altered. GLP-1 is released from the L cells located in the distal small intestine and colon after meal ingestion [50]. Like GIP, GLP-1 is also rapidly cleaved by DPP-IV and thereby inactivated. It is particularly difficult to detect active GLP-1 concentrations in blood samples. Here, the measured levels were relatively low so that the results should be interpreted with caution.

All in all, comparison of amino acid profiles after meal ingestion revealed some differences between the diets. However, hormonal responses were similar among the groups indicating that variations in ingested amino acids as a part of a mixed meal do not play a major role. These data highlight the complexity of nutrient metabolism. In conclusion, a short-term high animal/plant protein diet induced no adverse effects on postprandial metabolism and had rather beneficial impact in view of insulin homeostasis in T2D patients.

5.3. Diet effects on liver fat

The levels of intrahepatic lipids (IHL) decreased considerably with comparable magnitude in both groups (48% in AP and 35.7% in PP) and independently of the modest body weight loss. Noteworthy, after the intervention IHL levels of 9 subjects fell below the definition limit for NAFLD of 5.56% [172]. Changes were associated with improvements of blood lipids, glucose and insulin homeostasis suggesting an important role of IHL reduction for the beneficial effects on glycemic control. Interestingly, decreases of stearic and γ-linolenic acid as well as lipogenic and Δ6 desaturase activity indices correlated with reduced IHL. Moreover, liver enzymes (AST, ALT, γ-GT) were diminished as well. The IHL reduction was not related to changes in body weight and fat mass, neither to VAT and NVAT and remained significant after adjustment to body weight reduction which indicates that this effect was rather due to diet than to changes in body composition.

The effect of high protein intake on IHL was reported in several animal and human studies. In mice, high protein diet led to significant reduction of liver fat compared to low protein diet [215, 216]. Further, protein supplementation prevented high fat diet induced development of fatty liver [217-219]. In healthy lean humans, supplementation with amino acid mixtures or high protein intake blunted the increase of IHL induced by fructose or high fat diet [84, 86, 87]. Bortolotti et al. observed 20% reduction of liver fat in 11 obese women after 4-week supplementation with 60 g/day whey protein in addition to their normal diet [85]. Initial mean IHL was around 7.8% and only 5 of them had IHL levels above 5% at baseline. In contrast, subjects in the present study had twice as high IHL content at onset, 25 had NAFLD. Importantly, the observed reduction was more pronounced (42% on average), which highlights the powerful effect of high protein intake independently of protein type.

There are several possible mechanisms underlying amino acid metabolism and liver fat content that should be discussed.

Many studies and reviews have confirmed that dietary protein increases thermogenesis to a greater extent than carbohydrates and fats [70, 72, 220, 221]. Possibly, high protein intake triggers protein catabolism since the body is not able to store excess amino acids. As a result, elevated amino acid oxidation leads to increased postprandial thermogenesis [72]. In rodents, it was already shown that high protein intake upregulates uncoupling proteins in liver and brown adipose tissue and thus energy expenditure and thermogenesis [108].

Amino acid catabolism is an energy requiring process taking place mainly in liver, for example urea synthesis and amino acid conversion into glucose [61]. Thus, high protein intake may trigger hepatic energy expenditure and hepatic lipid oxidation. Here, protein oxidation and urea synthesis and excretion were highly enhanced. Besides, it was shown

that the rate of β-oxidation in fatty liver is higher compared to non-fatty liver [40] which might explain rapid changes in liver fat content.

Furthermore, amino acids and their metabolites regulate the expression of genes involved in β-oxidation and lipid synthesis in the liver. Several rodent studies showed that supplementation with arginine, glutamine or citrulline prevented fructose-induced NAFLD by diminishing hepatic lipogenesis (*PPARα, SREBP1, CHREBP*), lipid peroxidation, and inflammation [222-225]. In mice, high protein diet led to IHL reduction via downregulation of lipogenic genes (*PPARγ, FAS, ACC1*) [217-219]. Other studies described diminished inflammation and cell stress in liver [215, 216]. Recent publication postulated an induction of AMPK by amino acids linking protein catabolism, ureagenesis, and β-oxidation in hepatocytes [226]. Thus, the high protein intake might have promoted the IHL reduction through the mechanisms cited above.

Interestingly, change of asparagine correlated negatively with reduction of IHL, VAT, and NVAT in the PP group supposing a beneficial role. Similarly, data from Takashina and colleagues published recently showed an association of asparagine with lower visceral obesity in humans [200].

Another aspect, that should be discussed, is the carbohydrate and fat intake during the diet. Dietary sugar derived 2-carbons are main precursors for newly synthetized fatty acids in the liver [227]. Since carbohydrate intake was not changed during the diet intervention (Figure 8), it can be assumed they did not play a role on hepatic *de novo* lipogenesis. Additionally, dietary fats enter the liver postprandially (~15% of total flux contain chylomicron remnants derived by the intestine and FFA derived after chylomicron lipolysis in plasma via LPL) [228]. Since dietary fat intake was lower than prior to enrollment (30 %EN vs 41 %EN), it is likely that the lower fat intake contributed to a lower lipid flux to the liver as well. Moreover, during the intervention, SFA and MUFA intake was lower, and the intake of PUFA was higher than before (Figure 9). SFA ingestion is associated with NAFLD, in which GIP seems to play an important role [229]. On the other hand, MUFAs might improve NAFLD but more evidence is needed to confirm this [230]. PUFAs, especially long chain ω-3 fatty acids (DHA), were shown to diminish TG content in liver and markers of the metabolic syndrome [231]. Unsaturated fatty acids might activate PPARs and thus lipid oxidation, while suppressing insulin resistance and inflammation. Another mechanism is the inhibition of SREBP1 leading to lower hepatic *de novo* lipogenesis and thereby liver fat accumulation [231]. Several human studies have shown liver fat reduction after ω-6 PUFAs rich diets [232, 233]. Thus, the intake of more PUFAs and less SFAs may have also contributed to the reduction of IHL.

In general, imbalance between lipid supply (*de novo* lipogenesis, FFA flux, chylomicron remnants) and lipid disposal (VLDL secretion and lipid oxidation) leads to increased fat

accumulation in the liver. FFA, taken up by the liver and converted to TG, are mainly derived from SAT [170]. Chronically increased FFA flux into the liver causes enhanced TG synthesis, gluconeogenesis and contributes to insulin resistance [37]. Here, most of the detected FFA species decreased significantly in both groups, accompanied by a slight reduction of total FFA levels and AdipoIR in the fasting state in the PP group. Hence, the high protein diet induced decrease of circulating FFA in both groups might have played a major role in IHL reduction as postulated in recent studies in animals and humans [234-236]. This is supported by the observed correlations between reductions of IHL and the saturated stearic acid. Remarkably, the polyunsaturated γ-linolenic acid correlated directly with IHL and inversely with insulin sensitivity which agrees with previously observed associations of γ-linolenic acid with NAFLD [237] and higher T2D risk [207]. Moreover, a change of the Δ6 desaturase activity index, which was also related to the risk of T2D [207], correlated positively with IHL.

The indices of enzyme activities showed diminished lipogenesis, elongase and desaturase activities in both groups. This was echoed by the mRNA expression analyses in SAT samples revealing decreases of *ACC1* and *SCD1* in AP and of *PGC1A* and *PPARG* in PP group, all genes involved in adipose tissue lipogenesis (defined as both the process of fatty acid synthesis and TG synthesis). With regard to lipolytic enzymes, *HSL* was significantly lower only in the PP group, *ATGL* was yet unchanged. Although expression of these genes was significant only in one of dietary groups studied, similar trends were found in the other group, and this can be explained rather by the small group size than by a role of protein type. The data might be an indication for improvement of insulin induced inhibition of lipolysis and is in line with the decrease of AdipoIR index in the fasting state in the PP group. Nevertheless, changes in mRNA expression do not necessarily lead to the equivalent protein changes in adipose tissue and do not necessarily mirror changes of fat metabolism in liver. Furthermore, although only around 14% of total FFA are derived from the liver as shown by isotope studies [170], the significant correlation between the reduced lipogenic index and IHL for the entire cohort points to diminished hepatic *de novo* lipogenesis. Overall, it could be concluded that high protein diets induced great reduction of IHL, whereby lower hepatic *de novo* lipogenesis as well as improved insulin sensitivity in adipose tissue and thus diminished lipolysis played an important role. Additional analysis of VDLD-fraction will provide more information about changes of hepatic lipid metabolism.

Because protein intake stimulates glucagon secretion [76] it appears likely that a decrease in postprandial insulin/glucagon ratio might contribute to TG breakdown and lipolysis, and suppress *de novo* lipogenesis in liver and adipose tissue. According to insulin and glucagon release during MTTs there was a postprandial increase of insulin/glucagon ratio and decrease of FFA in both groups and thus it contradicts this hypothesis.

Protein intake might increase synthesis of bile acids [84], that inhibit hepatic *de novo* lipogenesis and increase fat oxidation via farnesoid X receptor [238]. It would be interesting to estimate bile acids in this study also in order to investigate possible roles of particular amino acids.

In view of protein origin, relationships of IHL reduction with glucose, insulin and insulin sensitivity markers were more pronounced in the PP group, while with cholesterol and fatty acids in the AP group. Of note, significant correlations in the one group were similar in the other group, therefore differences are most likely due to small sample size.

Conclusively, high protein diet or rather a diet with 30 EN% protein, 40 EN% carbohydrates, and 30 EN% fat (SFA 10 EN%, MUFA 10 EN%, PUFA 10 EN%) potently reduced liver fat content with clinical relevance.

5.4. Diet effects on cardiovascular parameters and blood pressure

Levels of total, LDL, and HDL cholesterol decreased by approximately 15% in the entire cohort. High protein low carbohydrate diets have been associated with increased cardiovascular risk but in the context of habitual food patterns which are rich in saturated fats [239]. Meta-analysis reported no effect of high protein diets on blood lipids [64]. In the present study protein intake was exchanged for fat which might contribute to cholesterol lowering effects. Moreover, participants consumed more PUFA and less SFA and MUFA. Replacement of SFA with MUFA and PUFA is linked to lower all-cause mortality as recently shown in a large cohort study [240]. Thus, the decreased fat intake and the favorable fatty acid composition of the diets might have contributed to the improved blood lipid profile. Furthermore, intake of legumes with low GI was associated with reduced cardiovascular risk [128]. Nevertheless, there was no difference between the AP and PP interventions, so that a possible role of protein type on blood lipids could not be assumed.

A meta-analysis from 2013 reported that high protein diet interventions improve blood pressure in patients with T2D [64]. Interestingly, in the present study systolic and diastolic blood pressure were significantly decreased only in the PP group, which differed significantly from the AP group. This difference between the protein types might be due to higher baseline blood pressure levels in the PP group, which was significant for diastolic blood pressure. Another possible explanation is the different amino acid composition of the two diets. For instance, Mariotti et al. showed that ingestion of a meal extra containing arginine, which is a precursor of the vasodilator nitric oxide (NO), increases it's synthesis in healthy men [241]. Thus, the higher intake of arginine in the PP group might contributed to the beneficial effect on blood pressure as shown in several supplementation studies [161].

5.5. Diet effects on renal function

Deteriorative effects of high protein intake on kidney function, particularly in T2D, are still controversial with heterogeneous results [89-93]. In the present study the GFR was estimated from serum levels of creatinine and cystatin C. For the period of 6 weeks high protein diets did not diminish the GFR in diabetic patients without severe kidney disease and with initial GFR above 70 ml/min/1.73m^2. Urinary albumin excretion was not altered, and even decreased in patients with microalbuminuria. Glomerular hyperfiltration, on the other hand, is an absolute increase of the GFR occurring physiologically after high protein load [242]. Still, no indications of hyperfiltration were seen over the 6 weeks. In agreement, two previous studies did not observe any adverse effects of high protein diets on the GFR in T2D patients with microalbuminuria or nephropathy [92, 93]. However, longitudinal studies examining renal complications in detail are needed to clarify chronic effects of high protein diets in patients with impaired kidney function.

