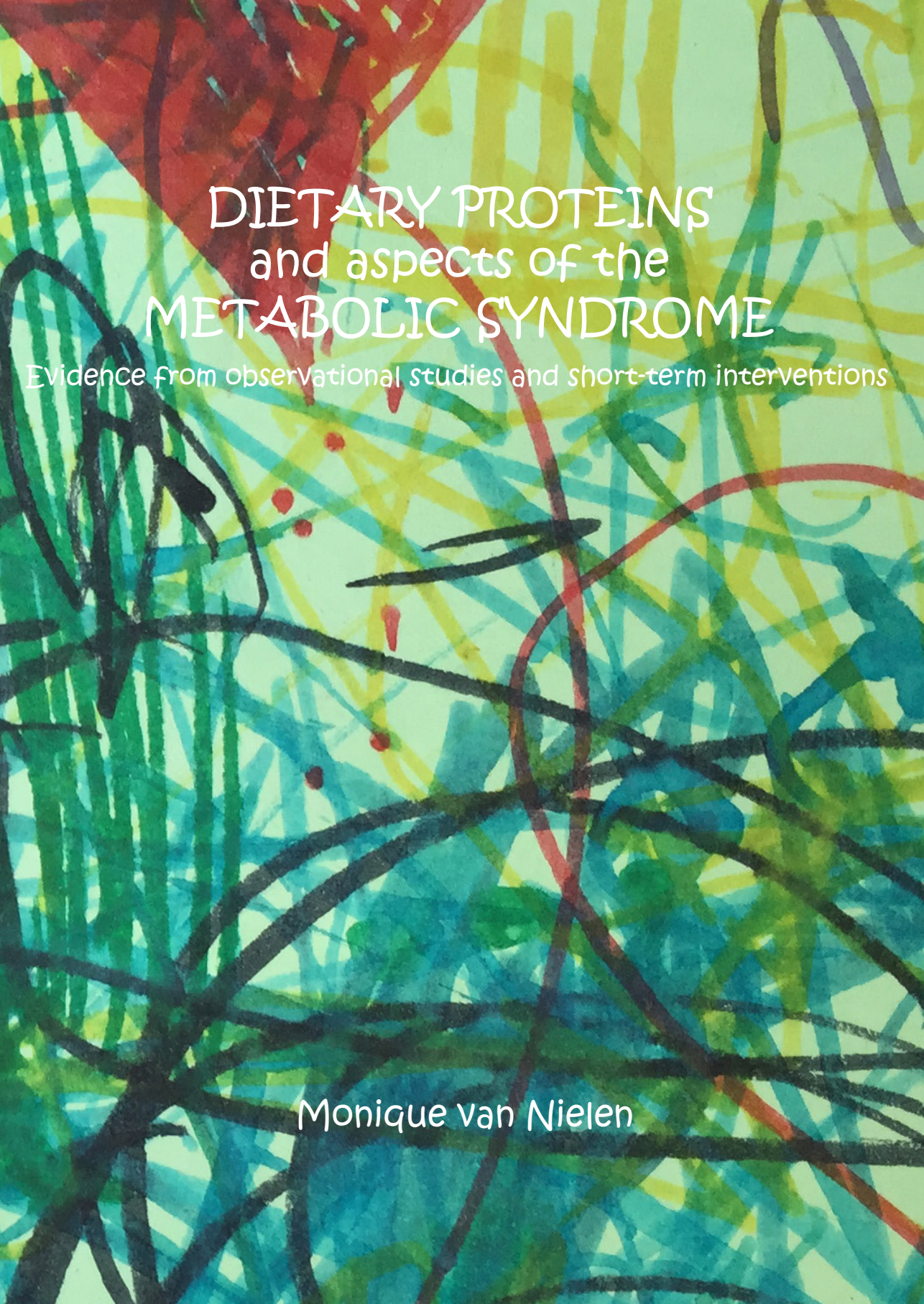
DIETARY PROTEINS
and aspects of the
METABOLIC SYNDROME
Evidence from observational studies and short-term interventions
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Thesis

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ABSTRACT

Background Type 2 diabetes (T2D) and cardiovascular diseases (CVD) are important causes of morbidity and mortality worldwide. The metabolic syndrome (MetS) identifies people at elevated risk of T2D and CVD by its mutual risk factors, such as abdominal obesity, atherogenic dyslipidemia, raised blood pressure and impaired glucose tolerance. Improvements in individual aspects of MetS could be risk-reducing for T2D and CVD and could thus be clinically relevant. Besides by using drug therapy, this can be achieved by lifestyle changes, such as weight loss, increasing physical activity and changes in dietary composition. In addition to general dietary recommendations, such as diets rich in fiber, fruits and vegetables and low in refined grains and saturated fatty acids, increasing dietary protein and soy intake seem promising approaches to prevent MetS. Short-term trials report positive effects of dietary protein intake on weight loss and weight maintenance after weight loss. The postprandial and short-term effect of protein and soy consumption on insulin resistance, glucose homeostasis, and other aspects of MetS are not frequently studied in humans in energy balance. Also, the long-term association between dietary protein intake and T2D incidence is uncertain, it even seemed risk-increasing in prior research.

Objectives We evaluated the impact of dietary protein intake on T2D incidence, aspects of MetS and other cardio-metabolic risk factors, by observational studies (long-term) and interventions (short-term). We studied not only total protein intake, but also specific protein types, more specifically soy protein and arginine-rich protein. We explored the long-term association between total, animal, and plant protein intake and the incidence of T2D. We further investigated the effects of a 4-week strictly controlled weight-maintaining moderate-high-protein diet rich in soy on insulin sensitivity and other cardio-metabolic risk factors. Next, we investigated if inflammatory markers were also changed as a possible pathway through which dietary protein affects cardio-metabolic risk factors. Lastly, we examined whether protein, and more specific arginine-rich protein, added to a high fat meal improved postprandial metabolism and cardiovascular risk factors.

Methods The association between dietary protein intake and T2D incidence was studied in the EPIC-InterAct case-cohort study ($n_{incident\ cases}$= 12,403; $n_{subcohort}$=16,154).
In a randomized crossover trial of 2 4-week periods diets with a moderate-high-protein content, i.e. 22 energy percent (En%) protein, 27En% fat, and 50En% carbohydrate, were studied (n=15). In a diet with protein from mixed sources (HP_{mix}) we partly replaced meat products with soy products (HP_{soy}) to investigate the effect of soy protein intake on insulin resistance, glucose homeostasis, and other aspects of MetS.
A high-fat challenge test was used to study postprandial metabolic markers, inflammatory markers and arterial stiffness (n=18). We compared the postprandial response after a high-fat liquid control meal (95g fat) without protein with meals with 30g added protein.

Results Intake of total protein (per 10 g: HR 1.06 [95% CI 1.02–1.09], $P_{trend} < 0.001$) and animal protein (per 10 g: HR 1.05 [95% CI 1.02–1.08], $P_{trend} < 0.001$) was associated with higher incidence of T2D, after adjustment for main confounders including other dietary factors.
Partly replacing meat with soy in a moderate-high-protein diet resulted in greater insulin sensitivity (FSIGT: S_I:34 ± 29 vs. 22 ± 17 $(mU/L)^{-1}min^{-1}$, P=0.048; disposition index:4974 ± 2543 vs. 2899 ± 1878, P=0.038). After HP_{soy} total cholesterol was 4% lower than after HP_{mix} (4.9 ± 0.7 vs. 5.1 ± 0.6 mmol/L, P=0.001) and LDL cholesterol was 9% lower (2.9 ± 0.7 vs. 3.2 ± 0.6 mmol/L, P=0.004). The summary score for inflammation was lower after HP_{soy} compared with HP_{mix} (z-score: -0.2 ± 0.3 vs. -0.1 ± 0.2, P=0.04), after excluding participants with CRP>6mg/L and extreme outliers. Individual inflammatory markers were not significantly different.
Adding protein to a high-fat meal increased the postprandial insulin response. No differences between arginine-rich and protein low in arginine on postprandial responses were seen. Intact proteins and hydrolysates resulted in similar responses.

