Effect of dietary protein on lipid and glucose metabolism: implications for metabolic health

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Abstract

Background: Diet is an important factor in the development of the Metabolic Syndrome (Mets) and type 2 Diabetes Mellitus. Accumulation of intra hepatic lipid (IHL) can result in non-alcoholic fatty liver disease (NAFLD), which is sometimes considered the hepatic manifestation of Mets. Manipulation of the dietary macronutrient composition – altering either fat or simple carbohydrates – has the potential to change lipid storage in the liver. Protein also has this ability, however human data is scarce. Moreover, high dietary protein intake is linked with an increased type 2 Diabetes risk. Therefore, it is essential to study the metabolic consequences of changes in macronutrient composition focussing on altering dietary protein quantity.

Objective: In this thesis the effects of dietary protein on metabolic health focusing on lipid and glucose metabolism were investigated in both observational studies as well as in a human dietary intervention trial.

Methods: In an observational study (n=1283), Fatty Liver Index (FLI) was calculated and related to macronutrient consumption from dietary assessment data. In a controlled dietary intervention, participants (n=27) were assigned to either a control-diet for 4 weeks, or a high-fat, hypercaloric diet, with either a high-protein or a normal-protein content for two weeks, and vice versa. Measurements of IHL (^{1}H-MRS) and blood plasma glucose and lipid concentrations were performed, both in the fasting following a meal.

Results: In the observational study, the prevalence of fatty liver as indicated by an FLI>60, was 22.0%. Compared to persons with a normal FLI score of <30, protein intake was positively related with high FLI score >60 (OR: 1.26 per 1 en%, 95%CI 1.16-1.37). This was in particular the case for protein intake from animal sources. In the dietary intervention study, the high-protein diet compared to the normal-protein diet resulted in lower IHL and plasma TG concentrations (IHL: 0.35 ± 0.04 % vs. 0.51 ± 0.08 %; p=0.08; TG: 0.65 ± 0.03 vs. 0.77 ± 0.05 mmol/L; p=0.07). Furthermore, after the meal challenge the free fatty acids (FFA) response was significant different between all three intervention diets

(p=0.03). Moreover, the postprandial glucose response was significantly lower after adaptation to NP compared with HP (p=0.03), without differences in the postprandial insulin responses (p=0.37).

Conclusions: From data of the intervention study and observational studies reported in this thesis, it can be concluded that dietary protein intake is associated with alterations in metabolic profile, with both favourable and potential unfavourable health outcomes. On the short term increasing dietary protein in healthy subjects improved lipid metabolism, as seen by lower TG and IHL levels, but not glucose metabolism. On the long term, however, a high-protein intake was related to a fatty liver, and associated to insulin resistance.

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Chapter 1

General introduction

The growing obesity epidemic and its consequences on metabolic health

Worldwide, the number of people suffering from obesity continues to increase (1). In 2005, 33% of the world's adult population was either overweight or obese, and if the current trend continues this could be more than 57% in 2030 (2). Obesity is associated with metabolic disturbances like insulin resistance, high blood pressure and dyslipidaemia (3). These metabolic disturbances together with (central) obesity are named the Metabolic Syndrome (MetS), also known as insulin resistance syndrome, deadly quartet or syndrome X. The International Diabetes Federation indicated that 25% of the world adult population suffered from MetS in 2006 (4), and numbers are rising, in relation with the increasing global trend in obesity (2). After many years of discussion and different definitions, the current criteria of MetS are: having central obesity with a waist circumference specified per ethnicity, plus having at least two of the following metabolic disturbances: raised circulating triglycerides (TG), reduced HDL-cholesterol, hypertension or raised fasting plasma glucose (3, 5).

It is believed that the main contributor or central phenomenon of MetS is a disrupted insulin metabolism, due to insulin resistance and hyperinsulinemia acting in close conjunction (3), resulting in various disturbed metabolic processes. When a surplus of nutrients are digested, the substrates from catabolism "fill up" the TCA cycle, called anaplerosis, leading to a more incomplete oxidation of other metabolites like fatty acids and amino acids. In this state glucose is also no longer being totally used as energy substrate for the TCA cycle (6, 7) and blood glucose levels stay high and keep stimulating insulin secretion eventually leading to hyperinsulinemia (7). Hyperinsulinemia has a large effect on the progress of insulin resistance by making tissues less sensitive to the action of insulin. Hyperinsulinemia is thought not only to be related to insulin resistance and type 2 diabetes, but also to the other characteristics of MetS, like hypertension and cardiovascular diseases (8). Furthermore, insulin resistance is often seen together with a state of chronic low grade inflammation, possibly linking hyperinsulinemia and MetS (8, 9).

Intra hepatic lipid accumulation

Intra hepatic lipid (IHL) accumulation might be considered as the hepatic manifestation of MetS (10). Excess IHL accumulation is referred to as non-alcoholic fatty liver disease (NAFLD), as this is not the consequence of an excessive alcohol use, but a result of dysfunction in liver handling of energy nutrients (11). The spectrum of disease of NAFLD comprises different stages ranging from a fatty liver (5% of the hepatocytes have triglycerides stored), to steatohepatitis, fibrosis and possibly leading to a

hepatocarcinoma (11). It is estimated that 20-30% of the Western population has NAFLD (11), with a higher prevalence in males compared to females (12). Fat accumulation in the liver impairs normal liver function (13, 14) and is correlated with hypertriglyceridemia and a reduced suppressive effect of insulin on hepatic glucose production, which contributes to hyperglycemia (15).

Several mechanisms can be involved in the excessive accumulation of lipids in the liver (**figure 1.1**). The main contributor, as in the MetS, is thought to be insulin resistance. First of all, insulin resistance suppresses the inhibitory effect of insulin on adipose tissue lipolysis leading to increased circulating free fatty acids (FFA) concentrations. These FFA can easily accumulate in the liver as TG (9, 14). Moreover, a surplus of circulating FFA decreases hepatic ß-oxidation and leads to decreased VLDL secretion, also contributing to an increased IHL (16). Second, hyperinsulinemia also increases hepatic *de novo lipogenesis* (DNL), by stimulating hepatic lipogenic enzymes (14). Increased DNL can also be caused by a surplus of dietary carbohydrates leading to increased lipid accumulation as well (17). Lastly, the role of increased insulin resistance on IHL accumulation might also be mediated by adipose tissue-derived hormones like adiponectin and leptin (10). Adiponectin is thought to protect the liver from lipid accumulation, however this hormone is usually low in insulin resistant patient, as its levels are inversely related to adipose tissue mass (10). Leptin is known as the satiety hormone and high leptin levels also protect the liver from lipid accumulation, however a state of insulin resistance often coincides with a state of leptin resistance. Furthermore, insulin action is inhibited by increased leptin concentrations (10) (figure 1.1).

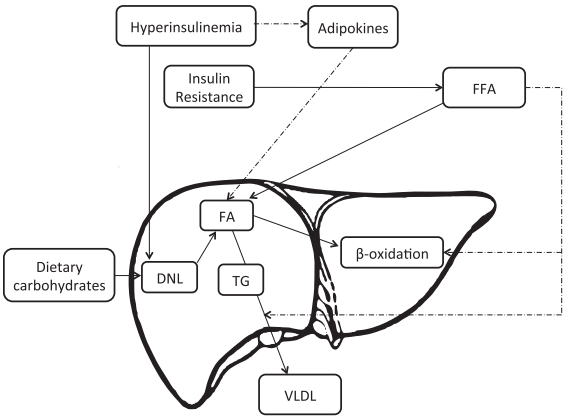

Figure 1.1. Mechanisms of IHL accumulation; dashed lines resemble inhibition. 1) Insulin resistance leads to suppressed effects of insulin on lipolysis and thus increased levels of FFA. 2) Hyperinsulinemia increases DNL leading to increased stores of FA in the liver. 3) Dietary carbohydrates can increase DNL. 4) Adipokines leptin and adiponectin protect the liver from IHL accumulation, however in insulin resistant states their effects are inhibited.

The golden standard for measuring fat accumulation in the liver is histopathological quantification of liver biopsies, but this is a very invasive method and not often used in clinical research (18). Fortunately, there are other, less invasive techniques, like computed tomography (CT), ultrasound (US) and proton magnetic resonance spectroscopy (^{1}H-MRS), which can be used as well. ^{1}H-MRS is the preferred method for quantifying liver fat, due to its non-ionizing radiation properties, and is applied in the present thesis. In short, protons from chemical structures can be aligned by using a strong homogenous magnetic field. Thereafter, giving a radiofrequency pulse the protons will flip with another orientation of their spin nuclei and after excitation, going back to their original alignment with the magnetic field, a radiofrequency pulse can be detected and translated into spectra. Thereafter, the amount of protons can be quantified using special software. Hepatic ^{1}H-MRS is based on the chemical structure of triglycerides like their double bounds and the amount of H-molecules (protons) (19, 20). In a validation study, quantifying liver fat by ^{1}H-MRS correlated very well with the golden standard, histopathological quantification (3T MR, r=0.85; p<.0001) (18) and (1.5T MR, r=0.93; p<.0001) (21). The sensitivity and specificity for detection of steatosis as measured with ^{1}H-MRS were 100% and 87% respectively (18). Measuring liver fat by imaging techniques is time consuming and expensive and not feasible for large observational studies. Therefore, several surrogate formulas or indices predicting fatty liver were suggested (12, 22, 23). 'The fatty liver index' (FLI) (12), was evaluated and proven to be simple and effective in predicting fatty liver and insulin resistance (24, 25). Although ^{1}H-MRS remains a better measure for quantifying liver fat the FLI has a sensitivity of 0.76 [0.50;0.93] and a specificity of 0.83 [0.72;0.90] for ruling in or out steatosis (25).

High-protein diets and the implications for metabolic health

Diet influences the risk of MetS, diabetes and fatty liver (26, 27). This thesis mainly focuses on dietary protein and its effects on lipid and glucose metabolism. Moreover, dietary protein has also metabolic effects on body composition and microbiota composition.

Many different diets for weight reduction and improvement of insulin sensitivity are being considered. In recent years, high-protein diets have become increasingly popular as a way to reduce obesity and improve metabolic risk factors in the general population (28). When comparing two well-known high-protein diets, the Atkins diet and the Zone diet, to two high-carbohydrate dietary strategies for weight reduction, the high-protein diets were more successful although total energy intake was not different among the different diet groups (29). Although, others did not find an additional effect

on weight loss for dietary protein (30). What makes high-protein such popular and a seemingly successful dietary strategy in reducing obesity and its co-morbidities? High-protein diets are known to increase satiety, therefore, people tend to eat less energy and thereby losing body weight when consuming a high-protein diet. A recent meta-analyses showed an acute and increased effect on satiety of high-protein (>20 en%) intake as compared to a normal-protein intake (<20 en%), this effect could be due to the lower postprandial ghrelin response which was observed after the high-protein intake (31). Besides increasing satiety, consuming a high-protein diet can have several other metabolic consequences which will be described in more detail below, i.e. affecting insulin secretion and lipid metabolism, changing body composition, increasing energy expenditure and altering microbiota composition.

Dietary protein and insulin

Dietary protein is known to promote insulin secretion. Insulin secretion induced by a protein meal can improve glucose use by peripheral tissues and thus enhances glucose clearance from the blood (32). Indeed, during conditions of elevated postprandial amino acid levels in healthy subjects, where subjects were infused with amino acids, insulin and glucagon secretion were stimulated (33). But the long term effects, are not entirely understood and it is suggested that hyper-aminoacidemia could promote diabetes via hyperinsulinemia leading to pancreatic beta cell exhaustion (34). This might be an explanation why long term high dietary protein intake, especially of animal origin, was found to be associated with type 2 diabetes in several longitudinal studies (35-38). This discrepancy requires an evaluation of present literature, since high-protein diets are advocated as being beneficial for metabolic health to the general population.

Dietary protein and intra hepatic lipids

Dietary protein can influence the lipid content of the liver. It is known that macronutrient manipulation has the potential to change lipid storage in the liver (27). In humans, a high-fat diet increased IHL after only a few days; the diet-induced increase of IHL was associated with an increase in markers of insulin resistance (39). The opposite was also found: a low-fat diet reduced liver fat accumulation, along with a reduction in fasting insulin levels (39). High-protein diets also have the potential to manipulate IHL. Mice fed a high-protein, high-fat diet, had a lower lipid content compared to mice given a normal-protein, high-fat diet (40). Changes in hepatic metabolism, due to adaptation to a high-protein diet, included a down regulation of lipogenesis and increased gluconeogenesis and glycogenesis (41, 42). In humans, studies evaluating the effect of

increasing protein intake on IHL are scarce, although initial results are promising (43-45). Supplementation with whey protein as well as a whole diet approach, showed that increasing protein intake was able to reduce IHL in human volunteers (43, 44). Since, further description of the possible mechanisms is essential, and to show a 'proof of principle' regarding high-protein intake and liver fat accumulation, additional human trials involving healthy volunteers are warranted to give more insight on the effects of diet on IHL accumulation.

Dietary protein, body composition and lipid metabolism

Since dietary protein affects insulin secretion and IHL, it might be expected that whole body lipid metabolism is altered after consuming a high-protein diet. High-protein diets increase thermogenesis and thereby increase energy expenditure possibly explaining weight loss (29, 46). Moreover, changes in body composition are also attributed to high-protein diets. In weight loss studies, a high-protein diet resulted in a reduced loss of lean body mass and increased fat mass loss (47). Also during overfeeding, diets higher in protein content (15 en% and 25 en%) significantly increased lean body mass, while a low-protein diet (5 en%) did not give such an increment (46). Furthermore, the direct effects of increasing dietary protein on lipid metabolism are thought to be beneficial for plasma lipid profiles (see for review (48)). First of all, dietary proteins are supposed to have hypocholesterolemic features, probably by increasing hepatic bile acid synthesis. In addition, dietary protein also lowers the activity of lipogenic enzymes. However, on this topic there is limited human data available (48). The insulinogenic properties of protein also affects lipid metabolism by, among others, inhibiting FFA release from the adipose tissue and increasing DNL in the liver (14). However, in a state of insulin resistance these actions of insulin are no longer effective. The controversies regarding the possible detrimental effects of dietary protein on glucose metabolism, via enhanced insulin secretion, together with the effects of dietary protein on lipid metabolism make dietary protein an important topic for research.

Dietary protein and microbiota

Recently, changes or differences in microbiota composition were identified to play an important role in obesity and MetS as well (49-51). In humans most of the gut microbiota belong to the two phyla Firmicutes or Bacteriodetes. In obese patients the amount of Firmicutes is increased, whereas the amount of Bacteriodetes is decreased (49). Additionally, changing the microbiota composition by transplantation from a lean donor to an obese patient improved metabolic profile of the recipient (52). Microbiota

composition can also be modified by changing dietary composition. A Western-type of diet can change the microbiota resulting in increased Firmicutes and decreased Bacteriodetes (53). Furthermore, the possible detrimental effect on metabolic health of long term high-protein diets are thought be mediated via the microbiota, which produce toxic substances like amines and sulphur affecting the intestinal epithelium (51). Thus microbiota are an interesting field of research in combating obesity and MetS, possibly via dietary intervention.

Branched chain amino acids and metabolic health

In addition to the above mentioned topics regarding metabolic health, especially insulin resistance, and high-protein diets, there is another matter, concerning the building blocks of dietary protein: amino acids (AA). In states of obesity and insulin resistance it is reported that circulating concentrations of specific AA are elevated (6). These are the sulphur containing AA, methionine (essential AA) and cysteine (conditionally essential AA), and the aromatic AA phenylalanine (essential AA) and tyrosine (conditionally essential AA) (6). Furthermore, branched chain amino acids (BCAA) have been known for decades to be elevated in insulin resistant and obese patients (6, 54). However, to date the question still remains whether high blood concentrations of BCAA are a cause, via their insulinotropic effects resulting in sustained hyperinsulinemia; or consequence, due to increased muscle breakdown as often seen in insulin resistant states, of insulin resistance (6). In this thesis BCAA will be elaborated on. The three BCAA are valine, leucine and isoleucine. All are essential amino acids and cannot be endogenously synthesized; they must be obtained from the diet. The BCAA content is about 20% in mixed protein sources (47) and of the dietary BCAAs absorbed, about 80% reaches the blood circulation (47). Several studies identified an association between an increased risk of insulin resistance and/or type 2 diabetes and plasma BCAA levels (34, 54-57). Circulating BCAA concentrations might be elevated due to decreased catabolism, increased dietary intake or increase release from stores. It is essential to get better understanding of what is the relationship between high levels of BCAA, dietary intake and (patho)physiological consequences, in order to be able to give better dietary advice to the (obese) population.

Rationale and objectives of this thesis

The objective of this thesis is to give more answers on the consequence of high-protein diets on metabolic health. First, there is a need to get better understanding on what the effect of dietary protein is on glucose metabolism; thereafter the effect of increased

dietary protein on lipid metabolism has to be studied. This together might give a better and more complete view on dietary proteins and metabolic health. Moreover, metabolic health is thought to be dependent on microbiota composition. The effects of high-protein diets on microbiota are thought to be unfavourable, therefore, we wanted to know the effects of a high-protein diet on microbiota and whether this can be linked to our findings on glucose and lipid metabolism. Lastly, the controversies regarding BCAA, insulin resistance and dietary protein need to be further elucidated to learn what the interactions are. This all combined results in the main objective of this thesis: to identify the effects of dietary protein on metabolic health focusing on lipid and glucose metabolism—with implications for metabolic disorders, like liver fat accumulation and insulin resistance, body composition and gut microbiota alterations.

The specific study aims per chapter are as follows:

- To discuss human studies addressing high dietary protein intake and insulin action, with special attention for BCAA. Additionally, to highlight the (patho) physiological consequences of high-protein diets regarding insulin action, and in particular the role of the mTOR pathway.
- To assess the relationship between macronutrient intake, food pattern, diet quality and fatty liver as scored by the 'Fatty Liver Index' FLI, in a large cross-sectional analysis of a ongoing Dutch longitudinal study, with a well characterized population.
- To evaluate effect of increasing protein intake, at the expense of carbohydrates, on intra hepatic lipids (IHL), circulating triglycerides (TG) and body composition in healthy humans when consuming a high-fat, hypercaloric diet.
- To evaluate the effects of a two week adaptation to a high-protein, high-fat, hypercaloric diet on the microbiota composition of healthy young volunteers.
- To evaluate the effect of a two week short-term adaptation to a high-protein diet on postprandial carbohydrate and lipid metabolism in healthy human subjects, by applying a meal challenge (MC).
- To investigated the association of BCAA with detailed body composition parameters, including abdominal visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT).

Outline of this thesis

Central in this thesis is a strictly-controlled dietary intervention study in young healthy adults. But first, in **chapter 2**, the controversies found in the relation between high-protein diets and insulin resistance are reviewed. Subsequently, in **chapter 3**, we tried

to unravel, in a longitudinal study, in a well described Dutch population, the question whether long term high-protein intake would affect intra hepatic lipid content. **Chapter 4** provides the results of our human dietary intervention study. In that study we investigated what the short-term effect of a high-protein hypercaloric diet is on IHL and fasting lipid metabolism in healthy young adults. As mentioned above microbiota are a hot topic in obesity and might be of great importance in the obesity epidemic. Therefore, in **chapter 4b**, we studied the effects of increasing the protein content in the hypercaloric-diet on microbiota composition in healthy young subjects. **Chapter 5** further explores the short-term adaptation to the high-protein hypercaloric diet, by applying a meal challenge and evaluating postprandial lipid and carbohydrate metabolism. In **chapter 6**, in a different population (e.g. lean and obese subjects) the relationship between circulating BCAA and visceral adiposity and, as well as measures of insulin sensitivity was explored. Finally, in **chapter 7**, the general discussion, I will give an answer on the main objective and provide directions for future research.

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Chapter 2

High dietary protein intake, reducing or eliciting insulin resistance?

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Abstract

Dietary proteins have an insulinotropic effect and thus promote insulin secretion, which indeed leads to enhanced glucose clearance from the blood. On the long term, however, a high dietary protein intake is associated with increased risk of type 2 diabetes. Moreover, branched-chain amino acids (BCAA), a prominent group of amino acids, were recently identified to be associated with diabetes. Observational data and intervention studies do not point in the same direction regarding the effect of protein intake on insulin sensitivity and diabetes risk. Therefore, the first aim of this review will be to discuss human studies addressing high dietary protein intake and insulin action, with special attention for BCAA. In the second part, we will highlight the (patho) physiological consequences of high-protein diets regarding insulin action, in particular the role of the mechanistic target of the rapamycin pathway.

Introduction

Insulin resistance is defined as tissues no longer being sensitive to the physiological actions of insulin, in particular glucose uptake. The insulin-mediated uptake of glucose can decrease when tissues are chronically over-exposed to high levels of insulin. Thus, prolonged hyperinsulinemia can lead to insulin resistance and eventually type 2 diabetes mellitus (1). Lifestyle factors like physical activity and diet have a key role in the development of insulin resistance. Many different diets for weight reduction and improvement of insulin sensitivity are advocated. Diets high in protein content and low in carbohydrates, such as the Atkins diet or Zone diet, have demonstrated a positive effect on body composition and body weight. However, the effect of those high-protein diets on insulin sensitivity are somewhat controversial. In the beginning of the 20th century, Jacobsen (2) identified a high-protein diet as an insulin sensitivity improving strategy. Dietary proteins have an insulinotropic effect and thus promote insulin secretion, which indeed leads to enhanced glucose clearance from the blood (3). In the long term, however, a high dietary protein intake has been associated with an increased risk of type 2 diabetes (4-7). Moreover, branched-chain amino acids (BCAA), a prominent group of amino acids, and a significant part of dietary protein, were recently strongly suspected to be associated with diabetes (8, 9). Observational data and intervention studies do not point in the same direction regarding the effect of protein intake on insulin sensitivity and diabetes risk. Therefore, the aim of this review was first to discuss human studies addressing high dietary protein intake and insulin action, with special attention for BCAA. In the second part, we wanted to highlight the (patho)physiological consequences of high-protein diets regarding insulin action, and in particular the role of the mechanistic target of the rapamycin (mTOR) pathway.

Methods: Selection of relevant studies

There are many human studies available on high-protein intake and insulin sensitivity. However, comparing these studies is difficult. This is mainly due to differences in the control diet used, duration of the study, energy balance, that is, with or without weight loss, the source and amount of protein used and differences in the type of subjects included: non-obese, overweight, non-diabetic and diabetic subjects. Comparing studies can be made easier when studies are organised by those factors. For the first part of this overview article, recent - year 2000 and onwards - human intervention

studies were selected in which a dietary protein content of >20 energy percentage (En%) was used and which measured insulin sensitivity. The present review aims to give a broad overview on the subject, but does not intend to be a complete systematic review. Furthermore, for a better comparison, studies were arranged by the duration of the intervention: short term (less than 6 months of intervention) versus long term (more than 6 months of intervention, and including observational studies). Within these topics, a distinction was made between the type of subjects prescribed the diets, followed by interventions with weight loss. For all studies mentioned the amount of protein is expressed En% considering that a high-protein diet refers to a protein content of >20 En%. For a summary of the studies cited in this overview article on the effect of high-protein diets on insulin action in humans, see **table 2.1**.

Table 2.1. Overview of effects of high-protein diets on insulin action in human studies

Duration of the high-protein diet (>20 en%)	Type of subjects	Effects on insulin resistance (IR)
1 week – 6 months (intervention studies)	Healthy	• No effect on IR (10-12) • Reduction in daily plasma insulin (13)
	Obese	• Improves IR(14) • No effect on IR (15, 16) • Reduced IR (17) • Improves IR if weight loss is present (22-24) • No effect on IR if weight loss is present (25-27)
	Insulin resistant, DM2	• Improves IR (18, 19) • Decreased postprandial glucose concentration (20) • No effect on IR if weight loss is present (28-31)
>6 months (longitudinal studies)	NA	• Increased DM2 risk (4-6, 33). • Reduction in DM2 risk (34)

High dietary protein diets and insulin action

Short-term, energy balanced, high-protein diets

In healthy non-obese volunteers, data on the short-term consequences of manipulating protein intake on insulin action are limited and showing only minor effects. When healthy subjects were fed a high-fat diet (39.4 En%), high in protein content (25.7 En%), for 2 weeks, this did not have any effect on insulin and glucose homeostasis when compared with a normal protein (15.4 En%) high-fat diet (37.7 En%) (10). Similarly, in a young and an old group of healthy subjects, a high-protein diet did not affect insulin sensitivity, and the acute insulin response to glucose also did not vary between age

group or between diets. Here, 10 days of a high-protein diet (21 En% in the young group and 24 En% protein in the old group) was compared with a normal protein diet (11 En% in the young group and 12 En% protein in the old group). Furthermore, the fat content differed between diets, whereas the carbohydrate content was kept constant at 50 En% (11). In the same way, two months of a high-protein diet (29 En%) did not change insulin action and secretion compared with a normal protein diet (16 En%) with a similar fat content (30 En%) in healthy subjects (12). However, an acute high-protein diet (35 En%, 1 week), with very little carbohydrates (5 En%) caused an instant postprandial reduction in daily plasma insulin (13).

In overweight and obese subjects, the short-term effect of increasing protein, without weight loss, is somewhat more diverse. Increasing dietary protein to 35 En% for 12 weeks with a whey supplement, compared with subjects supplemented with glucose (16 En% protein), resulted in improved insulin sensitivity in overweight subjects; carbohydrates were exchanged for protein, whereas fat intake was kept constant (29 En%) (14). Yet, in a similar population, no effect on insulin sensitivity was seen after six weeks of a diet restricted in carbohydrates (13 En%), which were replaced by proteins (29 En%). However, although carbohydrates were replaced by protein, the fat (and consequently the energy) content of the test diets were also increased. Thus, the fact that insulin sensitivity did not change could be due to the increased fat content of the diet and could have less to do with the exchange of protein for carbohydrate (15).

Similarly, after a period of initial weight loss, a low-fat diet (24 En%) supplemented with either casein or whey (35 En% protein) did not alter insulin sensitivity compared with a high-carbohydrate diet (16 En% protein, 63 En% carbohydrate) (16). However, Weickert et al. demonstrated reduced insulin sensitivity after 6 weeks on a high-protein (25-30 En%), reduced-carbohydrate (40-45 En%) diet. This diet contained high amounts of legumes and dairy products, and was compared with a high-fibre diet (protein 15 En%, carbohydrate 55 En%) (17). Yet, the observed effect weakened after 18 weeks (17). In type 2 diabetic patients, 5 weeks on a high-protein (30 En%), low-carbohydrate (20 En%) diet without weight loss improved insulin sensitivity compared with a normal protein (15 En%), normal carbohydrate (55 En%) diet. No differences were observed between the two diets in fasting insulin levels (18, 19). The same high-protein diet did result in a decrease in both postprandial glucose and in the overnight fasting glucose concentration (20). Therefore, in short-term studies without weight loss in healthy, as well as in overweight and type 2 diabetic subject, effects of high-protein diets on insulin sensitivity are inconclusive. In addition, when dietary protein is increased by decreasing the carbohydrate content of the diet, it is difficult to determine which is responsible for any effects on insulin sensitivity.

Short-term, energy-restricted, high-protein diets

In overweight, obese and type 2 diabetic subjects, the majority of studies using high-protein energy-restricted diets have focused on weight loss. Weight loss, however, is known to have a strong beneficial influence on insulin sensitivity (21). Improved insulin resistance was observed when a high-protein (27 En%) energy-restricted diet at the same time contained a low amount of carbohydrates (17 En%) compared with the baseline diet (protein 18 En%, carbohydrates 42 En%) (22). Eight weeks of a high-protein (30 En%), reduced-carbohydrate (33 En%) energy restricted weight-loss diet improved insulin sensitivity, compared with other weight loss diets (protein 19 En%, carbohydrate 51 En%), which were either high in fatty fish and legumes, or a balanced control diet (23). The same was observed after a 10-week energy-restricted diet relatively high in both fibre and protein (24 En%; carbohydrate 45 En%) compared with standard dietary advice (protein 19 En%; carbohydrate 46 En%) (24). However, Rizkalla et al. (25) showed that HOMA-IR improved more on a conventional energy-restricted weight-loss diet (21 En% protein, 44 En% carbohydrate), whereas ß-cell function improved more after the high-protein weight-loss diet (33 En% protein, 40 En% carbohydrate). In overweight subjects, a high-protein (30 En%) very low carbohydrate (4 En%) energy-restricted diet did not offer any metabolic advantage on insulin sensitivity over a moderate-carbohydrate weight-loss diet (26, 27). In addition, in studies considering obese type 2 diabetic subjects and aiming at weight loss, high-protein energy-restricted diets with carbohydrates exchanged for proteins showed no additional effect on insulin sensitivity above energy restriction (28-30). Sargrad et al. (31) even found a high-carbohydrate (51 En%; protein 19 En%) energy-restricted diet to be superior to a high-protein diet (27 En%; carbohydrate 43 En%) in terms of improving glycaemic control and insulin resistance.

It might be concluded that the improved insulin sensitivity with high-protein energy-restricted diets in overweight, obese and type 2 diabetic subjects is, at least partly, dependent on weight loss. This was also confirmed by a recent meta-analysis considering high-protein energy-restricted diets, concluding that there is no effect of protein intake on glucose homeostasis; this effect was not adjusted for the extra weight loss of 0.79 kg in the high-protein diets compared to the standard protein diets (32).

Long-term protein intake

In healthy subjects, fed for 6 months in energy balance, a diet high in protein (24 En%) compared with a normal protein diet (10 En%) induced a state of higher insulin

resistance and glucose intolerance (33). Thus, in this study, a long-term consumption of high-protein diet in healthy subjects seems to decrease insulin sensitivity. Moreover, in observational studies, long-term high dietary protein intake is associated with an increased risk of developing metabolic syndrome or diabetes type 2 (4-6). However, the nurses' health study observed that diets lower in carbohydrate and higher in protein and fat were not associated with an increased risk for diabetes type 2 (34). Even a slight reduction of the risk was observed when vegetable sources of protein and fat were chosen. It was suggested that reducing the glycaemic load of a diet was the beneficial underlying factor of the reduced diabetes risk (34).

Recently, attention was attracted by several studies pointing to an association between plasma BCAA levels and an increased risk of insulin resistance and/or type 2 diabetes (8, 9, 35, 36). The three BCAA are valine, leucine and isoleucine, which are all considered essential amino acids. The BCAA content of mixed protein sources is about 20% (37). Higher levels of BCAA can be found in whey protein and to a lower extend in cod protein (14, 37, 38), with leucine being the most abundant. Of the dietary BCAA ingested, about 80% reaches the blood circulation (37, 39, 40). High-protein intake or the intake of high levels of BCAA as supplements increases blood plasma concentrations of BCAA at least in shorter-term dietary interventions (11, 41, 42). Newgard et al. (9) found a linear relationship between increased levels of plasma BCAA and decreased insulin sensitivity, as measured by an increase of the HOMA index. Levels of plasma BCAA were higher in obese as compared with lean Caucasian and African-American subjects (9). Similar results were found in an all Asian population, where increased levels of BCAA were observed in subjects diagnosed with insulin resistance by a high HOMA index (35). This inverse relationship between insulin sensitivity and BCAA levels, however, does not prove cause-effect relationships, as, BCAA levels may be high just because of insulin resistance. Since, tissues are no longer sensitive for the lowering effect of insulin on proteolysis leading to increased levels of circulating BCAA. Conversely, reducing body weight improves insulin sensitivity, thereby decreasing proteolysis resulting in decreased levels of circulating BCAA.