Protein type did not seem to exert different effects on parameters of kidney function. Interestingly, serum creatinine decreased in the PP group in contrast to cystatin C levels which led to significantly different GFR results calculated with the two equations. Since cystatin C is independent of age, sex, BMI, muscle mass, protein intake, it was considered as more eligible marker for the present study. The combined equation is more accurate than equations based on either of the two parameters alone as reported by Inker and colleagues [151]. The change of creatinine only in the PP group suggests specific metabolic effects of the plant foods. Creatine is synthesized from glycine, arginine, and methionine in liver and further metabolized in muscle to creatinine. Dietary creatine is almost exclusively found in meat and fish, with low levels in dairy foods [243], which would explain the diminished creatinine levels in the group consuming mainly plant foods. Additionally, the lower methionine availability in the PP group, confirmed also in the postprandial profiles, might contribute to changes in creatinine metabolism.

Moreover, non-dairy animal protein is suspected to affect kidney function in contrast to dairy and vegetable protein [126, 127]. The AP diet contained not only non-dairy but also dairy and egg protein and exerted, like the plant protein diet, no adverse effect, which concurs with these observations. Noteworthy, enhanced renal function might be associated with improvements of insulin sensitivity, blood pressure and weight loss [244]. Although no associations between the changes of these parameters were found in the current study, it could explain the lack of adverse effects of high protein diets.

In addition, serum uric acid levels decreased in both intervention groups without indication of specific role of protein origin. Elevated blood uric acid levels lead not only to gout but also to the formation of renal calculi [245] and are related to increased risk of T2D and cardiovascular disease [163, 164]. High circulating levels are caused by lower excretion and/or increased synthesis and is induced by high fructose intake [245]. The role of dietary protein on uric acid levels was studied in large observational studies. The consumption of purine-rich animal foods (meat, seafood) was associated with higher uric acid levels, while purine-rich vegetables (peas, beans, lentils) and total protein intake were not. In contrast, diary product consumption was inversely correlated with elevated serum uric acid [246, 247]. In agreement, both high protein diets, AP and PP, did not induce a raise in uric acid levels, quite the opposite. The ameliorated uric acid concentrations might also be a result of the improved metabolic state in both groups.

Hence, consuming protein appears innocuous for short term periods but long-term studies looking into the impact of high protein diets on kidneys disease are still needed to establish dietary recommendations.

5.6. Diet effects on FGF21 and adiponectin

An unexpected finding was the extensive reduction of the hepatokine FGF21 by around 50% in the entire cohort. This is particularly puzzling in view of reported studies in which FGF21 analog application improved insulin levels, body weight, and dyslipidemia [248, 249]. Nevertheless, serum FGF21 is elevated in obesity, NAFLD and T2D, which was seen also in current cohort [168, 169, 250], hypothesizing FGF21 resistance in peripheral tissues of patients with metabolic disorders. Therefore, mRNA expressions of FGF21 downstream proteins were investigated in subcutaneous adipose tissue biopsies. FGF receptor 1 *(FGFR1)* expression was reduced only in the PP group, while *FGFR2* and β-Klotho (*KLB*) did not change. Hence, there were no indications for improved FGF21 sensitivity in adipose tissue. Furthermore, FGF21 is associated with increased metabolic stress and is regulated by ER-stress induced cascades incorporating inositol-requiring enzyme 1 (IRE1), X-box binding protein 1 (XBP1), and activating transcription factor 4 (ATF4) [251]. Thus, a decrease in metabolic stress might lead to restoration of the elevated FGF21 concentrations. We had access only to adipose tissue where gene expression of *XBP1* and *ATF4* did not vary. Still, as FGF21 is produced mainly in the liver, reduced ER-stress in adipose tissue is not necessarily accountable for lower FGF21 levels in circulation. It is likely to think that the great IHL reduction was accompanied by decrease of hepatic ER-stress and thus FGF21 expression and secretion as seen in mice receiving high protein diets [215, 216]. Indeed, absolute reduction of FGF21 correlated positively with absolute reduction of IHL in the entire

cohort ($\rho = 0.363$, $p = 0.049$). Another possible mechanism of protein-stimulated FGF21 might include increased amino acids supply, their intermediates or ammonia flux. This requires further investigations.

Regarding nutrients, protein restriction upregulates FGF21 via uncharged tRNA activating eIF2α-GCN2-ATF4 signaling [205, 252]. Another interesting finding is the fructose-induced stimulation of FGF21 via ChREBP followed by suppressed alcohol and sweet preference [253]. The fructose-dependent increase of FGF21 was recently confirmed in humans [254]. Genome-wide studies found SNPs in human *FGF21* gene associated with choices of dietary macronutrients: higher carbohydrate and lower fat and protein consumption [255, 256]. Thus, the change of FGF21 levels might be due to manipulation of protein and fat intake within the intervention period. It could be speculated that FGF21 participates in the regulation and preference of protein intake and in the same time protein intake regulates FGF21 in a feedback loop.

Levels of adiponectin in serum decreased in the PP group. This was confirmed by significantly lower gene expression in adipose tissue samples. Reductions in the AP group did not reach significance, but a diet specific effect was not indicated. Adiponectin is secreted by adipocytes into the bloodstream and transported to target tissues. In muscle, adiponectin binds to its receptor and activates a signal cascade allowing translocation of GLUT4 to the cell membrane [257]. Thus, adiponectin directly enhances glucose uptake in skeletal muscle independently of insulin. Moreover, adiponectin stimulates β-oxidation via activation of AMPK and PPARα thereby decreasing DAG levels and improving insulin sensitivity in muscle cell [258]. However, adiponectin synthesis decreases with increasing fat mass. It serves as a biomarker of the metabolic syndrome and low circulating levels correlate with high BMI, inflammation markers, impaired insulin sensitivity, and increased blood lipids [166]. Since fat mass as well as insulin sensitivity, IHL and other parameters of the metabolic syndrome improved in all individuals, the decrease of adiponectin was not expected. Interestingly, FGF21 binds to the FGFR1/β-klotho complex in adipose tissue and thereby upregulates adiponectin secretion and reduces accumulating ceramides [259]. Hence, the diminished circulating FGF21 might explain the observed decrease of adiponectin on mRNA and protein levels. In fact, mRNA expression of *FGFR1* was lowered in SAT samples in PP group, further supporting this hypothesis.

Additionally, adiponectin circulates in form of three different oligomers that exert distinct physiological functions in the body. Human studies observed differential regulation of adiponectin oligomers by weight loss and by hyperinsulinemic and hyperlipidemic states

[260, 261]. A modulation of adiponectin oligomer composition by high protein intake might explain the decrease of total adiponectin. However, this needs further investigation.

5.7. Diet effects on inflammatory state

Gene expression levels in adipose tissue revealed no diet specific changes of inflammatory markers. CRP and IL-18 levels in blood were reduced in the AP group, while TNFα in the PP diet. Noteworthy, IL-12p70 increased in the PP group unlike the AP diet. In the pathogenesis of obesity and insulin resistance, the production and release of cytokines from adipose tissue is increased [36]. Epidemiologic studies showed elevated blood levels of CRP [181] and cytokines [182] in T2D patients. In a large dietary intervention study higher protein content appeared to interfere with a further decrease of CRP compared with the low-protein diet [262]. This was not observed in the current study, since CRP levels decreased in all subjects. mTOR regulates inflammatory responses in blood cells differentially. It triggers secretion of anti-inflammatory signals by inducing signal transducer and activator of transcription 3 (STAT3) and suppresses proinflammatory cytokines via inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and caspase-1 in monocytes. However, it stimulates type I interferons in dendritic cells and IFNγ in T-cells [263-265]. Weichhart et al. studied mTOR role in immunity in human PBMCs observing increased IL-6, IL-12p40, IL-12p70, TNFα and decreased IL-10 levels after mTOR inhibition by rapamycin [188]. This supports the decrease of serum IL-18 and TNFα in the AP and PP group, respectively. Concordantly, after 6 weeks of diet intervention *ex vivo* stimulation of whole blood with LPS showed diminished IL-18 release in the AP and tendency of increased secretion of IL-10 in the PP and IFNγ in the AP group. However, no activation of mTOR was found in white blood cells of a subgroup, neither after chronic nor after acute high protein load. Nevertheless, the lack of effect might be due to the low sample size of selected subgroups ($n_{AP,PP}$ = 4). From these data it cannot be concluded whether one of the protein types had more beneficial role on inflammation or whether mTOR regulation was involved. Moreover, overall improvement in metabolism may have contributed to diminished inflammation.

5.8. Study limitations

There are some limitations of the study that should be discussed.

Firstly, despite the isocaloric dietary regimen, participants in both groups lost weight which was more pronounced in the AP group and led to significant difference between the groups. BMI was also reduced but the difference between the groups did not reach significance. Unfortunately, this adds a confounder to the diet induced changes of metabolic parameters. It is difficult to tease out effects of weight on baseline risk factors from effects of the high protein diets. Further, this impedes the comparison between AP and PP groups.

A second limitation might be the moderate number of participants and statistical power. This could be an argument that the lack of differences between animal and plant protein is due to low power, rather than truly due to no effect. This also affects the baseline balance between intervention groups (for example, the higher initial blood pressure in the PP group). Therefore, larger cohorts are needed to confirm and validate present data.

Thirdly, subjects continued to take their medications to control their glucose homeostasis and in order to avoid individual changes throughout the study. Subjects were also randomized according medical treatment to exclude the different influence of medications and their form on results and investigation procedure. However, medications could interfere with effects of the dietary interventions and thus decrease the ability to detect true effects of dietary protein.

Moreover, the study showed the beneficial effects of a short-term high protein intake in people with T2D. Longer time frames are needed to show the durability of the responses and possible group differences. Importantly, long-term effects on kidney function and eventual adverse effects must be determined.

Besides, the beneficial responses may be age-related since the cohort was over 60 years old and age-related effects might play a particular role in protein intake. Only Caucasian individuals were enrolled in the study, which diminishes the generalizability of the results.

More men than women participated in the study which also certainly limits the generalizability of the results. Still, males and females were equally distributed in both groups so that gender effect was eliminated.

6. Summary

Dietary approaches contribute to the prevention and treatment of type 2 diabetes. High protein diets were shown to exert beneficial as well as adverse effects on metabolism. However, it is unclear whether the protein origin plays a role in these effects. The LeguAN study investigated in detail the effects of two high protein diets, either from plant or animal origin, in type 2 diabetic patients. Both diets contained 30 EN% protein, 40 EN% carbohydrates, and 30 EN% fat. Fiber content, glycemic index, and composition of dietary fats were similar in both diets. In comparison to previous dietary habits, the fat content was exchanged for protein, while the carbohydrate intake was not modified. Overall, both high protein diets led to improvements of glycemic control, insulin sensitivity, liver fat, and cardiovascular risk markers without remarkable differences between the protein types.

Fasting glucose together with indices of insulin resistance were ameliorated by both interventions to varying extents but without significant differences between protein types. The decline of HbA_{1c} was more pronounced in the plant protein group, whereby the improvement of insulin sensitivity in the animal protein group. The high protein intake had only slight influence on postprandial metabolism seen for free fatty acids and indices of insulin secretion, sensitivity and degradation. Except for GIP release, ingestion of animal and plant meals did not provoke differential metabolic and hormonal responses despite diverse circulating amino acid levels.