Conclusion High total and animal protein intake was associated with modestly elevated T2D incidence in a large cohort of European adults. In contrast, a moderate-high-protein diet for 4 weeks improved many cardio-metabolic risk factors. Partly replacing meat with soy in this moderate-high-protein diet had clear advantages regarding insulin sensitivity and total and LDL cholesterol, and it improved the overall inflammatory state, although not showing clear benefits at individual inflammation markers. We hypothesized to see an origin of these short-term health effects in postprandial properties of arginine-rich protein. However, arginine-rich protein was not superior to a protein low in arginine added to a high-fat meal, regarding postprandial excursions in glucose, insulin, lipids and inflammatory markers.
In view of the rapidly increasing prevalence of MetS and T2D, limiting iso-energetic diets high in dietary proteins, particularly from animal sources, should be considered as on the long-term protein intake seems to increase T2D and CVD risk. However, at the short-term partly replacing meat with soy in a moderate high-protein diet could be preventive for several aspects of MetS, such as improvements in insulin sensitivity, total and LDL cholesterol and possibly a reduced inflammatory state.

CONTENT

Chapter 1

1

General introduction

The metabolic syndrome

The metabolic syndrome (MetS) is a clustering of clinical features associated with an increased risk of type 2 diabetes (T2D) and cardiovascular disease (CVD). It includes abdominal obesity, disturbed glucose homeostasis, increased blood lipids, and increased blood pressure (BP). Insulin resistance is considered pathophysiological important, and is sometimes included in the list of features. Synonyms for MetS are syndrome X, cardio-metabolic syndrome, insulin resistance syndrome and Reaven's syndrome. Since the introduction of the concept in 1920 various definitions of the syndrome have been published. In 2005 the International Diabetes Federation tried to launch a single unifying definition [1], and these criteria were used within our research (**Table 1**).

Rough estimates indicate that approximately 1 million Dutch adults between 28-59 years of age fulfil the criteria of MetS [2]. In Europe, the age-standardized percentage of men with MetS according to the classic 2001 NCEP-ATPIII criteria ranged from 42.7% in the Italian CHRIS cohort to 78.2% in the Finnish DILGOM cohort, and for women from 24% in the CHRIS study to 64.8% in the Finnish Health2000 cohort [3]. Prevalence rates of MetS increase rapidly with age and in obese populations. In ten large European cohorts only 12% of all obese participants did not have any metabolic abnormalities according to the strict definition of MetS, as well as no previous diagnosis of CVD [3]. Obesity without characteristics of MetS is more common in women than men. MetS is associated with a high risk of T2D and/or CVD, with its subsequent morbidity and mortality, which puts a heavy burden on our health care system.

Table 1. The 2005 International Diabetes Federation definition of the metabolic syndrome [4]

For persons to be defined as having the metabolic syndrome they must have:	
Central obesity (defined by waist circumference)	≥ 94 cm for men ≥ 80 cm for women
Plus any two of the following four factors:	
Raised TG level	≥ 150 mg/dL (1.7 mmol/L) *or specific treatment for this lipid abnormality*
Reduced HDL cholesterol	< 40 mg/dL (1.03 mmol/L) for men < 50 mg/dL (1.29 mmol/L) for women *or specific treatment for this lipid abnormality*
Raised blood pressure	systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg *or treatment of previously diagnosed hypertension*
Raised fasting plasma glucose	≥ 100 mg/dL (5.6 mmol/L) *or previously diagnosed type 2 diabetes*

The metabolic syndrome and type 2 diabetes risk

Diabetes mellitus is a condition in which the body is unable to keep fasting glucose concentrations within the normal range of 4 to 7 mmol/L and/or is unable to attain a 2-hour glucose <11.1 mmol/l after an oral glucose tolerance test, i.e. 75 g glucose dissolved in water. This inability occurs when problems arise with the hormone insulin that regulates blood glucose, either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. The two most common forms of diabetes are type 1 and type 2 diabetes, of which T2D covers 90% of all diabetes cases in the world. With increasing obesity and sedentary lifestyles the prevalence of T2D is rising worldwide. The latest estimates from IDF suggest that 387 million people had diabetes in 2015, a prevalence of 8.3%. This means that 1 out of every 12 people in the world are living with diabetes. About half of them do not even know they have it. The prevalence of T2D is expected to increase to 592 million by 2035 [5].

Obesity and MetS have been found to be major risk factors for T2D. Many large clinical trials and meta-analyses reported that the presence of MetS, regardless of which definition was used, was highly predictive of new T2D [6]. A meta-analysis, based on the results of 16 cohorts in various populations, found an average estimated summary RR of 3.5 to 5.2 for incident T2D with any MetS criteria [7]. T2D risk increases with the number of components of MetS. Compared with participants without components of MetS, estimates of RR for those with four or more MetS components ranged from 10.9 to 24.4 [7]. Many studies report that out of the components of MetS impaired fasting glucose (IFG) seems to be the strongest predictor for T2D. Based on limited evidence it has even been suggested that MetS beyond its components, in particular IFG, may not add to the prediction of incident T2D and thus to the clinical value of diagnosing MetS to identify people at elevated risk of T2D. MetS components on a continuous scale could be more effective and possibly improve MetS' predictive ability for T2D [7]. In our trials we studied fasting insulin, and dynamic measures of glucose tolerance and insulin sensitivity as indicators of T2D risk, in addition to the Mets component fasting glucose.

The metabolic syndrome and cardiovascular disease risk

CVD is one of the leading causes of morbidity and mortality worldwide. CVD includes hypertension, coronary/ischemic heart disease (disease of the blood vessels supplying the heart muscle; myocardial infarction), cerebrovascular disease (disease of the blood vessels supplying the brain; stroke), peripheral artery disease (disease of blood vessels supplying the arms and legs; claudication) and deep vein thrombosis and pulmonary embolism (blood clots in the leg veins, which can dislodge and move to the heart and lungs). An estimated 17.3 million people died from CVDs in 2008 and by 2030 more than 23 million people will die annually from CVDs [8].

Obesity and MetS have been found to be major risk factors for CVD. Many studies reported 2-3-fold increased cardiovascular mortality in participants with MetS [6]. Increased CVD risk seems to be nearly two times higher for women than for men and tends to gradually increase with the number of components of MetS in both men and women. MetS however seems less strongly associated with cardiovascular events compared to T2D risk, with a RR of about 1.5 to 2.0 which is less than half the RR for T2D, likely because some MetS components (particularly fasting glucose and waist circumference) are more strongly associated with T2D risk [7]. In our trials, we studied arterial stiffness, markers of inflammation and endothelial function as indicators of CVD risk, in addition to MetS component hypertension.