It was suggested that this relationship might be due to increased protein turnover and/or decreased rates of BCAA catabolism; increased BCAA levels may also be indicative of sarcopenia (35). The consequence of weight loss on insulin resistance could even be predicted by decreased circulating BCAA levels (43). Thus, plasma BCAA, which also represents BCAA catabolism, was correlated with insulin resistance, with low BCAA concentrations predicting improved insulin sensitivity when subjects had moderately lost weight (35). Furthermore, in longitudinal studies, it has been shown that elevated

plasma levels of BCAA and aromatic amino acids were good predictors of future development of type 2 diabetes (8, 36). It has been suggested that hyperaminoacidaemia could promote diabetes via hyperinsulinaemia leading to pancreatic beta cell exhaustion (8). On the other hand, another study demonstrated that peripheral insulin response was improved after weight loss in insulin-resistant offspring, yet this response was independent of plasma BCAA (44). Wurtz et al. (45) also found no association between HOMA-IR and fasting amino-acid levels, indicating that amino acids do not have a role in the pathogenesis of insulin resistance (45). Besides, whether high blood concentrations of BCAA could be a cause or consequence of insulin resistance remains unclear (46). A recent meta analyses - which included 15 randomized controlled trials of more than 12 months - on the long-term effect of diets high in protein (>25 En% of protein) showed neither a positive nor a negative effect on glycaemic control compared to diets low in protein content (10-15 En%) in both healthy and insulin-resistant subjects (47).

In conclusion, studies considering high-protein diets and insulin action are not univocal. A beneficial effect on insulin sensitivity with high-protein diets is mainly observed in overweight and insulin-resistant subjects, when weight loss is present. Next to weight loss, these effects are most frequently explained by the insulinotropic properties of amino acids or the decreased glycaemic load of the high-protein diets. In healthy subjects, short-term manipulation of protein intake did not affect insulin action, whereas a high-protein diet seems to be deleterious when the intake is prolonged. This is in line with the observed association between plasma BCAA levels, insulin resistance and diabetes risk. In the long term, increased insulin secretion and consequent hyperinsulinemia might lead to reduced hepatic insulin sensitivity. Increased hepatic glucose output results in a decreased glucose control, although a direct effect in insulin action in insulin-sensitive tissue can also have a role.

Physiological pathways linking protein intake and insulin action

In the second part of this review, first the insulinotropic effect of dietary proteins will be discussed, thereafter the topic of BCAA and insulin resistance will be highlighted. Finally, the relationship of BCAA and insulin resistance will be linked via the mTOR pathway.

Insulinotropic effect of dietary proteins

It is well known that dietary proteins promote insulin secretion, which leads to enhanced glucose clearance from the blood by peripheral tissues (3). Many intervention studies have confirmed this effect and underscored that amino acids have an important role in

mediating insulin and glucagon secretion (48, 49). It has been shown that whey protein and its hydrolysates resulted in a direct postprandial increment of insulin levels, some to a larger extent than others (50-52). In lean healthy subjects, a single high-protein test meal (50 En% protein, 40 En% carbohydrate, 10 En% fat) with either whey, casein or soy protein lowered peak glycaemia significantly, as compared with a carbohydrate test meal (1.2 En% protein, 95.5 En% carbohydrate, 3.3 En% fat). Plasma insulin was significantly higher after a whey protein-rich meal compared with a high-glucose meal (50). Moreover, this higher insulin level was still present after 330 minutes when consuming whey, whereas this was not the case for glucose, casein and soy meals (50). Indeed, when conditions of elevated postprandial amino-acid levels were artificially created in healthy subjects, insulin and glucagon secretion were stimulated (48). However, elevation of plasma amino acids to postprandial levels also caused insulin resistance by direct inhibition of muscle glucose transport and/or glucose phosphorylation with subsequent reduction in rates of glycogen synthesis. Thus, on the other hand, amino acids might have a role in the modulation of peripheral insulin sensitivity and contribute to insulin resistance (53). The stimulating effect of protein on insulin might be a strategy in insulin-resistant subjects (54). However, it could be harmful in healthy subject in the long run, owing to prolonged hyperinsulinemia, which can lead to decreased insulin sensitivity.

BCAAs and insulin resistance

In humans, high levels of plasma BCAA, which might partly be derived from dietary protein, are linked to insulin resistance and diabetes via insulin secretion and subsequent hyperinsulinaemia (8, 9). Although high levels of BCAA can be found in whey protein (38), the relationship between diet, circulating BCAA and insulin resistance deserves further exploration. A cause-effect relationship is not established yet and that it is still discussed whether plasma BCAA levels reflect long-term protein intake (35, 45).

When looking at the short-term effect of high-protein intake, circulating BCAA increased 25% after a 22 En% high-protein diet in young subjects without affecting insulin sensitivity (11). On the other hand, insulin secretion was increased when supplementing lean subjects with BCAA-rich whey protein, compared to supplementing with either egg, fish or turkey protein, but postprandial AA profiles were not measured in this study (55). In addition, a 4-week cod protein diet improved insulin sensitivity compared with an animal meat and milk protein diet with the same amount of protein; the lower concentration of BCAA in the cod, compared with the other animal protein source, was

proposed to explain this observation, but this conclusion remains speculative as cod protein also has a relatively high BCAA content compared with usual mix protein diet (that is >20%) (56). In the long term, 6 months of supplementation with leucine did not improve glycaemic control in elderly type 2 diabetic men (57). Similarly, no differences in glycaemic levels after increasing BCAA intake were observed in patients with chronic hepatitis C and insulin resistance (42). Here it was concluded that BCAA might even have a beneficial effect on glycated hemoglobin values in insulin-resistant subjects. Given the fact that plasma amino acids in the postprandial state also respond to insulin secretion with more sustained long-lasting changes than glucose levels, it was proposed that plasma BCAA might even serve as better indicators of impaired insulin sensitivity in prediabetic states than glucose levels (58).

Increased BCAA can also be a consequence of insulin resistance. An important player in this light is BCKD (branched-chain α-ketoacid dehydrogenase), the rate-limiting enzyme in BCAA oxidative catabolism. BCKD activity is inhibited by insulin, and as a result, BCAA catabolism is inhibited (46, 59). Elevated plasma-free fatty acid levels, commonly observed in insulin-resistant subjects, reduce BCKD activity as well (46). Thus, it might be suggested that raised blood BCAA in insulin resistance actually reflects reduced BCKD activity and is a consequence rather than a cause of insulin resistance (46). However, although *in vitro* data support this concept, *in vivo* studies are nonconsistent, as no decrease of leucine oxidation was observed with hyperinsulinaemia-euleucinaemia (60).

Yet, in rats fed a high-fat BCAA diet, insulin resistance was induced accompanied by activation of the mTOR pathway (61). The mTOR pathway is a nutrient-sensing pathway, which integrates nutrient sensing and insulin signalling to coordinate cell growth and metabolism (**figure 2.1**) (62, 63), and it could have a crucial role in understanding the association between BCAA and insulin action. BCAA and other nutrients activate mTOR. Among other signals, this activation can lead, in a negative feedback loop, to phosphorylation of the insulin receptor substrate 1 (IRS1), leading to decreased insulin sensitivity. This provides a potential link between BCAA and insulin resistance or diabetes risk.

The mechanistic target of rapamycin (mTOR) pathway

mTOR is a serine-threonine protein kinase widely expressed in various tissues and involved in many important cellular functions. It was first identified in yeast as a target of rapamycin (64)a. In mammals, two multiprotein complexes containing mTOR were identified (63). mTORC1 is composed of a number of proteins, which include mTOR itself (the catalytic subunit of the complex), RAPTOR (regulatory-associated protein

of mTOR), mLST8 (mammalian lethal with Sec13 protein 8), PRAS40 (proline-rich AKT substrate 40kDa) and Deptor (DEP-domain-containing mTOR interacting protein). Similarly, the mTORC2 complex is composed of mTOR, mLST8 and Deptor, present in mTORC1, but also contains Rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein) and Protor-1 (protein observed with Rictor-1). In contrast to mTORC1, mTORC2 is insensitive to the mTOR inhibitor rapamycin (65).

The regulation of the mTOR complexes is intricate and yet not fully understood. It is activated in the fed state initiating anabolism and energy storage (66). mTORC1 integrates signals from nutrients such as amino acids and glucose but seems to be more particularly sensitive to amino acid signalling and especially leucine (figure 2.1).

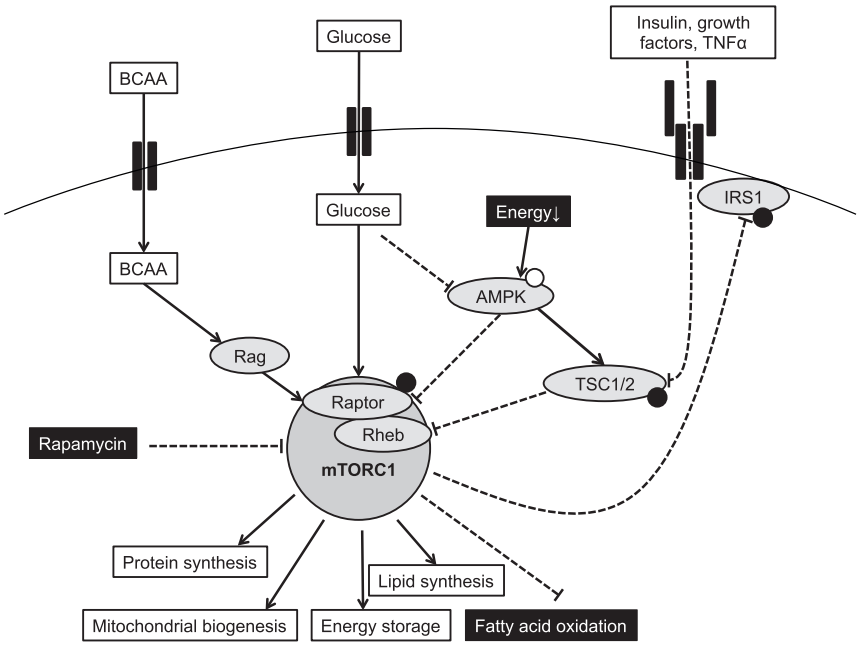

Figure 2.1. Nutrient-sensing pathways of mTORC1. BCAA and glucose activate mTORC1, resulting in the induction of protein synthesis, mitochondrial biogenesis, energy storage and lipid synthesis. Fatty acid oxidation and activity of the IRS1 is inhibited. Insulin, growth factors and TNF α activate mTORC1 via the phosphorylation (P) and inhibition of the mTOR inhibitor TSC1/2 (tuberous sclerosis complex heterodimer). Rapamycin and a drop in energy inhibit mTORC1 action.Raptor, regulatory-associated protein of mTOR; Rheb, Ras homolog enriched in brain; AMP-activated protein kinase (AMPK); White boxes and solid flashes represents activation; black boxes and dotted lines represents inhibition.

It is suggested that leucine activates mTOR by translocation Rag proteins and their binding to Raptor (67). This leads to a relocation of the complex to a perinuclear region containing the activator Rheb. An endogenous suppressor of mTORC1 is the tuberous sclerosis complex heterodimer (TSC1/2) which, when active, inhibits Rheb (66, 68). Endogenous signals such as growth factors (Insulin, IGF), hormones and TNF lead to an activation of mTORC1 by phosphorylating and thereby deactivating TSC1/2. In turn, in a negative feedback loop activation of mTORC1 causes phosphorylation of IRS1. This inhibits the association with the insulin receptor and thereby lowers insulin sensitivity. In the fasted state, mTOR activity is supressed. During energy depletion AMP-activated protein kinase is phosphorylated and inactivates mTOR either directly or via activation of TSC1/2. mTORC1 is considered to be an important regulator of many cellular processes, especially cell growth and metabolism. It positively regulates protein and lipid synthesis, as well as mitochondrial biogenesis and metabolism.

MTOR-pathway in the liver

The liver acts as a buffer for the peripheral availability of nutrients, most importantly in maintaining blood glucose levels. The mTOR pathway is suggested to have an important role in this regulatory metabolism. In the past years, several genetic engineered mice elucidated mTOR function in the liver. During fasting, liver mTORC1 is responsible for providing peripheral organs with ketone bodies as an energy source (69). Adaptation to a high-protein diet leads to increased mTOR phosphorylation and, consequently, its activation in the liver of rats (70). Overactivation of the mTOR pathway in turn was reported to cause higher sensitivity toward hepatic steatosis when fed a high-fat diet (71). Furthermore, it was associated with decreased hepatic insulin sensitivity (62). Leucine deprivation on the other hand lowered mTOR phosphorylation and improved insulin sensitivity (72). Loss of mTORC1 function in the liver enhanced glucose tolerance and increased insulin sensitivity (73). As a consequence of deregulated hepatic insulin signalling, mTORC1-dependent effects on lipid metabolism were reported (74). In primary hepatocytes, mTORC1 activation lead to initiation of lipogenesis via SREBP1c (75), whereas inhibition by rapamycin increased fatty acid oxidation (76). Data in human are scarce. However, it is suggested that post-transplantational side effects after treatment with rapamycin impairs lipid and glucose metabolism via mTORC1-dependent mechanisms (77).

MTOR-pathway in the muscle

In the muscle, mTORC1 mainly has a role in protein synthesis (78), and muscle-specific loss of function of mTORC1 by depletion of Raptor causes musclar dystrophy. This is not the case with loss of mTORC2 function (79). In addition, mTORC1 is lately also suspected to be involved in muscular insulin resistance (78) as in glucose homeostasis. It was suggested that amino acids activate the mTOR pathway and thereby inhibit insulin-stimulated glucose uptake into skeletal muscle via phosphorylation of IRS1 (78). This was demonstrated *in vitro* and could be reversed by treatment with the mTOR inhibitor rapamycin (80, 81). Also *in vivo*, rats fed a high-fat BCAA diet developed insulin resistance accompanied by increased phosphorylation of mTOR in IRS1 in skeletal muscle (9). In humans, amino acid-induced insulin resistance in skeletal muscle was assessed during a hyperinsulinemic clamp (80). In muscle biopsies, a combination of amino acid and insulin infusion strongly increased IRS1 phosphorylation, indicating inhibited insulin-dependent glucose uptake (80).

Concluding remarks

It is possible that healthy individuals consuming a high-protein non-restricted diet, containing more than 20 En% of protein (that is, consuming more than the Population Reference Intake of 0.83 g protein/kg/d), can lead to hyperinsulinaemia and in the long term can cause insulin resistance. However, as a dietary strategy, high-protein non-energy-restricted diets, containing more than 20 En% of protein, might be helpful for obese people in reducing body weight and subsequently increasing insulin sensitivity also owing to the insulinotropic effect of dietary protein. Yet, the results on circulating BCAA levels in relation to insulin resistance should be further explored on the effect of diet on these metabolic biomarkers. It is still discussed whether there is a relationship between long-term protein intake and BCAA plasma levels and whether high BCAA levels are a cause or consequence of insulin resistance. One possible mechanism can be the activation of mTOR by nutrients (for example, BCAA) leading to a phosphorylation of the IRS1. This supresses IRS1 function and might lead to a decrease in insulin sensitivity. The role of mTOR activation by BCAA in insulin resistance is not clear yet. However, activation of this pathway should be more explored when looking at the effect of high-protein diets on insulin resistance.

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Chapter 3

The relationship of macronutrients, food groups and diet quality with non-alcoholic fatty liver in a general Dutch population

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Abstract

Non-alcoholic fatty liver disease (NAFLD), the accumulation of triglycerides within hepatocytes, is considered the hepatic manifestation of the metabolic syndrome. Diet is known to affect liver fat accumulation in humans. The objective was to assess the relationship between dietary intake and fatty liver as scored by the validated Fatty Liver Index (FLI), in a large cross-sectional study among a general Dutch adult population. A total of 1283 participants of the NQ-plus study, aged 20-70y, were included. At baseline, anthropometrics, blood sampling, and dietary assessment with validated FFQ were performed. FLI was calculated from BMI, waist circumference, triglycerides and gamma-glutamyltransferase. Associations were adjusted for energy intake, alcohol intake, age, sex, education, smoking and prevalence of hypertension and diabetes. Additionally, the Dutch Healthy Diet index (DHD)-score was assessed.

In this population (age: 53.6 ± 11.2y; BMI: 25.9 ± 4.0 kg/m^2; FLI 35.6 ± 27.8), the prevalence of fatty liver as indicated by an FLI>60, was 22.0%. Compared to persons with a normal FLI score of <30, protein intake was positively associated with high FLI score >60 (OR: 1.26 per 1 en%, 95%CI 1.16-1.37). This was especially clear for protein intake from animal sources (OR 1.28, 95%CI 1.19−1.38). Furthermore, the DHD-index, was significantly lower in the high FLI group (FLI<30: 63.4 ± 10.8 vs. FLI 30-60: 62.1 ± 10.1 vs. FLI>60: 59.3 ± 11.1; p<.0001). Subjects in the highest FLI-category were more likely to be male, have a higher BMI and a larger waist circumference. Furthermore, these subjects consumed more protein, especially from animal origin. Additionally, people in the highest FLI group had a lower DHD-score reflecting a lower adherence to the Dutch healthy eating guidelines. Results are in line with previous studies on protein intake and diabetes risk.

Introduction

Non-alcoholic fatty liver disease (NAFLD), the accumulation of triglycerides within hepatocytes, is a rapidly growing health problem, and considered as the hepatic manifestation of the metabolic syndrome (1). The spectrum of disease of NAFLD comprises different stages ranging from a fatty liver, to steatohepatitis, fibrosis, cirrhosis, and eventually leading to hepatocarcinoma (2). NAFLD can lead to a state of cell dysfunction and cell death, and hence a diminished functional capacity of the liver characterized by a reduced insulin clearance and a diminished suppression of hepatic glucose production by insulin among others (3-5). It is estimated that 20-46% of the general adult Western population suffers from NAFLD (2, 6). In obese persons and diabetic patients the prevalence of NAFLD is even higher with estimates ranging from 24%-98% and 30%-56% respectively (7). Furthermore, a fatty liver is more prevalent in males compared to females as was shown in an Italian population (8).

Diet is known to affect liver fat accumulation in humans. In patients with NAFLD, a diet containing more carbohydrates and less fat, but equal amounts of protein and calories, was associated with a greater histological severity of NAFLD (9). Furthermore, an association between high glycaemic index diet and liver steatosis has been suggested (10). Especially a high intake of simple carbohydrates such as fructose, mostly by consumption of soft drinks, is thought to be related to the development of NAFLD and non-alcoholic steatohepatitis (NASH) (11-14). On the other hand, a recent meta-analysis indicated that the relationship between fructose intake and NAFLD might be confounded by energy intake (15).

Fatty acids are other nutrients of interest in NAFLD. It was shown that NASH patients had a higher consumption of saturated fatty acids compared to healthy controls (16). However, a more recent cross-sectional study did not observe a higher intake of dietary fat in NAFLD and NASH patients compared to healthy, overweight controls (17). Yet, a higher fat content of the diet can rapidly increase the intrahepatic fat content, as was observed in a dietary intervention study in 10 obese women (18).

Ricci et al. (19) concluded from a cross-sectional data in obese subjects, that particularly a high carbohydrate intake was associated with an increased risk of NAFLD, whereas a high animal protein intake was associated with insulin resistance (19). However, this study was performed in 63 patients only (19). Altogether, associations between long term dietary intake and NAFLD have been investigated, but these studies mainly focussed on carbohydrate and fat intake, whereas protein intake might have been overlooked in that regard (20). However, in experimental studies we (21) and others (22) have shown that

increasing the protein content of the diet, at the expense of carbohydrates, reduced the intrahepatic liver content.

Besides a focus on macronutrients, it is necessary to keep in mind that humans do not exclusively consume a certain nutrient, but eat a complete diet. Therefore, it is important to take the dietary pattern into consideration. For instance, NAFLD patients consumed more fast food than subjects without signs of NAFLD (6). To our knowledge only one study so far investigated the association of dietary patterns and hepatic liver fat content. This population-based German study indicated that dietary patterns associated with a fatty liver were characterized by a lower intake of tea, confectionary, fats, bread, breakfast cereals and cheese, and by a higher intake of soups, beer, wine, juice, poultry and eggs (23).

Liver fat can be measured non-invasively by using imaging techniques like magnetic resonance imaging (MRI) or computed tomography (CT). This is, however time consuming and expensive and not feasible in large observational studies. Therefore, several surrogate formulas and indices predicting fatty liver are proposed and validated (8, 24-26), amongst which the fatty liver index (FLI) by Bedogni et al. (8). The objective of the present study was to assess the relationship between dietary intake and fatty liver as scored by the 'Fatty Liver Index' FLI, in a large cross-sectional analysis of an ongoing Dutch longitudinal study, with a general Dutch population.

We hypothesized that a high-carbohydrate intake would be associated with a high FLI, whereas a high-protein intake would be associated with a low FLI. Furthermore, dietary intake can also be scored for assessment of nutrient density and dietary quality of the complete diet. The Dutch Healthy Diet index (DHD-index) was developed to evaluate dietary quality according to the Dutch Guidelines for a Healthy diet, and to assess nutrient density in a Dutch population (27). The association between the DHD-index and NAFLD has not been considered so far. Therefore, adherence to the Dutch Guidelines for a Healthy diet, as assessed by the DHD-index, was hypothesized to be associated with a lower FLI.

Participants and Methods

Study design and Population

For the present study, baseline data of the ongoing Dutch Nutrition Questionnaires plus (NQplus) study were used. NQplus is a large longitudinal observational study with multiple aims, conducted within the surroundings of Wageningen. The aims of the

study are to study dietary factors and intermediate health outcomes. The NQplus study was approved by the ethical committee of Wageningen University and is conducted according to the guidelines laid down in the declaration of Helsinki.
Men and women aged 20 to 70 years and who were able to speak and write Dutch were eligible to be included in the NQplus study. Recruitment for participants living in Ede, Wageningen, Renkum or Arnhem was done via the 'EetMeetWeet' research panel. The 'EetMeetWeet' research panel is a randomly selected sample of subjects interested to answer questionnaires on nutrition and health.
Baseline measurements consisted of 2 methods of dietary assessment: FFQ and 24-hour recalls, anthropometric measurements, blood sampling which were repeated after 1 and 2 years.
Between May 2011 and February 2013, 2048 men and women were included in the NQplus study. For the present analysis, subjects were excluded because of missing data needed to calculate FLI (n=177); missing FFQ data (n=289); missing physical activity data (n=245); implausible energy intake (energy intake females <500, >3500; energy intake males <800, >4200; n=12); self-reported known liver disease (n=27); missing data on liver disease (n=11); and those with a daily alcohol consumption of >60g/d (n=10). As a result, the analytical sample comprised 1283 subjects.

Fatty liver index
Fatty liver index (FLI) was calculated according to the formula generated by Begoni et al. (8).

$$FLI = (e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(\text{gamma glutamyltransferase}) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(\text{gamma glutamyltransferase}) + 0.053*\text{waist circumference} - 15.745}) * 100$$

Thereafter, subjects were divided into three groups according to their FLI: FLI < 30, FLI 30-60, FLI > 60. Where a score of <30 can rule out and a score of >60 can rule in fatty liver. FLI, with the original cut-off values has a sensitivity of 0.76 [0.50;0.93] and a specificity of 0.83 [0.72;0.90] for ruling in or out steatosis. The optimal cut-off value was 29.2 having a sensitivity of 0.82 [0.56;0.96] and a specificity of 0.61 [0.49;0.72] (26).

Anthropometric measurements
All measurements were done following a standardized protocol by trained research assistants. Height was measured to the nearest 0.5 cm without shoes with a stadiometer

(SECA, Germany). Weight was measured without shoes and sweaters and empty pockets to the nearest 0.1 kg on a digital scale (SECA, Germany). Waist circumference was assessed by tape (SECA 201, Germany) twice to the nearest 0.5 cm and averaged.

Blood collection and Biochemical analyses

After an 10 hour overnight fast 24 ml of blood was drawn from an antecubital vein using venepuncture. Blood was immediately centrifuged and plasma was stored at -80°C until further analyses.

All analyses were done using a Dimension Vista 1500 automated analyser (Siemens, Erlangen, Germany) or a Roche Modular P800 chemistry analyser (Indianapolis, USA), except for haemoglobin (Hb), haemoglobin A1c (HbA1c), fasting glucose, haematocrit (Ht), erythrocytes and leucocytes.

Total cholesterol, HDL-cholesterol and triglycerides were determined with enzymatic methods (28). LDL-cholesterol was calculated with the Friedewald equation (29). Catalytic activity concentration of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and gamma glutamyltransferase (Gamma-GT) levels were measured by international federation of clinical chemistry reference procedures at 37 °C degrees. HbA1c was determined with HPLC measurement technology using an ADAMS™ A1c HA-8160 analyser (A. Menarini Diagnostics).

Diet assessment

A validated 180-item semi-quantitative general food frequency questionnaire (FFQ) was used to assess usual dietary intake (30).

Average daily nutrient intake was calculated by multiplying frequency of consumption by portion size and nutrient content per gram using the Dutch food composition table of 2011 (31). Food groups were calculated based on this 180-item FFQ. The following food groups were analysed: animal protein sources: total meat; processed meat, red meat, poultry, eggs, fish, yoghurt, cheese, milk, dairy desserts; vegetable protein sources: legumes, nuts and seeds, soy and vegetarian products; carbohydrates and fibres: vegetables, fruit, potatoes, whole grain bread, whole grain pasta and rice; mono- and disaccharides: soft drinks, juices, sugar and confectionary; fats: oils and dressings, margarine, fat and butter, cakes and cookies and snacks.

Dutch Healthy Diet Index

The Dutch Healthy Diet (DHD) Index consists of ten components, for which subjects can receive a score as was published by van Lee et al. (27). In brief, the ten components

of the DHD index are: physical activity per week, vegetable, fruit and fruit juice, dietary fibre, fish, saturated fatty acids, mono- and trans- fatty acids, number of food occasions, sodium intake per day and alcohol intake per day. For each of these components a score of 0 to 10 points can be given according to their threshold and cut-off values (27). For the present study, the DHD-index was calculated using the 180-item FFQ (30). This FFQ did not assess number of food occasions; therefore, the maximum score was 90 points.

Covariate assessment

Health and lifestyle questionnaires at baseline included information on age (years), sex, education (low, middle, high), hypertension (yes, no, do not know), smoking (never, former, current) and diabetes (yes, no). Information about the participants' usual physical activity over the past four weeks was assessed using the Short QUestionnaire to ASsess Health enhancing physical activity (SQUASH) (32). The SQUASH consists of three main queries: days per week, average time per day, and intensity. Total minutes of activity were calculated for each question by multiplying frequency by duration. Activity scores for separate questions were calculated by multiplying total minutes of activity by the intensity score. Total activity score was calculated by taking the sum of the activity scores for separate questions.

Statistical analyses

Statistical analyses were performed with SAS 9.3 (SAS 9.3; 2002-2010 by SAS Institute Inc., Cary, NC, USA). General characteristics are expressed as mean ± SD. Normality of distribution was assessed using univariate analyses, checking for skewness. Parameters that were not normally distributed were log-transformed before analyses. Crude macronutrient intake and DHD-scores across FLI-categories were estimated using general linear models (ANCOVA). Moreover, linear regression with stepwise selection was performed to determine prediction of FLI by the following factors: total protein (energy-percent: En%), carbohydrate (En%) and fat (En%), alcohol intake (En%), total caloric intake (Kcal/day), physical activity (number of occasions per week moderate intensity physical activity for more than 30 minutes), age (years), gender, education (low, middle, high), self-reported hypertension (yes, no, do not know), self-reported diabetes (yes, no) and smoking status (never, former, current). Next, odds ratios (OR) were calculated by multinomial logistic regression to assess whether persons with the highest FLI had an increased odds of having a higher or lower intake of a certain macronutrient compared to persons with a FLI below 30.

Finally, selected food group intakes (mean ± SE) across FLI-categories were estimated using general linear models. Adjustments were made for energy intake (kcal/dag), alcohol intake (En%), age (years), sex, education (low, middle, high), hypertension (yes, no, do not know), smoking (never, former, current) and diabetes (yes, no). P-values were considered significant if $p<0.05$.

Results

Table 3.1. Subject characteristics (n=1283)[1].

	All subjects	FLI < 30	FLI 30 -60	FLI ≥ 60	p-value for trend
N	1283	679	322	282	
FLI[2]	35.64 ± 27.79	13.52 ± 7.85	44.24 ± 8.62	79.08 ± 11.32	
BMI[3] (kg/m²)	25.91 ± 4.00	23.47 ± 2.21	26.69 ± 2.14	30.89 ± 3.92	
WC[4] (cm)	91.63 ± 12.40	83.06 ± 7.40	95.67 ± 5.74	107.77 ± 8.67	
WC (male; cm)	96.80 ± 10.80	87.51 ± 6.11	96.79 ± 5.09	107.92 ± 8.98	
WC (female; cm)	85.76 ± 11.45	80.45 ± 6.83	92.88 ± 6.16	107.35 ± 7.74	
TG[5] (mmol/L)	1.21 ± 0.76	0.87 ± 0.34	1.33 ± 0.47	1.89 ± 1.16	
GGT[6] (U/L)	24.72 ± 25.77	16.46 ± 8.68	25.87 ± 17.40	43.31 ± 44.61	
Sex (% (#) male)	53 (682)	37 (251)	69 (221)	74 (210)	<.0001
Age (years)	53.60 ± 11.21	50.66 ± 12.01	56.48 ± 9.47	57.39 ± 8.86	<.0001
Physical activity (# of activities per day >30 minutes moderate active)	2.95 ± 2.95	3.30 ± 3.00	2.73 ± 2.92	2.36 ± 2.74	<.0001
Diabetes (% (#))	3 (42)	1 (6)	4 (13)	8 (23)	<.0001
Hypertension (% (#))	25 (317)	16 (108)	25 (81)	45 (128)	<.0001
Alcohol intake (g/day)	11.22 ± 11.78	9.40 ± 10.11	12.56 ± 12.48	14.07 ± 13.76	<.0001
Smoking					
Never (% (#))	45 (581)	53 (359)	42 (136)	31 (86)	<.0001
Former (% (#))	37 (474)	31 (209)	40 (130)	48 (135)	<.0001
Current (% (#))	7 (92)	5 (33)	7 (24)	12 (35)	<.0001
Education					
Low (% (#))	7 (88)	4 (29)	7 (24)	12 (35)	<.0001
Middle (% (#))	30 (379)	28 (193)	31 (99)	31 (87)	0.39
High (% (#))	63 (812)	67 (455)	61 (198)	56 (159)	0.001
Total Cholesterol (mmol/L)	5.93 ± 1.04	5.30 ± 1.02	5.52 ± 1.03	5.46 ± 1.08	0.024
HDL-cholesterol[7] (mmol/L)	1.57 ± 0.45	1.75 ± 0.44	1.44 ± 0.37	1.25 ± 0.30	<.0001
LDL-cholesterol[8] (mmol/L)	3.28 ± 0.92	3.14 ± 0.89	3.48 ± 0.91	3.37 ± 0.95	0.0003
HbA1c[9] (%)	36.26 ± 4.56	35.14 ± 3.24	36.69 ± 5.49	38.47 ± 5.16	<.0001
ALAT[10] (U/L)	27.33 ± 14.86	22.99 ± 11.34	28.79 ± 11.51	36.09 ± 20.50	<.0001
ASAT[11] (U/L)	22.72 ± 8.32	21.63 ± 7.14	22.77 ± 8.65	25.78 ± 9.90	<.0001

[1]Data are presented as means ± SD; [2]FLI: fatty liver index; [3]BMI: body mass index; [4]WC: waist circumference; [5]TG: triglycerides; [6]GGT: gamma glutamyltransferase: [7]HDL: high-density lipoprotein; [8]LDL: low-density lipoprotein; [9]HbA1c: glycated haemoglobin; [10]ALAT: alanine-aminotransferase; [11]ASAT: aspartate-aminotransferase.