The animal protein diets led to a selective increase of fat-free mass and decrease of total fat mass, which was not significantly different from the plant protein diet. Moreover, the high protein diets potently decreased liver fat content by 42% on average which was linked to significantly diminished lipogenesis, free fatty acids flux and lipolysis in adipose tissue. Moderate decline of circulating liver enzymes was induced by both interventions. The liver fat reduction was associated with improved glucose homeostasis and insulin sensitivity which underlines the protective effect of the diets.

Blood lipid profile improved in all subjects and was probably related to the lower fat intake. Reductions in uric acid and markers of inflammation further argued for metabolic benefits of both high protein diets. Systolic and diastolic blood pressure declined only in the PP group pointing a possible role of arginine.

Kidney function was not altered by high protein consumption over 6 weeks. The rapid decrease of serum creatinine in the PP group was noteworthy and should be further investigated. Protein type did not seem to play a role but long-term studies are warranted to fully elucidate safety of high protein regimen.

Varying the source of dietary proteins did not affect the mTOR pathway in adipose tissue and blood cells under neither acute nor chronic settings. Enhancement of whole-body insulin sensitivity suggested also no alteration of mTOR and no impairment of insulin sensitivity in skeletal muscle.

A remarkable outcome was the extensive reduction of FGF21, critical regulator of metabolic processes, by approximately 50% independently of protein type. Whether hepatic ER-stress, ammonia flux or rather macronutrient preferences is behind this paradoxical finding remains to be investigated in detail.

Unlike initial expectations and previous reports plant protein based diet had no clear advantage over animal proteins. The pronounced beneficial effect of animal protein on insulin homeostasis despite high BCAA and methionine intake was certainly unexpected assuming more complex metabolic adaptations occurring upon prolonged consumption. In addition, the reduced fat intake may have also contributed to the overall improvements in both groups.

Taking into account the above observed study results, a short-term diet containing 30 EN% protein (either from plant or animal origin), 40 EN% carbohydrates, and 30 EN% fat with lower SFA amount leads to metabolic improvements in diabetic patients, regardless of protein source.

7. References

1. Timper, K. and M.Y. Donath, *Diabetes mellitus Type 2--the new face of an old lady.* Swiss Med Wkly, 2012. **142**: p. w13635.
2. Rask-Madsen, C. and C.R. Kahn, *Tissue-specific insulin signaling, metabolic syndrome, and cardiovascular disease.* Arterioscler Thromb Vasc Biol, 2012. **32**(9): p. 2052-9.
3. Iozzo, P., et al., *Independent influence of age on basal insulin secretion in nondiabetic humans. European Group for the Study of Insulin Resistance.* J Clin Endocrinol Metab, 1999. **84**(3): p. 863-8.
4. Cowie, C.C., et al., *Prevalence of diabetes and impaired fasting glucose in adults in the U.S. population: National Health And Nutrition Examination Survey 1999-2002.* Diabetes Care, 2006. **29**(6): p. 1263-8.
5. Poulsen, P., et al., *Increased risk of type 2 diabetes in elderly twins.* Diabetes, 2009. **58**(6): p. 1350-5.
6. Medici, F., et al., *Concordance rate for type II diabetes mellitus in monozygotic twins: actuarial analysis.* Diabetologia, 1999. **42**(2): p. 146-50.
7. Poulsen, P., et al., *Heritability of type II (non-insulin-dependent) diabetes mellitus and abnormal glucose tolerance--a population-based twin study.* Diabetologia, 1999. **42**(2): p. 139-45.
8. Kaprio, J., et al., *Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland.* Diabetologia, 1992. **35**(11): p. 1060-7.
9. Newman, B., et al., *Concordance for type 2 (non-insulin-dependent) diabetes mellitus in male twins.* Diabetologia, 1987. **30**(10): p. 763-8.
10. Grarup, N., et al., *Genetic susceptibility to type 2 diabetes and obesity: from genome-wide association studies to rare variants and beyond.* Diabetologia, 2014. **57**(8): p. 1528-41.
11. Herder, C. and M. Roden, *Genetics of type 2 diabetes: pathophysiologic and clinical relevance.* Eur J Clin Invest, 2011. **41**(6): p. 679-92.
12. Desiderio, A., et al., *Epigenetics: spotlight on type 2 diabetes and obesity.* J Endocrinol Invest, 2016.
13. Abdullah, A., et al., *The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies.* Diabetes Res Clin Pract, 2010. **89**(3): p. 309-19.
14. Modan, M., et al., *Effect of past and concurrent body mass index on prevalence of glucose intolerance and type 2 (non-insulin-dependent) diabetes and on insulin response. The Israel study of glucose intolerance, obesity and hypertension.* Diabetologia, 1986. **29**(2): p. 82-9.
15. Hartemink, N., et al., *Combining risk estimates from observational studies with different exposure cutpoints: a meta-analysis on body mass index and diabetes type 2.* Am J Epidemiol, 2006. **163**(11): p. 1042-52.
16. Carey, V.J., et al., *Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study.* Am J Epidemiol, 1997. **145**(7): p. 614-9.
17. Vazquez, G., et al., *Comparison of body mass index, waist circumference, and waist/hip ratio in predicting incident diabetes: a meta-analysis.* Epidemiol Rev, 2007. **29**: p. 115-28.
18. Malik, V.S., et al., *Sugar-sweetened beverages and risk of metabolic syndrome and type 2 diabetes: a meta-analysis.* Diabetes Care, 2010. **33**(11): p. 2477-83.
19. Malik, V.S., et al., *Sugar-sweetened beverages, obesity, type 2 diabetes mellitus, and cardiovascular disease risk.* Circulation, 2010. **121**(11): p. 1356-64.

20. Riserus, U., W.C. Willett, and F.B. Hu, *Dietary fats and prevention of type 2 diabetes.* Prog Lipid Res, 2009. **48**(1): p. 44-51.
21. Lee, I.M., et al., *Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy.* Lancet, 2012. **380**(9838): p. 219-29.
22. Perseghin, G., et al., *Increased glucose transport-phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects.* N Engl J Med, 1996. **335**(18): p. 1357-62.
23. Brunner, E.J. and M. Kivimaki, *Epidemiology: work-related stress and the risk of type 2 diabetes mellitus.* Nat Rev Endocrinol, 2013. **9**(8): p. 449-50.
24. Heraclides, A., et al., *Psychosocial stress at work doubles the risk of type 2 diabetes in middle-aged women: evidence from the Whitehall II study.* Diabetes Care, 2009. **32**(12): p. 2230-5.
25. Kivimaki, M., et al., *Antidepressant medication use, weight gain, and risk of type 2 diabetes: a population-based study.* Diabetes Care, 2010. **33**(12): p. 2611-6.
26. Barnard, K., R.C. Peveler, and R.I. Holt, *Antidepressant medication as a risk factor for type 2 diabetes and impaired glucose regulation: systematic review.* Diabetes Care, 2013. **36**(10): p. 3337-45.
27. Taylor, E.N., F.B. Hu, and G.C. Curhan, *Antihypertensive medications and the risk of incident type 2 diabetes.* Diabetes Care, 2006. **29**(5): p. 1065-70.
28. Wannamethee, S.G., et al., *Smoking as a modifiable risk factor for type 2 diabetes in middle-aged men.* Diabetes Care, 2001. **24**(9): p. 1590-5.
29. Ronnemaa, T., et al., *Smoking is independently associated with high plasma insulin levels in nondiabetic men.* Diabetes Care, 1996. **19**(11): p. 1229-32.
30. Targher, G., et al., *Cigarette smoking and insulin resistance in patients with noninsulin-dependent diabetes mellitus.* J Clin Endocrinol Metab, 1997. **82**(11): p. 3619-24.
31. Touma, C. and S. Pannain, *Does lack of sleep cause diabetes?* Cleve Clin J Med, 2011. **78**(8): p. 549-58.
32. Knowler, W.C., et al., *Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin.* N Engl J Med, 2002. **346**(6): p. 393-403.
33. *<WHO - Definition and diagnosis of diabetes_2006.pdf>.*
34. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2010. **33 Suppl 1**: p. S62-9.
35. International Expert, C., *International Expert Committee report on the role of the A1C assay in the diagnosis of diabetes.* Diabetes Care, 2009. **32**(7): p. 1327-34.
36. Samuel, V.T. and G.I. Shulman, *Mechanisms for insulin resistance: common threads and missing links.* Cell, 2012. **148**(5): p. 852-71.
37. Samuel, V.T. and G.I. Shulman, *The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux.* J Clin Invest, 2016. **126**(1): p. 12-22.
38. Cornell, S., *Continual evolution of type 2 diabetes: an update on pathophysiology and emerging treatment options.* Ther Clin Risk Manag, 2015. **11**: p. 621-32.
39. Roden, M., *The liver in focus.* Diabetologia, 2016. **59**(6): p. 1095-7.
40. Iozzo, P., et al., *Fatty acid metabolism in the liver, measured by positron emission tomography, is increased in obese individuals.* Gastroenterology, 2010. **139**(3): p. 846-56, 856 e1-6.
41. Koliaki, C., et al., *Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis.* Cell Metab, 2015. **21**(5): p. 739-46.
42. DeFronzo, R.A., et al., *Characterization of renal glucose reabsorption in response to dapagliflozin in healthy subjects and subjects with type 2 diabetes.* Diabetes Care, 2013. **36**(10): p. 3169-76.
43. Kahn, B.B., *Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance.* Cell, 1998. **92**(5): p. 593-6.

44. Prentki, M. and C.J. Nolan, *Islet beta cell failure in type 2 diabetes.* J Clin Invest, 2006. **116**(7): p. 1802-12.
45. Weir, G.C. and S. Bonner-Weir, *Five stages of evolving beta-cell dysfunction during progression to diabetes.* Diabetes, 2004. **53 Suppl 3**: p. S16-21.
46. Hull, R.L., et al., *Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes.* J Clin Endocrinol Metab, 2004. **89**(8): p. 3629-43.
47. Rhodes, C.J., *Type 2 diabetes-a matter of beta-cell life and death?* Science, 2005. **307**(5708): p. 380-4.
48. DeFronzo, R.A., *Current issues in the treatment of type 2 diabetes. Overview of newer agents: where treatment is going.* Am J Med, 2010. **123**(3 Suppl): p. S38-48.
49. Meyer, C., et al., *Abnormal renal and hepatic glucose metabolism in type 2 diabetes mellitus.* J Clin Invest, 1998. **102**(3): p. 619-24.
50. Freeman, J.S., *Role of the incretin pathway in the pathogenesis of type 2 diabetes mellitus.* Cleve Clin J Med, 2009. **76 Suppl 5**: p. S12-9.
51. Nauck, M.A., B. Baller, and J.J. Meier, *Gastric inhibitory polypeptide and glucagon-like peptide-1 in the pathogenesis of type 2 diabetes.* Diabetes, 2004. **53 Suppl 3**: p. S190-6.
52. Cade, W.T., *Diabetes-related microvascular and macrovascular diseases in the physical therapy setting.* Phys Ther, 2008. **88**(11): p. 1322-35.
53. Brownlee, M., *The pathobiology of diabetic complications: a unifying mechanism.* Diabetes, 2005. **54**(6): p. 1615-25.
54. Terranova, C.O., et al., *Effectiveness of lifestyle-based weight loss interventions for adults with type 2 diabetes: a systematic review and meta-analysis.* Diabetes Obes Metab, 2015. **17**(4): p. 371-8.
55. Chen, L., et al., *Effect of lifestyle intervention in patients with type 2 diabetes: a meta-analysis.* Metabolism, 2015. **64**(2): p. 338-47.
56. Gong, Q.H., et al., *Lifestyle interventions for adults with impaired glucose tolerance: a systematic review and meta-analysis of the effects on glycemic control.* Intern Med, 2015. **54**(3): p. 303-10.
57. Yang, X.J., et al., *The influence of intensive lifestyle intervention on patients with isolated impaired fasting glucose: a meta-analysis.* J Adv Nurs, 2016.
58. Huang, X.L., et al., *Efficacy of lifestyle interventions in patients with type 2 diabetes: A systematic review and meta-analysis.* Eur J Intern Med, 2016. **27**: p. 37-47.
59. Mann, J.I., et al., *Evidence-based nutritional approaches to the treatment and prevention of diabetes mellitus.* Nutr Metab Cardiovasc Dis, 2004. **14**(6): p. 373-94.
60. Evert, A.B., et al., *Nutrition therapy recommendations for the management of adults with diabetes.* Diabetes Care, 2014. **37 Suppl 1**: p. S120-43.
61. Berg, J.M., J.L. Tymoczko, and L. Stryer, *Biochemistry*. 7th ed ed. 2012, New York: W.H. Freeman. xxxii, 1054, 132 p.
62. Hutson, S.M., A.J. Sweatt, and K.F. Lanoue, *Branched-chain [corrected] amino acid metabolism: implications for establishing safe intakes.* J Nutr, 2005. **135**(6 Suppl): p. 1557S-64S.
63. Layman, D.K., et al., *Protein in optimal health: heart disease and type 2 diabetes.* Am J Clin Nutr, 2008. **87**(5): p. 1571S-1575S.
64. Dong, J.Y., et al., *Effects of high-protein diets on body weight, glycaemic control, blood lipids and blood pressure in type 2 diabetes: meta-analysis of randomised controlled trials.* Br J Nutr, 2013. **110**(5): p. 781-9.
65. Gannon, M.C. and F.Q. Nuttall, *Effect of a high-protein, low-carbohydrate diet on blood glucose control in people with type 2 diabetes.* Diabetes, 2004. **53**(9): p. 2375-82.
66. Newgard, C.B., et al., *A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance.* Cell Metab, 2009. **9**(4): p. 311-26.