Link between obesity, the metabolic syndrome, type 2 diabetes, cardiovascular diseases and dietary proteins

In the pathogenesis of T2D and MetS excess accumulation of fat in adipose tissue and non-adipose tissues, such as skeletal muscle and in particular the liver, is considered crucial [9, 10]. Increased fat accumulation can result in so-called 'lipotoxicity' when lipid accumulation exceeds innate storage capacity. This lipotoxicity can affect insulin action and glucose metabolism, drive inflammation, and alter cytokine and adipokine secretion from adipose tissue [11]. Fat accumulation in skeletal muscle contributes to hyperglycemia and hypertriglyceridemia, due to reduced insulin-stimulated glucose uptake and glycogen synthesis [12] and reduced suppression of insulin on hepatic glucose production [13]. Lipotoxicity in adipose tissue can play a role in the pathogenesis of insulin resistance as it is associated with a low-grade inflammatory state, by production of inflammatory cytokines, such as TNF-α and IL-6, and altered adipokine secretion, such as leptin, adiponectin, resistin [11, 14]. Thus, lipotoxicity and low-grade inflammation are prominent features of obesity, and can link obesity to MetS, insulin resistance, glucose intolerance, T2D and CVD.

Dietary protein intake and the metabolic syndrome

In order to prevent people from developing MetS it is important to know which risk factors are related to characteristics of MetS. It is known that diet can play an important role in the pathogenesis, treatment and prevention of MetS. In recent years, diets with increased protein content have become increasingly popular as a means to reduce obesity [16]. High-protein energy-restricted diets are reported to result in a greater weight loss compared to other diets, as dietary proteins are thermogenic and increase satiety. Although high-proteins diets may result in a greater weight loss compared to other diets, it has also been highlighted that the type of diet seems unimportant for weight loss [17]. Besides for weight loss, high-protein diets are also popular to improve cardio-metabolic risk factors. Dietary proteins increase insulin secretion and can have a direct effect on insulin resistance [18]. Several studies suggest a beneficial effect of high-protein diets on insulin resistance and

glucose homeostasis, independent of weight loss [19, 20]. Varying the energy content of fat and carbohydrate seems to only marginally influence this effect [21, 22]. Besides effects on glucose metabolism, dietary protein intake is also associated with cardio-metabolic risk factors, such as blood lipid profile [23], reduced intrahepatic lipids (IHLs) [24, 25], and possibly inflammation [26] and BP [27].

Various protein sources, cardio-metabolic risk factors and type 2 diabetes incidence

Aside from the total protein content of the diet, the dietary protein source can be influential. High intake of protein from animal origin may increase T2D incidence [28], and is associated with increased risk of fatal and non-fatal cardiovascular outcomes [29]. However, protein from dairy [30, 31] and fish [32] may reduce T2D risk. And although all dietary proteins affect glucose metabolism by increasing insulin secretion and glucose uptake, effects are different by protein source. Animal protein, more specific whey protein [33-35] and protein from fish [36], could be superior over other protein sources. Plant protein seems to give a less strong insulin response, while long-term risks of high plant protein are less evident. High plant protein intake is even associated with lower BP [37, 38] and lower CVD mortality in healthy adults [39].

Soy protein intake, cardio-metabolic risk factors and type 2 diabetes incidence

Soy protein is well-studied, because it improves circulating blood lipids and is associated with weight loss [40]. It is further suggested to have anti-inflammatory properties by reducing lipotoxicity, and may thereby improve insulin resistance and glucose homeostasis [11, 41]. Increased intake of legumes, and soybean in particular, has been found to lower T2D risk [42] and concentrations of total and LDL cholesterol [43]. But the number of human studies addressing the role of dietary soy in insulin resistance, glucose homeostasis, T2D and MetS is limited. Like all dietary proteins, soy protein increased post-prandial insulin concentrations and reduced the glycemic response [44, 45]. On short-term soy consumption improved glycemic control and insulin resistance, as measured by the HOMA-index, in postmenopausal women [46]. In diabetic patients, both men and women, long-term soy protein intake reduced glycaemia [47]. So far, only one epidemiological study explored the long-term association of soy protein and MetS as such, and not its individual components [48]. Soy protein tended to be associated with reduced MetS risk in women, but elevated risk in men. In this sex-specific effect estrogen-like activity of one its constituents, e.g. isoflavones, could be involved [48]. The effect of soy and/or isoflavones on markers of (vascular) inflammation was studied more frequently, reflecting the interest for soy and CVD risk. However, results are inconclusive, some reporting reduced

inflammation [49, 50], while others did not [51, 52]. The majority of studies was performed in postmenopausal women and did not address insulin action and glucose metabolism.

Postprandial effects of dietary protein intake

Differences between short-term and long-term effects of dietary proteins may relate to the postprandial phase, which is associated with low-grade inflammation and T2D and CVD risk [53, 54]. Postprandial metabolism is gaining attention, because although the effects are temporarily and reversible, people spent most of their waking hours in the postprandial state. People may have disturbed postprandial values, while still able to maintain normal fasting values, making it valuable for early risk assessment. The postprandial response is disturbed in people with characteristics of MetS, e.g. characterized by deceased glucose tolerance and/or diminished ability to respond to high-fat intake. A high-fat meal increases TG, which is believed to trigger low-grade inflammation and deteriorate vascular function. These effects may be counteracted by various dietary components. For example, the amino acid arginine is suggested to have anti-inflammatory properties, by reducing lipotoxicity and/or affecting postprandial metabolism. Arginine is highly available in soy protein, thus beneficial effects of soy protein could be partly driven by arginine. The effect of arginine is most likely mediated via the Nitric Oxide (NO) pathway. Beside its effect on vascular function, which can also improve glucose homeostasis, NO can have a variety of metabolic effects which reduce lipotoxicity and inflammation. Studies on L-arginine so far indicated both long-term effects, as well as acute postprandial actions, especially when metabolism is already challenged, as in diabetic patients, or after a high-fat meal. Whether arginine-rich proteins are equally effective is not known and a careful examination of the effect of arginine-rich protein on postprandial (dys)metabolism and inflammation is hardly performed.

Aim and outline of the thesis

High-protein diets increase weight loss and the odds of successful weight maintenance, and as mentioned earlier they are popular to improve cardio-metabolic risk factors. Although most short-term studies report beneficial effects of dietary protein intake on insulin sensitivity, high-protein intake is associated with a higher T2D and CVD incidence in long-term observational studies [28, 55, 56]. Suggested beneficial acute effects of dietary protein intake on insulin secretion and glycemic control [57] do not seem to persist mid- and long-term [58, 59].

Research on the effects of high-protein diets in energy balance, so not during and after weight loss, is scarce, although it is important. A possible risk of the positive campaign on high-protein diets is that the intake of protein will increase, for example when people continue to have an increased dietary protein intake after quitting a weight loss or weight maintenance diet. This could be hazardous as high-protein diets are still discussed to

potentially have negative effects on kidney function [39, 60, 61]. High-protein intake (above 0.8 g protein/kg body weight/day) is also associated with other disorders and health risks in adults [62] and evidence on low-carbohydrate high-protein diets (20-23En%) is suggestive for higher all-cause mortality [39]. Less information is available about the possible impact of moderately-high-protein diets on health.
For that reason this thesis aims to evaluate the impact of moderate-high dietary protein intake on T2D incidence, aspects of the metabolic syndrome and other cardio-metabolic risk factors, by observational studies (long-term) and interventions (short-term). It is the aim not only to look at total protein intake, but also effects of specific protein sources, more specifically soy protein and arginine-rich protein in people in energy balance.