In this general Dutch population, the prevalence of a fatty liver as indicated by an FLI>60, was 22.0%. **Table 3.1** shows the subject characteristics per FLI-group. In brief, those with a higher FLI were more likely to be older, male, current smoker, and to have a lower educational level. Moreover, they were more likely to have been diagnosed with diabetes or hypertension, had higher plasma concentrations of cholesterol, ALAT, and ASAT and an increased HbA1c.

Table 3.2 depicts the absolute macronutrient intake across FLI-groups. Subjects in a higher FLI-group had a higher protein intake and a lower intake of carbohydrates and dietary fibre. Fat intake was not statistically different among groups. Adherence to the Dutch dietary guidelines, as measured with the DHD-index, was significantly different among the FLI-groups, with the higher FLI groups having a lower score (63.4 ± 10.8 vs. 62.1 ± 10.1 vs. 59.3 ± 11.1; p<.0001).

Table 3.2. Crude dietary intakes and DHD index of all subjects across categories of FLI[1].

	All subjects	FLI < 30	FLI 30 -60	FLI ≥ 60	p-value for trend
N	1283	679	322	282	
FLI[2]	35.64 ± 27.79	13.52 ± 7.85	44.24 ± 8.62	79.08 ± 11.32	
Energy intake (Kcal/d)	2056.6 ± 563.8	2055.1 ± 552.6	2093.0 ± 577.8	2018.4 ± 573.9	0.36
Protein intake (EN%)	14.81 ± 2.19	14.49 ± 2.13	15.05 ± 2.15	15.31 ± 2.27	<.0001
Animal protein (EN%)	8.15 ± 2.39	7.68 ± 2.36	8.43 ± 2.18	8.94 ± 2.42	<.0001
Vegetable protein (EN%)	6.69 ± 1.24	6.83 ± 1.28	6.64 ± 1.11	6.38 ± 1.24	<.0001
Total carbohydrate (EN%)	42.91 ± 5.69	43.58 ± 5.63	43.08 ± 5.61	41.08 ± 5.56	<.0001
Mono- and disaccharide (EN%)	18.82 ± 4.97	19.36 ± 5.00	18.86 ± 4.77	17.47 ± 4.86	<.0001
Polysaccharide (EN%)	24.08 ± 4.54	24.21 ± 4.70	24.22 ± 4.25	23.60 ± 4.45	0.06
Dietary fibre (EN%)	4.74 ± 1.09	4.89 ± 1.15	4.67 ± 0.98	4.49 ± 1.01	<.0001
Total fat (EN%)	35.56 ± 5.28	35.73 ± 5.22	34.86 ± 5.31	35.97 ± 5.34	0.51
SFA[3] (EN%)	12.16 ± 2.54	12.20 ± 2.53	11.90 ± 2.48	12.35 ± 2.64	0.42
MUFA[4] (EN%)	12.82 ± 2.39	12.88 ± 2.43	12.57 ± 2.41	12.98 ± 2.27	0.53
PUFA[5] (EN%)	7.53 ± 1.93	7.61 ± 1.95	7.38 ± 1.87	7.52 ± 1.95	0.48
DHD-index[6]	62.2 ± 10.8	63.4 ± 10.8	62.1 ± 10.1	59.3 ± 11.1	<.0001

[1]Data are presented as means ±SD; [2]FLI: fatty liver index; [3]SFA: saturated fatty acids; [4]MUFA: mono-unsaturated fatty acids; [5]PUFA: poly-unsaturated fatty acids; [6]DHD-index: Dutch Healthy Diet index.

Table 3.3 displays the multivariable analysis of dietary intake and FLI. Adjustments were made for energy intake, physical activity, alcohol intake, gender, age, education, presence of hypertension and diabetes, and smoking (Table 3.3). Compared to persons with a FLI <30, 1 En% increase in protein intake corresponded with an increased odds of 26% of being in the highest FLI-group (OR: 1.26 (95%CI 1.16-1.37)). This was mainly due

to animal protein intake (OR 1.28 (95%CI 1.19–1.38), while for vegetable protein intake a decreased odds (OR 0.80 (95%CI 0.70–0.92)) was found. An inverse association was also observed for total carbohydrate intake (OR 0.94 (95%CI 0.90–0.97); while total fat intake was positively associated with presence of high FLI (OR 1.04 (95%CI 1.00–1.07)), especially mono-unsaturated fatty acids (MUFA) (OR 1.08 (95%CI 1.01–1.16)).

Table 3.3. Odds ratio's per En% increase of macronutrient intake for FLI 30-60 and FLI ≥ 60 versus FLI <30 across categories of FLI[1].

	FLI <30	FLI 30 -60 OR (95% CI)	FLI ≥ 60 (OR) (95% CI)
N	679	322	282
FLI[2]	13.52 ± 7.85	44.24 ± 8.62	79.08 ± 11.32
Protein intake (EN%)	1.0 (ref)	1.20 (1.11–1.29)	1.26 (1.16–1.37)
Animal protein (EN%)	1.0 (ref)	1.18 (1.10–1.26)	1.28 (1.19–1.38)
Vegetable protein (EN%)	1.0 (ref)	0.93 (0.83–1.05)	0.80 (0.70–0.92)
Total carbohydrate (EN%)	1.0 (ref)	1.00 (0.97–1.03)	0.94 (0.91–0.97)
Mono- and disaccharide (EN%)	1.0 (ref)	0.99 (0.96–1.02)	0.94 (0.90–0.97)
Polysaccharide (EN%)	1.0 (ref)	1.00 (0.97–1.04)	0.98 (0.94–1.02)
Dietary fibre (EN%)	1.0 (ref)	0.92 (0.80–1.07)	0.79* (0.67–0.94)
Total fat (EN%)	1.0 (ref)	0.98 (0.95–1.01)	1.04 (1.00–1.07)
SFA[3] (EN%)	1.0 (ref)	0.95 (0.90–1.01)	1.04 (0.98–1.11)
MUFA[4] (EN%)	1.0 (ref)	0.97 (0.91–1.04)	1.08 (1.01–1.16)
PUFA[5] (EN%)	1.0 (ref)	0.95 (0.88–1.03)	1.02 (0.93–1.10)
DHD-index[6]	1.0 (ref)	1.04 (0.99–1.02)	0.98 (0.96–1.00)

[1]Adjusted for: energy intake, physical activity, alcohol intake, gender, age, education, hypertension, diabetes and smoking.[2]FLI: fatty liver index; [3]SFA: saturated fatty acids; [4]MUFA: mono-unsaturated fatty acids; [5]PUFA: poly-unsaturated fatty acids; [6]DHD-index: Dutch Healthy Diet index.

The stepwise linear regression analyses assessing the prediction of FLI as continuous variable indicated that gender was the largest predictor in the model, explaining 13% of variance (R=0.13, p<.0001), followed by hypertension (R=0.07, p<.0001). Moreover, 5% of total variance in FLI was explained by a higher animal protein intake (R=0.05, ß=1.87, p<.0001); a lower mono- and disaccharide intake (R=0.008, ß=-0.67, p=0.0001); and a lower saturated fatty acid intake (R=0.002, ß=-0.85, p=0.05). The remaining dietary factors did not reach the significance level when entered in the model. The total model explained 31.5% of the total variance in FLI.

Table 3.4. Adjusted food group intakes of all subjects across categories of FLI[1].

	FLI < 30	FLI 30 -60	FLI ≥ 60	p-value for trend
N	679	322	282	
FLI[2]	13.52 ± 7.85	44.24 ± 8.62	79.08 ± 11.32	
Animal protein sources				
Total Meat (g/d)	61.8 ± 1.52	74.5 ± 2.11	79.3 ± 2.38	<.0001
Processed meat (g/d)	19.5 ± 0.75	23.5 ± 1.05	25.0 ± 1.19	0.0002
Red meat (g/d)	31.9 ± 0.87	38.9 ± 1.21	41.1 ± 1.37	<.0001
Poultry (g/d)	10.1 ± 0.34	11.7 ± 0.48	12.6 ± 0.54	0.0002
Eggs (g/d)	14.0 ± 0.58	14.2 ± 0.81	16.7 ± 0.91	0.02
Fish (g/d)	21.5 ± 0.65	22.4 ± 0.90	23.0 ± 1.02	0.23
Yogurt (g/d)	100.0 ± 3.54	100.8 ± 4.92	86.9 ± 5.56	0.06
Cheese (g/d)	28.7 ± 0.93	28.6 ± 1.29	30.8 ± 1.46	0.25
Milk (g/d)	163.7 ± 5.95	179.1 ± 8.28	180.2 ± 9.35	0.16
Dairy desserts (g/d)	23.3 ± 1.18	24.2 ± 1.64	24.2 ± 1.86	0.69
Vegetable protein sources				
Nuts and seeds (g/d)	17.9 ± 0.71	17.5 ± 0.99	16.9 ± 1.11	0.47
Legumes (g/d)	14.2 ± 0.88	13.6 ± 1.23	12.0 ± 1.39	0.20
Soy and vegetarian products (g/d)	15.7 ± 1.49	7.7 ± 2.07	7.6 ± 2.33	0.006
Carbohydrates and fibre				
Vegetables (g/d)	157.3 ± 3.38	148.8 ± 4.70	154.7 ± 5.31	0.69
Potatoes (g/d)	66.9 ± 1.90	63.4 ± 2.64	59.6 ± 2.98	0.05
Fruit (g/d)	184.9 ± 4.73	184.9 ± 6.58	173.6 ± 7.43	0.22
Whole grain bread (g/d)	114.2 ± 2.01	109.4 ± 2.80	104.2 ± 3.16	0.01
Whole grain pasta and rice (g/d)	20.6 ± 0.98	19.4 ± 1.36	18.4 ± 1.54	0.26
Mono- and disaccharides				
Soft drinks (g/d)	43.0 ± 4.09	60.5 ± 5.69	67.6 ± 6.42	0.002
Juices (g/d)	55.9 ± 3.20	63.1 ± 4.44	62.7 ± 5.02	0.28
Sugar and confectionary (g/d)	31.4 ± 0.84	26.9 ± 1.16	23.9 ± 1.32	<.0001
Fats				
Oils and dressing (g/d)	5.0 ± 0.21	4.5 ± 0.29	4.6 ± 0.32	0.26
Margarine, fat and butter (g/d)	24.5 ± 0.56	24.6 ± 0.77	24.7 ± 0.87	0.85
Cake and cookies (g/d)	31.5 ± 0.90	32.1 ± 1.25	31.1 ± 1.41	0.82
Snacks (g/d)	50.2 ± 1.39	51.5 ± 1.94	57.4 ± 2.19	0.009

[1]Data are presented as means±SEM. Adjusted for: energy intake, physical activity, alcohol intake, gender, age, education, hypertension, diabetes and smoking [2]FLI: fatty liver index.

Adjusted food group intake across the FLI-categories is presented in **table 3.4**. Subjects in the higher FLI-group had a significantly higher intake of sources of animal protein, i.e. meat and eggs, and consumed more soft drinks and snacks. Moreover, the higher FLI-group had a significant lower intake of vegetable protein sources, i.e. potatoes, whole

wheat bread and soy based products. Remarkably, sugar and confectionary intake and yoghurt consumption were also lower in the high FLI group (Table 3.4).

Discussion

The objective of the present study was to assess the relationship of macronutrient intake, foods consumption and diet quality with the presence of fatty liver as scored by the 'Fatty Liver Index' FLI, in a cross-sectional study of a well-characterized Dutch study population. To our knowledge this is the first larger study assessing the association of macronutrient intake and diet quality with fatty liver. We observed that subjects in the highest FLI-category were more likely to be male, and to have a higher BMI and a larger waist circumference. Furthermore, these subjects consumed more protein, especially from animal origin, less carbohydrates and less dietary fiber. Additionally, people in the highest FLI group had a lower DHD-score reflecting a lower adherence to the Dutch healthy eating guidelines. Subjects in the highest FLI group had a higher intake of foods of animal sources and a lower intake of foods from vegetable sources. Also soft drink intake was significantly higher in this group.

Opposite findings regarding protein intake have been found in intervention studies. We and others showed in dietary intervention studies that increasing dietary protein decreases intra hepatic lipid accumulation (21, 22). Suggested mechanisms for these observations include an increased energy need of the liver for catabolizing the protein, enhanced VLDL secretion (21, 33), and the regulating capacity of amino acids on metabolic genes, like SREBP-1 and PPAR-γ controlling hepatic lipid metabolism (34). However, these mechanisms cannot underlie the long-term detrimental effects on hepatic lipid accumulation as found in the present observational study. In the present study, a higher intake of protein, especially from animal sources, was related to a higher FLI. In contrast, a high intake of protein from vegetable sources was related to a lower FLI. Potential mechanisms for the present observations on long-term, are probably more linked with the effects as found on insulin resistance (35, 36). Dietary proteins are known to stimulate insulin secretion; however, on the long-term the hyperinsulinemia might lead to insulin resistance (36). In addition, insulin resistance suppresses the inhibitory effect of insulin on lipolysis leading to increased circulating free fatty acids (FFA) concentrations which accumulate in the liver as TG (4, 37) and decreases hepatic beta-oxidation leading to decreased VLDL secretion (38). Furthermore, high levels of circulating insulin also increase hepatic *de novo lipogenesis* (DNL), by stimulating

hepatic lipogenic enzymes (4), leading to increased lipid accumulation as well (39). This inconsistency between observational data and intervention studies regarding protein intake and liver fat suggest that acute and long-term effects of an increased protein intake do differ and that other lifestyle factors should be taken into account when formulating health-advice.

Intake of total carbohydrates and dietary fiber were lower in the higher FLI groups. The results on dietary fiber may indicate that more healthy dietary habits may account for our findings, also since we did find a lower DHD-score in the highest FLI-group. With regard to total carbohydrate intake, we saw a decreased intake of mono- and disaccharides with increased FLI scores. Also a lower intake of the sugar and confectionary food group was reported in subjects with the highest FLI score. However, soft drinks consumption was higher in subjects with high FLI. It is known that high-carbohydrate intake, with a high glycaemic index, is associated with a higher intra hepatic lipid content (11-14). A possible explanation for the present result might be that with each increase in BMI point there is a significant under reporting of sugar intake from sweets, candy and cookies (40). Fat intake did not show any significant differences among the FLI-group. Although an increased MUFA intake increased the odds of being in the highest FLI group, it should be noted that MUFA in The Netherlands 23% comes from meat and meat products and 12% from dairy products; all from animal origin (41).

The observation that persons with a higher FLI consumed more foods from animal sources, while persons with a low FLI consumed more foods from vegetable sources shares some similarities with dietary observations in diabetes. Consumption of foods from animal origin, especially processed meat, was found to be associated with an increased risk of type 2 diabetes (35). This supports the notion that an increased accumulation of fat in the liver can be considered as the hepatic manifestation of the metabolic syndrome (1). Moreover, it was reported that an increase in fat and protein from animal origin was associated with an increased risk of raised liver enzymes and insulin resistance (19), although an increased risk of a high non-alcoholic fatty liver disease fibrosis score (NFS) was associated with high carbohydrate and fat intake (19).

Because not only one macronutrient is responsible for an increased risk of a high FLI, the relationship with the Dutch Healthy Diet index (DHD index) was explored. The DHD index consists of 10 components, namely: vegetable, fruit and fruit juice, dietary fibre, fish, saturated fatty acid (SFA) , trans fatty acid (TFA) , acidic drinks and foods intake moments (ADF), sodium, alcohol intake and physical activity (27). We did find a relationship between FLI and DHD-index, which might indicate that the index is sensitive enough to predict organ health. Recently, it was reviewed that there were

no associations between the 'Healthy Eating Index' nor the 'Dietary Diversity Score' and fatty liver disease, but this was largely due to small sample sizes (n=116-995) (42). Furthermore, the present results might be explained by the inclusion of physical activity in the DHD-index, and reflecting more a healthy lifestyle and not just dietary habits.

The fatty liver index (FLI) was used to classify subjects in categories of amount of liver fat content. FLI is a surrogate measure, but has shown to be a good measure to rank and predict fatty liver in participants enrolled in this study. The golden standard to quantify liver fat is a biopsy but, liver fat can also be measured non-invasively by using imaging techniques such as MRI or CT. These techniques are however time consuming and expensive and therefore not feasible in larger observational studies. For that reason, several surrogate formulas or indices predicting fatty liver are proposed (8) (24, 25). Three validated indices are: 'the fatty liver index' (FLI) (8), the 'NAFLD-liver-fat- score' (NAFLD-LFS) (24) and the hepatic steatosis index (HSI) (25). These indices were evaluated and proven to be simple and effective in predicting fatty liver and insulin resistance (26, 43), although proton-magnetic-resonance-spectroscopy (^{1}H-MRS) remains a better measure for quantifying liver fat (26). The NAFLD-LFS requires fasting serum insulin, which was unavailable in the present study. Furthermore, the HSI was derived and validated only in an Asian population, having different cut-off points for BMI and waist circumference. Therefore, we used the FLI for indexing fatty liver (8). FLI has a sensitivity of 0.76 [0.50;0.93] and a specificity of 0.83 [0.72;0.90] for ruling in or out steatosis, as assessed in a group of non-diabetic subjects from a general population (26). Because those analyses where performed in a different population we adjusted in our models for energy intake, physical activity, alcohol intake, age, sex, level of education, hypertension, smoking and diabetes to assure that subjects would be classified correctly according to their FLI and not based on other possible confounding factors. Physical activity is known to decrease in a positive effect on liver fat content (44).

To conclude, in this cross-sectional study we observed that a higher intake of protein, especially from animal sources, was related to a higher FLI, as was the consumption of foods from animal source food groups, in particularly meat. Low dietary fiber intake and an increased soft drink consumption were also observed in the high FLI-group, suggesting poorer dietary habits in this group which can also be concluded from their lower DHD-index scores. Our results are in line with results on diabetes risk and protein intake.

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Chapter 4

Increasing protein intake modulates lipid metabolism in healthy young men and women consuming a high-fat, hypercaloric diet

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Abstract

The objective of this study was to evaluate effect of increasing protein intake, at the expense of carbohydrates, on intra hepatic lipids (IHLs), circulating triglycerides (TGs), and body composition in healthy humans when consuming a high-fat, hypercaloric diet. A crossover randomized trial with a parallel control group was performed. After a 2-wk run-in period, participants were assigned to either the control-diet (n=10; 27.8 energy percent (En%) fat, 16.9 En% protein, 55.3 En% carbohydrates) for 4 wk, or a high-fat, hypercaloric diet (n=17; >2 MJ/d) crossover trial with 2 periods of 2 wk, with either high-protein (HP) (37.7 En% fat, 25.7 En% protein, 36.6 En% carbohydrates) or normal-protein (NP) (39.4 En% fat, 15.4 En% protein, 45.2 En% carbohydrates) content. Measurements were performed after 2 wk of run-in (baseline), after 2 wk of intervention (period 1), and after 4 wk of intervention (period 2). A trend toward lower IHL and plasma TG concentrations during the HP compared to the NP condition compared with the NP condition was observed (IHL: 0.35 ± 0.04 % vs. 0.51 ± 0.08 %; p=0.08; TG: 0.65 ± 0.03 vs. 0.77 ± 0.05 mmol/L, p=0.07; for HP and NP respectively). Fat mass was significantly lower (10.6 ± 1.72 kg vs. 10.9 ± 1.73 kg; p=0.02) on the HP diet compared with the NP diet, whereas fat-free mass was higher (55.7 ± 2.79 kg vs. 55.2 ± 2.80 kg; p=0.003). This study indicated that an HP, high-fat, hypercaloric diet affects lipid metabolism. It tends to lower the IHL and circulating TG concentrations and significantly lowers fat mass and increases fat-free mass compared with an NP, high-fat, hypercaloric diet.

This trial was registered at www.clinicaltrials.gov as NCT01354626

Introduction

In recent years, high-protein (HP) diets have become increasingly popular as a way to reduce obesity and improve metabolic risk factors in the general population (1). Rodent data revealed that the metabolic adaptation to an HP diet included a down regulation of lipogenesis and increased gluconeogenesis and glycogenesis in liver (2, 3). Increasing dietary protein intake also has the potential to reduce intrahepatic lipid (IHLs) (4). Indeed, our group recently showed that mice fed an HP, high-fat diet (50 energy percent (En%) protein, 35 En% fat), had a lower liver lipid content compared with mice given a normal-protein (NP), high-fat diet (5). IHL accumulation can be regarded as the hepatic manifestation of the metabolic syndrome and is correlated with hypertriglyceridemia and a reduced suppression of insulin on hepatic glucose production, contributing to hyperglycemia.

Hepatic steatosis, the first stage of so-called non-alcoholic fatty liver disease (NAFLD), is quite prevalent in Western societies, particular in societies with increased obesity and/or type 2 diabetes mellitus prevalence (6).

Dietary manipulation has the potential to change lipid storage in the liver (4). In humans, a high-fat diet increased IHLs after only a few days (7). This was associated with an increase in markers of insulin resistance (7). A low-fat diet reduced liver fat accumulation and fasting blood insulin concentrations (7). Studies evaluating the effect of increasing protein intake on IHLs in humans are limited, although initial results appear promising (8-10). Increasing protein intake to 23 En% in a whole-diet approach blunted the effect of a 4-d high-fat diet on IHLs in healthy human volunteers (8). Whey protein supplementation (60 g/d) for 4 wk, without changing the habitual diet, substantially lowered IHLs in obese women (9). Moreover, supplementation with a mixture of the amino acids, leucine, isoleucine, valine, lysine and threonine, at 6.77 g, 3 times per day, for 6 d blunted the effect of fructose-induced increment in IHLs (10).

The main objective of the present study was to evaluate the effects of increasing protein intake on markers of lipid metabolism in healthy humans. Protein intake was increased at the expense of carbohydrates in a high-fat, hypercaloric diet (HD) with unsaturated and saturated fats and dietary fiber comparable across diets. Markers of lipid metabolism considered were circulating TGs, body composition, and especially IHLs. Last, we evaluated adipose tissue gene expression that might reflect the uptake and use of circulating TGs. A group of healthy participants given a balanced control diet (CD) was included as a reference.

Participants and Methods

Participants

Twenty-nine healthy, young, lean, Caucasian, men and women participated in this strictly-controlled dietary intervention study. Participants had no family history of type 2 diabetes and were not taking any medication. The experimental protocol was approved by the Medical Ethical Committee of Wageningen University. The sample size calculation was based on the following: in young healthy men, the addition of protein to the diet attenuated the increase in IHLs by 22% ±32% ($p<0.02$) (8). In addition, we assumed a SD of 0.7% in our healthy young population without overweight based on data from literature (11), and we aimed to include 10 participants per condition.

Study design

The total dietary intervention lasted for 6 wk. All participants started with a 2-wk run-in period on a weight-maintaining CD (27.8 En% fat, 16.9 En% protein, 55.3 En% carbohydrates) to get familiarized with the dietary regimen and to adapt to the same diet. Thereafter, participants were randomly assigned to either the HD group (n=19) or to the CD reference group (n=10). Stratified randomization was performed by an independent research assistant using a computer-generated table of random numbers. Participants were unaware of their assigned diets until the end of the study, although subjects could have guessed their study status based on the diets. After run-in, participants in the HD group were overfed with 2 MJ/d for the following 4 wk of intervention. Within the HD group, a randomized crossover design consisting of 2 periods of 2 wk was applied: 1) a 2-wk HP intervention; and 2) a 2-wk NP intervention **(figure 4.1)**. Participants started randomly with either the NP condition or the HP condition and crossed to the other condition after 2 wk. Parallel to the HD group, the reference group continued on the weight-maintaining CD for another 4 wk.

Measurements of IHL content, fasting blood glucose, insulin and TG concentrations were performed after 2 wk of run-in (baseline), 2 wk of intervention (period 1), and 4 wk of intervention (period 2). Body composition, adipose tissue gene expression, and resting energy expenditure (REE) were measured after each intervention period (periods 1 and 2) (figure 4.1). All measurements were performed after an overnight fast at the research facilities of Wageningen University.

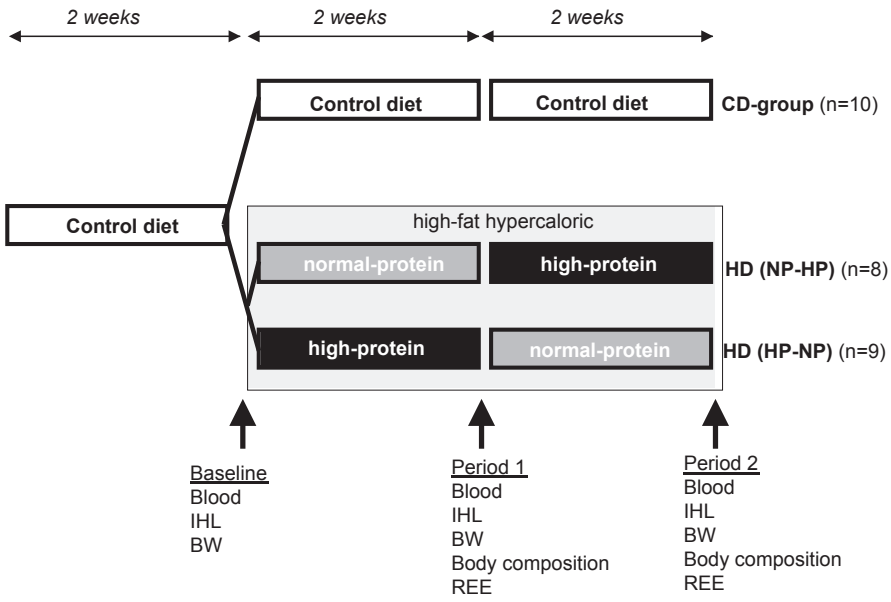

Figure 4.1. Study design and composition of the diets. Measurements are indicated as follows: 1)after 2 wk of run-in (Baseline); 2) after 2 wk of intervention (Period 1); and 3) after 4 wk of intervention (Period 2). BW, body weight; CD, Control diet; HD, high-fat, hypercaloric diet; HP, high-protein condition (within the high-fat, hypercaloric diet); IHL, intra hepatic lipid; NP, normal-protein condition (within the high-fat, hypercaloric diet); REE, resting energy expenditure.

Dietary intervention

Diets where strictly controlled. During the entire 6-wk study period, participants consumed foods covering 90% of their designated needs. The remaining 10% had to be selected from a predefined free-choice list. All food items chosen from the free-choice list were recorded. Participants came to the research facility every working day during lunchtime. They consumed a hot meal, which was weighed to the nearest gram by the research dieticians. Breakfast, evening bread meals, snacks, beverages and all meals for the weekends were provided in take-home packages. Participants were carefully instructed how to prepare the take-home meals. All the foods were precalculated for macronutrient composition and energy content for each individual participant by the research dieticians. Participants were instructed to eat all the provided food and not to change their physical activity pattern for the duration of the study.

Body weight was measured twice a week on a calibrated scale. During the run-in period, energy intake was adjusted in case of weight change. Duplicate portions of a mean daily energy amount of 11 MJ of each intervention diet were collected each day, pooled per intervention group, and analysed for energy, measured by bomb-calorimetry, macronutrients, FAs, and dietary fibre composition (**table 4.1**). Protein contribution was 16.9 En%, 15.4 En%, and 25.7 En% for the CD, NP diet, and HP diet, respectively. Protein was of mixed origin: dairy, animal, and plant sources, with the contribution of dairy protein more prominent in the HP condition (~55% compared with 30% for both other diets). The contribution of SFAs was kept at ~10 En%, as dietary guidelines recommend, and accounted for 9.5 En% in the CD, and 11.5 En% in the HD both the HP and NP conditions. Dietary compliance was assessed by completion of a diary by the participants, return of emptied food packages, and measurement of 24h-urine urea concentration by kinetic UV assay (Roche Diagnostics). Physical activity was controlled through a regular check by the dieticians, who informed participants about the allowed physical activity. Moreover, a diary on physical activity had to be fill out every day. Physical activity amount was calculated as total energy expenditure/ basal metabolic rate. Total energy expenditure was estimated form energy balance during the 2-wk run-in, and basal metabolic rate was estimated by the Schofield equation (12)

Table 4.1. Nutrient composition of the diets[1].

Diet	CD	NP	HP
Energy, *MJ/d*	12.1	14.5	14.4
Protein, *En%*	16.9	15.4	25.7
Fat, *En%*	27.8	39.4	37.7
SFA, *En%*	9.60	11.5	11.5
MUFA, *En%*	9.10	13.8	11.9
PUFA, *En%*	7.70	11.9	12.1
Cholesterol, *mg/MJ*	26.9	23.9	25.1
Carbohydrate[2], *En%*	55.3	45.2	36.6
Fibres, *g/MJ*	3.60	3.10	2.70

[1]*Values are means of duplicate portions. CD, Control diet; En%, energy percent; HP, high-protein condition (within the high-fat, hypercaloric diet); NP, normal-protein condition (within the high-fat, hypercaloric diet).*
[2]*Carbohydrate content includes dietary fibre.*

Intrahepatic lipids (IHLs)

IHL content was measured by image-guided single-voxel spectroscopy (SVS), a quantitative version of ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS). Measurements were performed on a 3.0 tesla MR-scanner (Syngo MR B17; Siemens) with a flexible

receiver and transmitter body coil. In brief, the voxel (30x30x20mm) was placed in the right hepatic lobe, avoiding big structures and the proximity of subcutaneous adipose tissue, using scout images in all 3 planes. Shimming of the magnetic field to optimize magnetic field homogeneity was performed manually. Spectra were acquired using a point-resolved spectroscopy (PRESS) sequence (bandwidth, 1200 Hz; echo time, 30 ms; repetition time, 4000 ms; 1024 points; 32 averages).

Participants were asked to breathe to the rhythm of the measurement and to be at end-expiration when they hear the sound of gradient switching. An H_2O signal was suppressed using prepulses (60 Hz; flip angle, 90°). In addition, a reference spectrum was acquired without H_2O suppression to determine H_2O signal intensity (16 averages). At visits 2 and 3, the voxel was placed visually at the same location in the liver using the images of the first visit. Post-processing of the spectral data were done by magnetic resonance user interface software (jMRUI version 4.0, build 162) (13). After manual phasing of the spectra, the observed lipid peak [methylene (CH_2)] and H_2O peak were fitted by a Gaussian line shape. For the lipid spectra, the residual H_2O peak was removed using an Hankel Lanczos Singular Values Decomposition Filter (HLSVD) filter.

The lipid and H_2O signals were analysed using the AMARES (advanced method for accurate, robust, and efficient spectral fitting) algorithm, which enables the inclusion of prior knowledge (14). The H_2O peak was identified at ~0 mg/kg and the lipid peak at approximately -3.5 mg/kg for CH_2 (15). A correction for T2 relaxation was used according to values derived from the literature: 3.69 for H_2O and 1.62 for CH_2 (16). IHLs were expressed as percentage, according to the following: $((CH_2) / (CH_2 + H_2O)) *100$.

Body composition, REE plasma analyses and insulin sensitivity

Body composition was measured by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, 70KeV, enCORE version 13.40; GE Healthcare) to assess fat mass and fat free mass.