67. Wang, T.J., et al., *Metabolite profiles and the risk of developing diabetes.* Nat Med, 2011. **17**(4): p. 448-53.
68. Larsen, T.M., et al., *Diets with high or low protein content and glycemic index for weight-loss maintenance.* N Engl J Med, 2010. **363**(22): p. 2102-13.
69. Soenen, S., et al., *Relatively high-protein or 'low-carb' energy-restricted diets for body weight loss and body weight maintenance?* Physiol Behav, 2012. **107**(3): p. 374-80.
70. Leidy, H.J., et al., *The role of protein in weight loss and maintenance.* Am J Clin Nutr, 2015.
71. Martens, E.A. and M.S. Westerterp-Plantenga, *Protein diets, body weight loss and weight maintenance.* Curr Opin Clin Nutr Metab Care, 2014. **17**(1): p. 75-9.
72. Paddon-Jones, D., et al., *Protein, weight management, and satiety.* Am J Clin Nutr, 2008. **87**(5): p. 1558S-1561S.
73. Westerterp-Plantenga, M.S., et al., *Dietary protein, metabolism, and body-weight regulation: dose–response effects.* International Journal of Obesity, 2006. **30**: p. S16-S23.
74. Westerterp-Plantenga, M.S., S.G. Lemmens, and K.R. Westerterp, *Dietary protein - its role in satiety, energetics, weight loss and health.* Br J Nutr, 2012. **108 Suppl 2**: p. S105-12.
75. Pasiakos, S.M., et al., *Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial.* FASEB J, 2013. **27**(9): p. 3837-47.
76. Gannon, M.C., et al., *Effect of protein ingestion on the glucose appearance rate in people with type 2 diabetes.* J Clin Endocrinol Metab, 2001. **86**(3): p. 1040-7.
77. Gannon, M.C. and F.Q. Nuttall, *Amino acid ingestion and glucose metabolism--a review.* IUBMB Life, 2010. **62**(9): p. 660-8.
78. Frid, A.H., et al., *Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects.* Am J Clin Nutr, 2005. **82**(1): p. 69-75.
79. Lindgren, O., et al., *Incretin effect after oral amino acid ingestion in humans.* J Clin Endocrinol Metab, 2015. **100**(3): p. 1172-6.
80. Patil, P., et al., *Food protein-derived bioactive peptides in management of type 2 diabetes.* Eur J Nutr, 2015. **54**(6): p. 863-80.
81. Nuttall, F.Q. and M.C. Gannon, *Metabolic response of people with type 2 diabetes to a high protein diet.* Nutr Metab (Lond), 2004. **1**(1): p. 6.
82. Gannon, M.C., et al., *An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes.* Am J Clin Nutr, 2003. **78**(4): p. 734-41.
83. Nuttall, F.Q., et al., *Effect of the LoBAG30 diet on blood glucose control in people with type 2 diabetes.* Br J Nutr, 2008. **99**(3): p. 511-9.
84. Bortolotti, M., et al., *High protein intake reduces intrahepatocellular lipid deposition in humans.* Am J Clin Nutr, 2009. **90**(4): p. 1002-10.
85. Bortolotti, M., et al., *Effects of a whey protein supplementation on intrahepatocellular lipids in obese female patients.* Clin Nutr, 2011. **30**(4): p. 494-8.
86. Theytaz, F., et al., *Effects of supplementation with essential amino acids on intrahepatic lipid concentrations during fructose overfeeding in humans.* Am J Clin Nutr, 2012. **96**(5): p. 1008-16.
87. Rietman, A., et al., *Increasing protein intake modulates lipid metabolism in healthy young men and women consuming a high-fat hypercaloric diet.* J Nutr, 2014. **144**(8): p. 1174-80.
88. Brenner, B.M., T.W. Meyer, and T.H. Hostetter, *Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease.* N Engl J Med, 1982. **307**(11): p. 652-9.
89. Robertson, L., N. Waugh, and A. Robertson, *Protein restriction for diabetic renal disease.* Cochrane Database Syst Rev, 2007(4): p. CD002181.

90. Pan, Y., L.L. Guo, and H.M. Jin, *Low-protein diet for diabetic nephropathy: a meta-analysis of randomized controlled trials.* Am J Clin Nutr, 2008. **88**(3): p. 660-6.
91. Nezu, U., et al., *Effect of low-protein diet on kidney function in diabetic nephropathy: meta-analysis of randomised controlled trials.* BMJ Open, 2013. **3**(5).
92. Pomerleau, J., et al., *Effect of protein intake on glycaemic control and renal function in type 2 (non-insulin-dependent) diabetes mellitus.* Diabetologia, 1993. **36**(9): p. 829-34.
93. Pedersen, E., D.R. Jesudason, and P.M. Clifton, *High protein weight loss diets in obese subjects with type 2 diabetes mellitus.* Nutr Metab Cardiovasc Dis, 2014. **24**(5): p. 554-62.
94. Levine, M.E., et al., *Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population.* Cell Metab, 2014. **19**(3): p. 407-17.
95. Weickert, M.O., et al., *Effects of supplemented isoenergetic diets differing in cereal fiber and protein content on insulin sensitivity in overweight humans.* Am J Clin Nutr, 2011. **94**(2): p. 459-71.
96. Malloy, V.L., et al., *Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction.* Aging Cell, 2006. **5**(4): p. 305-14.
97. Stone, K.P., et al., *Mechanisms of increased in vivo insulin sensitivity by dietary methionine restriction in mice.* Diabetes, 2014. **63**(11): p. 3721-33.
98. Lees, E.K., et al., *Methionine restriction restores a younger metabolic phenotype in adult mice with alterations in fibroblast growth factor 21.* Aging Cell, 2014. **13**(5): p. 817-27.
99. Laplante, M. and D.M. Sabatini, *mTOR signaling in growth control and disease.* Cell, 2012. **149**(2): p. 274-93.
100. Polak, P. and M.N. Hall, *mTOR and the control of whole body metabolism.* Curr Opin Cell Biol, 2009. **21**(2): p. 209-18.
101. Mannaa, M., et al., *mTOR and regulation of energy homeostasis in humans.* J Mol Med (Berl), 2013. **91**(10): p. 1167-75.
102. Howell, J.J. and B.D. Manning, *mTOR couples cellular nutrient sensing to organismal metabolic homeostasis.* Trends Endocrinol Metab, 2011. **22**(3): p. 94-102.
103. Avruch, J., et al., *Amino acid regulation of TOR complex 1.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E592-602.
104. Tremblay, F., et al., *Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability.* Diabetes, 2005. **54**(9): p. 2674-84.
105. Tremblay, F., et al., *Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance.* Proc Natl Acad Sci U S A, 2007. **104**(35): p. 14056-61.
106. Gran, P., et al., *Muscle p70S6K phosphorylation in response to soy and dairy rich meals in middle aged men with metabolic syndrome: a randomised crossover trial.* Nutr Metab (Lond), 2014. **11**(1): p. 46.
107. Smith, G.I., et al., *Protein Ingestion Induces Muscle Insulin Resistance Independent of Leucine-Mediated mTOR Activation.* Diabetes, 2015. **64**(5): p. 1555-63.
108. Tremblay, F., et al., *Role of dietary proteins and amino acids in the pathogenesis of insulin resistance.* Annu Rev Nutr, 2007. **27**: p. 293-310.
109. Barnard, N., S. Levin, and C. Trapp, *Meat consumption as a risk factor for type 2 diabetes.* Nutrients, 2014. **6**(2): p. 897-910.
110. Pan, A., et al., *Changes in red meat consumption and subsequent risk of type 2 diabetes mellitus: three cohorts of US men and women.* JAMA Intern Med, 2013. **173**(14): p. 1328-35.
111. Pan, A., et al., *Red meat consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis.* Am J Clin Nutr, 2011. **94**(4): p. 1088-96.