Based on the overall aim, the following research questions were formulated:

- Is long-term high-protein intake positively associated with T2D incidence, while in contrast high-protein intake on short-term has beneficial effects on aspects of MetS, e.g. insulin sensitivity?
- Does soy protein intake compared to meat protein beneficially affect aspects of MetS?
- Does arginine, as a typical soy amino acid, have a potential role in these effects by improving postprandial dysmetabolism?

To answer the first question the association of dietary protein intake and incidence of T2D was investigated in a large-scale European case-cohort study in **chapter 2**. **Chapter 3** covers the first and second question with results of a randomized crossover trial about partly replacing meat protein with soy protein on cardio-metabolic risk factors, such as insulin resistance, glycemic control, blood lipids and other cardio-metabolic risk factors, in postmenopausal women with abdominal overweight. Additional results of this trial on the low grade inflammatory state associated with MetS are given in **chapter 4**. The postprandial effects of arginine-rich protein added to a high-fat meal on metabolic control, inflammation and endothelial function in men with characteristics of MetS in **chapter 5** were used to try to answer the last question. Finally, the overall findings are discussed and conclusions and recommendations for future research are made in **chapter 6**.

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Abstract

Objective The long-term association between dietary protein and type 2 diabetes incidence is uncertain. We aimed to investigate the association between total, animal, and plant protein intake and the incidence of type 2 diabetes.

Research design and methods The prospective European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct case-cohort study consists of 12,403 incident type 2 diabetes cases and a stratified subcohort of 16,154 individuals from eight European countries, with an average follow-up time of 12.0 years. Pooled country-specific hazard ratios (HRs) and 95% CI of prentice-weighted Cox regression analyses were used to estimate type 2 diabetes incidence according to protein intake.

Results After adjustment for important diabetes risk factors and dietary factors, the incidence of type 2 diabetes was higher in those with high intake of total protein (per 10 g: HR 1.06 [95% CI 1.02–1.09], $P_{trend} < 0.001$) and animal protein (per 10 g: 1.05 [1.02–1.08], $P_{trend} = 0.001$). Effect modification by sex ($P < 0.001$) and BMI among women ($P < 0.001$) was observed. Compared with the overall analyses, associations were stronger in women, more specifically obese women with a BMI >30 kg/m^2 (per 10 g animal protein: 1.19 [1.09–1.32]), and nonsignificant in men. Plant protein intake was not associated with type 2 diabetes (per 10 g: 1.04 [0.93–1.16], $P_{trend} = 0.098$).

Conclusions High total and animal protein intake was associated with a modest elevated risk of type 2 diabetes in a large cohort of European adults. In view of the rapidly increasing prevalence of type 2 diabetes, limiting iso-energetic diets high in dietary proteins, particularly from animal sources, should be considered.

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Chapter 2

2

Dietary protein intake and incidence of type 2 diabetes in Europe:

The EPIC-InterAct case-cohort study

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INTRODUCTION

Dietary proteins are advocated to have positive effects on weight loss and weight maintenance due to properties related to satiety and diet-induced thermogenesis [1]. Doubling the relative protein content of the diet under ad libitum conditions for 12 weeks reduces food intake and can lower body weight by >6% [2]. Therefore, increasing protein intake seems a promising approach to tackle the obesity epidemic and therewith to reduce the incidence of chronic diseases.

In contrast, long-term observational studies report an association of high protein intake with a higher risk of type 2 diabetes [3,4]. Suggested beneficial acute effects of dietary protein on insulin secretion and glycemic control [5] do not seem to persist mid- and long-term [6,7]. An iso-energetic high-protein diet compared with a low-protein high-fiber diet reduced insulin sensitivity after 6 weeks [6], and participants with a habitually high- compared with a normal-protein diet showed signs of reduced insulin sensitivity [7]. The few epidemiological studies that addressed the association between protein intake and type 2 diabetes all found an increased type 2 diabetes risk with high protein and/or meat protein intake [3,4,8–10]. However, most of these studies had small sample sizes, ranging from 140 [8,9] to 1,200 [10] participants. Of two large cohort studies with >35,000 participants [3,4], Sluijs et al. [3] did not observe a significant association for total protein after adjustment for BMI and waist circumference in the Dutch cohort of European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct. Based on subanalyses, the authors concluded that only for participants with a BMI <25 kg/m^2 does high protein intake increase type 2 diabetes risk [3].

Besides total and animal protein, prior research suggests that the protein source could be of relevance. Type 2 diabetes risk is associated with higher meat consumption, particularly red and processed meat [11–13]. Type 2 diabetes risk is reported to be lower in subjects with high dairy use [14–17] and/or high plant product consumption, especially of legumes [18] and nuts [19]. Data on the relation between fish consumption and type 2 diabetes reached mixed conclusions [20–22], and a recent study in the EPIC-InterAct case cohort reported no association [23]. It is unclear whether it is the protein or other nutrients in such food groups that explain the association with type 2 diabetes.

Within the setting of EPIC-InterAct, we were able to study the association between protein intake and risk of type 2 diabetes in a large case cohort in eight countries in Europe: the largest cohort of type 2 diabetes so far [24]. The characteristics of this study made it possible to explore the association between protein intake and type 2 diabetes according to plant or animal origin and by protein source.

RESEARCH DESIGN AND METHODS

Study design

The participants, methods, study design, and measurements have previously been described [24]. Briefly, the InterAct project was initiated to investigate how genetic and lifestyle behavioral factors, particularly diet and physical activity, interact to lead to type 2 diabetes. As part of the wider InterAct project, consortium partners have established a case-cohort study of incident type 2 diabetes (EPIC-InterAct case cohort) based on cases occurring in EPIC cohorts between 1991 and 2007 in 8 of 10 EPIC countries. A case-cohort study is comparable with a cohort study but it is more efficient, as it uses a random sample of the cohort to compare cases with. The case-cohort design combines the advantages of a prospective cohort with the efficiency and power of a large case-control study.

Type 2 diabetes case ascertainment

We followed a pragmatic high-sensitivity approach for case ascertainment with the aim of 1) identifying all potential incident type 2 diabetes cases and 2) excluding all individuals with prevalent type 2 diabetes. Prevalent and incident type 2 diabetes was identified using multiple sources of evidence including self-report, linkage to primary care registers and secondary care registers, medication use (drug registers), hospital admissions, and mortality data. Further details have previously been published [24].

Subcohort

A subcohort of 16,835 individuals was randomly selected stratified by center. After exclusion of 548 individuals with prevalent type 2 diabetes, 129 individuals without information on reported diabetes status, and 4 individuals with postcensoring type 2 diabetes, 16,154 subcohort individuals remained.

Participants

We used a case-cohort design, including incident diabetes cases (n = 12,403) and a representative subcohort (n = 16,154, including 778 cases of incident type 2 diabetes) selected from the EPIC cohort [24]. After exclusion of participants with missing information on dietary data (n = 117; 70 case subjects, 47 subcohort) or other missing covariates, i.e., physical activity, educational, and smoking status (n = 790; 357 case subjects, 433 subcohort), and participants who fell in the top or bottom 1% of the "energy intake/energy requirement ratio" (n = 619; 339 case subjects, 280 subcohort), our analysis included 26,253 participants (10,901 incident type 2 diabetes case subjects and a subcohort of 15,352 participants including 736 cases of incident type 2 diabetes).