REE was measured by indirect calorimetry with a ventilated hood using a canopy (Deltatrac II, Datex-Ohmeda B.V., GE Healthcare). Participants lay down on a bed for ≥20 min before measurements were performed. A canopy was placed over their head, and the measurement started. After 10 min, oxygen consumption (VO_2) was recorded for 5 min, and these values were averaged to calculate REE according to the formula $3.9 (VO_2) + 1.1 (0.85*VO_2)/1000$ (17), and a respiration quotient of 0.85 was assumed because CO_2 was not recorded.

Fasting blood samples were collected from an antecubital vein in EDTA-containing tubes. Glucose was analysed by the hexokinase method (Roche Diagnostics), TGs

were determined photometrically (Roche Diagnostics), and insulin was measured ELISA (Mercodia). Insulin and glucose concentrations were used to calculate insulin sensitivity using the HOMA-IR: [glucose (mmol/L) x insulin (µU/L)] / 22.5 (18).

Adipose tissue gene expression

Subcutaneous adipose tissue samples were obtained caudally from the umbilicus under local anaesthetic (1% lidocaine) with a small liposuction cannula after an overnight fast. The samples were rinsed with PBS to eliminate blood, immediately frozen in liquid nitrogen, and stored at –80°C. Total RNA was isolated with TRIzol reagent (Invitrogen), purified (RNeasy Micro kit; Qiagen), and quantified (Nanodrop ND 1000; Nanodrop technologies). Quality was determined by Bioanalyzer (Agilent 2100 Bioanalyser; Agilent Technologies). Total RNA with a RNA integrity number of 7.26 ± 0.12 was then reverse transcribed (cDNA synthesis kit; Promega) and analysed by standard qPCR (SensiMix SYBR; Bioline) on a CFX384 Real-Time System (Bio-Rad). Primers were designed using Beacon design version 6.7 (Premier Biosoft). Primer sequences are available on request. Expression of key genes involved in FA uptake, *de novo* lipogenesis (DNL), lipid storage, lipolysis, and ß-oxidation and of adipokines was investigated. The mRNA expression of all genes was normalised to ribosomal protein, large, P0 (*RPLP0)* expression.

Statistical analyses

All data are expressed as mean ± SEMs. Data were judged on normality; if data were not normally distributed, a log transformation was done before analysing the data. We analysed the data using a random intercept model (SAS PROC MIXED), including diet and period as independent variables. Additionally, the effect of protein intake within the HD group was assessed using a paired-samples *t* test to test the equality of the means between the HP and NP conditions (HP vs. NP). All p values were considered significant if $p<0.05$. Statistical analyses were performed with SAS 9.2 (2002-2008; SAS Institute).

Results

Participants

Two participants dropped out for personal reasons after the run-in period, before starting the intervention diets. Only the data from the remaining 27 participants (17 participants in the HD group and 10 participants in the CD group) were analyzed. Baseline participant characteristics can be found in **table 4.2**. Participant recruitment

and screening were done from August 2011 until November 2011. The intervention had phased inflow of participants and was performed from October 2011 until March 2012. No differences in any of the baseline parameters were observed between the HD and CD groups. The diets were well received by the participants and compliance was good, as measured by urea concentration in the 24-h urine samples after periods 1 and 2. After the CD, urine urea concentration was 222 ± 26.7 mmol/L (period 1) and 234 ± 25.7 mmol/L (period 2) (p=0.7). Within the HD group, urine urea concentration was substantially higher after the HP condition compared with the NP condition (440 ± 29.9 mmol/L vs. 272 ± 25.7 mmol/L, respectively; p<0.0001).

Table 4.2. Participant characteristics[1].

	Value
Subjects (males), n (n)	27 (19)
Age, *y*	22.8 ± 0.74
BMI, *kg/m²*	21.5 ± 0.29
Body weight, *kg*	68.6 ± 1.58
IHL, *% of H_2O peak*	0.40 ± 0.05
Fasting plasma TGs, *mmol/L*	0.96 ± 0.05
Fasting plasma glucose, *mmol/L*	5.20 ± 0.11
Fasting plasma insulin, µU/L	5.15 ± 0.48
Physical activity amount, *TEE/BMR*	1.74 ± 0.04

[1]Data are means ± SEMs. BMR, basal metabolic rate; IHL, intrahepatic lipid; TEE, total energy expenditure.

IHLs and fasting TGs.

IHL measurements were analysed in 8 participants of the CD group and 15 participants of the HD group, because not all spectra could be considered as a result of poor quality of the spectra and/or very low IHL content. Mean IHL concentrations were low at baseline (0.40 ± 0.05 % of the H_2O peak).

In general, IHLs tended to increase over time (p=0.08) (**figure 4.2A**), particularly for those consuming the CD. When comparing all 3 groups, no clear effect of diet was seen (p=0.18) (figure 4.2A). However, a trend toward a lower IHL content on the HP condition compared with the NP condition was observed (p=0.08) (**figure 4.2B**).

Overall, fasting TG concentration was significantly different between the diets (p=0.003), with no effect of time (p=0.79) (**figure 4.3A**). After the HP HD, participants had a significantly lower TG concentration compared with those on the CD (p=0.0007). Furthermore, the fasting TG concentration tended to be lower with the HP condition compared with the NP condition (p=0.07) (**figure 4.3B**).

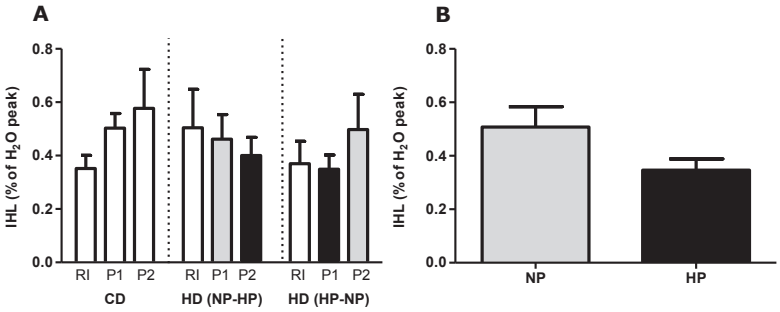

Figure 4.2. IHL content in healthy adult participants. Data are means ± SEMs. All dietary groups (A). After a 2-wk RI CD, participants consumed either a CD for 4 wk (n=8) or an HD differing in protein content, (NP vs. HP) in random order (NP-HP, n=7; HP-NP, n=8) for 4 wk. Data of the HD group pooled (n=15): NP vs. HP conditions (B). CD, control diet; HD, high-fat, hypercaloric diet; HP, high-protein; IHL, intrahepatic lipid; NP, normal-protein; P1, period 1; P2, period 2; RI, run-in.

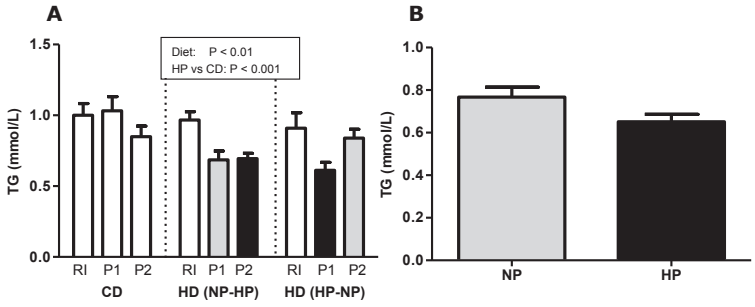

Figure 4.3. Fasting plasma TG concentrations in healthy adult participants. Data are means ± SEMs. All dietary groups (A). After a 2-wk RI CD, participants consumed either a CD for 4 wk (n=10) or an HD differing in protein content, (NP vs. HP) in random order (NP-HP, n=8; HP-NP, n=9) for 4 wk. Data of the HD group pooled (n=17): NP vs. HP conditions (B). CD, control diet; HD, high-fat, hypercaloric diet; HP, high-protein; IHL, intrahepatic lipid; NP, normal-protein; P1, period 1; P2, period 2; RI, run-in.

Body weight, body composition, insulin sensitivity and energy expenditure

Body weight did not differ significantly between dietary groups (p=0.30), but a trend toward an increase in time was observed (p=0.09) due to a slight expected increase in the HD groups (**Supplemental table 4.1**). Body weight was not significantly different between the HP and NP conditions (p=0.22) (**table 4.3**). However, fat-free mass was significantly higher after 2 wk of the HP condition compared with the NP condition (p=0.003) (table 4.3), whereas fat mass was significantly lower with the HP condition compared with the NP condition (p=0.02) (table 4.3). No differences were observed

between all 3 dietary groups or both protein conditions in REE, fasting glucose, fasting insulin, or insulin sensitivity as measured by HOMA-IR (Supplemental table 4.1, table 4.3).

Adipose tissue gene expression

The response to the HP and NP conditions of a selection of key genes from different lipid metabolic pathways was assessed in subcutaneous adipose tissue of 16 participants; in 1 participant, not enough adipose tissue was obtained (**Supplemental table 4.2**). No differences were observed in the expression of genes involved in FA uptake [lipoprotein lipase (*LPL*), angiopoietin-like 4 (*ANGPTL4*), fatty acid binding protein 4 (*FABP4*), cluster of differentiation 36 (*CD36*), and Caveolin 1 (*CAV1*)], nor for genes involved in DNL [glucose-transporter type 4 (*GLUT4*), fatty acid synthase (*FASN*), insulin receptor substrate1, (*IRS1*), and sterol regulatory element binding transcription factor1, transcript variant 1 and 2 (*SREBF1a and SREBF1c*)] -or lipid storage [diacylglycerol O-acyltransferase 2 (*DGAT2*), and peroxisome proliferator-activated receptor gamma (*PPARG*)]. Also, gene expression of hormone sensitive lipase (*HSL*) and carnitine palmitoyltransferase 1B (*CPT1B*), leptin and adiponectin were not different between protein conditions.

Table 4.3. Effect of increasing dietary protein content on metabolic markers in the HD-group[1]

	NP	HP
Body weight, *kg*	69.5 ± 1.80	69.8 ± 1.85
Lean mass, *kg*	55.1 ± 2.79	55.7* ± 2.78
fat mass, kg	10.9 ± 1.73	10.6* ± 1.72
REE, *kcal/min*	1.11 ± 0.04	1.11 ± 0.04
Fasting plasma glucose, *mmol/L*	5.04 ± 0.08	5.05 ± 0.09
Fasting plasma insulin, µU/L	4.21 ± 0.62	3.95 ± 0.63
HOMA-IR, *mmol/L x µU/ml*	0.95 ± 0.14	0.90 ± 0.15

1Data are means ± SEMs and are pooled from the NP and HP conditions, n=17. * Significantly different compared with the NP condition p<0.05.HD, high-fat, hypercaloric diet; HP, high-protein condition (within high-fat, hypercaloric diet); NP, normal-protein condition (within high-fat, hypercaloric diet); REE, resting energy expenditure.

Discussion

The main objective of the present study was to investigate the effect of increasing protein intake, at the expense of carbohydrates, on markers of lipid metabolism in healthy adults. The results indicated that, after 2 wk, an HP diet compared with an NP diet substantially affected body composition, i.e. lowered fat mass, increased fat-free

mass, tended to lower circulating TGs and reduced IHLs. Accordingly, it appeared that, in the HD group, the surplus of energy derived from fat did not result in increased IHLs, plasma TGs, or adipose tissue, nor did it result in changes in glucose metabolism.

The results showed a considerable effect of diet on fasting blood TG concentrations, with lower TGs after the HD compared with the CD. A trend toward a lower TG concentration was seen in the HP condition compared with the NP condition in the context of the HD. Furthermore, the lower TG concentration was not the only change during the HP condition in the present study. Remarkably, a substantial reduction of adipose tissue mass was seen also when comparing the HP condition with the NP condition. This occurred without any changes in adipose tissue gene expression of genes involved in lipid uptake or storage. Besides this reduction of fat mass, fat-free mass turned out to be 0.5 kg higher during the HP condition compared with the NP condition, despite a comparable total body mass. Although lifestyle was not completely controlled, participants did not report any extra physical activity. An increase of fat-free mass was previously observed during an HP HD (>40% energy, 25 En% protein) (19). It was stated that the surplus of energy in that diet was used for diet-induced thermogenesis due to the high-protein turnover and protein storage for increasing lean body mass. In the present study, other metabolic markers, including insulin, glucose, and insulin sensitivity, did not differ between the dietary conditions, although HP diets are known to have an insulinotropic effect and modulate glucose metabolism (20).

It is known that IHLs can be influenced rapidly by dietary changes. Previous studies showed that a high-fat diet increased IHLs by 35%, and similarly, a low-fat diet decreased IHLs by 20% in only 2 wk (7). Additionally, a higher protein intake was able to attenuate the effect of a high-fat diet in 4 d (8). Similarly, we also observed that increasing the protein content of the diet to 26 En% was associated with a ~30% lower IHL content, although the difference was not significant ($p=0.08$). However, it should be noted that, at the start of the dietary intervention, all our participants had a very low IHL content (~0.5 %). Additionally, to generate more statistical power, we did not separately analyze male and female participants. Some studies suggested that females have a lower IHL content compared with males (21, 22), although others had no sex-related differences in IHLs (23). The suggestion of lower IHLs when increasing protein intake is consistent with previous observations in both humans (8-10) and rodents (3, 5), and different mechanisms can be proposed.

One mechanism that could explain the observed effects on lipid metabolism is an increase of energy needs in the liver due to HP feeding. However, in the present study, whole body REE was measured, and no differences in energy expenditure between

dietary conditions were detected. Conversely, in other studies, REE did increase after 1.5 d of adaptation to an HP diet, consisting of 30 En% protein (24) and after 8 wk of an HP of 25 En% of protein (19). It is likely that postprandial and 24-h energy expenditure may have been increased with the HP diet, but this was not assessed in the present study. Another mechanism could be a reduced DNL rate after an HP diet. An increased flux of amino acids reaching the hepato-portal area in HP diet conditions might induce an increased amino acid deamination and production of amino acid-derived carbon skeletons. From mice data it was suggested that the carbon skeletons derived from amino acids are poorly transferred to glucose and not converted to FAs. Therefore, DNL rates might be lower or even absent after an HP diet (5). A decreased rate of hepatic lipogenesis after an HP diet was also observed in rats; this was in parallel with a lower hepatic expression of *Fasn*, which is the rate-limiting enzyme of hepatic lipogenesis (3). Moreover, expression of sterol receptor element binding protein-1c (*Srebp1c*) was found to be lower; this modulated insulin and thereby also prevented hepatic lipid accumulation (3, 25). In humans, it was also concluded previously that hepatic DNL is highly sensitive to dietary changes (26). The observed lower blood TGs in the present study might be a result of lower DNL due to the lower carbohydrate content of the HP diet compared with both the NP and CD. Finally, an increase in the release of bile acids in response to the high-fat diet could play a role. Bile acids are formed in the liver out of cholesterol and can affect lipid, glucose, and energy metabolism via the bile acid receptors farnesoid X receptor and G protein-coupled bile receptor 1. Activation of the farnesoid X receptor and G protein-coupled bile receptor 1 receptors by the secreted bile acids were shown to reduce liver lipid concentrations by increasing liver lipid oxidation (27). However, no increase in bile acid concentrations was observed previously after a HP, high-fat diet (8). In the present study, bile acids were not measured.

This study was conducted using a realistic dietary approach during a strictly-controlled dietary intervention instead of supplementing protein. Compliance was ensured by means of diaries and urinary urea excretion. This approach gave the opportunity to study the effect of nutrition in a real-life setting under controlled conditions. The crossover design used did not have a washout period, because we considered 2 wk a long enough period to adapt to a diet. Adding a control group gave insight into IHL responses to a normal, weight-maintaining diet. This was of interest since both IHL and circulating TG concentrations were higher on this presumably healthy diet compared with both HD conditions. Therefore, it suggests not only a positive effect of increasing protein, but also a more negative effect of high carbohydrate intake on these metabolic markers. In the experimental design, participants were young, lean and healthy. Thus, they are

expected to have a flexible metabolism that is able to adapt easily, possibly within 14 d - the duration per intervention period - to dietary changes. This might explain the relatively small changes observed in our study compared with the somewhat larger changes in IHLs observed previously in intervention of 4 d only (8). Those results might be a consequence of participants not being fully adapted to the diet yet. Furthermore, the fat content in the HD conditions was just <40 En%, which is considered a high-fat content, because dietary guideline advice a fat intake <30 En%. The contribution of SFAs was at a normal level (<10-12 En%) and comparable between CD and HD. However, national food surveys revealed that 46% of the Dutch population do have a fat intake >35 En% (28). The habitual dietary fat intake of our participants resembled this (data not shown). Therefore, we cannot exclude that this relatively high habitual fat intake could have attenuated the consequences of our experimental HD, although the contrast with the low-fat CD, which contained 27 En%, was considered to be big enough, and, in addition, the participants were overfed by 2 MJ/d. For future research, it might be of importance to study participants who are already suffering to some degree from metabolic syndrome or have a fatty liver and consequently decreased metabolic flexibility. This could give information on the effect of HP diets on reversing increased IHL content and possibly the positive effects on other metabolic markers, such as insulin sensitivity and body composition.

Hence, it might be concluded that, by increasing protein content, some of the additional energy provided by the HD was expended through protein and amino acid processing in the liver and in other peripheral tissues. In addition, the results of this study may suggest that the effect on lipid metabolism observed in the HP HD condition most likely originates from decreased liver lipogenesis and not from a higher release of TGs from the liver to the circulating blood, or from a direct effect of HP on adipose tissue.

In conclusion, our study indicated that the HP HD condition resulted in changes in lipid metabolism, namely a trend toward lower IHL content and circulating TGs, substantially lower fat mass, and higher fat-free mass compared with the NP HD condition. These effects may possibly be attributed to a lower DNL and to increased energy needs by the liver when consuming HP diets.

Acknowledgements

The authors' responsibilities were as follows: AR, MM, DT, JS and FK designed and developed the protocol; AR recruited the subjects, conducted the clinical trial and the data analysis; AR and MM measured IHL by ^{1}H-MRS developed the technique and analysed the data; LA, BB and JS analysed the gene expression in the adipose tissue and analysed the corresponding data; ES was responsible for and developed the dietary intervention; AR analysed the data; AR, MM, DT, JS interpreted the data; AR, MM, DT and JS wrote the manuscript. All authors read and approved the final manuscript. None of the authors reported any conflicts of interest.

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Supplemental Table 4.1. Effect of increasing dietary protein content on metabolic markers[1]

	CD (n=10)			HD (NP-HP) (n=8)			HD (HP-NP) (n=9)		
	Run in	Period 1	Period 2	Run in	Period 1	Period 2	Run in	Period 1	Period 2
Body weight, *kg*	68.1 ± 3.02	68.6 ± 2.96	68.4 ±3.03	70.3 ± 3.21	70.4 ± 3.12	71.2 ± 3.20	67.6 ± 2.11	68.5 ± 2.14	68.8 ± 2.14
Lean mass, *kg*	-	52.0 ± 3.31	52.1 ± 3.43	-	56.3 ± 4.29	57.0 ± 4.41	-	54.5 ± 3.71	54.2 ± 3.84
fat mass, *kg*	-	12.9 ± 1.91	12.7 ± 1.90	-	10.7 ± 1.93	10.8 ± 2.00	-	10.5 ± 2.83	11.1 ± 2.89
REE[2], k*cal/min*	-	1.06 ± 0.06	1.04 ± 0.06	-	1.16 ± 0.06	1.15 ± 0.05	-	1.08 ± 0.05	1.07 ± 0.04
Fasting plasma glucose, *mmol/L*	5.19 ± 0.12	5.07 ± 0.04	5.08 ± 0.06	5.14 ± 0.10	4.98 ± 0.11	5.14 ± 0.12	5.27 ± 0.30	4.97 ± 0.13	5.09 ± 0.11
Fasting plasma insulin, µU/L	4.71 ± 0.70	4.01 ± 0.62	4.23 ± 0.85	5.90 ± 0.86	4.75 ± 1.14	4.93 ± 1.13	4.97 ± 1.00	3.08 ± 0.57	3.74 ± 0.62
HOMA-IR[3], *mmol/L x µU/ml*	1.10 ± 0.17	0.91 ± 0.14	0.96 ± 0.19	1.36 ± 0.21	1.05 ± 0.26	1.13 ± 0.26	1.19 ± 0.24	0.69 ± 0.13	0.86 ± 0.15

[1]Data are mean ± SEM; CD, Control diet; HD, high-fat-hypercaloric diet; HP, high-protein condition (within high-fat-hypercaloric diet); NP, normal protein condition (within high-fat-hypercaloric diet).

[2]REE, resting energy expenditure.

[3]HOMA-IR, homeostatic model assessment-Insulin Resistance index.

Supplemental Table 4.2. Effect of increasing dietary protein content on adipose tissue gene expression in the HD-group[1]

	HD (n=16)	
	NP	HP
Fatty acid uptake		
LPL	1.77 ± 0.18	1.67 ± 0.18
FABP4	1.31 ± 0.09	1.35 ± 0.08
CD36	2.16 ± 0.35	2.05 ± 0.34
Caveolin1	1.45 ± 0.17	1.30 ± 0.12
Angptl4	0.98 ± 0.08	1.08 ± 0.10
De *novo* lipogenesis		
GLUT4	2.27 ± 0.23	2.19 ± 0.16
FAS	1.53 ± 0.18	1.43 ± 0.16
IRS1	1.06 ± 0.20	1.09 ± 0.21
SREBP1a	0.88 ± 0.19	0.73 ± 0.12
SREBP1c	1.15 ± 0.27	0.86 ± 0.14
Lipid storage		
DGAT2	2.10 ± 0.43	1.75 ± 0.31
PPARG	1.51 ± 0.14	1.47 ± 0.13
Lipolysis and ß-oxidation		
HSL	1.45 ± 0.16	1.35 ± 0.14
CPTB1B	1.39 ± 0.14	1.36 ± 0.09
Adipokines		
Leptin	1.09 ± 0.22	1.02 ± 0.20
Adiponectin	2.20 ± 0.19	1.96 ± 0.22

[1]Data are mean ± SEM. HD, high-fat-hypercaloric diet; HP, high-protein condition (within high-fat-hypercaloric diet); NP, normal protein condition (within high-fat-hypercaloric diet). Data are pooled from the NP and HP (n=16).

Chapter 4b

Influence of a high-protein, high-fat, hypercaloric diet on intestinal microbiota in healthy young adults

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Short communication

Abstract

In a strictly controlled dietary intervention study the influence of an enhanced protein intake on the intestinal microbiota was studied After a 2-wk run-in period, participants were assigned to either the control-diet (n=10; 27.8 energy percent (En%) fat, 16.9 En% protein, 55.3 En% carbohydrates) for 4 wk, or a high-fat, hypercaloric diet (n=17; >2 MJ/d) crossover trial with 2 periods of 2 wk, with either high-protein (HP) (37.7 En% fat, 25.7 En% protein, 36.6 En% carbohydrates) or normal-protein (NP) (39.4 En% fat, 15.4 En% protein, 45.2 En% carbohydrates) content. Faeces samples were collected after after 2 wk of intervention (period 1), and after 4 wk of intervention (period 2). Composition of microbiota was studied using the Bayesian ribosomal database project (RDP) Multiclassifier as well as BlastN comparisons against curated databases. For downstream analysis software packages, were applied together with generic scripts to obtain a differential analysis of the diet-related factors on the bacterial communities.
A large part of the microbiota in all three diets belonged to a stable and shared core of organisms that constituted approximately 60%. Among the part of the microbiota that varied between the diet groups certain species showed significantly altered abundances in the high-protein as well as in both HDs relative to the CD. Approximately 10% of those species belonged to the Firmicutes. Increased levels of Firmicutes are associated with obesity and a Western diet.

This trial was registered at www.clinicaltrials.gov as NCT01354626

Changes or differences in microbiota composition were identified to play an important role in obesity and the metabolic syndrome (MetS) (1-3). In humans most of the gut microbiota belong to the two phyla Firmicutes or Bacteriodetes. In obese patients the amount of Firmicutes is increased, whereas the amount of Bacteriodetes is decreased (1). Additionally, microbiota transplantation from a lean donor to an obese patient can improve the microbiota composition and metabolic health of the recipient (4).

Microbiota composition can also be modified by changing dietary composition. A Western-type of diet can change the microbiota unfavourably, resulting in increased Firmicutes and decreased Bacteriodetes (5). Furthermore, the possible detrimental effect on metabolic health of long term high-protein diets are thought be mediated via the microbiota, which produce toxic substances like amines and sulphur affecting the intestinal epithelium (3). Although protein being a principal diet component, its influence on the composition of the microbiota has not been very well studied yet (6).

Evaluating the possible mitigating role microbiota can have in these alteration of dietary protein on metabolism is of interest. Therefore, we wanted to investigate in our strictly controlled dietary intervention study (**chapter 4**) the influence of an increased protein and or energy intake on the intestinal microbiota. Faeces samples were collected after after 2 wk of intervention (period 1), and after 4 wk of intervention (period 2). Composition of microbiota was studied using the Bayesian ribosomal database project (RDP) Multiclassifier as well as BlastN comparisons against curated databases (Ribosomal Database Project and Living Tree Project) containing each reference sequences representing approximately 10^4 bacterial species. For downstream analysis several software packages, mothur and QIIME, were applied together with generic scripts to obtain a differential analysis of the diet-related factors on the bacterial communities.

We observed that the majority of the intestinal microbiota present in the three diet groups belonged to a shared core community that can be addressed as a stable bacterial community nucleus in the gut of the sampled subpopulation of young healthy participants. Among the part of the microbiota that varied between the diet groups certain species showed significantly altered abundances in the high-protein as well as in both HDs relative to the CD. Approximately 10% of those species belonged to the Firmicutes.

Results

Comparative rRNA-based analysis of the microbiota in the three sample groups
According to RDP classification on phylum level, differences between the three groups were not significantly different (at a level of $p < 0.05$) (**figure 4b.1**).

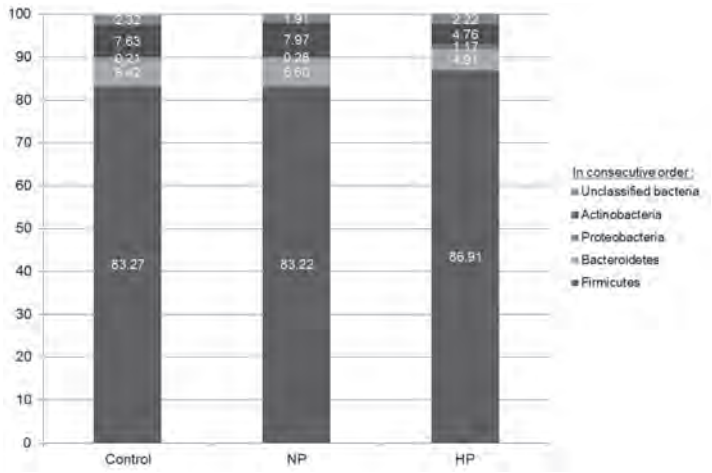

Figure 4b.1. Average abundances of the major phyla present in the microbiota. In total, the five groups account for 99.86, 99,99 and 99.98% of all sequence reads in the CD, NP and HP groups, respectively.

On family level, only four of a total of 58 families showed significant differences between at least two of the groups (**figure 4b.2, table 4b.1**). Most prominently were Veillonellaceae, which had abundances of 1.99, 1.88 and 0.26% in CD, HP and HP samples, respectively; high-protein and each of the other groups were significantly different (CD vs. HP: p=0.0236; NP vs. HP p=0.0143).
On genera level, the highest resolution level of the RDP classifier, in total 137 genera or genus-corresponding groups were identified as being present in at least one of the groups. However, only 10 of them showed significant differences between groups (**figure 4b.3, table 4b.1**). The summed abundances of these 10 genera accounted for only 11.1, 11.9 and 16.5% of the community members (read abundances) in the CD, NP and HP groups, respectively. Accordingly, the large majority of families and genera did not differ significantly in abundance between the three diets.
The RDP classification approach was limited by the failure to classify significant parts of the community, in total 28.3% of all reads, within the pre-defined taxonomic framework, when applying the default confidence threshold of 0.8. On family level

2.9% and on genus level 21.4% of the reads did not classify, the majority of them belonging to the most abundant families of Lachnospiraceae and Ruminococcaceae. Although application of a more relaxed threshold of 0.5 raised the rate of classified reads moderately from 71.7 to 88.8% (data not shown), an alternative approach using BlastN searches as identification scheme instead of the Bayesian RDP classification was utilized.

Table 4b.1. Families and genera identified with the RDP Multiclassifier that show significant differences between CD (n=20), NP (n=17) and HP (n=17)[1].

		Average			p - values		
	Rank	*CD (n=20)*	*NP (n=17)*	*HP (n=17)*	*CD vs. NP*	*CD vs. HP*	*NP vs. HP*
Family level (phylum)							
Lactobacillaceae (Firmicutes)	family	0.09	0.09	**0.00**	0.953	0.605	**0.0437**
Veillonellaceae (Firmicutes)	family	1.99	1.88	**0.26**	0.919	**0.0236**	**0.0143**
Actinomycetaceae (Actinobacteria)	family	0.03	0.05	**0.01**	0.448	0.0972	**< 0.0001**
Coriobacteriaceae (Actinobacteria)	family	**0.89**	1.33	1.57	0.165	**0.0488**	0.595
% sums		3.01	3.35	1.84			
Genus level (family)							
Lachnospiraceae incertae sedis (Lachnosp.)	genus	4.53	4.25	**6.62**	0.601	0.0534	**0.0365**
Dorea (Lachnospiraceae)	genus	2.07	2.86	**3.65**	0.279	**0.0180**	0.4820
Coprococcus (Lachnospiraceae)	genus	1.16	1.38	**2.36**	0.4	**0.00027**	**0.00751**
Clostridium IV (Ruminococcaceae)	genus	0.86	0.88	**2.62**	0.935	**0.00004**	**0.00132**
Flavonifractor (Ruminococcaceae)	genus	**0.14**	0.04	0.03	0.0732	**0.0234**	0.4430
Coprobacillus (Ruminococcaceae)	genus	**0.00**	0.01	0.01	0.338	**0.0489**	0.3370
Lactobacillus (Lactobacillaceae)	genus	0.09	0.09	**0.00**	0.966	0.6050	**0.0437**
Dialister (Veillonellaceae)	genus	1.88	1.84	**0.24**	0.968	0.0219	**0.018**
Actinomyces (Actinomycetaceae)	genus	0.03	0.05	**0.01**	0.486	0.0996	**0.00001**
Collinsella (Coriobacteriaceae)	genus	0.34	0.49	**0.92**	0.339	**0.0242**	0.1390
% sums		11.10	11.88	16.47			

[1]In the middle, average abundances; the most dissenting value is highlighted in bold. On the right, the p-values of the two-sided permutation tests are indicated. Values below the significance level of 0.05 are shown in bold.