112. Aune, D., G. Ursin, and M.B. Veierod, *Meat consumption and the risk of type 2 diabetes: a systematic review and meta-analysis of cohort studies.* Diabetologia, 2009. **52**(11): p. 2277-87.
113. Chen, M., et al., *Dairy consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis.* BMC Med, 2014. **12**: p. 215.
114. Aune, D., et al., *Dairy products and the risk of type 2 diabetes: a systematic review and dose-response meta-analysis of cohort studies.* Am J Clin Nutr, 2013. **98**(4): p. 1066-83.
115. Gao, D., et al., *Dairy products consumption and risk of type 2 diabetes: systematic review and dose-response meta-analysis.* PLoS One, 2013. **8**(9): p. e73965.
116. Yokoyama, Y., et al., *Vegetarian diets and glycemic control in diabetes: a systematic review and meta-analysis.* Cardiovasc Diagn Ther, 2014. **4**(5): p. 373-82.
117. Tonstad, S., et al., *Vegetarian diets and incidence of diabetes in the Adventist Health Study-2.* Nutr Metab Cardiovasc Dis, 2013. **23**(4): p. 292-9.
118. Tonstad, S., et al., *Type of vegetarian diet, body weight, and prevalence of type 2 diabetes.* Diabetes Care, 2009. **32**(5): p. 791-6.
119. Orlich, M.J., et al., *Vegetarian dietary patterns and mortality in Adventist Health Study 2.* JAMA Intern Med, 2013. **173**(13): p. 1230-8.
120. Hermansen, K., et al., *Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects.* Diabetes Care, 2001. **24**(2): p. 228-33.
121. Azadbakht, L., S. Atabak, and A. Esmaillzadeh, *Soy protein intake, cardiorenal indices, and C-reactive protein in type 2 diabetes with nephropathy: a longitudinal randomized clinical trial.* Diabetes Care, 2008. **31**(4): p. 648-54.
122. Pipe, E.A., et al., *Soy protein reduces serum LDL cholesterol and the LDL cholesterol:HDL cholesterol and apolipoprotein B:apolipoprotein A-I ratios in adults with type 2 diabetes.* J Nutr, 2009. **139**(9): p. 1700-6.
123. van Nielen, M., et al., *Partly replacing meat protein with soy protein alters insulin resistance and blood lipids in postmenopausal women with abdominal obesity.* J Nutr, 2014. **144**(9): p. 1423-9.
124. von Post-Skagegard, M., B. Vessby, and B. Karlstrom, *Glucose and insulin responses in healthy women after intake of composite meals containing cod-, milk-, and soy protein.* Eur J Clin Nutr, 2006. **60**(8): p. 949-54.
125. Gascon, A., et al., *Plasma lipoprotein profile and lipolytic activities in response to the substitution of lean white fish for other animal protein sources in premenopausal women.* Am J Clin Nutr, 1996. **63**(3): p. 315-21.
126. Knight, E.L., et al., *The impact of protein intake on renal function decline in women with normal renal function or mild renal insufficiency.* Ann Intern Med, 2003. **138**(6): p. 460-7.
127. Bernstein, A.M., L. Treyzon, and Z. Li, *Are high-protein, vegetable-based diets safe for kidney function? A review of the literature.* J Am Diet Assoc, 2007. **107**(4): p. 644-50.
128. Jenkins, D.J., et al., *Effect of legumes as part of a low glycemic index diet on glycemic control and cardiovascular risk factors in type 2 diabetes mellitus: a randomized controlled trial.* Arch Intern Med, 2012. **172**(21): p. 1653-60.
129. Hosseinpour-Niazi, S., et al., *Substitution of red meat with legumes in the therapeutic lifestyle change diet based on dietary advice improves cardiometabolic risk factors in overweight type 2 diabetes patients: a cross-over randomized clinical trial.* Eur J Clin Nutr, 2015. **69**(5): p. 592-7.
130. Sievenpiper, J.L., et al., *Effect of non-oil-seed pulses on glycaemic control: a systematic review and meta-analysis of randomised controlled experimental trials in people with and without diabetes.* Diabetologia, 2009. **52**(8): p. 1479-95.

131. Viguiliouk, E., et al., *Effect of Replacing Animal Protein with Plant Protein on Glycemic Control in Diabetes: A Systematic Review and Meta-Analysis of Randomized Controlled Trials.* Nutrients, 2015. **7**(12): p. 9804-24.
132. Wheeler, M.L., et al., *Animal versus plant protein meals in individuals with type 2 diabetes and microalbuminuria: effects on renal, glycemic, and lipid parameters.* Diabetes Care, 2002. **25**(8): p. 1277-82.
133. Lucotti, P., et al., *Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass.* Metabolism, 2009. **58**(9): p. 1270-6.
134. Tomoskozi, S., et al., *Isolation and study of the functional properties of pea proteins.* Nahrung, 2001. **45**(6): p. 399-401.
135. Jenkins, D.J., et al., *Glycemic index of foods: a physiological basis for carbohydrate exchange.* Am J Clin Nutr, 1981. **34**(3): p. 362-6.
136. Wolever, T.M. and D.J. Jenkins, *The use of the glycemic index in predicting the blood glucose response to mixed meals.* Am J Clin Nutr, 1986. **43**(1): p. 167-72.
137. Atkinson, F.S., K. Foster-Powell, and J.C. Brand-Miller, *International tables of glycemic index and glycemic load values: 2008.* Diabetes Care, 2008. **31**(12): p. 2281-3.
138. Levis, S.P., C.A. McGowan, and F.M. McAuliffe, *Methodology for adding and amending glycaemic index values to a nutrition analysis package.* Br J Nutr, 2011. **105**(8): p. 1117-32.
139. Aston, L.M., et al., *Developing a methodology for assigning glycaemic index values to foods consumed across Europe.* Obes Rev, 2010. **11**(1): p. 92-100.
140. Salmeron, J., et al., *Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women.* JAMA, 1997. **277**(6): p. 472-7.
141. Meyer, K.A., et al., *Carbohydrates, dietary fiber, and incident type 2 diabetes in older women.* Am J Clin Nutr, 2000. **71**(4): p. 921-30.
142. Wannamethee, S.G., et al., *Associations between dietary fiber and inflammation, hepatic function, and risk of type 2 diabetes in older men: potential mechanisms for the benefits of fiber on diabetes risk.* Diabetes Care, 2009. **32**(10): p. 1823-5.
143. Frayn, K.N., *Calculation of substrate oxidation rates in vivo from gaseous exchange.* J Appl Physiol Respir Environ Exerc Physiol, 1983. **55**(2): p. 628-34.
144. Slein, M.W., G.T. Cori, and C.F. Cori, *A comparative study of hexokinase from yeast and animal tissues.* J Biol Chem, 1950. **186**(2): p. 763-80.
145. Pivovarova, O., et al., *Hepatic insulin clearance is closely related to metabolic syndrome components.* Diabetes Care, 2013. **36**(11): p. 3779-85.
146. Mari, A., et al., *A model-based method for assessing insulin sensitivity from the oral glucose tolerance test.* Diabetes Care, 2001. **24**(3): p. 539-48.
147. Matsuda, M. and R.A. DeFronzo, *Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp.* Diabetes Care, 1999. **22**(9): p. 1462-70.
148. Yeckel, C.W., et al., *Validation of insulin sensitivity indices from oral glucose tolerance test parameters in obese children and adolescents.* J Clin Endocrinol Metab, 2004. **89**(3): p. 1096-101.
149. Takkunen, M.J., et al., *Longitudinal associations of serum fatty acid composition with type 2 diabetes risk and markers of insulin secretion and sensitivity in the Finnish Diabetes Prevention Study.* Eur J Nutr, 2016. **55**(3): p. 967-79.
150. Levey, A.S., et al., *A new equation to estimate glomerular filtration rate.* Ann Intern Med, 2009. **150**(9): p. 604-12.
151. Inker, L.A., et al., *Estimating glomerular filtration rate from serum creatinine and cystatin C.* N Engl J Med, 2012. **367**(1): p. 20-9.
152. Baylin, A., et al., *Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: comparison with adipose tissue and plasma.* Am J Epidemiol, 2005. **162**(4): p. 373-81.

153. Liu, L., et al., *Free fatty acid metabolic profile and biomarkers of isolated post-challenge diabetes and type 2 diabetes mellitus based on GC-MS and multivariate statistical analysis.* J Chromatogr B Analyt Technol Biomed Life Sci, 2010. **878**(28): p. 2817-25.
154. Machann, J., et al., *Follow-up whole-body assessment of adipose tissue compartments during a lifestyle intervention in a large cohort at increased risk for type 2 diabetes.* Radiology, 2010. **257**(2): p. 353-63.
155. Wurslin, C., et al., *Topography mapping of whole body adipose tissue using A fully automated and standardized procedure.* J Magn Reson Imaging, 2010. **31**(2): p. 430-9.
156. Schwenzer, N.F., et al., *Quantitative analysis of adipose tissue in single transverse slices for estimation of volumes of relevant fat tissue compartments: a study in a large cohort of subjects at risk for type 2 diabetes by MRI with comparison to anthropometric data.* Invest Radiol, 2010. **45**(12): p. 788-94.
157. Machann, J., et al., *Hepatic lipid accumulation in healthy subjects: a comparative study using spectral fat-selective MRI and volume-localized 1H-MR spectroscopy.* Magn Reson Med, 2006. **55**(4): p. 913-7.
158. Chow, S., et al., *Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations.* Cytometry A, 2005. **67**(1): p. 4-17.
159. Ikeda, K., et al., *Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections.* J Histochem Cytochem, 1998. **46**(3): p. 397-403.
160. Catalan, V., et al., *Validation of endogenous control genes in human adipose tissue: relevance to obesity and obesity-associated type 2 diabetes mellitus.* Horm Metab Res, 2007. **39**(7): p. 495-500.
161. Zhu, Q., et al., *Effect of L-arginine supplementation on blood pressure in pregnant women: a meta-analysis of placebo-controlled trials.* Hypertens Pregnancy, 2013. **32**(1): p. 32-41.
162. Richette, P. and T. Bardin, *Gout.* Lancet, 2010. **375**(9711): p. 318-28.
163. Kim, S.Y., et al., *Hyperuricemia and risk of stroke: a systematic review and meta-analysis.* Arthritis Rheum, 2009. **61**(7): p. 885-92.
164. Dehghan, A., et al., *High serum uric acid as a novel risk factor for type 2 diabetes.* Diabetes Care, 2008. **31**(2): p. 361-2.
165. Diez, J.J. and P. Iglesias, *The role of the novel adipocyte-derived hormone adiponectin in human disease.* Eur J Endocrinol, 2003. **148**(3): p. 293-300.
166. Trujillo, M.E. and P.E. Scherer, *Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome.* J Intern Med, 2005. **257**(2): p. 167-75.
167. Owen, B.M., D.J. Mangelsdorf, and S.A. Kliewer, *Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21.* Trends Endocrinol Metab, 2015. **26**(1): p. 22-9.
168. Chavez, A.O., et al., *Circulating fibroblast growth factor-21 is elevated in impaired glucose tolerance and type 2 diabetes and correlates with muscle and hepatic insulin resistance.* Diabetes Care, 2009. **32**(8): p. 1542-6.
169. Gallego-Escuredo, J.M., et al., *Opposite alterations in FGF21 and FGF19 levels and disturbed expression of the receptor machinery for endocrine FGFs in obese patients.* Int J Obes (Lond), 2015. **39**(1): p. 121-9.
170. Nielsen, S., et al., *Splanchnic lipolysis in human obesity.* Journal of Clinical Investigation, 2004. **113**(11): p. 1582-1588.
171. Laidlaw, S.A., et al., *Plasma and urine taurine levels in vegans.* Am J Clin Nutr, 1988. **47**(4): p. 660-3.

172. Szczepaniak, L.S., 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): p. E462-8.
173. Lappas, M., *Effect of pre-existing maternal obesity, gestational diabetes and adipokines on the expression of genes involved in lipid metabolism in adipose tissue.* Metabolism, 2014. **63**(2): p. 250-62.
174. Lee, B.C. and J. Lee, *Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance.* Biochim Biophys Acta, 2014. **1842**(3): p. 446-62.
175. Yadav, A., et al., *Role of leptin and adiponectin in insulin resistance.* Clin Chim Acta, 2013. **417**: p. 80-4.
176. de Kreutzenberg, S.V., et al., *Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms.* Diabetes, 2010. **59**(4): p. 1006-15.
177. Suryawan, A., et al., *A molecular model of human branched-chain amino acid metabolism.* Am J Clin Nutr, 1998. **68**(1): p. 72-81.
178. Pagliassotti, M.J., et al., *Endoplasmic reticulum stress in obesity and obesity-related disorders: An expanded view.* Metabolism, 2016. **65**(9): p. 1238-46.
179. Kovsan, J., et al., *Altered autophagy in human adipose tissues in obesity.* J Clin Endocrinol Metab, 2011. **96**(2): p. E268-77.
180. Kolb, H. and T. Mandrup-Poulsen, *An immune origin of type 2 diabetes?* Diabetologia, 2005. **48**(6): p. 1038-50.
181. Festa, A., et al., *Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study.* Diabetes, 2002. **51**(4): p. 1131-7.
182. Spranger, J., et al., *Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study.* Diabetes, 2003. **52**(3): p. 812-7.
183. Herder, C., et al., *Immunological and cardiometabolic risk factors in the prediction of type 2 diabetes and coronary events: MONICA/KORA Augsburg case-cohort study.* PLoS One, 2011. **6**(6): p. e19852.
184. Herder, C., et al., *Low-grade inflammation, obesity, and insulin resistance in adolescents.* J Clin Endocrinol Metab, 2007. **92**(12): p. 4569-74.
185. Carstensen, M., et al., *Accelerated increase in serum interleukin-1 receptor antagonist starts 6 years before diagnosis of type 2 diabetes: Whitehall II prospective cohort study.* Diabetes, 2010. **59**(5): p. 1222-7.
186. Dasu, M.R., et al., *Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects.* Diabetes Care, 2010. **33**(4): p. 861-8.
187. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, *Obesity induces a phenotypic switch in adipose tissue macrophage polarization.* J Clin Invest, 2007. **117**(1): p. 175-84.
188. Weichhart, T., et al., *The TSC-mTOR signaling pathway regulates the innate inflammatory response.* Immunity, 2008. **29**(4): p. 565-77.
189. Bray, G.A., et al., *Effect of dietary protein content on weight gain, energy expenditure, and body composition during overeating: a randomized controlled trial.* JAMA, 2012. **307**(1): p. 47-55.
190. Veldhorst, M.A., M.S. Westerterp-Plantenga, and K.R. Westerterp, *Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet.* Am J Clin Nutr, 2009. **90**(3): p. 519-26.
191. Hall, W.L., et al., *Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite.* Br J Nutr, 2003. **89**(2): p. 239-48.
192. Tome, D., *Digestibility issues of vegetable versus animal proteins: protein and amino acid requirements--functional aspects.* Food Nutr Bull, 2013. **34**(2): p. 272-4.