All EPIC study participants gave written informed consent, and the study was approved by the national ethics committees and the International Agency for Research on Cancer.

Protein intake and other variables

Dietary intake, over the 12 months before enrollment, was assessed by self- or interviewer-administered dietary questionnaires (mainly food frequency questionnaires [FFQ]), developed and validated within each country, to estimate the usual individual food intakes of the study participants (for more detail, see Riboli et al. [25]). Protein intake (g/day) was adjusted for total energy intake by the residual method [26] and categorized in quintiles according to the data of the subcohort. As part of EPIC, standardized information on lifestyle exposures was collected by self-administered national questionnaires at baseline [25]. Physical activity during work and leisure time was classified in four categories according to the Cambridge Physical Activity Index [27]. Weight, height, and waist circumference were recorded using a standard protocol during a visit at the research center, except in Oxford (U.K.) and France, where only self-reported height and weight were available [25].

Statistical analysis

The association between energy-adjusted protein intake and type 2 diabetes risk was examined in hazard ratios (HRs) and 95% CIs using Cox proportional hazards models adapted to case-cohort designs according to the Prentice-weighted method [28]. We stratified all analyses by country, mainly because of the large dietary heterogeneity between countries, specifically between northern and southern Europe, e.g., relatively high protein intake in Spain and low protein intake in Germany and Sweden. We used random-effect meta-analyses to pool the country-specific HRs. Between-country heterogeneity was tested by I^2 statistic. Linear associations between protein intake and type 2 diabetes were estimated per 10-g increment of protein intake and by analyzing linear trends across protein intake categories using the median value of each quintile as a continuous variable. We adjusted for type 2 diabetes risk factors and nutritional factors using a stepwise approach, with age as the underlying time scale. Model 1 included protein intake, total energy (kilocalories per day), center, and sex. In model 2, we added type 2 diabetes risk factors, i.e., smoking status (never, former, or current), education (low, secondary, or high), physical activity (inactive, moderately inactive, moderately active, or active), and alcohol use (0, >0–6, >6–12, >12–24, or >24 g/day). Model 3 was additionally adjusted for soft drinks, tea, coffee, and the following residual adjusted nutrients: fiber, saturated fatty acids, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and cholesterol. It was not adjusted for carbohydrates to estimate the effect of increasing protein at the expense of carbohydrates. Model 4 was additionally adjusted for BMI (measured as weight in kilograms divided by the square of height in meters) and waist circumference (centimeters).

Effect modification was examined by various dietary and lifestyle factors, i.e., sex, BMI, waist circumference, physical activity, smoking, menopausal status, hyperlipidemia, and

hypertension. A country-specific multivariate Wald test was used to evaluate interactions by continuous interaction terms. In case of significant interactions, HRs were stratified. The association between protein intake and type 2 diabetes risk was estimated within each country by sex (11,241 men; 15,012 women) and by BMI (normal: BMI <25 kg/m^2, *n* = 8,317; overweight: BMI 25–30 kg/m^2, *n* = 10,951; obese: BMI ≥30 kg/m^2, *n* = 6,985) and meta-analyzed. Models for sources of animal and plant protein and sequentially excluding main sources of protein, e.g., meat, were designed to explore the contribution of various protein sources to the associations of protein type with type 2 diabetes risk. Finally, sensitivity analyses were performed by excluding individuals who might have made dietary adjustments and/or lifestyle changes because of chronic disease at baseline (i.e., hypertension, hyperlipidemia, myocardial infarction, and/or stroke) and by excluding misreports of energy according to Goldberg criterion categories, defined as "under-reporters" with a ratio of energy intake to basal metabolic rate <1.14 and "over-reporters" with a ratio of >2.1 [29]. Data analysis was performed using SAS 9.2, and the meta-analyses were conducted in STATA11.

Results

Our analysis of this case-cohort study consisted of 10,901 incident type 2 diabetes cases and a subcohort of 15,352 participants (including 736 type 2 diabetes cases) with an mean ± SD follow-up time of 12.0 ± 2.3 years. FFQ-based median estimated energy-adjusted total protein intake was 90.4 g/day for men and 91.0 g/day for women, mainly consisting of animal protein. It was highest in Spain (102.5 g/day) and lowest in Germany and Sweden (respectively, 80.0 and 80.8 g/day) (**Figure S1**).

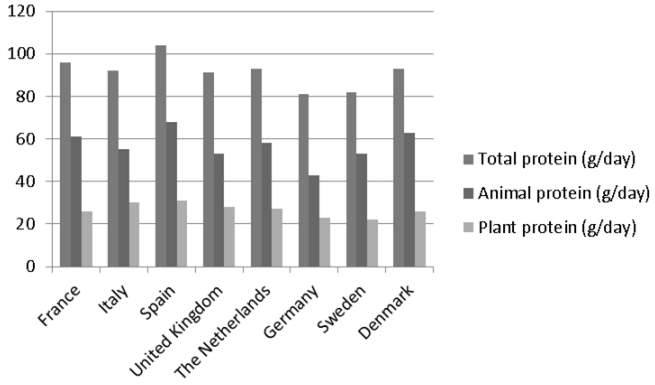

Figure S1. Mean FFQ estimated daily protein intake (g/day), energy-adjusted by the residual method, in the 8 countries of the EPIC-InterAct subcohort

Main animal protein sources in order of proportion were meat, dairy, and fish; main plant protein sources were bread, pasta and rice, potatoes, and vegetables (**Table 1**). In the subcohort, participants with high intake of total protein (highest vs. lowest quintile) had

higher mean BMI and waist circumference and higher intake of MUFAs, dietary fiber, dietary cholesterol, calcium, and β-carotene, whereas educational level and mean intake of carbohydrates, saturated fatty acids (SFAs), coffee, tea, and soft drinks were lower (Table 1). Additionally, with increasing protein intake women were less physically active, drank less alcohol, and were less often smokers, whereas men were more often smokers. With increasing quintiles of total protein intake, the number of type 2 diabetes cases in the subcohort increased (Table 1).

High total protein intake was associated with a 13% higher incidence of type 2 diabetes (HR 1.13 [95% CI 1.08–1.19]) for every 10-g increment after adjustment for energy, center, sex, type 2 diabetes risk factors, and dietary factors (**Table 2; Figure S2**). Animal protein intake showed comparable results (1.12 [1.07–1.17]). Additional adjustment for waist circumference and BMI attenuated the associations to some extent for total protein to a 6% higher incidence of type 2 diabetes (1.06 [1.02–1.09]) and to 5% for animal protein (1.05 [1.02–1.08]). Analyzing total and animal protein intake by quintile (high vs. low) showed comparable results (1.17 [1.00–1.38], $P_{trend} < 0.001$ and 1.22 [1.06–1.40], $P_{trend} < 0.001$). Between-country heterogeneity was low (I^2 0.0–45.3%) (Figure S2).