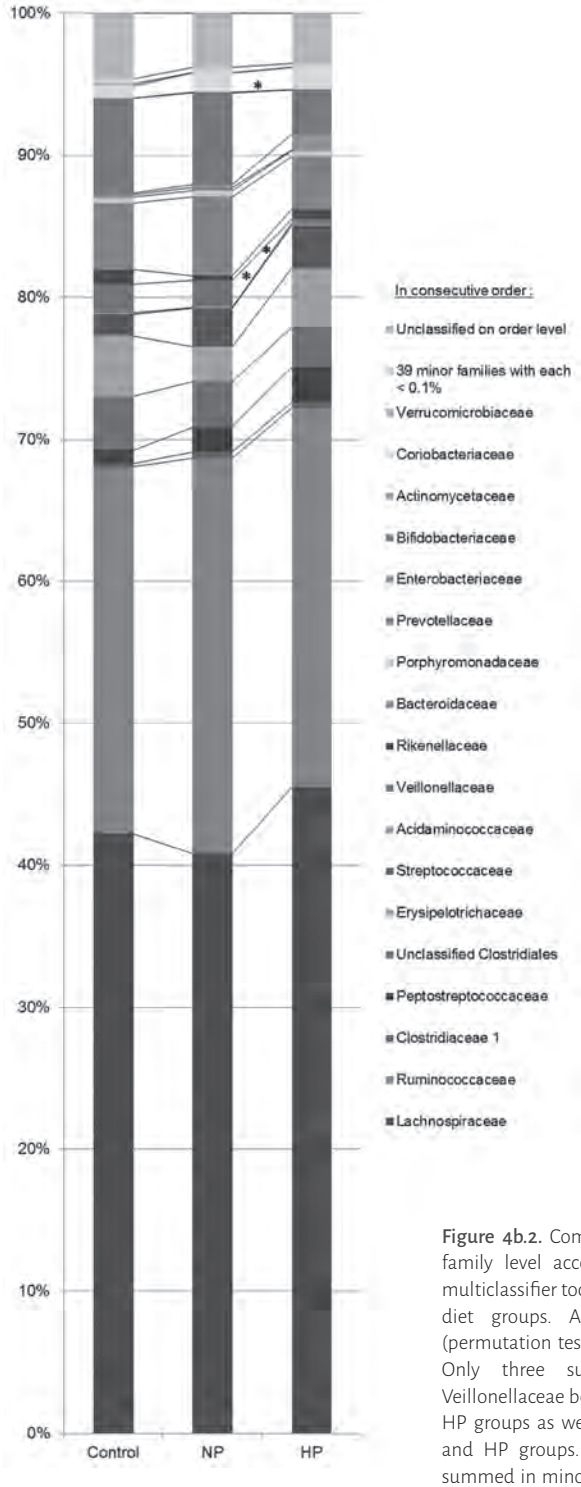

Figure 4b.2. Comparison of microbiota composition on family level according to classification with the RDP multiclassifier tool (c = 0.8). Distribution among the three diet groups. Asterisks mark significant differences (permutation test, $p < 0.05$) between two of the groups. Only three such differences are indicated: for Veillonellaceae between CD and HP and between NP and HP groups as well as for Coriobacteriaceae between CD and HP groups. Differences for Lactobacillaceae (sub-summed in minor families) and Actinomycetaceae could not be indicated due to small group sizes.

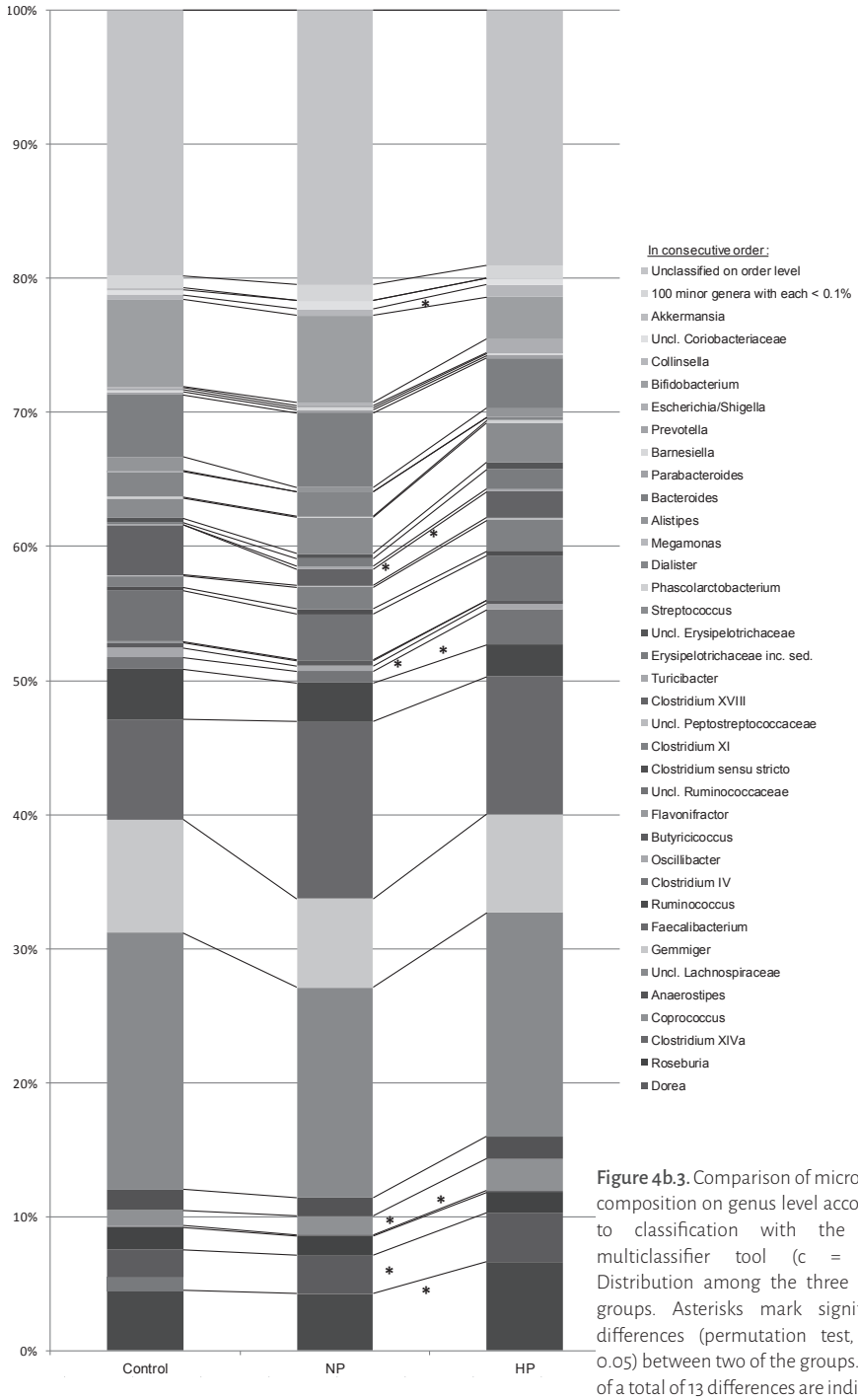

Figure 4b.3. Comparison of microbiota composition on genus level according to classification with the RDP multiclassifier tool (c = 0.8). Distribution among the three study groups. Asterisks mark significant differences (permutation test, $p < 0.05$) between two of the groups. Nine of a total of 13 differences are indicated (see table 1 for details).

BlastN comparisons were performed against a database representative on species level, LTP 111 with 9,701 reference sequences. Altogether, there were 575 reference sequences that were best hits for at least one of the 313,287 queried reads. A total of 309,911 reads (98.9%) matched with 182 reference sequences that had abundances of at least 50 reads. GU562446 *Gemmiger formicilis*, AJ413954 *Faecalibacterium prausnitzii* and EF036467 *Blautia wexleri* were the most abundant best hits in all three diet groups with total population shares of 10.8, 10.4 and 7.9% for CD, NP and HP samples, respectively. Although, *F. prausnitzii* was the most abundant best hit in the NP and HP diet groups (Data not shown).

Actual identities of query and reference sequences in fact varied largely among each group of sequence types sharing a common reference indicating significant within-group heterogeneity of present microbial groups. To mirror these variabilities, a 97% cut-off value, a limit considered usually as indicative for species affiliation, was applied. This separated "matching reads" closely enough related to the reference sequence to address them as the respective species from less similar reads that likely pertain to yet undescribed species. Using this approach, 27.8% of the sequence types, representing in total 61.8% of all reads (193 k), had sequence identities above 97%. The other 72.2% sequence types representing 38.2% (120 k) of all reads had identities below the 97% threshold. All reads belonging to the latter group were then clustered at 97% identity and used for the differential analysis (below) together with the >97% best-matching sequence types.

The degree of infra-group heterogeneity among the 182 best-matching reference sequences was quite dissimilar; whereas for *F. prausnitzii* 83.8% and for *B. wexleri* 83.4% of all best-matching reads had identities above 97% with AJ413954 and EF036467, respectively, this value was only at 41.6% for the reads best matching GU562446 *G. formicilis*.

Diversity and cluster analysis

Neither alpha nor beta diversity indices (Sobs, Shannon, Chao, Simpson) showed any diet-specific differences or patterns (data not shown).

Cluster analysis with FastUniFrac based on the entire microbiota did not result in consistent grouping of the samples according to diets with neither of the two data sets, one containing 14,731 phylotypes each of which containing at least 2 reads and a second data set with 465 phylotypes with at least 100 reads per cluster. Also the algorithms used, QIIME or usearch/FastTree, had no effect on the result. These negative results indicated that the majority of the communities were quite similar thus hampering a clear clustering based on the differences.

Differential evaluation of the influence of hypercaloric and high-protein content in the diet 'protein-responsive' versus 'fat-responsive' taxa

To evaluate the effect of the hypercaloric, high-fat diet component versus the CD and of the HP addition to the hypercaloric diet pairwise comparisons CD – NP, CD – HP, and NP – HP of abundances of "identified species" and 97%-operational taxonomic units (OTUs) were performed.

Considering the species-matching above 97% identity clusters as well as clustering of double de-replicated end trimmed reads into 97% OTUs resulted in the identification of diet-induced distribution patterns (data not shown). In total 9, 9 and 2 taxa showed specific patterns of significant differences for the HP group only, both hypercaloric groups, NP and HP, in a parallel manner or in a differential manner that was indicative for an effect of either the protein or the fat component of the diet or a combined effect of both (supplemental tables 1 and 2).

The microbiota of the three experimental groups shared a common core of 42 basic members that was present in stable manner in the bacterial communities in all three groups. Based on BlastN and classification comparisons, a total of 29 species plus 13 OTUs could be identified as belonging to this core group (supplemental tables 1 and 2). The sequence reads corresponding to these species and OTUs make up between 38.7 and 41.8% and between 16.2 and 19.3% of all reads, respectively, in the three diet groups. In total, the stable core community comprised between 54.9, 58.2 and 60.1% of the microbiota for CD, NP and HP diets, respectively.

Consequently, the rest of the microbiota, corresponding to up to 40% of all sequence reads was variable in its abundance. Species belonging to this ex-core group varied in abundance or were even completely absent in some of the samples or experimental groups.

Among the groups increasing in abundance in the group with increased protein content were *Coprococcus comes* (Lachnospiraceae), *Eubacterium siraeum*, *Clostridium leptum*, *Clostridium bartlettii* and three OTUs belonging to the family Clostridiaceae. Decreased abundances in the HP diet were observed for *Eubacterium eligens* and an OTU closely related to *Ruminococcus champanellensis*. In the group of species and OTUs that changed consistently in both HD-groups (NP and HP), indicative of a response to the high-fat content, were *Dorea longicatena* (Lachnospiraceae) and *Bacteroides eggerthii* as well as a Coriobacteriaceae OTU upshifted. Whereas *Blautia stercoris* (Unclassified Clostridiales), *Alistipes onderdonkii* (Rikenellaceae) and four OTUs belonging to different families (supplemental table 3), were present in significantly smaller abundances (all $p < 0.05$). Finally, among the species/OTUs that showed a double reinforcing abundance response

were only two, both experiencing a downshift, *Dialister invisus* (Veillonellaceae) and an OTU related to *Clostridium hathewayi*.

Altogether these variable species and OTUs summed up to between 5.0 and 6.2% and between 3.4 and 5.7% of all reads, totalling to a diet-affected share of the microbiota of 10.7, 9.2 and 11.3% for the CD, NP and HP samples, respectively.

To summarize, a large part of the microbiota in all three diets belonged to a stable and shared core of organisms. This core constituted approximately 60% of all sequence reads from all three diet groups. Among the part of the microbiota that varied between the diet groups certain species and OTUs showed significantly altered abundances in the high-protein as well as in both HDs relative to the CD. Therefore, these groups are possibly indicative for protein and/or energy-related changes of the microbiota. From these groups, approximately 10% of the sequence reads, belonged to the Firmicutes. Increased levels of Firmicutes are associated with obesity and a Western diet (1, 5).

Detailed description of methods

DNA extraction and pyrosequencing

Total DNA from faecal samples was extracted like described previously (8). Pyrosequencing was carried out at LifeSequencing (Paterna, Spain) on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Basel, Switzerland) yielding in total 468,232 reads for the 54 sequenced samples (20 control diet, CD, 17 normal-protein, hypercaloric, NP and 17 high-protein, hypercaloric diet, HP).

Taxa and operational taxonomic units (OTU) identification

Sequence analysis for the 54 samples was done like outlined in Moya et al. (9). Briefly, raw sequences were trimmed to amplified sequence parts (cutting off all bases belonging to adapters and primers) with cutadapt (version 0.3.2,Martin (10)). Using mothur (version 32, Schloss et al. (11)) and usearch (version 7, Edgar et al. (12), sequences were quality-filtered (minimum average phrap value 25, minimum length 300 bases, no ambiguities, maximum homopolymer length 8) and cleared for chimeras (usearch). The resulting 313,265 reads were used for the analysis. Prior to further analysis, reads were de-replicated, each sample separately.

Taxonomic assignation of pyrosequencing reads was done in two ways, using the RDP MultiClassifier tool (version 1.1; Wang et al. (13)) and via BlastN comparisons against a database containing 9,701 16S rDNA reference sequences, which corresponds to approximately one sequence per bacterial species (Living Tree Project database, version

111, (14)). For downstream analysis of the BlastN data only reference sequences showing "best hits" values with at least 50 total reads (sum of all 54 samples) were considered.

Diversity analyses and sample cluster analysis

Species diversity indices like Sobs, Shannon, Chao and Simpson were calculated using mothur (11).

To perform cluster analysis on a sample basis the sample-specific sets of all 313 k non-chimeric reads were de-replicated and aligned using mothur under default conditions. The alignment was end-trimmed to a core of approximately 550 bp, the sequences again de-replicated on a per-sample basis to eliminate sequence-end length variations and finally clustered at 97% sequence identity separately for each sample resulting in ca. 300 k total reads.

For subsequent selection of OTUs only sequence types with at least two copies in the respective sample were considered. In total, this excluded on average 6% of the reads, in total maintaining 282,804 reads.

Subsequent sample cluster analysis was performed using the FastUniFrac tool (http://unifrac.colorado.edu) after OTU selection (cluster analysis) in two ways: using the QIIME pipeline (Caporaso et al.(15)) and, alternatively, using usearch (version 7, Edgar et al.(12) for OTU selection, and FastTree (Price et al. (16)) for calculation of the guidance tree and with two data sets: one containing 14,731 phylotypes (97% identity-clustered) represented by at least 2 reads and a second with 465 phylotypes with at least 100 reads per cluster.

Differential analysis to detect dietary effects on microbiota composition

The differential analysis pipeline was applied to the BlastN-classified match data. BlastN best hits were separated along a 97.0% threshold (identity compared to its best hit). All sequence type matches with its closest reference sequence above this cut-off were considered as species identification. All sequences below the 97% species identity threshold (reads not assignable to a reference species) were clustered at 97% using the cluster_smallmem command of mothur. Resulting OTUs as well as the species-affiliated sequence types were subjected to the following protocol based on the analysis of abundance ratios: Calculated mean abundances of BlastN-identified species as well as of remaining 97%-clustered phylotypes (OTUs) for the three diets were compared. Differences in proportional abundances of more than factor 2 (or less than 0.5) between two diets were considered as significant. Pairwise comparisons among the three diet

groups enabled to elucidate specific effects of the HD and the HP component. A differing abundance by at least the factor 2/0.5 in the HP group versus similar abundances (relative differences smaller than factor 2) in both, the CD and NP groups, is indicative of an effect of the high-protein content, resulting either in an upshift or in a downshift of certain taxa. A likewise difference between the CD on one hand and similar respective values for NP and HP diets on the other hand was considered indicative for an effect of the hypercaloric character of the NP and HP diets versus the normal-caloric CD diet.

Statistical analysis

For comparison of the three experimental groups, CD, NP and HP, samples were pooled and mean (+/- SD) abundances for OTUs, obtained either via Bayesian RDP multi-classification or by BlastN comparisons using a 1-species-1-sequence database (LTP version 111), compared. Statistically significant differences in taxon abundances obtained by pyrosequencing were determined using a permutation test (DAAG package version 1.18 for R). A p-value of <0.05 was considered significant.

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Chapter 5

Two weeks of adaptation to a high-protein, high-fat, hypercaloric diet alters the postprandial carbohydrate and fat response

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Abstract

The western dietary pattern is known for frequent meals, high intake of foods high in saturated fat and protein content, resulting in a postprandial state for an important part of the day. The objective of this study was to evaluate the consequences of 2 weeks adaptation to a high-protein (HP) hypercaloric, high-fat diet vs. a normal-protein (NP) hypercaloric, high-fat diet or a control diet on post-prandial glucose and lipid metabolism. Seventeen healthy male and female subjects were adapted for two weeks in a crossover design to a high-protein, hypercaloric diet (37.7 En% fat; 25.7 En% protein; 36.6 En% carbohydrates; +2 MJ/d) and a normal-protein, hypercaloric diet (39.4 En% fat; 15.4 En% protein; 45.2 En% carbohydrates; +2 MJ/d). Additionally, a parallel group adapted to a normal-protein, normal-caloric diet (CD-group; n=10; 27.8 En% fat; 16.9 En% protein; 55.3 En% carbohydrates) was included. After two weeks adaptation to the different conditions, post-prandial lipid and glucose metabolism were measured following a meal challenge (MC; 3MJ; 40 En% fat; 15 En% protein; 45 En% carbohydrates). iAUCs showed a significant difference in free fatty acids (FFA) response after the MC between the three intervention diets (p=0.03) and a trend towards a bigger response after adaptation to NP compared with HP was observed (p=0.06). Glucose response to the MC was significantly lower after adaptation to NP compared with HP (p=0.03), without differences in the postprandial insulin responses (p=0.37). Adaptation to a HP diet induced a lower level of circulating FFA during the MC suggesting a possible beneficial alteration in postprandial lipid metabolism. In addition, adaptation to a HP diet could slightly impair post-prandial glucose homeostasis. Results highlight that analysis of post-prandial glucose and lipid metabolism after a MC is a sensitive approach to identify slight modifications of metabolic pathways induced by dietary protein.

This trial was registered at www.clinicaltrials.gov as NCT01354626.

Introduction

The Western dietary pattern has been associated to an increased risk of the metabolic syndrome. Besides, the western diet is known for high intake of foods from animal origin with a high saturated fat and protein content (1) and frequent meals and snacks (2), resulting in a postprandial state for an important part of the day. Therefore, this period is of particular importance. The postprandial metabolism is characterized by circulating metabolites of glucose metabolism following shortly after a meal and having a short appearance, whereas circulating lipid metabolites have a prolonged effect and stay in the circulation for hours after meal ingestion (2).

The influence of high dietary protein intake on glucose and lipid metabolism is complex, as some results indicate a higher risk of metabolic syndrome and insulin resistance on the long term (3, 4). Others have shown that high-protein diets have a potential to improve glucose metabolism by an acute stimulating effect on postprandial insulin secretion, that is known to also affect lipid metabolism (5). A 4-day adaptation regimen to a high-protein diet compared with a normal-protein diet did not reduce the post-prandial inhibition of lipid oxidation observed after a meal challenge (MC) with a high-protein content (6). In contrast, increasing protein content of a high-fat, hypercaloric diet was shown to reduce the intra hepatic lipid content, to reduce fasting plasma triglyceride concentrations (7, 8) and to reduce body fat mass (8), indicating that a high-protein diet could improve lipid metabolism.

To better understand the effects of increased protein ingestion in the postprandial phase, the objective of this study was to evaluate whether the effect on glucose and lipid metabolism of adaptation to a high-protein diet is related to modifications of the postprandial response to a MC. For that purpose healthy subjects were adapted for two weeks in a crossover design to high-protein, hypercaloric diet compared with normal-protein, hypercaloric diet. A parallel group adapted to a normal-protein, normal-caloric diet was included to also have a reference to normal feeding conditions. After two weeks adaptation to the different conditions, post-prandial lipid and glucose metabolism were measured following a MC containing normal levels of protein, carbohydrate and fat.

Participants and Methods

Participants

Twenty-nine healthy, young, lean, Caucasian, male and female subjects participated in this strictly-controlled dietary intervention study. Participants had no family history of type 2 diabetes and were not taking any medication. The experimental protocol was approved by the Medical Ethical Committee of Wageningen University, The Netherlands. Subject recruitment and screening was done from August 2011 until November 2011. The intervention had phased inflow of participants and was performed from October 2011 until March 2012.

Study design

As described in a previous publication (8) the total dietary intervention lasted for 6 weeks. All participants started on a two-week run-in period on a weight maintaining diet. Thereafter, participants were randomly assigned to either the high-fat, hypercaloric diet group (HD-group; n=17; + 2 MJ per day), consisting of a 2x2-week cross-over design with two separate intervention periods of two weeks each: a high-protein condition (HP; 37.7 En% fat; 25.7 En% protein; 36.6 En% carbohydrates) and a normal-protein condition (NP; 39.4 En% fat; 15.4 En% protein; 45.2 En% carbohydrates). Parallel, the reference group consumed a weight maintaining control diet (CD-group; n=10; 27.8 En% fat; 16.9 En% protein; 55.3 En% carbohydrates) for another four weeks. Details on the diets can be found elsewhere (8). Measurements of fasting blood glucose, insulin and triglycerides concentrations were performed after the run-in period (week 2), after period 1 (week 4) and after period 2 (week 6).

The MC was performed after period 1 and period 2. The measurements after the run-in period are considered as baseline characteristics. All measurements were performed after an overnight fast at the research facilities of Wageningen University.

Meal Challenge

After an overnight fast participants came to the research facility. First, a blood sample was drawn from an antecubital vein (baseline). Thereafter, a venous catheters was inserted in a vene on the dorsal side of the hand, in retrograde direction for sampling arterialized venous blood. The hand was put in a heated box and warmed till 55°C for 15 minutes before sampling, to assure arterialization of the venous blood. The catheter was kept patent by flushing with 0.9% NaCl. At regular time points (t= -15 (fasted) 0, 30, 60, 120, 180, 240, 360 minutes) blood samples were collected in heparinized

syringes. Plasma was separated and stored at -80 °C till analysis. At t=0, a liquid meal (low fat yoghurt: 114.3g; water: 121.3g; low-fat curd cheese: 116.7g; whipped cream: 102.7g; strawberry syrup: 88.7g; sugar: 24.5g; whey protein powder: 15.2g) presented as a milkshake was consumed (3MJ; 40 En% fat; 15 En% protein; 45 En% carbohydrates).

Biochemical analysis

Plasma biochemical analysis of free fatty acid (FFA), glycerol and ß-hydroxybutyrate, cholesterol and HDL cholesterol was performed on Olympus AU 400 robot (Centre d' Explorations Fonctionnelles Intégré (CEFI), Bichat, France). Glucose was analysed by the hexokinase method (Roche Diagnostics, Indianapolis, US); triglycerides were determined photometrically (Roche Diagnostics, Indianapolis, US); and insulin was measured by an enzyme linked immuno-assay kit (ELISA) (Mercodia, Uppsala, Sweden).

Statistical analyses

All data are expressed as mean ± SEM. Incremental area's under the curve (iAUC's) were calculated using the trapezoid method (9).

Data were judged on normality; if data were not normally distributed a log transformation was done before analysing the data. We analysed the data using a random intercept model (SAS PROC MIXED) including diet and period as independent variables. Additionally, the effect of protein intake within the high-fat, hypercaloric diet group was assessed using a paired samples t-test to test the equality of the means between the high-protein and normal-protein condition (HP vs. NP). All P-values were considered significant if $p<0.05$. Statistical analyses were performed with SAS 9.3 (SAS 9.3; 2002-2010 by SAS Institute Inc., Cary, NC, USA).

Results

Participants

Two participants dropped out after the run-in period for personal reasons, before starting the intervention diets. Only data of the remaining 27 participants (17 participants HD-group, 10 participants CD-group) were considered. Baseline subject characteristics can be found in **table 5.1** and were reported previously (8). No differences in any of the baseline parameters were observed between the HD- and CD-group.

Table 5.1. Subject characteristics[1]

	Value
N (# male)	27 (19)
Age (y)	22.8 ± 0.74
BMI (kg/m^2)	21.5 ± 0.29
Body weight (kg)	68.6 ± 1.58
IHL (% of H_2O peak)	0.40 ± 0.05
Fasting glucose (mmol/L)	5.20 ± 0.11
Fasting insulin (mU/L)	5.15 ± 0.48
Fasting triglycerides (mmol/L)	0.96 ± 0.05
Fasting FFA (mmol/L)	0.52 ± 0.05
Fasting ß-hydroxybutyrate (mmol/L)	0.07 ± 0.01
Fasting Glycerol (umol/L)	77.54 ± 5.86
Fasting Total Cholesterol (mmol/L)	4.59 ± 0.18
Fasting HDL (mmol/L)	1.15 ± 0.05
Physical activity level[2]	1.74 ± 0.04

[1]data are presented as means ± SEM.
[2]Pysical activity level (PAL) was estimated form energy intake when fed in energy balance during the 2 weeks run-in, and basal metabolic rate as estimated by the Schofield equation.

Fasted blood concentrations

The fasted blood concentrations were assessed for all subjects at the beginning of each MC, data are shown in **table 5.2**. Fasting glucose increased over the intervention periods (p=0.04), in particular in the HD-groups. Comparing all three groups, no effect of diet was seen (p=0.90). Furthermore, fasting insulin did not show a period neither a diet effect (period: p= 0.25; diet: p=0.81). However, fasting triglyceride concentration was significantly different between diets (p=0.003), with no effect of time (p=0.89). Both the HP and NP diet within the HD-group showed a significant lower triglyceride concentration compared with the CD-diet (resp. p=0.0009 and p=0.04). Fasting free fatty acids concentration decreased over the intervention period in all groups (p=0.003).

Table 5.2. Fasted plasma concentrations, determined from venous blood[1].

	CD		HD-1		HD-2		P-value	
	P1	P2	P1 (NP)	P2 (HP)	P1(HP)	P2(NP)	Diet	Period
Glucose (mmol/L)	5.07 ± 0.04	5.08 ± 0.06	4.98 ± 0.11	5.14 ± 0.12	4.97 ± 0.13	5.09 ± 0.11	0.90	0.04
Insulin (mU/L)	4.01 ± 0.62	4.22 ± 0.85	4.74 ± 1.14	4.93 ± 1.13	3.08 ± 0.57	3.74 ± 0.62	0.81	0.25
TG (mmol/L)	1.03 ± 0.10	0.85 ± 0.08	0.69 ± 0.06	0.70 ± 0.04	0.61 ± 0.06	0.84 ± 0.06	0.003*	0.89
FFA (mmol/L)	0.40 ± 0.07	0.25 ± 0.03	0.41 ± 0.08	0.22 ± 0.05	0.32 ± 0.08	0.21 ± 0.03	0.65	0.003
BHB (mmol/L)	0.07 ± 0.02	0.05 ± 0.01	0.09 ± 0.03	0.05 ± 0.01	0.12 ± 0.04	0.04 ± 0.01	0.45	0.02
Glycerol (umol/L)	40.8 ± 4.43	34.9 ± 4.69	45.2 ± 6.84	34.2 ± 6.22	36.0 ± 6.37	37.2 ± 6.57	0.45	0.18
Total Cholesterol (mmol/L)	4.79 ± 0.30	4.33 ± 0.25	4.69 ± 0.42	4.35 ± 0.18	4.98 ± 0.32	5.51 ± 0.59	0.20	0.61
HDL (mmol/L)	1.19 ± 0.09	1.11 ± 0.07	1.33 ± 0.08	1.32 ± 0.15	1.24 ± 0.09	1.25 ± 0.07	0.20	0.52

[1]Data are mean ± SEM; CD, Control diet; HD, high-fat, hypercaloric diet; HP, high-protein condition (within high-fat, hypercaloric diet); NP, normal-protein condition (within high-fat, hypercaloric diet). Results from: P1 (period 1) after 2 weeks of intervention, P2 (period 2) after 4 weeks of intervention.* Significant difference HP vs. CD: p=0.0009; NP vs. CD p=0.04.

Yet, comparing the three groups, no effect of diet was seen (p=0.65). Fasting ß-hydroxybutyrate concentration decreased over the intervention period (p=0.02), in particular in the HD-groups. However, also no effect of diet was seen (p=0.45).
Fasting glycerol, HDL, and total cholesterol did not show a period neither a diet effect (glycerol: period: p=0.18; diet: p=0.45; HDL: period: p= 0.52; diet: p=0.20; total cholesterol: period: p= 0.61; diet: p=0.20).

Postprandial responses of glucose and insulin

Figure 5.1a displays the response curve of blood glucose during the MC after two weeks of adaptation to all three diets per period. iAUCs indicated a difference close to significance in glucose response between the three diets (p=0.05), but no effect of period (p=0.56) (**figure 5.1b**). Furthermore, glucose response to the MC was significantly lower after adaptation to the NP compared with the HP condition (p=0.03; **figure 5.1c**).

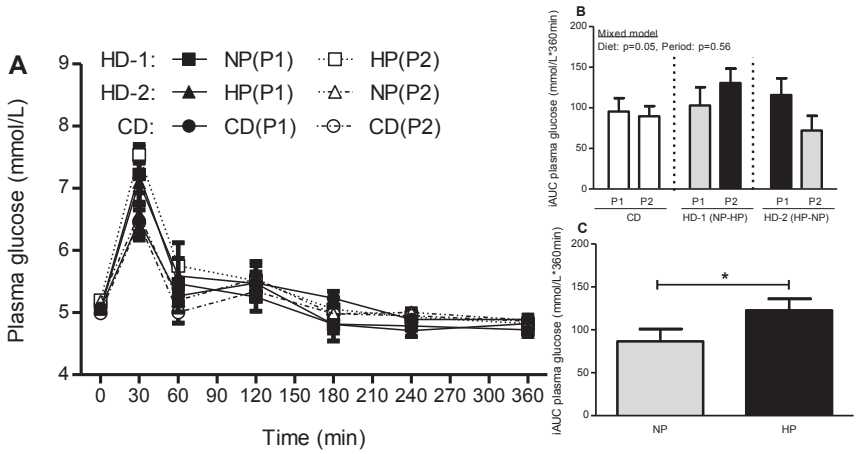

Figure 5.1. Data are mean ± SEM. A) response curve of plasma glucose during the meal challenge in all dietary groups; subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the glucose response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

However, no differences were observed in the insulin responses (**figure 5.2a**). Moreover, as can be concluded from the insulin iAUCs, there was no effect for diet or period (diet: p=0.48; period: p=0.17; **figure 5.2b**). Also no differences were observed between the postprandial insulin responses after adaptation to either the HP or NP diet (p=0.37; **figure 5.2c**).

A significant correlation between iAUC glucose and iAUC of insulin was observed (R=0.31; p=0.02).