193. Gausseres, N., et al., *[15N]-labeled pea flour protein nitrogen exhibits good ileal digestibility and postprandial retention in humans.* J Nutr, 1997. **127**(6): p. 1160-5.
194. Lynch, C.J., et al., *Leucine is a direct-acting nutrient signal that regulates protein synthesis in adipose tissue.* Am J Physiol Endocrinol Metab, 2002. **283**(3): p. E503-13.
195. Lynch, C.J., et al., *Tissue-specific effects of chronic dietary leucine and norleucine supplementation on protein synthesis in rats.* Am J Physiol Endocrinol Metab, 2002. **283**(4): p. E824-35.
196. Cuthbertson, D., et al., *Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.* FASEB J, 2005. **19**(3): p. 422-4.
197. Pereira, S., et al., *Insulin resistance of protein metabolism in type 2 diabetes.* Diabetes, 2008. **57**(1): p. 56-63.
198. van Vliet, S., N.A. Burd, and L.J. van Loon, *The Skeletal Muscle Anabolic Response to Plant- versus Animal-Based Protein Consumption.* J Nutr, 2015. **145**(9): p. 1981-91.
199. Lamming, D.W. and D.M. Sabatini, *A Central role for mTOR in lipid homeostasis.* Cell Metab, 2013. **18**(4): p. 465-9.
200. Takashina, C., et al., *Associations among the plasma amino acid profile, obesity, and glucose metabolism in Japanese adults with normal glucose tolerance.* Nutr Metab (Lond), 2016. **13**: p. 5.
201. Warensjo, E., et al., *Biomarkers of milk fat and the risk of myocardial infarction in men and women: a prospective, matched case-control study.* Am J Clin Nutr, 2010. **92**(1): p. 194-202.
202. Mayneris-Perxachs, J., et al., *Plasma fatty acid composition, estimated desaturase activities, and their relation with the metabolic syndrome in a population at high risk of cardiovascular disease.* Clin Nutr, 2014. **33**(1): p. 90-7.
203. Franco, R.S., *Measurement of red cell lifespan and aging.* Transfus Med Hemother, 2012. **39**(5): p. 302-7.
204. Nakamura, H., et al., *Plasma amino acid profiles are associated with insulin, C-peptide and adiponectin levels in type 2 diabetic patients.* Nutr Diabetes, 2014. **4**: p. e133.
205. Laeger, T., et al., *FGF21 is an endocrine signal of protein restriction.* J Clin Invest, 2014. **124**(9): p. 3913-22.
206. Gallinetti, J., E. Harputlugil, and J.R. Mitchell, *Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR.* Biochem J, 2013. **449**(1): p. 1-10.
207. Yary, T., et al., *Serum n-6 polyunsaturated fatty acids, Delta5- and Delta6-desaturase activities, and risk of incident type 2 diabetes in men: the Kuopio Ischaemic Heart Disease Risk Factor Study.* Am J Clin Nutr, 2016.
208. Knebel, B., et al., *Specific Metabolic Profiles and Their Relationship to Insulin Resistance in Recent-Onset Type 1 and Type 2 Diabetes.* J Clin Endocrinol Metab, 2016. **101**(5): p. 2130-40.
209. Yamada, C., et al., *Association between insulin resistance and plasma amino acid profile in non-diabetic Japanese subjects.* J Diabetes Investig, 2015. **6**(4): p. 408-15.
210. Boirie, Y., et al., *Slow and fast dietary proteins differently modulate postprandial protein accretion.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14930-5.
211. Calbet, J.A. and D.A. MacLean, *Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans.* J Nutr, 2002. **132**(8): p. 2174-82.
212. van Loon, L.J., et al., *Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate.* Am J Clin Nutr, 2000. **72**(1): p. 96-105.
213. Larsson, H. and B. Ahren, *Glucose intolerance is predicted by low insulin secretion and high glucagon secretion: outcome of a prospective study in postmenopausal Caucasian women.* Diabetologia, 2000. **43**(2): p. 194-202.

214. Nauck, M.A., *Unraveling the science of incretin biology.* Eur J Intern Med, 2009. **20 Suppl 2**: p. S303-8.
215. Garcia-Caraballo, S.C., et al., *Prevention and reversal of hepatic steatosis with a high-protein diet in mice.* Biochim Biophys Acta, 2013. **1832**(5): p. 685-95.
216. Garcia Caraballo, S.C., et al., *High-protein diets prevent steatosis and induce hepatic accumulation of monomethyl branched-chain fatty acids.* J Nutr Biochem, 2014. **25**(12): p. 1263-74.
217. Petzke, K.J., A. Freudenberg, and S. Klaus, *Beyond the role of dietary protein and amino acids in the prevention of diet-induced obesity.* Int J Mol Sci, 2014. **15**(1): p. 1374-91.
218. Freudenberg, A., K.J. Petzke, and S. Klaus, *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): p. 519-28.
219. Freudenberg, A., K.J. Petzke, and S. Klaus, *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): p. 1524-30.
220. Ravn, A.M., et al., *Thermic effect of a meal and appetite in adults: an individual participant data meta-analysis of meal-test trials.* Food Nutr Res, 2013. **57**.
221. Halton, T.L. and F.B. Hu, *The effects of high protein diets on thermogenesis, satiety and weight loss: a critical review.* J Am Coll Nutr, 2004. **23**(5): p. 373-85.
222. Jegatheesan, P., et al., *Effect of specific amino acids on hepatic lipid metabolism in fructose-induced non-alcoholic fatty liver disease.* Clin Nutr, 2016. **35**(1): p. 175-82.
223. Jegatheesan, P., et al., *Citrulline and Nonessential Amino Acids Prevent Fructose-Induced Nonalcoholic Fatty Liver Disease in Rats.* J Nutr, 2015. **145**(10): p. 2273-9.
224. Sellmann, C., et al., *Oral citrulline supplementation protects female mice from the development of non-alcoholic fatty liver disease (NAFLD).* Eur J Nutr, 2016.
225. Sellmann, C., et al., *Oral Glutamine Supplementation Protects Female Mice from Nonalcoholic Steatohepatitis.* J Nutr, 2015. **145**(10): p. 2280-6.
226. Madiraju, A.K., et al., *Argininosuccinate synthetase regulates hepatic AMPK linking protein catabolism and ureagenesis to hepatic lipid metabolism.* Proc Natl Acad Sci U S A, 2016. **113**(24): p. E3423-30.
227. Rui, L., *Energy metabolism in the liver.* Compr Physiol, 2014. **4**(1): p. 177-97.
228. Donnelly, K.L., et al., *Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease.* Journal of Clinical Investigation, 2005. **115**(5): p. 1343-1351.
229. de Wit, N.J., et al., *Phenotyping the effect of diet on non-alcoholic fatty liver disease.* J Hepatol, 2012. **57**(6): p. 1370-3.
230. Assy, N., et al., *Olive oil consumption and non-alcoholic fatty liver disease.* World J Gastroenterol, 2009. **15**(15): p. 1809-15.
231. Masterton, G.S., J.N. Plevris, and P.C. Hayes, *Review article: omega-3 fatty acids - a promising novel therapy for non-alcoholic fatty liver disease.* Aliment Pharmacol Ther, 2010. **31**(7): p. 679-92.
232. Bjermo, H., et al., *Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: a randomized controlled trial.* Am J Clin Nutr, 2012. **95**(5): p. 1003-12.
233. Rosqvist, F., et al., *Overfeeding polyunsaturated and saturated fat causes distinct effects on liver and visceral fat accumulation in humans.* Diabetes, 2014. **63**(7): p. 2356-68.
234. Vatner, D.F., et al., *Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids.* Proc Natl Acad Sci U S A, 2015. **112**(4): p. 1143-8.
235. Perry, R.J., et al., *Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes.* Cell, 2015. **160**(4): p. 745-58.

236. Girousse, A., et al., *Partial inhibition of adipose tissue lipolysis improves glucose metabolism and insulin sensitivity without alteration of fat mass.* PLoS Biol, 2013. **11**(2): p. e1001485.
237. Puri, P., et al., *The plasma lipidomic signature of nonalcoholic steatohepatitis.* Hepatology, 2009. **50**(6): p. 1827-38.
238. Watanabe, M., et al., *Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c.* J Clin Invest, 2004. **113**(10): p. 1408-18.
239. Lagiou, P., et al., *Low carbohydrate-high protein diet and incidence of cardiovascular diseases in Swedish women: prospective cohort study.* BMJ, 2012. **344**: p. e4026.
240. Wang, D.D., et al., *Association of Specific Dietary Fats With Total and Cause-Specific Mortality.* JAMA Intern Med, 2016.
241. Mariotti, F., et al., *Meal amino acids with varied levels of arginine do not affect postprandial vascular endothelial function in healthy young men.* J Nutr, 2007. **137**(6): p. 1383-9.
242. Helal, I., et al., *Glomerular hyperfiltration: definitions, mechanisms and clinical implications.* Nat Rev Nephrol, 2012. **8**(5): p. 293-300.
243. Brosnan, J.T., R.P. da Silva, and M.E. Brosnan, *The metabolic burden of creatine synthesis.* Amino Acids, 2011. **40**(5): p. 1325-31.
244. Tirosh, A., et al., *Renal function following three distinct weight loss dietary strategies during 2 years of a randomized controlled trial.* Diabetes Care, 2013. **36**(8): p. 2225-32.
245. de Oliveira, E.P. and R.C. Burini, *High plasma uric acid concentration: causes and consequences.* Diabetol Metab Syndr, 2012. **4**: p. 12.
246. Choi, H.K., S. Liu, and G. Curhan, *Intake of purine-rich foods, protein, and dairy products and relationship to serum levels of uric acid: the Third National Health and Nutrition Examination Survey.* Arthritis Rheum, 2005. **52**(1): p. 283-9.
247. Choi, H.K., et al., *Purine-rich foods, dairy and protein intake, and the risk of gout in men.* N Engl J Med, 2004. **350**(11): p. 1093-103.
248. Talukdar, S., et al., *A Long-Acting FGF21 Molecule, PF-05231023, Decreases Body Weight and Improves Lipid Profile in Non-human Primates and Type 2 Diabetic Subjects.* Cell Metab, 2016. **23**(3): p. 427-40.
249. Gaich, G., et al., *The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes.* Cell Metab, 2013. **18**(3): p. 333-40.
250. Zhang, X., et al., *Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans.* Diabetes, 2008. **57**(5): p. 1246-53.
251. Jiang, S., et al., *Fibroblast growth factor 21 is regulated by the IRE1alpha-XBP1 branch of the unfolded protein response and counteracts endoplasmic reticulum stress-induced hepatic steatosis.* J Biol Chem, 2014. **289**(43): p. 29751-65.
252. De Sousa-Coelho, A.L., P.F. Marrero, and D. Haro, *Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation.* Biochem J, 2012. **443**(1): p. 165-71.
253. von Holstein-Rathlou, S., et al., *FGF21 Mediates Endocrine Control of Simple Sugar Intake and Sweet Taste Preference by the Liver.* Cell Metab, 2016. **23**(2): p. 335-43.
254. Dushay, J.R., et al., *Fructose ingestion acutely stimulates circulating FGF21 levels in humans.* Mol Metab, 2015. **4**(1): p. 51-7.
255. Chu, A.Y., et al., *Novel locus including FGF21 is associated with dietary macronutrient intake.* Hum Mol Genet, 2013. **22**(9): p. 1895-902.
256. Tanaka, T., et al., *Genome-wide meta-analysis of observational studies shows common genetic variants associated with macronutrient intake.* Am J Clin Nutr, 2013. **97**(6): p. 1395-402.
257. Mao, X., et al., *APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function.* Nat Cell Biol, 2006. **8**(5): p. 516-23.