For the association between total and animal protein intake and type 2 diabetes, effect modification by sex ($P < 0.001$) and by BMI among women ($P < 0.001$) was present. The association between 10-g increment of total and animal protein intake and type 2 diabetes was confirmed in women (1.10 [1.06–1.14] and 1.09 [1.05–1.14], respectively) in model 4 (**Figure 1; Table S1**). In men, no association was present (both total and animal protein, 1.02 [0.98–1.06]). Compared with overweight women (1.07 [1.01–1.14]) and normal-weight women (1.11 [0.99–1.25]), obese women had a stronger association between animal protein intake and type 2 diabetes (1.19 [1.09–1.32]) (**Table 3**). In the sensitivity analyses, exclusion of "under-reporters" (n = 8,096) and "over-reporters" (n = 1,206) did not change the overall and sex-specific associations for both total and animal protein intake and type 2 diabetes (data not shown). Excluding the effect of possible lifestyle changes as a result of a medical condition (i.e., baseline self-reported hypertension, hyperlipidemia, myocardial infarction, and stroke, n = 11,043; 3,641 case subjects and 7,402 subcohort) strengthened the associations between both total and animal protein intake and type 2 diabetes in women (1.15 [1.04–1.27] and 1.12 [1.03–1.22], respectively) (**Table S2**). No specific group of protein sources was accountable for the positive association between animal protein and type 2 diabetes; excluding protein from dairy, fish, or meat from total animal protein did not alter the association.

Plant protein intake per 10 g was not associated with type 2 diabetes, with an HR of 0.92 [0.80–1.04], $P_{trend} = 0.507$, in model 1 and 1.04 [0.93–1.16], $P_{trend} = 0.098$, in model 4 (Table 2; Figure S2) and 1.12 [0.98–1.29] in the sensitivity analysis additionally adjusting for possible lifestyle changes as a result of a medical condition (Table S2). No effect modification by sex or BMI was present.

Excluding specific groups of plant protein sources did not alter the overall absent association between plant protein and type 2 diabetes.

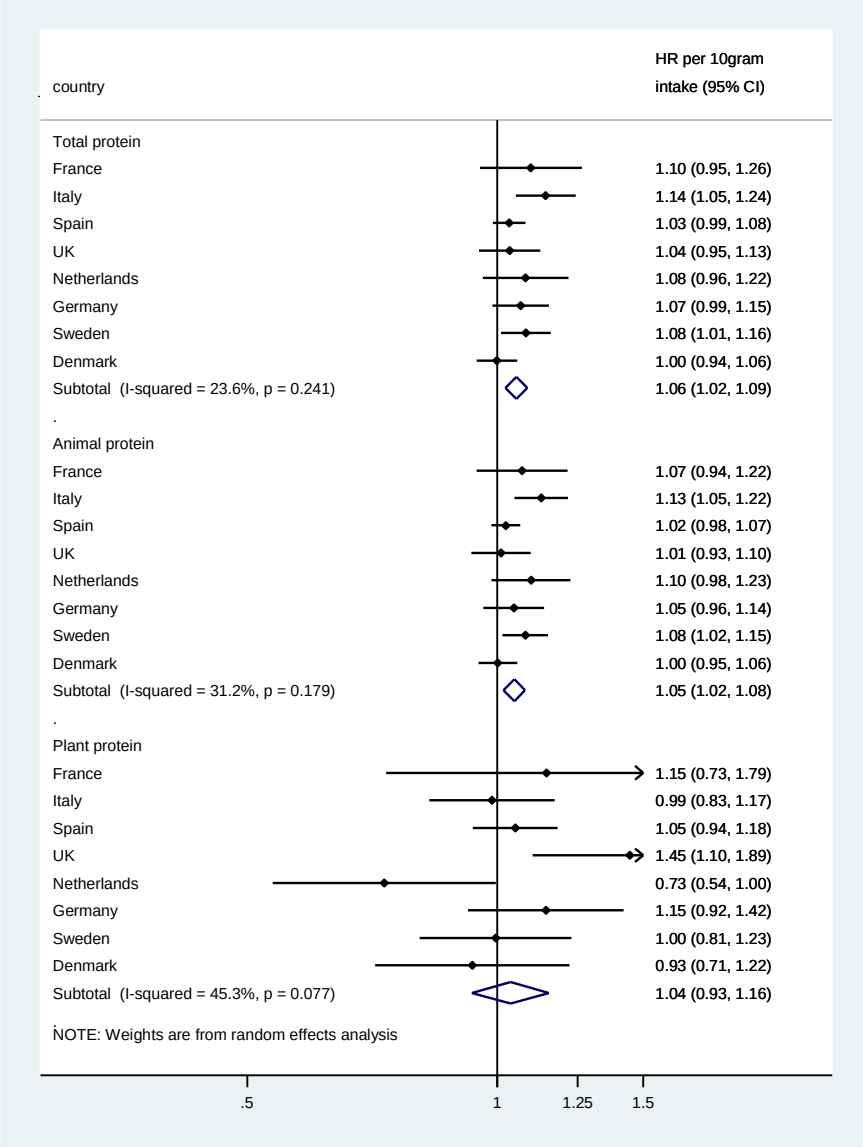

Figure S2. Overall meta-analysed (pooled country-specific) hazard ratios (HRs and 95%CI) of type 2 diabetes associated with 10g increments of total protein intake, animal protein intake and plant protein intake (FFQ estimated intake energy-adjusted by the residual method). The HRs are adjusted for energy, sex, smoking, education, physical activity, alcohol, fibre, SFA, MUFA, PUFA, cholesterol, soft drinks, tea, coffee, BMI and waist (not sex specific).

Table 1 Characteristics and dietary consumption of the EPIC-InterAct subcohort by categories of total protein intake* (*n* = 15,352) for **MEN**

Protein intake (g/day)*	Q1 68.9 (63-73)	Q3 89.0 (87-91)	Q5 113.5 (109-121)
N cases/n	45/1162	76/1162	105/1161
Characteristics			
Age (yrs)	53.0 ± 9.4	53.5 ± 9	51.4 ± 7.8
Follow-up (yrs)	11.8 ± 2.6	11.8 ± 2.7	12.2 ± 2.6
BMI (kg\m^2)	25.6 ± 3.5	26.5 ± 3.5	27.9 ± 3.6
BMI <25 (%)	44.6	32.9	20.2
BMI 25-30 (%)	44.8	52.9	54
BMI >30 (%)	10.7	14.2	25.8
Waist (cm)	92.8 ± 10.1	94.7 ± 9.6	97.9 ± 9.6
Family history of diabetes (%)			
Yes	15.8	17.1	11.7
Smokers (%)			
Never	34.5	30.7	30.2
Former	36.8	37.9	33.4
Smoker	28.7	31.4	36.4
Hypertension (%)			
Yes	18.1	18.9	18.1
Don't know	2.1	3.9	3.9
Hyperlipidemia (%)			
Yes	22.6	20.9	23.2
Don't know	9.3	12.9	7.3
Educational level (%)			
Long education (incl. University deg.)	28.5	24.5	19.0
Physical activity (%)			
Active	24.2	22.8	27.2
Postmenopausal (%)	-	-	-
Country (n)			
France (♀ 526) (%)	-	-	-
Italy (♂ 639, ♀ 1300) (%)	7.5	13.6	6.7
Spain (♂ 1333, ♀ 2154) (%)	2.5	15.5	58.7
United Kingdom (♂ 423, ♀ 661) (%)	7.8	7.6	4.2
The Netherlands (♂ 226, ♀ 1139) (%)	2.4	5.3	3.0
Germany (♂ 845, ♀ 1176) (%)	26.7	15.6	4.1
Sweden (♂ 1231, ♀ 1625) (%)	40.5	20.2	4.6
Denmark (♂ 1110, ♀ 964) (%)	12.6	22.3	18.7

Data are means ± SD or median (25th percentile–75th percentile) unless otherwise indicated.

♂ men, ♀ women; incl., including; Q, quintile.