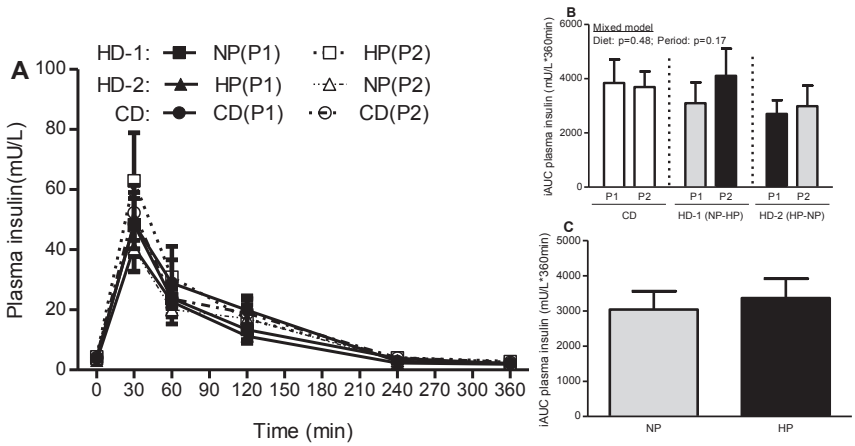

Figure 5.2. Data are mean ± SEM. A) response curve of plasma insulin during the meal challenge in all groups subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the insulin response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

Postprandial responses of TG and FFA

In **figure 5.3a** the response curve of blood TG during the MC can be found. iAUCs revealed an effect of the intervention period in TG response (p=0.002) but no effect of diet (p=0.24) (**figure 5.3b**). Also no differences were observed between the TG responses to the MC after adaptation to either the HP or NP diet (p=0.17; **figure 5.3c**).

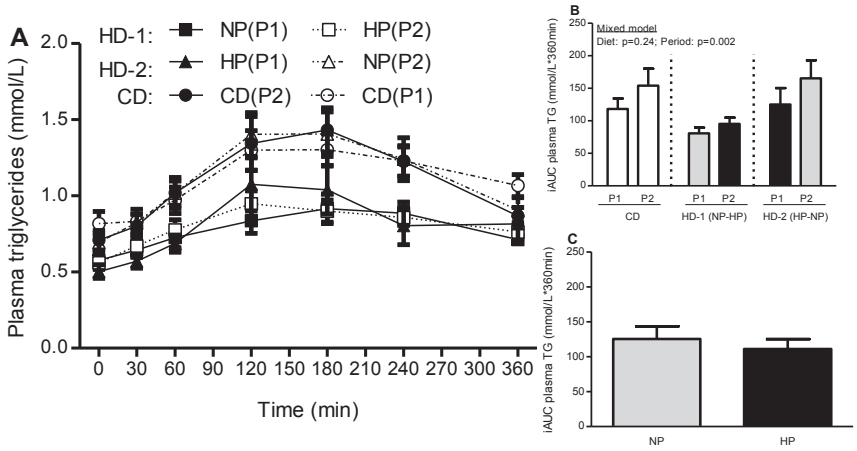

Figure 5.3. Data are mean ± SEM. A) response curve of plasma triglycerides (TG) during the meal challenge in all groups subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the TG response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

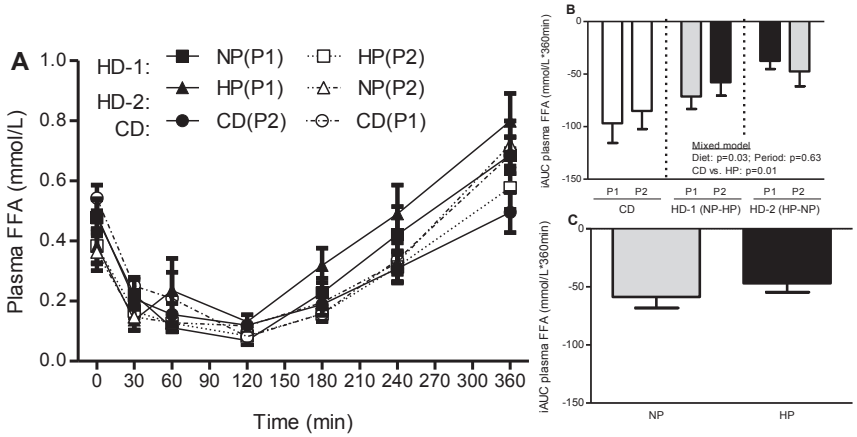

Figure 5.4. Data are mean ± SEM. A) response curve of plasma free fatty acids (FFA) during the meal challenge in all groups subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the FFA response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

Figure 5.4a shows the response curve of blood FFA during the MC. iAUCs showed a significant difference in FFA response between the three diets (p=0.03) but no effect

of the intervention period (p=0.63) (**figure 5.4b**), Comparing the diets separately, after the HP diet the FFA response to the MC was significantly smaller. Furthermore, in the FFA response to the MC a trend towards a larger response after adaptation to the NP compared with the HP condition was observed (p=0.06; **figure 5.4c**).

Postprandial responses of ß-hydroxybutyrate and glycerol

In **figure 5.5a** the response curve of blood ß-hydroxybutyrate during the MC is presented. As can be concluded from the iAUCs, there was no effect of adaptation to the either diet nor an effect of the period (diet: p=0.35; period: p=0.54; **figure 5.5b**). Also no difference was observed between the ß-hydroxybutyrate response to the MC after adaptation to either the HP or NP diet (p=0.44; **figure 5.5c**).

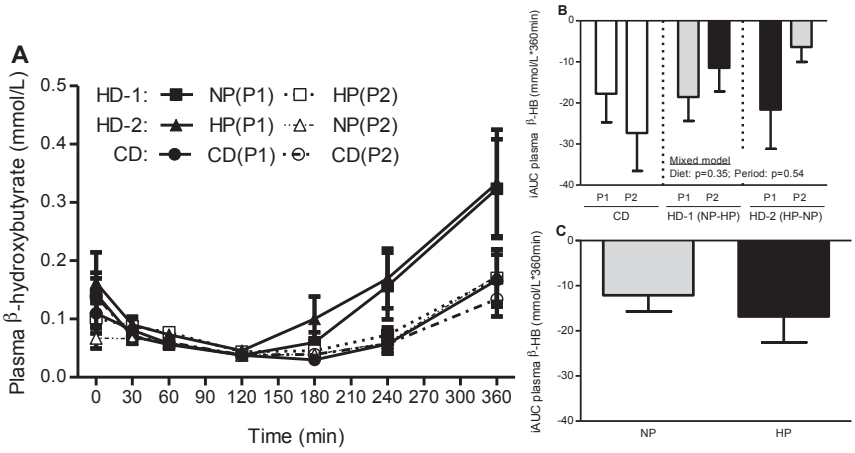

Figure 5.5. Data are mean ± SEM. A) response curve of plasma ß-hydroxybutyrate during the meal challenge in all groups; subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the ß-hydroxybutyrate response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

Figure 5.6a shows the response curve of blood glycerol during the MC. iAUCs showed a significant difference in glycerol response between the three diets (p=0.003) but no effect of period (p=0.46) (**figure 5.6b**). After the HP and NP diet the glycerol response to the MC was significantly smaller as compared with the CD (resp. p=0.002; p=0.006). However, glycerol response to the MC did not show a difference after adaptation to the NP compared with the HP condition (p=0.55; **figure 5.6c**).

Lastly, both HDL and total cholesterol showed no different responses to the MC after adaptation to neither diet (data not shown).

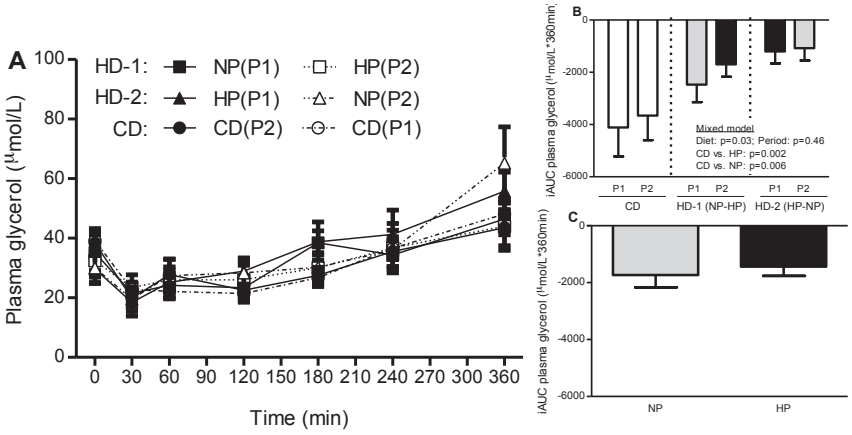

Figure 5.6. Data are mean ± SEM. A) response curve of plasma glycerol during the meal challenge in all groups; subjects where adapted to either a CD for 4 weeks (n=10) or 4 weeks of a hypercaloric diet (HD) differing in protein content, normal-protein (NP) vs. high-protein (HP) in random order (NP-HP, n=8; HP-NP, n=9), period 1 (P1) and period 2 (P2). B) iAUC of the glycerol response of all dietary groups. C) Data of the high-fat, hypercaloric diet group pooled (n=17), normal-protein (NP) vs. high-protein (HP) condition.

Discussion

This study addressed the consequences of 2 weeks adaptation to a high-protein, hypercaloric high-fat diet vs. a normal-protein, hypercaloric high-fat diet or a control diet on post-prandial glucose and lipid metabolism. We and others previously observed that increasing protein with a hypercaloric diet reduces intra hepatic lipid content and body fat mass (8), and mainly appears to improve fasting lipid metabolism (7, 8). The present results indicated that adaptation to a high-protein diet induces a lower level of circulating FFA during the MC suggesting a possible alteration in postprandial lipid metabolism. In addition, results obtained after the MC also suggest that adaptation to a high-protein diet could slightly impair post-prandial glucose homeostasis. These results highlight that analysis of post-prandial glucose and lipid metabolism after a MC is a sensitive approach to identify slight modifications of these metabolic pathways induced by dietary protein.

In an acute situation dietary protein is known to stimulate lipid metabolism, with lowering effects on cholesterol synthesis and lipogenic enzymes like fatty acid synthase

(10). Some studies on acute effects of dietary protein showed potential reducing effects on lipemia (11, 12), with different effects of different protein sources (13). Also, dietary protein acutely stimulates hepatic ß-oxidation and is suggested to slow down digestion and uptake kinetics of chylomicrons which are rich in triglycerides (10). These effects all are directly related to the acute postprandial effects of protein *per se*. The present study, in contrast, showed that a 2-week adaptation to the HP condition resulted in a lower decrease in FFA, when compared with the NP condition, after a MC of the same mixed macronutrient composition. Also, postprandial glycerol response only showed a lower decrease in glycerol after the HD compared with CD and no significant difference between the NP and HP diets. Other markers of lipid metabolism, like ß-hydroxybutyrate, HDL and total cholesterol were not altered after adaptation to the different diets. The results after the MC might suggest that not only in the fasted state, as reported previously (8), but also in the postprandial state, inhibition of lipolysis at the level of the adipose tissue is reduced after the HP diet, as seen by a lower level of circulating FFA. We were not able to observe any differences in adipose tissue gene expression of hormone sensitive lipase a key gene of lipolysis (8).

Limited information is available on the effects of adaptation to increased dietary protein on lipid metabolism (10, 14). In healthy men, 3 weeks daily supplementation of 20g soy protein isolate significantly decreased TG by 13.4% and remnant-like particles by 9.8% compared with baseline (15). These decreases were not apparent in the control group, and no differences in activity of LPL and hepatic lipase (HL) were observed between the control and the soy protein group (15). Suggested was that soy protein intake both inhibits the synthesis and secretion of TG-rich lipoprotein and possibly activates the receptor of these lipoproteins (15). Contrary, in obese humans, a 4-week adaptation to a high-protein diet (30 En% protein) did not result in differences in TG, lipoproteins and HDL cholesterol compared with a moderate protein (20En% protein diet) (16). Furthermore, in growing pigs, even an increase in FFA and TG serum levels after adaptation of approximately 25 weeks to a high-protein diet was observed (17). Lipoprotein lipase (LPL) activity and mRNA expression in adipose tissue were upregulated after the high-protein diet, indicating a higher breakdown of TG-rich lipoproteins (17).

Additionally, in the present study, adaptation to the HP-diet also slightly altered glucose homeostasis as shown by a higher iAUC for glucose after the MC as compared with the NP-diet. In contrast, Gannon et al. (18) reported that ingestion of amino acids together with glucose resulted in an acute decreased glucose response. However, the addition

of amino acids to glucose compared with ingestion of only glucose resulted in an insignificant different effect on insulin response (18).
High-protein diets can stimulate postprandial gluconeogenesis in the liver, with a reduced inhibition of glucose production by the meal (19). This potentially leads to an increased insulin demand, and consequently to beta cell exhaustion (19). In the present study insulin concentrations did not show a different postprandial response after adaptation to either diet. This is in line with results obtained in pigs, where a high level of dietary protein also had no effect on serum insulin and glucose (17). Our results might indicate that subjects adapted to a HP diet developed either a decreased clearance of carbohydrates or higher endogenous production of glucose and possibly had a lower sensitivity to insulin. However, we did see a correlation between insulin and glucose iAUC, indicating that there was an insulin response following the MC. Such a decreased glucose tolerance might be a transient situation, and the question is whether this effect will persist after longer adaptation to high-protein diets. Insulin can stimulate the uptake of fatty acids, catalysed by lipoprotein lipase (LPL) and it blocks lipolysis by inhibiting hormone sensitive lipase (20). However, glucose can also promote the LPL synthetic rate and increase the stimulatory effect of insulin. Yet, unlike insulin, glucose does not influence the LPL mRNA level (20).

Concluding, our results indicate that adaptation to a high-protein diet improves postprandial lipid metabolism. However, at the same time it could also slightly impair post-prandial glucose homeostasis and it might be the case that these observations are the result of combined effects on lipid and glucose metabolism, possibly mediated by insulin.

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The authors' responsibilities were as follows: AR, MM, DT, JS and FK designed and developed the protocol; AR recruited the subjects, conducted the clinical trial and the data analysis; ES was responsible for and developed the dietary intervention; DA and JP were responsible for the biochemical analyses; AR analysed the data; AR, MM, DT, DA interpreted the data; AR, MM, and DT wrote the manuscript.
All authors read and approved the final manuscript. None of the authors reported any conflicts of interest.
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Chapter 6

Associations between plasma branched chain amino acids, ß-aminoisobutyric acid and body composition

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Abstract

Plasma branched chain amino acids (BCAA) are elevated in obesity and associated with increased cardiometabolic risk. ß-aminoisobutyric acid (B-AIBA), a recently identified small molecule metabolite, is associated with decreased cardiometabolic risk. Therefore, we investigated the association of BCAA and B-AIBA with each other and with detailed body composition parameters, including abdominal visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). A cross-sectional study of lean (n=15) and obese (n=33) men and women. Detailed metabolic evaluations, including measures of body composition, insulin sensitivity and plasma metabolomics were completed. Plasma BCAA were higher (1.6±0.08 ($*10^7$) vs. 1.3±0.06 ($*10^7$) AU; p=0.005) in obese versus lean subjects. BCAA were positively associated with VAT (R=0.49; p=0.0006) and trended to an association with SAT (R=0.29; P=0.052). The association between BCAA and VAT, but not SAT, remained significant after controlling for age, gender and race on multivariate modeling (P<0.05). BCAA were also associated with parameters of insulin sensitivity (Matsuda index: R=-0.50; p=0.0004; glucose AUC: R=0.53; p<0.001; HbA1c: R=0.36; p=0.01). BCAA were not associated with B-AIBA (R=-0.04, p=0.79). B-AIBA was negatively associated with SAT (R=-0.37; P=0.01) but only trended to an association with VAT (R=0.27; P=0.07). However, neither relationship remained significant after multivariate modeling (P>0.05). Plasma B-AIBA was associated with parameters of insulin sensitivity (Matsuda index R=0.36; p=0.01; Glucose AUC: R=-0.30; p=0.04; HbA1c: R=-0.33; p=0.03).Plasma BCAA levels were positively correlated to VAT and markers of insulin resistance. Results suggest a possible complex role of adipose tissue in BCAA homeostasis and insulin resistance.

This trial was registered at www.clinicaltrials.gov as NCT00562796

Introduction

Worldwide, the number of people suffering from obesity continues to increase (1). Obesity is characterized by increases in both the abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). SAT is considered to have more protective properties in relation to cardiometabolic risk factors (2) whereas, VAT is detrimental to cardiometabolic health (3, 4).

Obesity is also associated with elevated levels of plasma branched chain amino acids (BCAA) (5). The BCAA, valine, leucine, and isoleucine, are associated with insulin resistance (6-11). Acute increases in plasma amino acids worsen insulin sensitivity (12), and higher levels of dietary protein intake is associated with impaired glucose metabolism (13). Furthermore, levels of baseline circulating BCAA predicted the development of incident diabetes in a large longitudinal cohort study (11) suggesting the relationship between BCAA and insulin resistance may be causal. However, the relationship between BCAA and detailed parameters of body composition, specifically VAT, has yet to be reported.

ß-aminoisobutyric acid (B-AIBA), a small molecule metabolite, was recently identified using a metabolomics approach as a possible novel myokine that increases browning of white adipocytes in response to physical activity and was found to be inversely associated with cardiometabolic risk including fasting glucose, insulin and HOMA in addition to triglycerides and cholesterol (14). As B-AIBA can be formed by catabolism of thymine and valine (14, 15) this may represent a possible pathway through which BCAA exert their metabolic effects. The catabolism of BCAA, valine in particular, could decrease circulating BCAA while increasing B-AIBA, both of which are associated with improved insulin resistance.

In the present study we investigated the association of BCAA and B-AIBA with each other and with detailed body composition parameters, including SAT and VAT, for the first time. We hypothesized that BCAA would be positively associated with VAT while B-AIBA would be negatively associated with VAT. This hypothesis was evaluated in a cross-sectional study of lean and obese men and women for whom detailed metabolic evaluations were performed.

Participants and Methods

Participants

Forty-eight lean (BMI <25 kg/m^2; n=15) and obese (BMI ≥30 kg/m^2; n=33) men and women from the Boston community were evaluated between November 2007 and March 2009 at the Massachusetts Institute of Technology and Massachusetts General Hospital Clinical Research Centre. Subjects were between the ages of 18 and 55 years and were otherwise healthy. Subjects receiving anabolic steroids, glucocorticoids, testosterone, hormone replacement, hormonal contraception, growth hormone or medication for diabetes mellitus treatment were excluded. Subjects with hemoglobin level less than 11 g/dl, creatinine above 1.5 mg/dl, aspartate aminotransferase more than 2.5-fold above the upper limit of normal, and chronic illness such as HIV were also excluded. Written informed consent was obtained from each subject before testing in accordance with the Committee on the Use of Humans as Experimental Subjects of the Massachusetts Institute of Technology and the Subcommittee on Human Studies at the Massachusetts General Hospital.

Body composition analyses

Anthropometric measurements including height, body weight, waist and hip circumference were obtained in triplicate by a trained nutritionist after an overnight fast. Total body fat percentage was determined by dual x-ray absorptiometry (DXA) testing using a Hologic-4500 densitometer (Hologic, Inc., Waltham, MA). DXA uses a 3-compartment model, partitioning tissue into lean, bone, and fat mass. 1-cm cross-sectional abdominal computed tomography (CT) scans were performed at the level of L4 to assess the distribution of abdominal SAT and abdominal VAT as previously described (16).

Biochemical analyses

Fasting blood samples were drawn and oral glucose tolerance tests (OGTTs) were performed using 75-g oral glucose challenge. Glucose and insulin were obtained at 0, 30, 60, 90 and 120 minutes. Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Matsuda Index (17) were calculated using the following equations:

$$\text{HOMA}=(\text{fasting glucose (mmol/l)} \times \text{fasting insulin (mIU/L)})/22.5$$

$$\text{ISI}_{(\text{Matsuda})} = 10000/\sqrt{(G_o \times I_o \times G_{mean} \times I_{mean})}$$

G_{mean} and I_{mean} were obtained from values at time points 0, 30, 60, 90 and 120 minutes. Measurement of fasting cholesterol profile was performed on a separate visit. Glucose and lipid levels were determined using standard methodology in the Massachusetts Institute of Technology clinical laboratory. Insulin was measured by a paramagnetic-particle chemi-luminescence immunoassay using the Beckman Access Immunoassay System (Beckman Coulter). The analytical sensitivity of the assay is 0.03 IU/ml, and the precision is 3–5.6%.

Metabolomic profiling

Metabolomic profiling was performed using liquid chromatography tandem mass spectrometry (LC-MS) for 65 polar metabolites including BCAA and B-AIBA from fasting plasma samples(11, 18). Polar metabolites were analyzed in the hydrophilic interaction liquid chromatography (HILIC)/ negative ion MS mode using targeted multiple reaction monitoring MS scans for optimal analytical sensitivity. To create this targeted profiling method, declustering potentials and collision energies were optimized for each metabolite by infusion of reference standards. In this method, the range of analyte signals spans at least four orders of magnitude dynamic range. Results are output in Arbitrary Units (AU). In general, the coefficients of variation (CVs) for repeated analyses are inversely proportional to the magnitude of the instrument response. Median CV was, determined using repeated analyses of a pooled plasma reference sample (r=10), and was 4.3%; >70% of metabolites had a CV ≤ 10%. The BCAA, leucine, isoleucine and valine were evaluated individually and summed and evaluated as total BCAA.

Dietary evaluation

Absolute intake of macronutrients including carbohydrates, proteins and fat were assessed by collection of a 4-day food record facilitated by a trained research dietician during direct interview. Data were analyzed using Nutrition Database Systems for Research (NDSR) software with the NDSR 2008 (version 2, University of Minnesota, Minneapolis, MN).

Statistical analyses

All data are expressed as mean ± SEM. Normality of distribution was assessed using Shapiro-Wilk analyses. Parameters that were not normally distributed were log transformed before analyses. All metabolites from the metabolomics assay were log transformed before analyses. A targeted statistical analysis using Pearson univariate regression analysis was performed to determine the relationship of BCAA and B-AIBA

with body composition and metabolic parameters. As a secondary analysis, a non-targeted metabolomics analyses was performed assessing the relationship of all 65 polar metabolites to various body composition parameters. Multivariate regression analysis was performed evaluating metabolomic parameters and body composition parameters that were significant on univariate analysis. For body composition parameters, the model controlled for age, gender and race. Additionally, to investigate whether a relationship between BCAA and VAT can be explained by insulin resistance Matsuda index was included in a multivariate model. For the metabolic parameters, the model included age, gender, race and BMI. For our primary end point assessing the relationship of BCAA to body composition parameters, p-values were considered significant if $p<0.05$, as this was a targeted analysis focusing specifically on BCAA. For the non-targeted secondary analyses evaluating all metabolites, p-values were considered significant if <0.00074 for Bonferroni correction for multiple comparisons. Statistical analyses were performed with SAS 9.2 (SAS 9.2; 2002-2008 by SAS Institute Inc., Cary, NC, USA).

Results

Subjects

A total of 48 subjects were evaluated. Fifteen subjects were lean and 33 subjects were obese. Subject characteristics can be found in **table 6.1.** Briefly, lean subjects were 44.2 ± 2.5 years, with a BMI of 22.6 ± 0.3 kg/m^2 and a waist circumference of 80.1 ± 2.1 cm, while obese subjects were 37.8 ± 1.7 years, with a BMI of 35.3 ± 0.8 kg/m^2 and a waist circumference of 110.8 ± 2.0 cm. The level of total plasma BCAA in lean subjects was 1.3 ± 0.06 (*10^7) AU versus 1.6 ± 0.08 (*10^7) AU (p=0.005) in obese subjects. The individual BCAA were also significantly different in lean versus obese subjects (isoleucine: 4.4 ± 0.3 (*10^6) AU vs. 5.6 ± 0.3 (*10^6) AU, p=0.006; leucine: 5.4 ± 0.2 (*10^6) AU vs. 6.5 ± 0.3 (*10^6) AU, p=0.02; valine: 3.1 ± 0.2 (*10^6) AU vs. 4.1 ± 0.2 (*10^6) AU, p=0.0006). Plasma B-AIBA levels in lean subjects were 2.5 ± 0.3 (*10^4) AU versus 1.9 ± 0.2 (*10^4) AU (p=0.08) in obese subjects.

Table 6.1. Subject characteristics (n=48)[1].

	Lean (n=15)	Obese (n=33)	p-value
Age (y)	44.2 ± 2.5	37.8 ± 1.7	0.07
Gender (# male)	8	13	0.28
Race (% Caucasian)	37.5	62.5	0.22
BMI (kg/m^2)	22.6 ± 0.3	35.3 ± 0.8	<0.0001
Waist circumference (cm)	80.1 ± 2.1	110.8 ± 2.0	<0.0001
VAT (cm^2)	53.0 ± 13.7	123.7 ± 10.3	<0.0001
SAT (cm^2)	139.4 ± 21.1	503.4 ± 30.4	<0.0001
HOMA-IR	0.6 ± 0.1	2.3 ± 0.5	<0.0001
Matsuda index	15.5 ± 1.8	7.0 ± 1.0	<0.0001
HbA1c (%)	5.5 ± 0.1	5.6 ± 0.1	0.38
Glucose AUC during OGTT (mg/dl*min)	1.3 ± 0.06 (*10^4)	1.6 ± 0.09 (*10^4)	0.02
Triglycerides (mg/dl)	49.7 ± 4.3	118.4 ± 13.2	<0.0001
HDL (mg/dl)	59.8 ± 2.2	45.7 ± 1.9	<0.0001
LDL (mg/dl)	99.3 ± 8.1	116.7 ± 5.6	0.09
BCAA			
Total BCAA (AU)	1.3 ± 0.06 (*10^7)	1.6 ± 0.08 (*10^7)	0.005
Isoleucine (AU)	4.4 ± 0.3 (*10^6)	5.6 ± 0.3 (*10^6)	0.006
Leucine (AU)	5.4 ± 0.2 (*10^6)	6.5 ± 0.3 (*10^6)	0.02
Valine (AU)	3.1 ± 0.2 (*10^6)	4.1 ± 0.2 (*10^6)	0.0006
B-AIBA (AU)	2.5 ± 0.3 (*10^4)	1.9 ± 0.2 (*10^4)	0.08

[1]Data are means ± SEM

Branched chain amino acids, and B-AIBA

Individual and total BCAA were not associated with B-AIBA (isoleucine: R=-0.007, p=0.96; leucine: R=-0.07, p=0.67; valine: R=-0.05, p=0.74; total BCAA: R=-0.04, p=0.79).

Branched chain amino acids, B-AIBA, amino acids and measures of body composition

Individual and total BCAA were significantly associated with BMI (all p<0.05), waist circumference (all p<0.01), and VAT (all p<0.005) as detailed in **table 6.2** and **figure 6.1**.

Table 6.2. Univariate regression analyses of body composition to BCAA and B-AIBA.

	BCAA	BCAA	B-AIBA	B-AIBA
	(R)	(p)	(R)	(p)
BMI	0.42	0.003	-0.28	0.054
Waist circumference	0.55	0.0004	-0.18	0.24
VAT (CT)	0.49	0.0006	-0.27	0.07
SAT (CT)	0.29	0.052	-0.37	0.01
Total fat (g) (DXA)	0.29	0.047	-0.34	0.02
Total fat% (DXA)	0.1	0.53	-0.42	0.004
Total lean% (DXA)	-0.12	0.43	0.37	0.01

Multivariate modeling correcting for age, gender and race continued to demonstrate a significant relationship between total BCAA and BMI (p=0.0002), waist circumference (p=0.0004) and VAT (p=0.002), independent of the other factors. When correcting for total fat mass, age, gender and race the significant relationship between total BCAA and VAT (p=0.04) remained, and was also dependent on total fat mass (p<0.0001), age (p=0.02) and gender (p=0.001). In multivariate modelling correcting for BMI, age, gender and race, the relationship between total BCAA and VAT trended toward significance (p=0.11), and was dependent on BMI (p<0.0001), age (p=0.01) and gender (p=0.04). Furthermore, in multivariate modeling including both VAT and Matsuda index VAT was no longer significant (p=0.61) while the relationship between the Matsuda index and BCAA remained significant (p=0.0001).

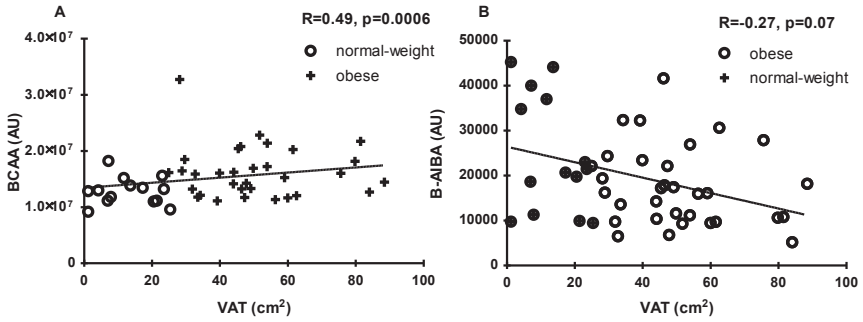

Figure 6.1. The correlation between plasma BCAA and B-AIBA vs. visceral adipose tissue (VAT). A. BCAA vs. VAT; R=0.49, p=0.0006. B. B-AIBA vs. VAT; R=-0.27, p=0.07. lean subjects are represented as open circles and obese subjects are represented as crosses.

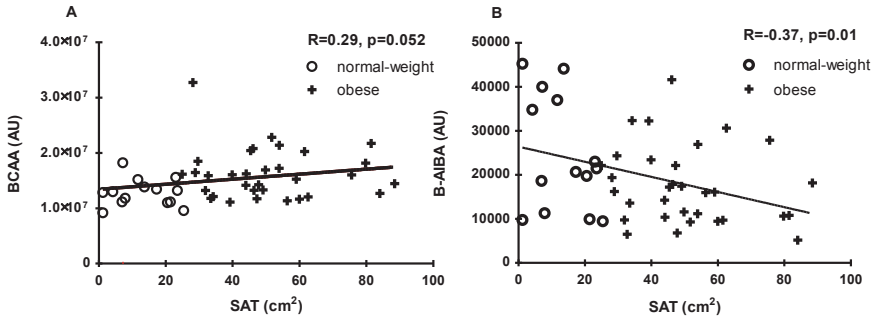

Figure 6.2. The correlation between plasma BCAA and B-AIBA vs. subcutaneous adipose tissue (SAT). A. BCAA vs. SAT; R=0.29, p=0.052. B. B-AIBA vs. SAT; R=-0.37, p=0.01. Lean subjects are represented as open circles and obese subjects are represented as crosses.

B-AIBA was significantly associated with SAT (R=-0.37; p=0.01), percent body fat (R=-0.42; p=0.004), total fat mass (R=-0.34; p=0.02) and percent lean body mass (R=0.37; p=0.01); and trended to an association with BMI and VAT (both p<0.10), but not waist circumference (**figures 6.1, 6.2** and **table 6.2**). However, none of these relationships remained significant upon multivariate modeling correcting for age, gender and race.

Branched chain amino acids, B-AIBA, amino acids and indices of insulin sensitivity

Individual and total BCAA were significantly associated with HOMA index (all p<0.005), Matsuda index (all p<0.005), glucose area-under-the-curve (AUC) during OGTT (all p<0.005), and HbA1c (all p<0.05) as detailed on **table 6.3** and **figure 6.3**.

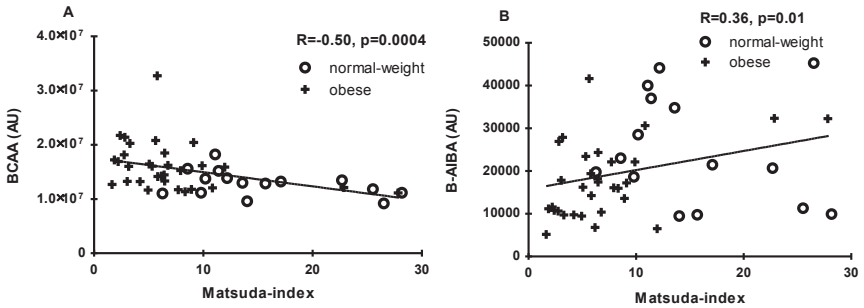

Figure 6.3. The correlation between plasma BCAA and B-AIBA vs. Matsuda index. A. BCAA vs. Matsuda index; R=-0.50; p=0.0004. B. B-AIBA vs. Matsuda index; R=0.36; p=0.01. Lean subjects are represented as open circles and obese subjects are represented as crosses.