258. Yoon, M.J., et al., *Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha.* Diabetes, 2006. **55**(9): p. 2562-70.
259. Holland, W.L., et al., *An FGF21-adiponectin-ceramide axis controls energy expenditure and insulin action in mice.* Cell Metab, 2013. **17**(5): p. 790-7.
260. Bobbert, T., et al., *Acute hyperinsulinaemia and hyperlipidaemia modify circulating adiponectin and its oligomers.* Clin Endocrinol (Oxf), 2009. **71**(4): p. 507-11.
261. Bobbert, T., et al., *Changes of adiponectin oligomer composition by moderate weight reduction.* Diabetes, 2005. **54**(9): p. 2712-9.
262. Gogebakan, O., et al., *Effects of weight loss and long-term weight maintenance with diets varying in protein and glycemic index on cardiovascular risk factors: the diet, obesity, and genes (DiOGenes) study: a randomized, controlled trial.* Circulation, 2011. **124**(25): p. 2829-38.
263. Weichhart, T. and M.D. Saemann, *The multiple facets of mTOR in immunity.* Trends Immunol, 2009. **30**(5): p. 218-26.
264. Saemann, M.D., et al., *The multifunctional role of mTOR in innate immunity: implications for transplant immunity.* Am J Transplant, 2009. **9**(12): p. 2655-61.
265. Katholnig, K., et al., *p38alpha senses environmental stress to control innate immune responses via mechanistic target of rapamycin.* J Immunol, 2013. **190**(4): p. 1519-27.

8. Appendix

Table 20. Exemplary food plans for both intervention groups.

	Animal Protein		Plant Protein	
protein powder	25 g	Inkospor Active Pro 80 *	25 g	**Pea protein** #
	250 g	Milk or yogurt (1.5% fat)	250 g	Carrot juice
Breakfast/	260 g	Whole-grain bread	30 g	**Toast** #
Snack/	60 g	Cream cheese (0,2% fat)	190 g	**Protein bread** #
Supper	75 g	Cheese (16% fat)	110 g	Whole-grain bread
	80 g	Camembert (12% fat)	12 g	butter
	50 g	Harz cheese	20 g	Cream cheese (16% fat)
	60 g	Egg (1)	30 g	Cream cheese (5% fat)
	30 g	Boiled ham	30 g	Cheese (16% fat)
	40 g	Rolled fillet of ham	40 g	Rolled fillet of ham
	40 g	Walnut	30 g	**Protein cookie** #
			20 g	Walnut
			250 g	Milk or yogurt (1.5% fat)
			50	Low fat curd cheese
Fruit/ Vegetable/ salad	300 g	Fruits (accordant to the parts substitution list)	360 g	Fruits (accordant to the parts substitution list)
	150 g	Beetroot	150 g	Beetroot
	100 g	Kohlrabi	150 g	Tomato
	150 g	Red pepper		
	30 g	Onion		
	13 g	Olive oil		
lunch	240 g	Potato in the skin	100 g	**Pasta with pea protein** #
	250 g	Low fat curd cheese	(raw)	
	50 g	Milk (1.5 % fat)	200 g	Chopped tomatoes (tinned)
	10 g	Chive	30 g	Onion
			12 g	Olive oil
drinks	600 g	Coffee	360 g	Coffee
	600 g	Tea	40 g	Evaporated milk (4% fat)
		Water		Water
Energy [kcal]		**2452**		**2417**
Protein [g (EN%)]		30.36		29.96
CH [g (EN%)]		39.58		39.75
Fat [g (EN%)]		30.06		30.30
SFA [g]		25.26		25.41
MUFA [g]		25.46		25.05
PUFA [g]		25.45		25.41

* Inkospor Active Pro 80 is a protein powder consisted of milk protein (Casein with Sojalecithine), whey protein and egg white protein as protein sources. # specially developed products which were sources for the major portion of plant protein.

Table 21. Food components of the meal tolerance tests for both groups.

Animal Protein Meal tolerance test	
24 g	Inkospor Active Pro 80 Vanilla*
150 g	Yoghurt 3.5% fat
70 g	Whole-grain bread
20 g	White cheese (Kiri) with yoghurt
20 g	Butter cheese 45% fat
100 g	Fruit pot plumb-apple
20 g	Cucumber
Plant Protein Meal tolerance test	
25 g	Pea protein enriched drink powder #
200 g	Carrot juice
70 g	Toast bread with pea protein
9 g	Butter
20 g	White cheese (Kiri) with yoghurt
90 g	Fruit pot apple-banana-peach
20 g	Cucumber

* Inkospor Active Pro 80 is a protein powder consisted of milk protein (Casein with Sojalecithine), whey protein and egg white protein as protein sources. # specially developed products which were sources for the major portion of plant protein.

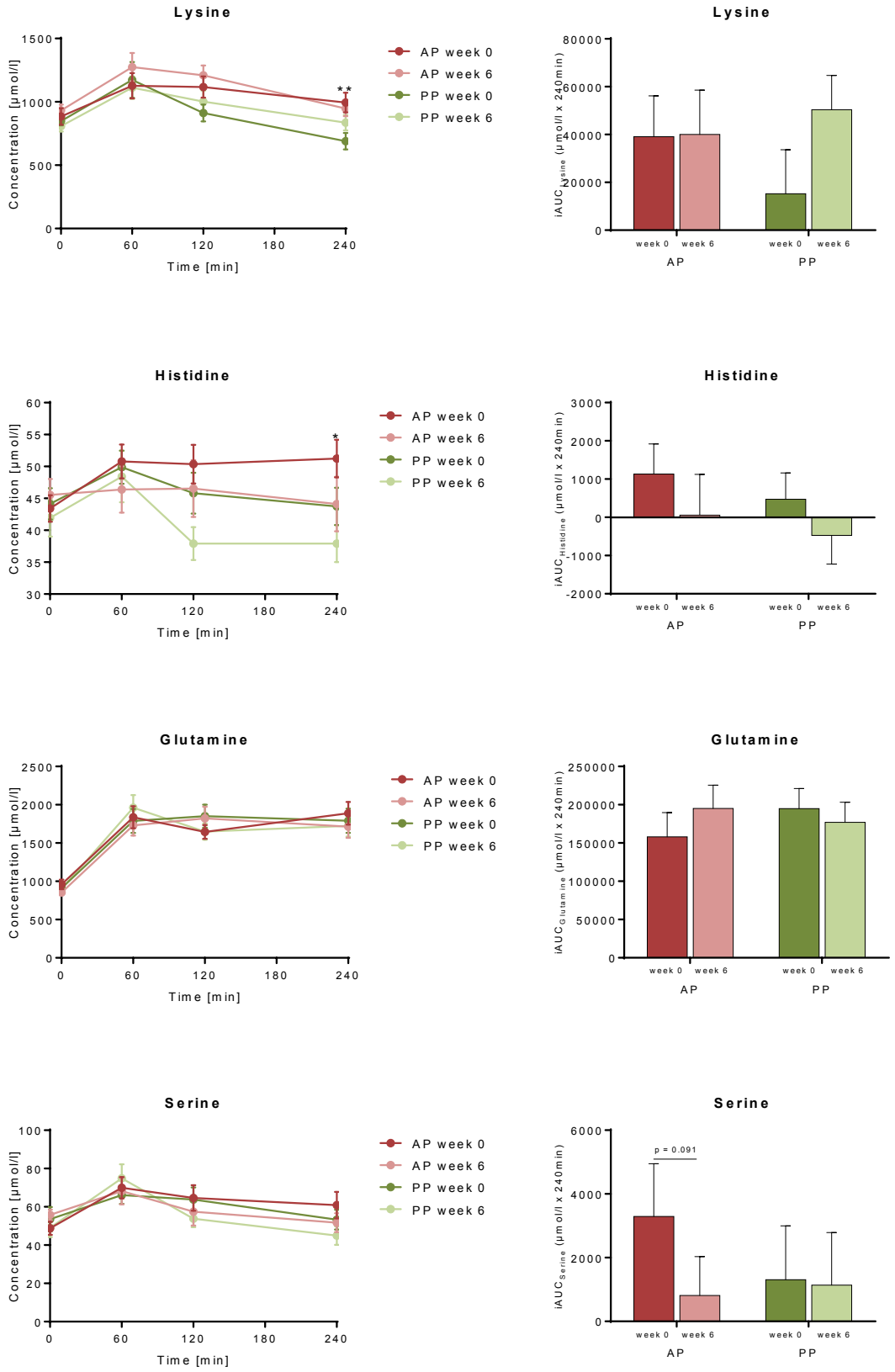

Figure 35. Postprandial time courses of lysine, histidine, glutamine, and serine. Postprandial 240-min time course and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; iAUC: incremental area under the curve; PP, plant protein. Values are presented as means ± SEM. *p < 0.05, **p < 0.01 different between AP and PP group at week 0.