Family history of diabetes was not collected in Italy, Spain, Heidelberg, or Oxford (missing n = 7,723).

*FFQ-estimated intake energy adjusted by the residual method.

Table 1 continued for **WOMEN**

Protein intake (g/day)*	Q1 73.5 (69-77)	Q3 90.6 (89-92)	Q5 109.5 (105-116)
N cases/n	51/1909	83/1909	96/1909
Characteristics			
Age (yrs)	51.1 ± 9.9	53 ± 8.9	51.3 ± 8.6
Follow-up (yrs)	11.8 ± 2.1	11.9 ± 2.2	12.3 ± 2.1
BMI (kg\m^2)	24.2 ± 3.9	25.7 ± 4.4	27.1 ± 4.8
BMI <25 (%)	66.4	50.3	36.8
BMI 25-30 (%)	25.1	33.7	40.4
BMI >30 (%)	8.5	16	22.7
Waist (cm)	77.6 ± 10.0	81.1 ± 10.7	84.5 ± 11.6
Family history of diabetes (%)			
Yes	20.4	22.1	21.4
Smokers (%)			
Never	52.9	57.1	59.8
Former	23.5	21.3	20.3
Smoker	23.7	21.7	20.0
Hypertension (%)			
Yes	16.9	18.8	18.5
Don't know	1.5	0.9	1.0
Hyperlipidemia (%)			
Yes	16.1	14.1	13.8
Don't know	6.4	7.1	4.8
Educational level (%)			
Long education (incl. University deg.)	23.5	17.7	14.4
Physical activity (%)			
Active	20.0	17.4	13.6
Postmenopausal (%)	45.8	51.7	41.1
Country (n)			
France (♀ 526) (%)	3.4	5.9	6.8
Italy (♂ 639, ♀ 1300) (%)	10.7	16.1	11.4
Spain (♂ 1333, ♀ 2154) (%)	3.1	19.1	49.9
United Kingdom (♂ 423, ♀ 661) (%)	6.9	6.0	8.6
The Netherlands (♂ 226, ♀ 1139) (%)	8.3	15.0	9.8
Germany (♂ 845, ♀ 1176) (%)	28.7	9.2	1.6
Sweden (♂ 1231, ♀ 1625) (%)	31.8	16.1	2.4
Denmark (♂ 1110, ♀ 964) (%)	7.0	12.5	9.6

Data are means ± SD or median (25th percentile–75th percentile) unless otherwise indicated.

♂ men, ♀ women; incl., including; Q, quintile.

Family history of diabetes was not collected in Italy, Spain, Heidelberg, or Oxford (missing n = 7,723).

*****FFQ-estimated intake energy adjusted by the residual method**.**

Table 1 Continued for **MEN**

Protein intake (g/day)*	Q1 68.9 (63-73)	Q3 89.0 (87-91)	Q5 113.5 (109-121)
N cases/n	45/1162	76/1162	105/1161
Dietary consumption			
Total energy (kcal/day)	2,564.2 ± 674	2,361.0 ± 594	2,664.3 ± 675
Total protein (energy %)	12.8 ± 1.2	16.5 ± 0.6	20.7 ± 1.9
Animal protein (energy %)	6.8 ± 1.7	10.2 ± 1.4	14.4 ± 2.5
From red meat (g/day)*	6.6 (3–12)	13.7 (8–21)	19.0 (11–28)
From processed meat (g/day)*	5.4 (3–8)	5.8 (3–9)	6.2 (3–12)
From poultry (g/day)*	2.6 (1–4)	4.8 (3–8)	9.8 (5–16)
From milk and dairy (g/day)*	5.3 (1–11)	7.0 (2–14)	7.5 (3–15)
From cheese (g/day)*	5.2 (2–9)	6.8 (3–12)	7.0 (3–15)
From fish (g/day)*	2.6 (1–5)	5.6 (3–9)	11.8 (6–19)
From eggs (g/day)*	1.2 (0–3)	2.0 (1–3)	3.0 (1–5)
Plant protein (energy %)	4.4 ± 1.3	5.0 ± 1.3	5.4 ± 1.4
From bread (g/day)*	10.0 (7–14)	11.0 (8–15)	11.4 (8–17)
From potatoes (g/day)*	2.0 (1–3)	2.2 (1–3)	1.7 (1–3)
From pasta and rice (g/day)*	1.2 (0–2)	1.6 (1–3)	1.8 (1–3)
From legumes (g/day)*	0.2 (0–1)	0.5 (0–2)	3.1 (0–6)
Total carbohydrates (energy %)	46.0 ± 7.7	43.1 ± 6.8	38.7 ± 6.6
Starch (energy %)	24.0 ± 6.6	24.6 ± 6.3	23.3 ± 6.1
Sugar (energy %)	21.0 ± 7.2	17.8 ± 5.8	15.0 ± 5.3
Total fat (energy %)	34.4 ± 7.0	34.1 ± 5.5	35.4 ± 5.6
Saturated fat (energy %)	14.1 ± 4.0	13.0 ± 3.4	11.9 ± 3.3
Monounsaturated fat (energy %)	12.3 ± 2.8	12.9 ± 2.9	14.7 ± 3.8
Polyunsaturated fat (energy %)	5.5 ± 2.0	5.4 ± 1.8	5.8 ± 2.2
Fiber (g)*	20.1 ± 7.0	22.2 ± 6.1	23.9 ± 7.5
Cholesterol (mg)*	274.4 ± 118.4	334.9 ± 108.3	422.6 ± 138.0
Calcium (mg)*	793 ± 286	942 ± 335	1,027 ± 502
Magnesium (mg)*	383.7 ± 107.8	379.5 ± 98.8	429.3 ± 110.3
Vitamin B1 (mg)*	1.4 ± 0.5	1.4 ± 0.5	1.8 ± 0.6
β-Carotene (µg)*	2,233 ± 2,049	2,597 ± 2,124	2,818 ± 2,483
Vitamin C (mg)*	109.8 ± 64.8	110.7 ± 59.1	140.7 ± 79.8
Vitamin D (mg)*	4.6 ± 3.0	4.6 ± 2.9	5.1 ± 3.8
Vitamin E (mg)*	12.5 ± 6.0	11.7 ± 5.2	14.7 ± 6.8
Soft drinks (g/day)*	37.9 (0–150)	15.9 (0–86)	0.0 (0–29)
Coffee (g/day)*	430 (190–621)	311 (100–611)	131 (43–450)
Tea (g/day)*	10.3 (0–150)	4.9 (0–146)	0.0 (0–12)
Alcohol (g)			
0 (%)	4.7	4.2	5.4
♂ 0–12, ♀ 0–6 (%)	42	39.1	39.6
♂ 12–24, ♀ 6–12 (%)	18.1	22.4	20.4
♂ >24, ♀ >12 (%)	35.3	34.3	34.5