Multivariate modeling correcting for age, gender, race and BMI continued to demonstrate a significant relationship between total BCAA and HOMA index (p=0.03). In this model, age and BMI also remained associated with BCAA (age: p=0.04; BMI: p=0.006). Multivariate modeling also demonstrated a significant relationship between total BCAA and Matsuda index (p=0.01). Age and BMI also remained associated with BCAA in this model (age: p=0.05; BMI: p=0.004). In additional multivariate modeling glucose AUC (p=0.003) and HbA1c (p=0.004) also remained significantly associated with BCAA independent of age, gender, race and BMI.

B-AIBA was negatively associated with HOMA index (R=-0.38; p=0.01), glucose AUC during OGTT (R=-0.30; p=0.04) and HbA1c (R=-0.33; p=0.03) and positively associated with Matsuda index (R=0.36; p=0.01) (table 6.3 and figure 6.3). However, these associations no longer remained significant after multivariate modeling correcting for age, gender, race and BMI.

Table 6.3. Univariate regression analyses of metabolic parameters to BCAA and B-AIBA.

	BCAA (R)	BCAA (p)	B-AIBA (R)	B-AIBA (p)
HOMA-IR	0.48	0.0007	-0.38	0.01
Matsuda index	-0.5	0.0004	0.36	0.01
Glucose AUC during OGTT	0.53	<0.001	-0.3	0.04
HbA1c	0.36	0.01	-0.33	0.03
Triglycerides	0.51	0.0003	-0.12	0.43
HDL	-0.43	0.003	0.21	0.16
LDL	0.26	0.08	-0.1	0.51

Branched chain amino acids, B-AIBA and dietary intake

Neither plasma BCAA, nor B-AIBA were associated with absolute dietary protein, carbohydrate or fat intake (data not shown).

Discussion

In the present study, we demonstrated, for the first time, a significant association between individual and total BCAA and VAT. We also confirmed the known association of BCAA with insulin resistance.

We showed, as hypothesized, a significant positive association between BCAA and VAT in this study. This suggests that adipose tissue may play an under-appreciated, but potentially significant role in BCAA homeostasis (6, 19). The three BCAA, valine, leucine

and isoleucine, are essential amino acids and cannot be endogenously synthesized. As dietary intake of proteins were not related to plasma BCAA levels in this study, the plasma levels of BCAA may primarily reflect inhibited catabolism of BCAA, as well as a decreased insulin sensitivity of the skeletal muscles leading to reduced inhibition of BCAA release by the skeletal muscle (7). The catabolism of BCAA begins with the transport of BCAA into the cell, initiated by branched chain amino acid aminotransferase (BCAT) in the mitochondrion (20). There are two forms of BCAT; mitochondrial (BCATm) and a cytosolic (BCATc). BCATm is found in nearly all tissues, including adipose tissue (21, 22). BCAT catalyzes reversible transamination of BCAA to form their α-keto acids (23, 24). The second step of BCAA catabolism is irreversible oxidative decarboxylation, catalyzed by branched-chain alpha-ketoacid dehydrogenase complex (BCKD), which is located in the mitochondrial matrix (20, 23, 24). BCKD is the rate limiting step in BCAA catabolism and its activity is decreased by increased acetyl-CoA concentration and $NADH/NAD^+$ ratio possibly due to ß-oxidation of FFA (6). Thus, increased FFA present in obesity may decrease the activity of BCKD, thereby decreasing the catabolism of BCAA. The importance of adipose tissue in BCAA catabolism has been demonstrated in several studies. Protein levels of BCATm and BCKDE1, one of the three catalytic components of BCKD, are reduced in *ob/ob* mice, diet induced obese mice and Zucker fatty rats (19, 25). Moreover, Herman et al. (22) demonstrated that transplanting adipose tissue from wild-type littermates to BCAT2 knock-out mice can reduce circulating BCAA (22). Human studies corroborate these findings. mRNA expression of BCKDHA, the human gene that encodes for the alpha subunit of E1 of BCKD, was reduced in adipocytes of obese-insulin-resistant human subjects compared to lean subjects (19). In addition, obese women with metabolic syndrome have lower mRNA expression of BCKD in VAT as compared to healthy obese women without disturbed glucose metabolism (19). Furthermore, obese patients who have undergone gastric bypass surgery to lose weight have increased levels of both BCATm and BCKDE1a mRNA expression in adipose tissue (25). These studies and our study all support a significant metabolic role of adipose tissue in BCAA homeostasis.

We demonstrated a significant association between B-AIBA and percent lean body mass. Although lean body mass as quantified by DXA consists of all non-fat, non-bone tissue, including but not limited to striated muscle, this association is consistent with the hypothesized role of B-AIBA as a muscle derived metabolite. We also demonstrated a significant negative association between B-AIBA and total body fat percentage and SAT, although these relationships were no longer significant upon multivariate analyses. These results suggest that increased plasma B-AIBA concentration is associated with

a more favourable body composition, e.g. more lean mass and more SAT versus VAT. BCAA and B-AIBA were not significantly associated with each other in our study. While the lack of association does not necessarily rule out a direct relationship between BCAA and B-AIBA, it does suggest complexity in the generation and metabolism of B-AIBA and furthermore suggests B-AIBA is not simply a breakdown product of valine (14, 15).

Our results also confirmed the association between BCAA and markers of insulin sensitivity (5-7, 9-11, 26) and lipid profile (27) as previously demonstrated. The relationship of BCAA with HOMA, Matsuda Index, glucose AUC after OGTT and HbA1c were strong and remained significant even after controlling for age, gender, race and BMI. Therefore, the role of insulin resistance in mediating the relationship between BCAA and VAT need to be considered. The addition of HOMA or the Matsuda Index to the multivariate regression model assessing the relationship between BCAA and VAT, resulted in the loss of statistical significance between BCAA and VAT suggesting that this relationship may be dependent upon insulin resistance.

In our study B-AIBA was not related to lipid parameters unlike previous report (14). Yet, B-AIBA did show an inverse relationship with markers of IR in our study.

Our study has some important limitations. First, we were not able to quantify the molar concentrations of BCAA and B-AIBA as no internal standard was used in the Metabolite Profiling. However, as the main focus of this investigation was the association between these metabolites and measures of body composition, the arbitrary units used are sufficient for that purpose and molar concentrations are not essential. Furthermore, with a relatively small sample size of 48 subjects, the study may not have been powered to detect true associations between BCAA, B-AIBA, and some measures of body composition or metabolism. Thus, lack of association in the present study should not be interpreted as absence of association.

The present study demonstrates an interesting relationship between BCAA and VAT. Shaham et al. (18) previously demonstrated that BCAA responses after OGTT remained higher in subjects with an impaired glucose tolerance compared to subjects with a normal glucose tolerance. This suggests that the inhibiting effect of insulin on proteolysis can be seen after an oral glucose challenge. Future research investigating the contribution of VAT to dynamic changes in BCAA in a post absorptive state such as after an OGTT may also be of interest. In addition, suppression of lipolysis has been suggested to be more sensitive to the actions of insulin compared to suppression of protein catabolism (18). Given the possible role of FFA in mediating BCAA catabolism (6), assessing the contribution of fasting, nocturnal and post-absorptive FAA in this context would also be warranted in future studies. Furthermore, consideration of the

role of liver fat in BCAA metabolism may also be necessary in future studies given the known association between VAT and hepatic steatosis (4) and the known role of liver in amino acids metabolism.

To conclude, we demonstrate, for the first time, a significant positive relationship of plasma BCAA levels to VAT, which persisted when adjusting for total fat mass, but not for BMI or Matsuda index. Furthermore, we once more showed a positive relationship of plasma BCAA and markers of insulin resistance. We also demonstrated a positive relationship of B-AIBA to lean body mass and a negative relationship to SAT and markers of insulin resistance. This study also adds further data supporting negative effects of VAT on cardiometabolic health. The results might suggest a more significant role of adipose tissue in BCAA homoeostasis than previously considered and indicates further research in the field is needed. Furthermore, our results on B-AIBA and markers of cardiometabolic health together with other published studies (14, 15) suggest that further research into the regulation of this small molecule metabolite and its physiologic significance is warranted.

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A.R. and T.L.S. analyzed the data, performed statistical analyses. C.C. and V.M. contributed to the metabolomics analyses and interpretation. A.R. and H.M. wrote the manuscript. T.L.S., M.M. and S.G. contributed to manuscript preparation. H.M. designed the study, conducted experiments, and oversaw all aspects of the study, data interpretation and manuscript preparation. None of the authors reported any conflicts of interest.

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Chapter 7

General discussion

Worldwide, the number of people suffering from obesity continues to increase (1). Obesity is associated with metabolic disturbances like insulin resistance, high blood pressure and, dyslipidaemia (2), together called the Metabolic Syndrome (Mets). Intra hepatic lipid (IHL) accumulation might be considered as the hepatic manifestation of MetS (3). Diet influences the risk of MetS, diabetes and fatty liver (4, 5) and many different diets for weight reduction and improvement of insulin sensitivity are being considered. In recent years, high-protein diets have become increasingly popular as a way to reduce obesity and improve metabolic risk factors in the general population (6). Therefore, this thesis focused on dietary protein and its effects on lipid and glucose metabolism. The main objective was: to identify the effects of dietary protein on metabolic health focusing on lipid and glucose metabolism – with implications for metabolic disorders, like liver fat accumulation and insulin resistance, body composition and gut microbiota alterations.

To date results on the metabolic health consequences of increased dietary protein intake remain inconclusive as is pointed out by several systematic reviews in varying populations in both observational and intervention studies (7-9), but with a slight health benefit for vegetable proteins versus animal protein (9)-. Also, among the studies described in this thesis, contradictory effects of protein on glucose and lipid metabolism were reported. Briefly, in this thesis it was concluded from a dietary intervention study that in healthy subjects a high-protein diet potentially improved lipid metabolism. This was shown by decreases in IHL, body fat mass, fasting TG and postprandial FFA concentrations. On glucose metabolism small detrimental effects were observed in the postprandial state after the high-protein condition compared with the normal-protein condition (**chapters 4 and 5**), with changes in microbiota composition as well (**chapter 4b**). From a observational study (**chapter 3**) we concluded that high-protein intake, especially from animal sources, is associated with high scores of the fatty liver index. Additionally, increased levels of circulating branched chain amino acids (BCAA) were associated with visceral adiposity and markers of insulin resistance (**chapter 6**). Furthermore, in a review on dietary protein and insulin sensitivity (**chapter 2**) it was described that long term high intakes of dietary protein were associated with unfavourable effects on insulin sensitivity, however in insulin resistant and overweight patients, as a short term dietary strategy, it might be feasible for increasing insulin secretion. Therefore, it is of utmost importance to further investigate and elucidate the possible mechanisms of dietary protein on metabolic health.

Main findings

Dietary protein and insulin

As mentioned above, observational data and intervention studies do not point in the same direction regarding the effect of protein intake on insulin action and diabetes risk. Therefore, we first reviewed human studies addressing high dietary protein intake and insulin action, and discussed the (patho) physiological consequences of high-protein diets regarding insulin action (**chapter 2**). Intervention studies showed that a high-protein, non-restricted diet can lead to hyperinsulinemia in healthy subjects. Contrary, observational studies indicated that high-protein intake might cause insulin resistance on the long term. Therefore, it was concluded that high-protein, non-energy restricted diets, might be helpful for insulin resistant and diabetic people in increasing insulin secretion, due to the insulinotropic effect of dietary protein. However, in healthy people long term high-protein intake is not recommended.

Dietary protein and intra hepatic lipids and lipid metabolism

Dietary protein can influence the lipid content of the liver. It is known that macronutrient manipulation has the potential to change lipid storage in the liver (5). First, in a cross-sectional study (**chapter 3**) we observed that subjects in the highest fatty liver index (FLI-) category consumed more protein, especially from animal origin, less carbohydrates and less dietary fiber. Furthermore, people in the highest FLI-group had a lower 'Dutch Healthy Diet Index' (DHD)-score, reflecting a lower adherence to the Dutch healthy eating guidelines. Our results were in line with results on diabetes risk and protein intake.

Additionally, a human trial was performed, for elucidating possible mechanisms and showing a 'proof of principle' regarding high-protein intake and liver fat accumulation. In this controlled dietary intervention, we showed that a 2 week high-protein, high-fat, hypercaloric diet resulted in changes of the lipid metabolism in healthy human volunteers. This was shown by a lower IHL content and circulating triglycerides, a significant lower fat mass and a higher fat free mass after the high-protein, high-fat, hypercaloric diet compared with the normal-protein, high-fat, hypercaloric diet (**chapter 4**). These effects might be attributed to a lower *De novo lipogenesis* (DNL) and to an increased energy need of the liver when consuming high-protein diets.

In addition, to evaluate whether adaptation to a two week high-protein diet would result in changes in glucose and lipid metabolism postprandially, a meal challenge was performed (**chapter 5**). The results indicated that adaptation to a high-protein

diet induced a lower level of circulating FFA during the meal challenge. This suggests a possible improvement in postprandial lipid metabolism. However, our results also showed that a high-protein diet could slightly impair post-prandial glucose homeostasis, even in this healthy population.

Dietary protein and microbiota

Since, microbiota are thought to play an important role in metabolism, we studied the influence of an increased protein intake on the intestinal microbiota as well. This was performed in the previous mentioned strictly-controlled dietary intervention among healthy volunteers. In **chapter 4b** we showed that a large part of the microbiota in all three diet groups belonged to a stable and shared core of organisms that constituted approximately 60% of all microbiota present. Among the part of the microbiota that varied between the diet groups, certain species showed significantly altered abundances in the high-protein as well as in both hypercaloric diets relative to the control diet. These groups of species are possibly indicative for protein and/or energy-related influences. About 10% of these groups of species belonged to the Firmicutes. Increased levels of Firmicutes are associated with obesity (10).

Branched chain amino acids and metabolic health

Plasma BCAA are elevated in obesity and associated with increased cardiometabolic risk. On the other hand BCAA are indispensable amino acids. In a cross-sectional study among normal-weight and obese subjects (**chapter 6**), a significant positive relationship of plasma BCAA levels to visceral adipose tissue (VAT), was demonstrated for the first time. Furthermore, we once more showed a positive relationship of plasma BCAA and markers of insulin resistance. The results might suggest a more significant role of adipose tissue in BCAA homeostasis than previously considered.

Methodological considerations

Intervention studies versus observational studies

This thesis includes data both from intervention as well as from observational studies. When comparing these different methodological approaches, conclusions on the consequences of increased dietary protein on metabolic health are not consistent. Being able to study the effect of dietary protein on lipid and glucose metabolism in both intervention and observational studies gives a more complete picture of

the consequences on the short and long term. For the effects of dietary protein on insulin resistance we reviewed human intervention studies and observational studies (**chapter 2**). It was concluded that increasing dietary protein as a strategy to improve insulin secretion might be beneficial in certain populations at the short term. However, observational studies indicated that long-term high dietary protein intake, especially from animal sources, was associated with an increased diabetes risk. Interestingly, our own intervention study (**chapter 4**) and observational study (**chapter 3**) revealed a comparable 'conflict' regarding high-protein intake and, in this case, fatty liver. In the intervention study, increasing protein intake resulted in a reduction of intra hepatic lipid accumulation. Contrary, in the observational study, a habitual diet with high-protein content was associated with a higher score on the fatty liver index. Methodological issues, like residual confounding, population under investigation and macronutrient balance of the diet, as well as different underlying metabolic mechanisms might be responsible for these contrasting observations between short-term intervention studies and long-term observational data. A challenge for the future is to better understand the metabolic consequences of increased dietary protein intake, taking these issues into consideration (see section 'implications for future research').

Subjects

In the intervention study 27 young healthy men and women (age 22.8 ± 0.74y; BMI 21.5 ± 0.29 kg/m^2) with no family history of type 2 diabetes, and not taking any medication were included. Such a population is highly metabolic flexible. Nevertheless, even in this young and healthy population we were able to show small differences in liver fat content, body fat mass, fasting and postprandial lipid metabolism and the postprandial handling of glucose. Metabolic flexibility might be defined as the ability to adjust fuel oxidation to fuel availability (11). Thus, to have the capacity to switch from mainly lipid oxidation in a fasted state with low insulin levels, to suppressed fat oxidation, increased glucose uptake and oxidation, and lipid storage during times of food consumption when insulin is high (11, 12). Metabolic flexibility can be measured postprandial by the changes in respiratory quotient after insulin stimulation (13), but it can also be seen as a more long-term process affecting metabolism as a whole. Since our intervention focussed on the differences in lipid and glucose metabolism after adaptation to different diets, it might be argued that having included subjects with high capacity to shift their fuel utilisation has faded out the results. However, in this study we wanted to show a 'proof of principle'. Including healthy subjects with a normal metabolic state really shows that macronutrient manipulation itself can result in alterations of

metabolism. These alterations were observed without interference of disturbing factors like insulin resistance. Nevertheless, subjects might have adapted faster than foreseen. Consequently, our measurements were performed too late to reveal more pronounced alterations in metabolism. Including metabolically challenged subjects, e.g. obese, diminished insulin sensitive or older subjects, would have given more pronounced outcomes.

Diets

In our intervention study we exchanged carbohydrates for protein in an isocaloric manner. Therefore, the observed effects might be due to either increased protein content or decreased carbohydrate content of the diet. A lower carbohydrate content might have influenced the insulin secretion more than the higher amino acid content, since glucose is the number one stimulus for insulin secretion by the pancreatic ß-cells (14). Furthermore, not all protein sources have the same ability to stimulate insulin secretion (15, 16), and some amino acids are faster absorbed in the gut than others (17). Consequently, the choice of protein source might be of importance for the results found on insulin secretion. In our study, subjects were given dietary protein from mixed protein sources, in order to get a reasonable normal diet, with a slight emphasis on dairy products.

Study design

Our study design consisted of a two week run-in period on the control diet, directly followed by a two week intervention period with the first dietary condition and thereafter a two week intervention period with the second dietary condition in a randomised cross-over-manner. We estimated that two weeks' time was sufficient for metabolic adaptation of the subjects. However, as argued above, two weeks might have been too long for this metabolic flexible population. Others did find effects in a similar population, but in a shorter 4 day intervention (18). On the other hand, to detect changes in body composition and fat accumulation in general more time is needed (19, 20). Furthermore, in between the intervention diets we did not include a 'wash-out' period. Yet, our gene data as well as the body composition data showed that there might have been a carry-over effect of the diets (**chapter 4**). Due to absent baseline measurements of both variables we cannot rule out what the effect of the preceding diet was, thus if a wash-out period was needed. Yet, it has to be said that a true wash-out is hard with a dietary intervention. However, an additional period with subjects receiving a control diet in between intervention diets, might have been feasible. Lastly, inclusion of a high-

protein, normal-fat group could have given us additional information on the role of protein in our diets, and for determining the effect of the caloric and carbohydrate content of the diets.

Dietary protein and metabolic health; the possible underlying physiological mechanisms

How can dietary protein potentially influence both lipid and glucose metabolism? That dietary protein has an acute insulinotropic effects has been known for decades (21) and ingestion or infusion of amino acids stimulates both insulin and glucagon (22, 23). This effect of amino acids on insulin can lead to enhanced glucose uptake (24), but also to hyperinsulinemia (25). Possibly, the increased insulin secretion by amino acids affects both glucose as well as lipid metabolism.

Protein, amino acids and glucose metabolism

Insulin is secreted by the pancreatic ß-cells and is highly controlled; glucose is the primary food component to enhance insulin secretion (14, 26). Amino acids are known to enhance insulin secretion as well. However, it is said that physiological levels of individual amino acids are not very potent insulin stimuli. Nevertheless, a combination of amino acids, derived from the diet, could stimulate insulin secretion via incretin-dependent mechanisms as was reviewed by Fu et al. (26). After ingestion of amino acids in the gut, incretin hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are secreted from the intestinal K-cells and L-cells which bind to their receptors on the ß-cells increasing insulin secretion (26). Indeed, others showed that dietary protein was able to increase incretin hormone release (27-29). However, it did not stimulate the release more than when given in combination with a glucose load (27) and it was questioned whether GLP-1 and GIP served a primary role as mediators of insulin release after protein ingestion (29). On the other hand, protein was more potent in reducing glucose responses compared with fat in a dose depended manner by increased insulin and GLP-1 secretion (28). Additionally, amino acids can also enter the TCA-cycle and thereby generating ATP. This all results in an increased insulin secretion (14). Furthermore, during fasting amino acids derived from skeletal muscle proteins ended up in the circulation leading to increased glucagon secretion, consequently, blood glucose levels were elevated initiating insulin secretion (14). The results from the intervention study, as presented in this thesis (**chapter 4**), showed no

differences in fasted insulin and glucose neither in HOMA after the high-protein diet compared with the normal-protein diet. However, when executing a meal challenge (**chapter 5**), it became apparent that after the high-protein diet subjects had a slightly impaired postprandial carbohydrate handling as indicated by higher blood glucose levels. Although, no differences in insulin response were observed. Here it could be concluded that subjects adapted to a high-protein diet developed either a decreased clearance of carbohydrates or higher endogenous production of glucose or a lower disposal of glucose combined with a possible lower sensitivity to insulin secretion. On the long term, observational studies have suggested that prolonged insulin secretion by amino acids from dietary protein might lead to ß-cell exhaustion and diminished insulin sensitivity (25).

Furthermore, amino acids might induce insulin resistance directly, as was elaborated on in **chapter 2**. Skeletal muscle is the largest organ for glucose uptake. Muscle glucose transport and/or glucose phosphorylation was inhibited by elevation of plasma amino acids to postprandial levels, this also caused subsequent reduction in rates of glycogen synthesis and might contribute to insulin resistance (30). In rats fed a high BCAA diet induced insulin resistance accompanied by increased phosphorylation of mechanistic target of rapamycin (mTOR), a nutrient sensing pathway in skeletal muscle (31). Activation of mTOR leads to, in a negative feedback loop, phosphorylation of the insulin receptor substrate 1 (IRS1), and thus to decreased insulin sensitivity (32, 33). In humans, amino acid induced insulin resistance in skeletal muscle was observed in muscle biopsies after a combination of amino acid and insulin infusion which increased IRS1 phosphorylation indicating inhibited insulin-dependent glucose uptake (34).

Amino acids and lipid metabolism

The effect of high-protein diets on lipid metabolism remains uncertain, and it is very likely that effects are a combination of different mechanisms. As mentioned before insulin secretion is enhanced by amino acids, and this can also affect lipid metabolism. Hepatic DNL, an important aspect in lipid metabolism, is highly sensitive to dietary changes in humans (35). It was proposed that dietary amino acids have the ability to both increase glyconeogenesis and inhibit DNL (36, 37). A decreased rate of hepatic lipogenesis after a high-protein diet was also observed in rats; this was in parallel with a lower hepatic expression of Fatty Acid Synthase (*Fasn*), which is the rate limiting enzyme of hepatic lipogenesis (38). Uebanso et al. (39) suggested two pathways being responsible for the reduction in liver lipids and circulating triglycerides in rats: a reduction of DNL as was shown by a down regulation of key genes, and, secondly, a reduction of VLDL-TG

production (39). However, In a study among obese men and women a 4-week weight maintaining diet high in protein (30 en%) no effects were found on insulin secretion and insulin action on lipid metabolism (40). Yet, we did observe changes in lipid metabolism after adaptation to a high-protein diet, as was indicated by a reduction in IHL and plasma TG, fasting as well as postprandial (**chapter 4 and 5**).

On the longer term, however, high levels of circulating insulin increase might increase hepatic DNL, by stimulating hepatic lipogenic enzymes, resulting in increased lipid accumulation in the liver (41). We showed in an observational study that habitual high-protein intake was associated with a higher score of the fatty liver index (**chapter 3**), indeed suggesting that prolonged high-protein intake can lead to increased fat accumulation in the liver. This might be mediated by insulin resistance, which suppresses the inhibitory effect of insulin on lipolysis leading to increased circulating FFA concentrations. Subsequently, increased FFA concentrations can accumulate in the liver as TG (41, 42) and can decrease hepatic beta-oxidation leading to decreased VLDL secretion (43).

The effects of dietary protein on total body energy metabolism might also cause changes in lipid metabolism. In mice an increased energy expenditure of a high-protein versus an isocaloric high-sucrose diet was shown (44). Another study in mice explained the reduction of hepatic lipid by an increase in total and resting energy expenditure (45). In our human intervention study we were not able to show effects of high-protein diet on energy metabolism (**chapter 4**). Yet, others did show an increased resting energy expenditure after 1.5 days of adaptation to a high-protein diet (30 En%) (46), and after 8 weeks of a high-protein (25 En% of protein) diet (47). Yet, it was reported that decreased hepatic lipid content was not caused by enhanced whole body lipolysis and fatty acid oxidation, in both human and mice (37, 48). Increasing dietary protein did result in a less efficient use of energy, due to dietary amino acids being used for acetyl-CoA production and mitochondrial oxidation rather than for storage (49, 50). We proposed that a lower DNL and an increased energy need of the liver when consuming high-protein diets were the main mechanisms (**chapter 4**). During the meal challenge (MC) the prior adaptation to the high-protein diet induced decreased concentrations of circulating FFA, suggesting a possible alteration in postprandial lipid metabolism. Possibly, this was an effect at the level of the adipose tissue where a reduction of post-prandial inhibition of lipid lipolysis occurred (**chapter 4 and 5**). On the other hand, when looking at the observational studies a high-protein intake was associated with a higher risk of having a fatty liver (**chapter 3**).

Concluding, dietary proteins are known to stimulate insulin secretion, however, on the long-term the hyperinsulinemia might lead to insulin resistance and possibly negatively affecting both glucose as well as lipid metabolism.

The role of dietary protein source

Not all protein sources have the same ability to stimulate insulin secretion. Compared with supplementing with either egg, fish or turkey protein, whey protein was a more potent insulin secretagogue in lean subjects (15). A study on the effects of whey versus soy protein ingestion showed that whey, an animal sourced protein, had a greater impact on plasma amino acids and insulin as compared with soy, a vegetable sourced protein (16). Furthermore, cod protein altered glucose metabolism more compared with an animal-meat-and-milk-protein diet in insulin resistant subjects suggesting an effect of the difference in amino acid composition of the diets (51).

Morifuji et al (52) showed that whey protein significantly decreased lipogenic enzyme activity and expression more compared with casein fed animals (52). On the long term, however, high-animal-protein intake has been associated with increased risk of type 2 diabetes, whereas vegetable protein intake showed a decreased risk of type 2 diabetes (53-56). Although, some suggest that reducing the glycaemic load of a diet is the beneficial factor underlying the reduced diabetes risk (56). We showed similar results for fatty liver: high-animal-protein intake was associated with a higher fatty liver index score, contrary vegetable protein intake was associated with a lower fatty liver index score (**chapter 3**). In the intervention study subjects were given dietary protein from mixed protein sources, in order to get a reasonable normal diet, with a slight emphasis on dairy products (**chapter 4 and 5**).

Individual amino acids can also affect metabolism differently, with a special role for the branched chain amino acids (BCAA), valine, leucine, and isoleucine. Obesity and insulin resistance are associated with elevated levels of plasma BCAA (25, 31, 57-61). Several hypotheses have been proposed to explain the role of BCAA in insulin resistance, however it remain inconclusive. As mentioned in **chapter 2 and 6**, high levels of plasma BCAA are linked to insulin resistance and diabetes via activation of the mTOR pathway and by their ability to promote insulin secretion, subsequently causing hyperinsulinemia (25, 31). Others suggested that raised blood BCAA in insulin resistance actually reflects decreased BCAA catabolism by reduced branched chain α-ketoacid dehydrogenase (BCKD) activity and thus is a consequence rather than a cause of insulin resistance (58). The catabolism of BCAA is strictly regulated via several mechanisms (**figure 7.1)** (58, 59). In addition to decreased catabolism, high dietary intake of BCAA may also increase

circulating BCAA levels as demonstrated by short term dietary intervention studies (62-64). The BCAA content of mixed protein sources is about 20% (65). Higher levels of BCAA can be found in whey protein and to a lower extend in cod protein (15, 65, 66), with leucine being the most abundant. However, the long term association between diet and plasma BCAA remains unclear (61, 67). Several hypotheses exist for the role of dietary intake and elevated BCAA concentrations. First, it was shown that insulin secretion increased when supplementing lean subjects with BCAA from rich whey protein (15). Second, over-consumption of BCAA, from high-protein diets, might result in increased flux of BCAA into the skeletal muscle and liver. This increase of BCAA in the liver and muscle might result in an increased catabolism leading to an increased production of intermediates of the TCA cycle.

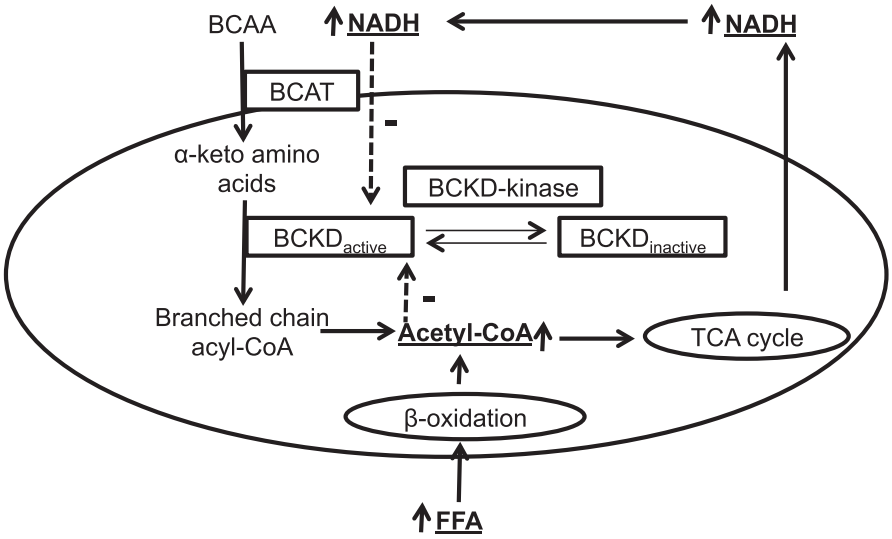

Figure 7.1. The catabolism of branched chain amino acids (BCAA) begins with the transport of BCAA into the mitochondrion, initiated by branched chain amino acid aminotransferase (BCAT). BCAT catalyses reversible transamination of BCAA to form their α-keto acids. The second step is irreversible oxidative decarboxylation, catalysed by branched-chain alpha-ketoacid dehydrogenase complex (BCKD), which is located in the mitochondrial matrix. BCKD is the rate limiting step in BCAA catabolism and its action is inhibited by phosphorylation through binding of BCKD kinase. The activity of BCKD is decreased by increased acetyl-CoA concentration and by NADH/NAD+ ratio due to ß-oxidation of free fatty acids (FFA).