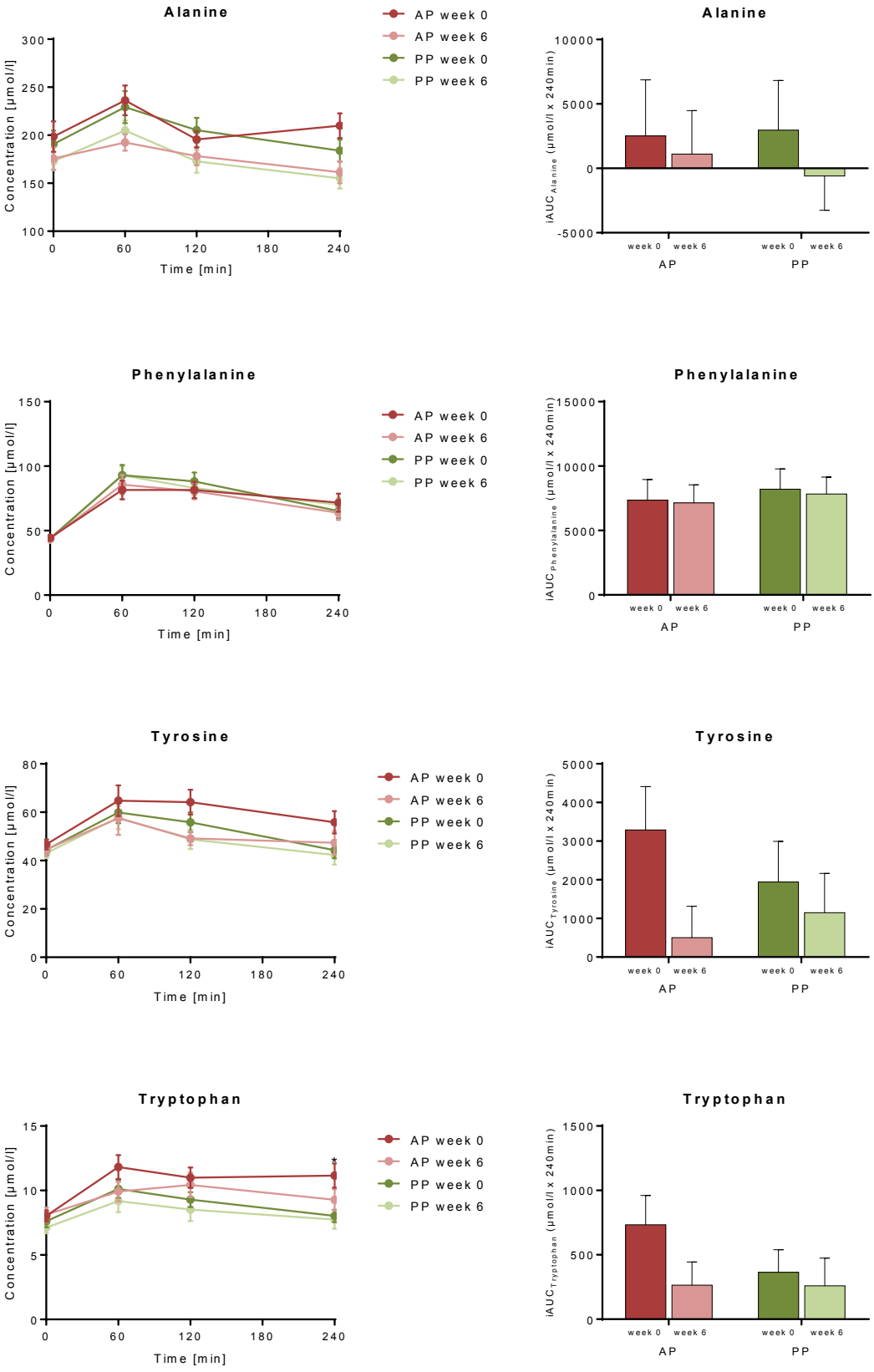

Figure 36. Postprandial time courses of alanine, phenylalanine, tyrosine, and tryptophan. Postprandial 240-min time course and iAUCs after ingestion of an animal/plant high protein test meal at week 0 and 6. Abbreviations: AP, animal protein; iAUC: incremental area under the curve; PP, plant protein. Values are presented as means ± SEM. *p < 0.05 different between AP and PP group at week 0.

9. Curriculum vitae

Publikationen

Markova M, Pivovarova O, Hornemann S, Sucher S, Frahnow T, Wegner K, Machann J, Petzke KJ, Hierholzer J, Lichtinghagen R, Herder C, Carstenberg-Kirberg M, Roden M, Rudovich N, Klaus S, Thomann R, Schneeweiss R, Rohn S, Pfeiffer AFH.: Isocaloric high animal or plant protein diets potently reduce liver fat in type 2 diabetes: a randomized clinical trial. [Gastroenterology, under revision].

Markova M, Sucher S, Pivovarova O, Pfeiffer AFH.: Proteinreiche Ernährung für die Gesundheit. LeguAN-Projekt: Tierisches oder pflanzliches Protein? Ernährung im Fokus. 2016; 07-08|16:190-5.

Murahovschi V, Pivovarova O, Ilkavets I, Dmitrieva RM, Docke S, Keyhani-Nejad F, Gogebakan O, Osterhoff M, Kemper M, Hornemann S, **Markova M**, Kloting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Bluher M, Pfeiffer AF, Rudovich N: WISP1 is a novel adipokine linked to inflammation in obesity. Diabetes. 2015;64(3):856-66

Lachenmeier DW, Monakhova YB, **Markova M**, Kuballa T, Rehm J.: What happens if people start drinking mouthwash as surrogate alcohol? A quantitative risk assessment. Food Chem Toxicol. 2013 Jan;51:173-8.

Schlee C, **Markova M**, Schrank J, Laplagne F, Schneider R, Lachenmeier DW.: Determination of 2-methylimidazole, 4-methylimidazole and 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole in caramel colours and cola using LC/MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2013 May 15;927:223-6.

Kongressbeiträge

DDG 2016 Berlin	Orale Präsentation Markova M, Hornemann S, Sucher S, Petzke KJ, Machann J, Pivovarova O, Pfeiffer AFH. “Fatty liver reduction and diminished lipolysis by high-protein intake without caloric restriction: a randomized controlled trial in type 2 diabetes.” Diabetologie und Stoffwechsel 2016; 11 - FV26
Diabetes Kongress 2016 Münster	Eingeladene orale Präsentation Markova M. “Proteinreiche Ernährung bei Typ-2-Diabetes - ein Risiko für die Niere?“
EASD 2015 Stockholm	Poster Präsentation Markova M, Hornemann S, Sucher S, Pivovarova O, Pfeiffer AFH. “Metabolic and molecular effects of a high-protein diet in subjects with type 2 diabetes.” Diabetologia 2015; 58 (Suppl 1):S335

DNSG 2015 Toronto	Orale Präsentation Markova M, Hornemann S, Sucher S, Pivovarova O, Pfeiffer AFH. “A high-protein diet reduces liver fat content and improves glomerular filtration rate in subjects with type 2 diabetes.” Short Oral Abstract 3
DDG 2015 Berlin	Poster Präsentation Markova M, Hornemann S, Sucher S, Kemper M, Herder C, Roden M, Prokisch H, Pivovarova O, Pfeiffer AFH. “A high-protein diet reduces liver fat content and improves glomerular filtration rate in subjects with type 2 diabetes”. Diabetologie und Stoffwechsel 2015; 10 - P157
DGE 2015 Lübeck	Poster Präsentation Markova M, Hornemann S, Sucher S, Kemper M, Pivovarova O, Pfeiffer AFH. “Effects of an isocaloric high-protein diet in subjects with type 2 diabetes: animal versus plant protein.” Exp Clin Endocrinol Diabetes 2015; 123 - P13-13
NuGO week 2014 Neapel	Poster Präsentation Markova M, Hornemann S, Kemper M, Herder C, Pivovarova O, Pfeiffer AFH. “Effects of an isocaloric high protein diet in subjects with type 2 diabetes: animal versus plant protein.” Poster 19
DGE 2014 Dresden	Poster Präsentation Markova M, Schiess S, Herder C, Pfeiffer AFH, Pivovarova O. “Analysis of the regulation of intracellular signaling pathways in peripheral blood cells in human studies.” Exp Clin Endocrinol Diabetes 2014; 122 - P160

Preise

- Posterpreis der DGE 2015 (Deutsche Gesellschaft für Endokrinologie)
- Danone-Reisestipendium 2015

10. Danksagung

Ich danke sehr herzlich Prof. Dr. Andreas F. H. Pfeiffer dafür, dass es mir überhaupt die Gelegenheit gegeben hat an diesem interessanten und wichtigen Thema zu arbeiten und dass er mir vertraut hat! Vielen Dank, dass ich in dem Feld der klinischen Forschung, was ich für äußerst wichtig halte, arbeiten dürfte! Danke für alle nette Gespräche, hilfreiche Diskussionen, und dass er immer geduldig, hilfsbereit, und so menschlich war! Ich bin auch sehr dankbar, dass ich die Möglichkeit hatte, an vielen nationalen und internationalen Kongressen teilzunehmen!

Ein besonderer Dank gilt der besten Betreuerin Frau Dr. Olga Pivovarova! Vielen Dank, dass sie mir so viel beigebracht hat, dass sie sich immer Zeit für alle mögliche Fragen und Probleme genommen hat, dass sie dabei immer Geduld hatte, dass ich jedes Mal, als ich an der Tür geklopft habe, auch reinkommen und mich setzen durfte, dass ich mich immer an sie wenden konnte!

Ein weiterer sehr besonderer Dank geht an die beste Bürokollegin Katharina Keßler, immer sonnig und strahlend! Mit ihrer süßen, netten und immer positiven Art hat sie unser Zusammenleben in dem kleinen Zimmer wirklich wohlig gemacht! Danke auch für die Diskussionen unserer Ergebnisse und jegliche Hilfe während dieser Zeit! Ich habe viel von ihr gelernt und ohne sie hätte ich es nie geschafft!

Eine weitere besondere Person, Farnaz Keyhani-Nejad! Erstmal Danke, dass sie mir immer geholfen hat! Mit ihrer lustigen Art hat sie Platz in meinem Herzen für immer gewonnen! Vielen Dank, dass ich sie kennen lernen und ihre Freundin werden durfte!

Sonja Schieß, die mir so viel geholfen hat und viele Tipps gegeben hat! Für die nette Zeit in Neapel! Eine Person, mit der ich immer diskutieren konnte, bei der ich mich beschweren konnte, von der ich viele Ratschläge holen konnte!

Turid Frahnow, ein sehr großer Dank, dass sie immer nett und hilfsbereit war! Und natürlich für das unendliche Geduld, als sie meine Statistikfragen beantwortet hat!

Dr. Rita Schüler bedanke ich für die große Hilfe und gute Tipps, auch für die immer nette Gespräche und die tolle Zeit in Toronto!

Auch an allen anderen Mitdoktoranden (Anne, Stephanie, Caroline, Ulrike, Valentina, Zeinab, Veronica, Nicole, Thomas, Li und Cheni) für die nette Zusammenarbeit, lustige Zeit beim Mittagessen! Genauso an Juliane, Kathleen und Yasmina, unsere liebe Studentinnen!

Dr. Martin Osterhoff, der bei allen möglichen Problemen hilfsbereit war! Dr. Stefan Kabisch, an dem ich mich immer wenden konnte, um mich über medizinische Fragen zu konsultieren!

Herzlichen Dank an Silke Hornemann und Stephanie Sucher, ohne die die LeguAN-Studie nie durchgeführt werden konnte! Vielen Dank, dass beide immer geduldig und hilfsbereit waren, als ich Fragen hatte! Sonst hätte ich es nie geschafft! Ein sehr großer Dank geht an das Ambulanzteam – Alex, Stephi, Nicki, Melanie und Katja, die das ganze durchgeführt haben! Danke auch dafür, dass sie immer nett waren, auch wenn es mehreren Probanden parallel gab und etwas stressiger war!

Die besten TA's Katrin, Tanja und Andrea! Für alle Messungen, die sie für mich gemacht haben, für die Hilfe im Labor, für die nette Gespräche in der Küche!

Berit und June, die ich so oft aufgrund meiner voller Inkompetenz in administrativen Sachen genervt habe! Vielen Dank, dass sie immer so nett und lieb waren!

Gabby & Elly, zwei der wunderbarsten Frauen, die ich kenne, und meine beste Freundinnen schon seit der achten Klasse, danke ich für die Mühe meine Arbeit zu lesen und Fehler auf Englisch zu korrigieren. Vielen Dank 1000 Mal!

Der größte Dank geht an meinen Eltern, die mich die ganze Zeit unterstützt, geliebt und an mich geglaubt haben! Mein Bruder, der mir unermüdlich gesagt hat, jedes Mal als ich mich beschwert habe, dass ich es schaffe und nicht aufgeben durfte! Mein Freund, Ivan, für seine ständige Unterstützung, weise Tipps, und dass er immer ein offenes Ohr für alle meine kleine Probleme hatte!

Herzlichen Dank!!!

11. Erklärung

Hiermit erkläre ich, Mariya Markova, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Arbeit wurde bisher an keiner anderen Hochschule eingereicht.

Mariya Markova Potsdam, August 2016