Lastly, a potential cause of increased circulating BCAA levels can be increased protein turnover. Increased release of BCAA from skeletal muscle, the principle source of protein in the human body, might partly be caused by sarcopenia, which is often associated with

insulin resistance. Filho et al (68) found a positive correlation between the amount of BCAA in the muscle and plasma BCAA concentrations. Thus, circulating levels of BCAA might reflect levels of BCAA stored in muscle.
As mentioned in **chapter 6**, we did not observe a relationship of BCAA with dietary protein intake. This might suggest that indeed the source of high circulating BCAA in states of obesity and insulin resistance is from endogenous sources. This might be caused by a decreased catabolism of BCAA due to inhibition of the catabolizing pathway by FFA derived from the VAT. However, more interventions using isotopic labelling of dietary as well as endogenous sources should be executed in order to solve the cause or consequence question.

Implications for future research

The work described in this thesis indicates that high dietary protein intake in short-term interventions may decrease liver fat accumulation and improve lipid metabolism and to a lesser extent glucose homeostasis. On the other hand, observational studies revealed a negative association between increased protein intake and diabetes risk or fatty liver. Furthermore, liver fat accumulation and insulin sensitivity, indicators of metabolic health, could be rapidly influenced by the dietary macronutrient composition.
To bridge the 'gap' between short-term effects and long-term observations future research on dietary proteins should focus on more long-term consequences of high-protein intake in relation to metabolic health, and address the (patho)physiological mechanisms. Ideally, a strictly controlled dietary intervention study should be performed for at least a year. This time-frame is (too) long, but is necessary to control for possible confounding factors like changes in physical activity or other dietary factors, which might affect the results from long-term observational studies. Also, detailed metabolic measures like hyperinsulinemic clamps for insulin sensitivity, liver fat assessment, and stressing of the metabolism by a mixed meal challenge to identify slight modifications of metabolic pathways induced by dietary protein should be a part of such a study. Furthermore, that study should comprise several arms with different macronutrient compositions (carbohydrate, C; fat, F; protein, P), e.g.: diet 1) 55 en% C, 30 en% F, 15 en% P; diet 2) 45 en% C, 30 en% F, 25 en% P; diet 3) 55 en% C, 20 en% F, 25 en% P. In this way both carbohydrate and fat are exchanged for protein, this might give more insight in the contribution of each macronutrient separately to the metabolic effects of a high-protein consumption. Consequently, this can answer the question whether

possible observed effects of prolonged high-protein intake solely can be contributed to protein or are results of low carbohydrate or fat intake.
The matrix or diet in which a protein or amino acid is consumed should receive attention as well e.g. high-fat diets are known to slow down gastric emptying and can affect bioavailability of amino acids from protein. Additionally, special attention should be given to different populations; starting with healthy subjects, and continuing with obese, insulin resistant and elderly subjects. Because, in this thesis it was concluded that different populations will likely result in different outcomes. Again, the need for more postprandial studies after adaptation to high-protein diets is underscored here. Since, people are in a postprandial state most of the day, and doing more postprandial studies might elucidate the effects of dietary proteins on metabolism better.
The observational studies in this thesis revealed once again a more detrimental effect of animal sourced proteins compared with vegetable sourced proteins. Therefore, the question on what the contribution of the different protein sources to metabolism is needs to be investigated further. This might be done by determining the fate of individual amino acids; e.g. will the amino acid be stored or used for fuel en thereby affecting insulin secretion. This can be either indirectly via increasing ATP when being used as substrate for the TCA cycle, or directly due to insulinotropic effects of the amino acid. The increased levels of BCAA as seen in obesity and insulin resistant states might also be a part of this. Stable isotope techniques using labelled amino acids might be a strategy for elucidating the origin of BCAA, which can come from either endogenous or dietary sources.

Implication for public health

Over the past 50 years a small decrease in protein intake was observed in de Dutch population, now being 12-16% of total energy intake, with a higher contribution of animal versus vegetable protein (69). Yet, with the increased attention for high-protein diets it is required that health professionals are aware of the different metabolic consequences of dietary protein. For example, as a dietary strategy to promote insulin secretion, in people with a diminished insulin sensitivity, a diet high in protein content can be considered. However, for weight loss, reduction in caloric intake, irrespective of the macronutrient accentuated, might be more important (70), although preservation of muscle mass requires keeping an adequate level of protein intake (71, 72). Therefore, it should be recommended that when high-proteins diets are advised the duration of

the diet as well as the patient characteristics are taken in to consideration. Additionally, looking at the results of the different chapters in this thesis, not only should we determine the recommended daily intake. For example, in **chapter 3**, we saw that people in the highest fatty liver index category had a higher protein intake compared with people in the lowest category. Therefore, more attention should be given to a safe upper level of intake for the general population.

Overall conclusion

The main objective of this thesis was: to identify the effects of dietary protein on metabolic health focusing on lipid and glucose metabolism – with implications for metabolic disorders, like liver fat accumulation and insulin resistance, body composition and gut microbiota alterations. To conclude, long term high levels of protein intake as well as acute increment of dietary protein intake can alter both lipid and glucose metabolism.

We showed in an intervention study and an observational study that dietary protein alters metabolic profile with potential health consequences. On the short term increasing dietary protein in healthy subjects favourably affects lipid metabolism, but not glucose metabolism. On the long term, however, a high-protein intake was associated with metabolic risk factors like diabetes and fatty liver. The insulinotropic effects of dietary protein were proposed to be of great importance in the effects of dietary protein on metabolic health. Still, the effects of protein on insulin secretion need to be studied more extensively, especially on the long term. Also, further clarification of the exact role of protein source, animal versus vegetable, and amino acid composition on metabolism is needed.

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Summary

Summary

Diet is an important risk factor of the Metabolic Syndrome (Mets), type 2 Diabetes Mellitus and non-alcoholic fatty liver disease. In this thesis the main focuses was on dietary protein and its effects on lipid and glucose metabolism. The objective was to identify the effects of dietary protein on metabolic health focusing on lipid and glucose metabolism—with implications for metabolic disorders, like liver fat accumulation and insulin resistance, body composition and gut microbiota alterations. This was addressed in observational studies and in a strictly controlled human dietary intervention study.
Observational data and intervention studies do not point in the same direction regarding the effect of protein intake on insulin action and diabetes risk. Therefore, we first reviewed human studies addressing high dietary protein intake and insulin action, and discussed the (patho) physiological consequences of high-protein diets regarding insulin action (**chapter 2**). Short-term intervention studies showed that a high-protein, non-restricted diet can induce hyperinsulinemia. Yet, in insulin resistant subjects this can result in improved glycaemic control. Contrary, observational studies indicated that high-protein intake might cause insulin resistance on the long term. Therefore, it was concluded that high-protein, non-energy restricted diets, might be helpful for insulin resistant and diabetic people in increasing insulin secretion, due to the insulinotropic effect of dietary protein. However, in healthy people long term high-protein intake is not recommended.

It is known that macronutrient manipulation has the potential to change lipid storage in the liver. First, in a cross-sectional study (**chapter 3**) we observed that subjects in the highest fatty liver index (FLI-) category, an index indicative for the amount of lipid in the liver, consumed more protein, especially from animal origin, less carbohydrates and less dietary fiber. Furthermore, people in the highest FLI-group had a lower 'Dutch Healthy Diet Index' (DHD)-score, reflecting a lower adherence to the Dutch healthy eating guidelines. Our results were in line with results on diabetes risk and protein intake.

Central in this thesis is a strictly-controlled dietary intervention study in young healthy adults (**chapters 4, 4b and 5)**. A crossover randomized trial with a parallel control group was performed. After a 2-week run-in period, participants were assigned to either the control-diet (n=10; 27.8 energy percent (En%) fat, 16.9 En% protein, 55.3 En% carbohydrates) for 4 week, or a high-fat, hypercaloric diet (n=17; >2 MJ/d) crossover trial with 2 periods of 2 week, with either high-protein (HP) (37.7 En% fat, 25.7 En% protein,

36.6 En% carbohydrates) or normal-protein (NP) (39.4 En% fat, 15.4 En% protein, 45.2 En% carbohydrates) content. Measurements of intra hepatic lipids (IHL), fasted glucose, insulin, triglycerides were performed after 2 week of run-in (baseline), after 2 week of intervention (period 1), and after 4 week of intervention (period 2). Additionally, faeces samples, and adipose tissue samples were collected, body composition was assessed and a meal challenge was executed after periods 1 and 2.

This human trial was performed, to elucidate possible mechanisms and show a 'proof of principle' regarding high-protein intake and liver fat accumulation (**chapter 4**). In this controlled dietary intervention, we showed that a 2 week high-protein, high-fat, hypercaloric diet resulted in changes in lipid metabolism in healthy human volunteers. This was shown by a lower IHL content and circulating triglycerides, a significant lower fat mass and a higher fat free mass after the high-protein, high-fat, hypercaloric diet compared with the normal-protein, high-fat, hypercaloric diet (**chapter 4**). These effects might be attributed to a lower *De novo lipogenesis* (DNL) and to an increased energy need of the liver when consuming high-protein diets. Since, microbiota are thought to play an important role in metabolism, we studied the influence of an increased protein intake on the intestinal microbiota in the strictly-controlled dietary intervention among healthy volunteers as well. In **chapter 4b** we described that a large part of the microbiota in all three diets groups - control, normal-protein, high-fat, hypercaloric and a high-protein, high-fat, hypercaloric diet group - belonged to a stable and shared core of organisms that constituted approximately 60% of the microbiota. Among the part of the microbiota that varied between the diet groups certain species showed significantly altered abundances in the high-protein as well as in both hypercaloric diets relative to the control diet. These altering groups of microbiota are indicative for possible protein and/or energy-related influenced changes. Of these altering groups of microbiota, about 10% of them belonged to the Firmicutes. Increased levels of Firmicutes are associated with obesity and a Western type of diet.

In addition, to evaluate whether adaptation to a two week high-protein diet would result in changes in glucose and lipid metabolism postprandially, a meal challenge was performed (**chapter 5**). The results indicated that adaptation to a high-protein diet induced a lower level of circulating FFA during the meal challenge. This suggests a possible improvement in postprandial lipid metabolism. However, our results also showed that a high-protein diet could slightly impair post-prandial glucose homeostasis, even in this healthy population.

Plasma BCAA are elevated in obesity and associated with increased cardiometabolic risk. On the other hand BCAA are indispensable amino acids. In a cross-sectional study among normal-weight and obese subjects (**chapter 6**), a significant positive relationship of plasma BCAA levels to visceral adipose tissue (VAT), was demonstrated for the first time. Furthermore, we once more showed a positive relationship of plasma BCAA and markers of insulin resistance. The results might suggest a more significant role of adipose tissue in BCAA homeostasis than previously considered.

From data of an intervention study and an observational study it can be concluded that dietary protein alters metabolic profile with potentially unfavourable health outcomes. On the short term increasing dietary protein in healthy subjects favourably affects lipid metabolism, but not glucose metabolism. In long term, observational studies, however, a high-protein intake was associated with metabolic risk factors related to diabetes and fatty liver. The insulinotropic effects of dietary protein were proposed to be of great importance in the effects of dietary protein and metabolic health. However, the long term effects of protein on insulin secretion need to be studied more extensively. Also, further clarification of the exact role of protein source, animal versus vegetable, and amino acid composition on metabolism needs to be elucidated.

Dutch summary

Nederlandse samenvatting

In dit proefschrift staat het effect van eiwit in de voeding op het glucose- en vetmetabolisme centraal. Het doel van de verschillende onderzoeken was het bepalen van de gevolgen van eiwit in de voeding op de zogenaamde 'metabole gezondheid'. De reden hiervoor is dat voeding een belangrijke rol speelt bij metabole gezondheid, zoals de accumulatie van vet in de lever en het ontstaan van diabetes type 2. Bekend is dat een hoge eiwitinname geassocieerd wordt met een verhoogd risico op het ontstaan van diabetes type 2. Daarnaast kan het veranderen van de macronutriënten-verhouding van de voeding, bijvoorbeeld het verhogen van het vet, of van de simpele koolhydraten, resulteren in veranderingen in de vetopslag van de lever. Eiwit heeft mogelijk ook dit effect, maar daarover zijn nog weinig gegevens van humaan onderzoek beschikbaar. Daarom hebben we gekeken naar de effecten van het veranderen van de hoeveelheid eiwit in de voeding op vetophoping in de lever, het glucose, lipide- en insulinemetabolisme, veranderingen in lichaamssamenstelling en op de darmflora. Deze aspecten van de metabole gezondheid zijn onderzocht middels een literatuurstudie, en in zowel een observationeel onderzoek als in een interventiestudie - een gecontroleerde voedingsproef - uitgevoerd bij gezonde jonge mensen.

Omdat er geen eenduidige resultaten uit bestaande literatuur waren met betrekking tot de effecten van eiwitinname op het glucosemetabolisme en insulineresistentie, is in eerste instantie een literatuuronderzoek uitgevoerd. Hierbij zijn de wetenschappelijke artikelen samengevat die gaan over de gevolgen van een verhoogde eiwitinname op het glucosemetabolisme en de fysiologische consequenties van een verhoogde eiwitinname op de effectiviteit van insuline (**hoofdstuk 2**). Insuline is een hormoon dat na een maaltijd afgegeven wordt in de bloedbaan om glucose uit de voeding op te nemen en te gebruiken in de verschillende weefsels, zoals spieren en organen. In deze literatuurstudie zagen we dat een verhoogde eiwitinname, vooral in korte-termijn studies van enkele dagen tot weken, bij gezonde mensen, kan leiden tot een hoge insulineconcentratie in het bloed. Bij gezonde mensen is dit niet wenselijk, maar bij mensen met een voorstadium van diabetes type 2 kan dit resulteren in een verbeterde opname van glucose uit het bloed. Daarentegen vonden we dat observationele studies een verhoogde eiwitinname relateren aan een verhoogde kans op het ontstaan van diabetes type 2. Daarom is in hoofdstuk 2 geconcludeerd dat een verhoogde eiwitinname in een normaal voedingspatroon (dus niet een voeding om af te vallen) gunstig kan zijn bij diabetes type 2 patiënten en mensen met een voorstadium van diabetes type 2, door

het stimulerende effect van eiwit op de insulineproductie en dus op de opname van glucose uit het bloed. Echter, bij gezonde mensen is voeding verrijkt met eiwit niet aan te raden, omdat in deze groep mensen stimulatie van insulineproductie uiteindelijk zou kunnen leiden tot ongevoeligheid voor insuline en dus verslechterde opname van glucose uit de bloedbaan.

Het was al bekend dat het manipuleren van de verhouding van macronutriënten - vet, eiwit en koolhydraten - in de voeding kan leiden tot veranderingen in de vetopslag van de lever. In **hoofdstuk 3** beschrijven we een observationele studie. Hierin werd een score die indicatief is voor de hoeveelheid opgeslagen vet in de lever, de fatty liver index (FLI-index), gerelateerd aan de macronutriënteninname van deze populatie. In deze studie zagen we dat mensen met de hoogste score op FLI een hogere eiwitinname hadden. Dit eiwit kwam vooral uit dierlijke voedingsmiddelen. Daarnaast aten mensen met een hoge FLI score minder koolhydraten en minder voedingsvezels. Ook hadden mensen met de hoogste FLI score een lagere 'Dutch Healthy Diet Index' (DHD) score. Deze score laat zien hoe goed men zich houdt aan de Nederlandse richtlijnen voor gezonde voeding. De resultaten van hoofdstuk 3 zijn in overeenstemming met de eerdere conclusies dat een verhoogde inname van eiwit gerelateerd is aan een verhoogd risico op diabetes type 2 en dus mogelijk ook op het ontstaan van leververvetting.

Centraal in dit proefschrift stond een interventiestudie, namelijk een volledig gecontroleerde voedingsproef bij gezonde jonge mensen (**hoofstukken 4, 4b en 5**). Dit hield in dat de deelnemers aan het onderzoek al hun dagelijkse voeding verstrekt kregen en verder niets anders mochten eten. Hierdoor kon er precies bepaald worden wat de deelnemers aten. Er werd een gerandomiseerde 'cross-over' proef met een parallelle controlegroep uitgevoerd. Nadat alle 27 deelnemers twee weken dezelfde voeding hadden ontvangen om te wennen aan het regime (de run-in periode) werden ze verdeeld in drie groepen. Tien deelnemers kregen vier weken een normale voeding, dit was de controlegroep (n=10; 27.8 energie procent van de voeding (En%) vet, 16.9 En% eiwit, 55.3 En% koolhydraten). Zeventien deelnemers zaten in de interventiegroep en kregen een voeding rijk in vet en hoog in energie (n=17; >2 MJ/d). Deze deelnemers begonnen eerst twee weken met een hoog-eiwit voeding rijk in vet (37.7 En% vet, 25.7 En% eiwit, 36.6 En% koolhydraten) of eerst twee weken op een normaal-eiwit voeding rijk in vet (39.4 En% vet, 15.4 En% eiwit, 45.2 En% koolhydraten). Na twee weken wisselden de deelnemers naar de voeding met de andere eiwithoeveelheid. De metingen werden gedaan na de twee weken run-in periode, na de eerste twee weken

van de proef (periode 1) en na de volgende twee weken van de proef (periode 2). De metingen bestonden uit het bepalen van de hoeveelheid vet in de lever en van de concentraties glucose, insuline en triglyceride in het bloed. Daarnaast namen we na periode 1 en 2 een monster van de ontlasting, een stukje van het vetweefsel, hebben we de lichaamssamenstelling gemeten en deden we een test waarbij het metabolisme extra werd belast, een zogenaamde meal challenge (MC).

Deze voedingsproef bij gezonde mensen werd uitgevoerd om bewijs te leveren dat een verhoogde eiwitinname effect heeft op de ophoping van vet in de lever. Daarnaast wilden we meer inzicht krijgen in de achterliggende mechanismen hiervoor (**hoofdstuk 4**). In dit hoofdstuk laten we zien dat twee weken een voeding verhoogd in eiwit, vet en energie resulteerde in veranderingen in het vetmetabolisme van de deelnemers. Dit concludeerden we omdat deze deelnemers een verminderde vetophoping in de lever, een lagere hoeveelheid triglyceride in het bloed, een verlaging van de lichaamsvetmassa, maar ook een hogere vetvrije massa hadden in vergelijking met de deelnemers die een voeding normaal in eiwit, maar wel verrijkt in vet en energie hadden gegeten. Deze effecten zouden toegeschreven kunnen worden aan een lagere *De novo lipogenesis* (DNL), dit is het maken van nieuwe vetten door de lever uit glucose. Een andere mogelijkheid voor de gevonden resultaten is dat de lever meer energie nodig heeft als er meer eiwit wordt geconsumeerd voor de afbraak van die eiwitten.
Omdat de darmflora ook een belangrijke rol speelt bij het metabolisme, hebben we ook gekeken naar de invloed van de eiwitrijke voeding op de darmflora in dezelfde voedingsproef. In **hoofdstuk 4b** beschrijven we dat een groot deel, ongeveer 60%, van de aanwezige darmflora hetzelfde is bij alle deelnemers ongeacht de voeding en behoort tot een onveranderlijke groep van micro-organismen. In het deel van de darmflora dat wel veranderde door de verschillende voedingen, waren er vooral verschillen door de verrijking met eiwit en energie in vergelijking met de controlevoeding. Van deze veranderende groepen micro-organismen behoorde ongeveer 10% tot de Firmicutes. De aanwezigheid van veel Firmicutes bacteriën is al vaker in relatie gebracht met overgewicht en obesitas en een Westers voedingspatroon.
Verder hebben we onderzocht tot welke metabole veranderingen de 2-weekse adaptatie aan een eiwit- en energieverrijkte voeding leidt. Met name hebben we gekeken naar de glucose- en vetstofwisseling in de periode na een maaltijd (**hoofdstuk 5**). De adaptatie van het metabolisme na twee weken van een eiwitrijke voeding resulteerde in lagere vrije vetzuren in het bloed. Dit veronderstelt een mogelijke verbetering in de

vetstofwisseling na een maaltijd. Aan de andere kant zagen we ook dat deze deelnemers een kleine verslechtering hadden in hun glucosemetabolisme na de maaltijd.

Uit meerdere onderzoeken is gebleken dat concentraties van bepaalde essentiële aminozuren in het bloed, namelijk de 'branched chain amino acids' (BCAA) verhoogd zijn bij mensen met obesitas. Ook worden BCAA's geassocieerd met een verhoogde kans op cardio-metabole aandoeningen. BCAA's zijn essentiële aminozuren, en moeten daarom verkregen worden uit eiwitten in de voeding. In een observationele studie bij mensen met een normaal gewicht en mensen met obesitas (**hoofdstuk 6**), zagen we voor het eerst een significante positieve relatie tussen BCAA concentraties in het bloed en visceraal vet, het vet dat ophoopt tussen de buikorganen. Daarnaast zagen we ook, net als in andere onderzoeken, een relatie tussen de BCAA's en tekenen van voorstadia van diabetes type 2. Onze resultaten suggereren een belangrijkere rol van het vetweefsel bij de regulering van de BCAA balans in het lichaam dan eerder werd aangenomen,.

Al deze resultaten leiden tot de volgende conclusies. Uit de resultaten van de gecontroleerde voedingsproef gecombineerd met de resultaten van de observationele studies kunnen we concluderen dat een verhoging van eiwit in de voeding het metabolisme verandert, met zowel mogelijk positieve als negatieve gevolgen voor de gezondheid. Het verhogen van de hoeveelheid eiwit kan bij gezonde mensen op de korte termijn leiden tot verbeteringen in het vetmetabolisme, maar ook tot een verslechtering van het glucosemetabolisme. Op de lange termijn, zoals gezien in de observationele studies, is een hoge eiwitinname geassocieerd met een toename van metabole risicofactoren zoals vetophoping in de lever en diabetes type 2. Wij veronderstellen dat de stimulerende effecten van eiwit op de insulinesecretie in het bloed een belangrijke factor is bij de gevonden resultaten. De effecten van verhoogde eiwitinname op de lange termijn moeten echter verder worden onderzocht. Daarnaast is het belangrijk dat de rol van de eiwitbron, dierlijk versus plantaardig, en de exacte rol van de aminozuursamenstelling op het metabolisme, verder worden onderzocht.

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Setting up and executing a human intervention trial cannot be done by one single person. Therefore, I would like to thank the dietetics department, the nursing and the laboratory staff of the division, the students that helped during the intervention and of course all the participants of the LiF-Pro study.

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A special thanks also to the PiNM group (Program in Nutrition an Metabolism group) of Massachusetts General Hospital in Boston U.S. It was a great opportunity to get additional research experience, I had a fantastic time and learned a lot. Dr. Hideo Makimura, thank you for all your time and efforts, the discussions with you were very valuable. Dr. Takara Stanley, and Professor Steven Grinspoon, thank you for all the help and giving me the chance to bring my research visit to a good ending.

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Na een dag stil zitten op kantoor kan ik wel wat lichaamsbeweging gebruiken en het liefst doe ik dat rennend (Zoomlopers) in de winter op het ijs (STW) of fietsend met 'De Brede Ruggen'. Ik wordt door deze mannen altijd uit de wind gehouden, soms een duwtje in de rug en hier en daar van (on)gevraagde adviezen voorzien. Bedankt voor alle leuke sportieve uurtjes.

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Annemarie

About the author

Curriculum Vitae

Annemarie Rietman was born on February 8th, 1985 in Zeist, The Netherlands. After completing secondary school at the 'Ichthus College' in Kampen, she started the Bachelor's programme 'Nutrition and Health' at Wageningen University in 2003. After having received her BSc in 2006, she enrolled in the Master's programmes 'Nutritional Physiology' and 'Nutrition in Health and Disease' at Wageningen University. During her Masters she did two masters theses entitled: 1) Short-term oral exposure to white wine transiently lowers serum free fatty acid at TNO (Netherlands Organization for Applied Scientific Research), Quality for Life, Zeist and 2) Effects of dietary sweeteners of blood lactate responses and their potential consequences for fatigue and appetite in collaboration with the research group 'Sports, Nutrition and Life Style', HAN University, Nijmegen and Department of Human and Animal Physiology, Wageningen University. Her research-internship on [$^{13}CO_2$] - Breath test to assess pathways of lactate clearance, was completed at the division of Nutrition Science, Warsaw University of Life Sciences (SGGW), Poland. Furthermore, she completed additional courses on Muscular Physiology, Sports psychology and philosophy at the faculty of Movement Sciences, VU University Amsterdam, The Netherlands.

In November 2010 Annemarie was appointed as a PhD candidate at the Division of Human Nutrition at Wageningen University. The main topic of her PhD research was: to study the effect of dietary protein on lipid and glucose metabolism with implications for metabolic health. This research was a collaboration between Wageningen University, AgroParisTech and the Dutch Dairy Association. As part of her PhD thesis Annemarie visited the research group 'Program in Nutritional Metabolism' of Harvard Medical School and Massachusetts General Hospital, Boston U.S. Here she had the opportunity to explore the effects of circulating branched chain amino acids on glucose and lipid metabolism, especially focussing on body composition in a well-described population. During here PhD project, Annemarie attended several national and international courses and conferences and she was involved in teaching activities. Furthermore, she was chair of the organizing committee of a study tour for the PhD students of the Division of Human Nutrition, to research institutes, universities and companies in Australia and chair of the committee to represent all PhD students from the Division of Human Nutrition, within this division. Currently, Annemarie is working as postdoctoral researcher in the research project of Professor Daniel Tomé on developing a stable isotope method to assess dietary protein quality.

List of Publications

Peer reviewed publications

Joosten MM, de Graaf C, **Rietman A**, Witkamp RF, Hendriks HFJ. *Short-term oral exposure to white wine transiently lowers serum free fatty acids*. Appetite, 2010. 55(1): p. 124-129. doi:10.1016/j.appet.2010.04.217

Rietman A, Schwarz J, Blokker BA, Siebelink E, Kok FJ, Afman, LA, Tomé D, Mensink M. *Increasing Protein Intake Modulates Lipid Metabolism in Healthy Young Men and Women Consuming a High-Fat Hypercaloric Diet*. The Journal of Nutrition, 2014. 144(8): p.1174-1180. doi: 10.3945/jn.114.191072.

Rietman A, Schwarz J, Tome D, Kok FJ, Mensink M. *High dietary protein intake, reducing or eliciting insulin resistance?* European Journal of Clinical Nutrition, 2014 doi: 10.1038/ejcn.2014.123. e-pub: 2 July 2014.

van Nielen M, Feskens EJM, **Rietman A**, Siebelink E, Mensink M. *Partly Replacing Meat Protein with Soy Protein Alters Insulin Resistance and Blood Lipids in Postmenopausal Women with Abdominal Obesity.* The Journal of Nutrition, 2014. 144(9): p. 1423-1429. doi: 0.3945/jn.114.193706.

Submitted papers

Rietman A, Stanley TL, Clish C, Mootha V, Mensink M, Grinspoon SK, Makimura H. *Associations between plasma branched chain amino acids, ß-aminoisobutyric acid and body composition.* Submitted

Rietman A, Sluik D, Feskens EJM, Kok FJ, Mensink M. *The relationship of macronutrients, food groups and diet quality with the 'fatty- liver-index' in a general Dutch population.* Submitted

Rietman A, Schwarz J, Azzout-Marniche D, Piedcoq J, Siebelink E, Kok FJ, Tomé D, Mensink M. *Two weeks of adaptation to a high-protein, hypercaloric high-fat diet alters the postprandial carbohydrate and fat response.* Submitted

Papers in preparation

Neef A, **Rietman A**, Palomares Faubel N, Siebelink E, Olivares M, Izquierdo L, Tomé D, Mensink M, Sanz Y. *Influence of a high-protein, high fat-hypercaloric diet on intestinal microbiota of healthy young adults: diet-specific indicator taxa versus stable core community.* Submitted

Abstracts and presentations

Rietman A, Schreurs VAM, Bujko J. *[$^{13}CO_2$] - Breath test to assess pathways of lactate clearance.* BASIS (Benelux Association of Stable Isotope Scientists) annual meeting, April 2010, poster presentation.

Rietman A, Schwarz J, Blokker BA, Siebelink E, Kok FJ, Afman, LA, Tomé D, Mensink M. *The effect of increased dietary protein on hepatic fat accumulation.* NWO (Dutch Nutritional Science Days), October 2012, oral presentation.

Rietman A, Schwarz J, Blokker BA, Siebelink E, Kok FJ, Afman, LA, Tomé D, Mensink M. *High protein diet results in lower intra hepatic lipid content.* NASO (The Netherlands Association for the Study of Obesity), April 2013, oral presentation.

Rietman A, Schwarz J, Blokker BA, Siebelink E, Kok FJ, Afman, LA, Tomé D, Mensink M. *High protein diet results in lower intra hepatic lipid content.* Experimental Biology, Boston, U.S.A., April 2013, oral presentation.

Blokker BA, **Rietman A**, Schwarz J, Siebelink E, Kok FJ, Muller M, Afman, LA, Mensink M. *Effect of high dietary protein intake on body fat mass and subcutaneous adipose tissue gene expression in humans* .Experimental Biology, Boston, U.S.A., April 2013, poster presentation.

Rietman A, Stanley TL, Clish C, Mootha V, Mensink M, Grinspoon SK, Makimura H. *Novel association between plasma branched chain amino acids and body composition.* NASO, April 2014, top abstract poster and oral presentation.

Rietman A, Stanley TL, Clish C, Mootha V, Mensink M, Grinspoon SK, Makimura H. *Novel association between plasma branched chain amino acids and body composition.* Endocrinology Society annual meeting, Chicago, U.S.A., June 2014, poster presentation.

Rietman A, Schwarz J, Siebelink E, Kok FJ, Tomé D, Mensink M. *Two weeks of adaptation to a high-protein hypercaloric high-fat-diet changes the postprandial carbohydrate and fat response after a mixed meal.* European Association for the Study of Diabetes (EASD) annual meeting, September 2014, poster and short oral presentation.

Rietman A, Sluik D, Feskens EJM, Kok FJ, Mensink M. *The relationship of macronutrients, food groups and diet quality with non-alcoholic fatty liver in a general Dutch population.* NASO, April 2015, oral presentation.

Overview of completed training activities

Discipline specific courses and activities		
Symposium 'New insights on dairy (nutrients) and our health'.	NZO, NL	2010
Mini symposia 'Research meetings NZO'.	NZO, NL	2010-2014
Mini symposia 'Food for thought'.	ZGV, NL	2010-2014
Symposium 'Lipid biology and lipotoxicity'.	Keystone, IRL	2011
Conference 'Dutch Nutrition Science Days'.	NWO, NL	2011-2013
Conference 'Experimental Biology'.	Experimental Biology, US	2013
Conference 'Dutch Association for the Study of Obesity'.	NASO, NL	2013-2015
Conference 'Endocrine Society Meeting'.	Endocrine Society, US	2014
Conference 'European Association for the Study of Diabetes'.	EASD, AT	2014
Course 'NutriScience'.	VLAG, NL	2013
Course 'Introduction to Metabolomics'.	University of Copenhagen, DK	2014
General courses and activities		
Graduate School VLAG, introduction week.	VLAG, NL	2011
Course 'Techniques for writing and presenting'.	WGS, NL	2012
Master Class 'Mixed Models'.	VLAG/HNE, NL	2013
Course 'Career Assessment'.	Meijer&Meijaard, NL	2014
Research visits to Harvard Medical School and MGH.	MGH, US	2013-2014
Optional Courses and activities		
Organizing and participating in the PhD study (VLAG/HNE) tour to Australia.	2013	
Research presentations for staff lectures inside and outside of the Division of Human Nutrition.	2010-2014	
Research Meetings at Harvard Medical School, MGH, Boston University, Tufts University.	2013-2014	
Dutch Academy of Nutrition Sciences (NAV) spring meeting.	2014	

Colophon

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