Table 1 Continued for **WOMEN**

Protein intake (g/day)*	Q1 68.9 (63-73)	Q3 89.0 (87-91)	Q5 113.5 (109-121)
N cases/n	51/1909	83/1909	96/1909
Dietary consumption			
Total energy (kcal/day)	2,061.2 ± 568	1,857.7 ± 485	2,005.1 ± 525
Total protein (energy %)	13.2 ± 1.2	17.2 ± 0.8	21.6 ± 2.4
Animal protein (energy %)	6.8 ± 1.8	10.6 ± 1.5	15.1 ± 2.8
From red meat (g/day)*	6.6 (4–10)	11.9 (8–17)	16.3 (10–23)
From processed meat (g/day)*	4.8 (3–7)	5.3 (3–8)	6.0 (4–10)
From poultry (g/day)*	2.7 (2–4)	5.1 (3–8)	10.3 (6–16)
From milk and dairy (g/day)*	5.4 (2–10)	8.5 (5–14)	10.9 (6–17)
From cheese (g/day)*	7.2 (4–10)	9.0 (6–13)	9.5 (5–16)
From fish (g/day)*	3.2 (2–5)	5.8 (3–9)	10.3 (6–16)
From eggs (g/day)*	1.5 (1–3)	2.2 (1–3)	2.9 (2–4)
Plant protein (energy %)	4.7 ± 1.4	5.2 ± 1.3	5.3 ± 1.3
From bread (g/day)*	10.4 (8–13)	11.4 (9–15)	11.2 (8–14)
From potatoes (g/day)*	1.5 (1–2)	1.6 (1–2)	1.4 (1–2)
From pasta and rice (g/day)*	1.5 (1–3)	1.9 (1–3)	1.9 (1–3)
From legumes (g/day)*	0.6 (0–1)	1.1 (1–2)	2.1 (1–4)
Total carbohydrates (energy %)	48.0 ± 7.2	45.1 ± 6.1	40.9 ± 6.2
Starch (energy %)	24.0 ± 6.4	24.1 ± 6.0	22.0 ± 5.8
Sugar (energy %)	23.4 ± 7.1	20.5 ± 5.8	18.5 ± 5.9
Total fat (energy %)	35.1 ± 6.4	34.9 ± 5.6	35.5 ± 5.9
Saturated fat (energy %)	14.4 ± 3.6	13.3 ± 3.1	12.6 ± 3.3
Monounsaturated fat (energy %)	12.5 ± 3.0	13.1 ± 3.6	14.2 ± 4.0
Polyunsaturated fat (energy %)	5.7 ± 1.9	5.6 ± 1.7	5.7 ± 2.2
Fiber (g)*	23.0 ± 6.6	23.5 ± 5.5	24.1 ± 5.9
Cholesterol (mg)*	288.0 ± 93.2	342.2 ± 91.6	405.5 ± 105.9
Calcium (mg)*	923 ± 248	1,054 ± 266	1,189 ± 454
Magnesium (mg)*	330.1 ± 93.6	315.8 ± 86.7	353.8 ± 96.5
Vitamin B1 (mg)*	1.2 ± 0.4	1.2 ± 0.4	1.4 ± 0.5
β-Carotene (µg)*	3,119 ± 2,491	3,195 ± 2,404	3,494 ± 2,917
Vitamin C (mg)*	129.9 ± 79.9	121.9 ± 60.3	141.0 ± 70.8
Vitamin D (mg)*	4.1 ± 2.2	4.1 ± 2.1	4.4 ± 2.2
Vitamin E (mg)*	11.7 ± 5.4	10.4 ± 4.2	11.9 ± 5.1
Soft drinks (g/day)*	13.3 (0–90)	2.4 (0–57)	0.0 (0–28)
Coffee (g/day)*	357 (120–580)	261 (89–500)	160 (52–450)
Tea (g/day)*	21.4 (0–250)	6.6 (0–238)	0.0 (0–119)
Alcohol (g)			
0 (%)	7.3	9	12.9
♂ 0–12, ♀ 0–6 (%)	46.6	53.6	55.4
♂ 12–24, ♀ 6–12 (%)	17.2	15	13.4
♂ >24, ♀ >12 (%)	28.9	22.4	18.4

Table 2: Meta-analysed (pooled) hazard ratios and 95%CI for the association between protein intake and type 2 diabetes in the EPIC-InterAct case-cohort.

	Q1 (low)	Q2	Q3	Q4	Q5 (high)	P_{trend}	Per 10 g
Total protein *N* (cases)	5,023 (2,048)	5,080 (2,144)	5,180 (2,265)	5,354 (2,425)	5,616 (2,755)		26,253 (11,637)
Median protein*	71.8	82.1	90	98.1	111		90
Model 1	1	1.08 (0.96–1.22)	1.20 (1.02–1.40)	1.40 (1.11–1.76)	1.63 (1.37–1.93)	<0.001	1.14 (1.09–1.19)
Model 2	1	1.10 (0.97–1.26)	1.19 (1.01–1.40)	1.38 (1.09–1.75)	1.57 (1.33–1.84)	<0.001	1.13 (1.09–1.18)
Model 3	1	1.12 (0.97–1.31)	1.19 (0.99–1.43)	1.39 (1.08–1.79)	1.55 (1.25–1.91)	<0.001	1.13 (1.08–1.19)
Model 4	1	1.00 (0.85–1.19)	1.02 (0.86–1.20)	1.13 (0.91–1.42)	1.17 (1.00–1.38)	<0.001	1.06 (1.02–1.09)
Animal protein *N* (cases)	4,950 (1,972)	5,034 (2,073)	5,207 (2,315)	5,351 (2,436)	5,711 (2,841)		26,253 (11,637)
Median protein*	36	47.4	55.6	64.3	78.1		55.6
Model 1	1	1.06 (0.95–1.19)	1.22 (1.08–1.37)	1.35 (1.18–1.54)	1.62 (1.35–1.94)	<0.001	1.12 (1.08–1.17)
Model 2	1	1.07 (0.94–1.21)	1.24 (1.08–1.41)	1.35 (1.18–1.54)	1.61 (1.34–1.93)	<0.001	1.12 (1.08–1.16)
Model 3	1	1.05 (0.90–1.22)	1.20 (1.01–1.42)	1.31 (1.11–1.56)	1.51 (1.20–1.91)	<0.001	1.12 (1.07–1.17)
Model 4	1	1.01 (0.85–1.20)	1.11 (0.96–1.29)	1.12 (0.99–1.27)	1.22 (1.06–1.40)	0.001	1.05 (1.02–1.08)
Plant protein *N* (cases)	5,536 (2,614)	5,243 (2,320)	5,144 (2,205)	5,128 (2,197)	5,202 (2,301)		26,253 (11,637)
Median protein*	18.6	23.1	26.2	29.6	35.9		26.2
Model 1	1	0.92 (0.82–1.04)	0.88 (0.78–0.99)	0.93 (0.73–1.19)	0.91 (0.72–1.15)	0.507	0.92 (0.80–1.04)
Model 2	1	0.94 (0.84–1.05)	0.89 (0.80–0.99)	0.94 (0.75–1.18)	0.92 (0.75–1.12)	0.416	0.91 (0.82–1.02)
Model 3	1	1.03 (0.94–1.13)	1.02 (0.92–1.13)	1.15 (0.99–1.34)	1.18 (1.02–1.36)	0.006	1.02 (0.95–1.10
Model 4	1	1.05 (0.92–1.19)	0.97 (0.83–1.15)	1.09 (0.91–1.31)	1.22 (0.98–1.52)	0.098	1.04 (0.93–1.16)

Model 1 includes age (= time scale) and covariates energy, center, and sex. Model 2, see model 1 plus covariates smoking, education, physical activity, and alcohol. Model 3, see model 2 plus covariates fiber, SFA, MUFA, PUFA, cholesterol, soft drinks, tea, and coffee (not adjusted for carbohydrates; i.e., a substitution model). Model 4, see model 3 plus covariates BMI and waist. Q, quintile.

*FFQ-estimated intake, adjusted for energy by the residual method

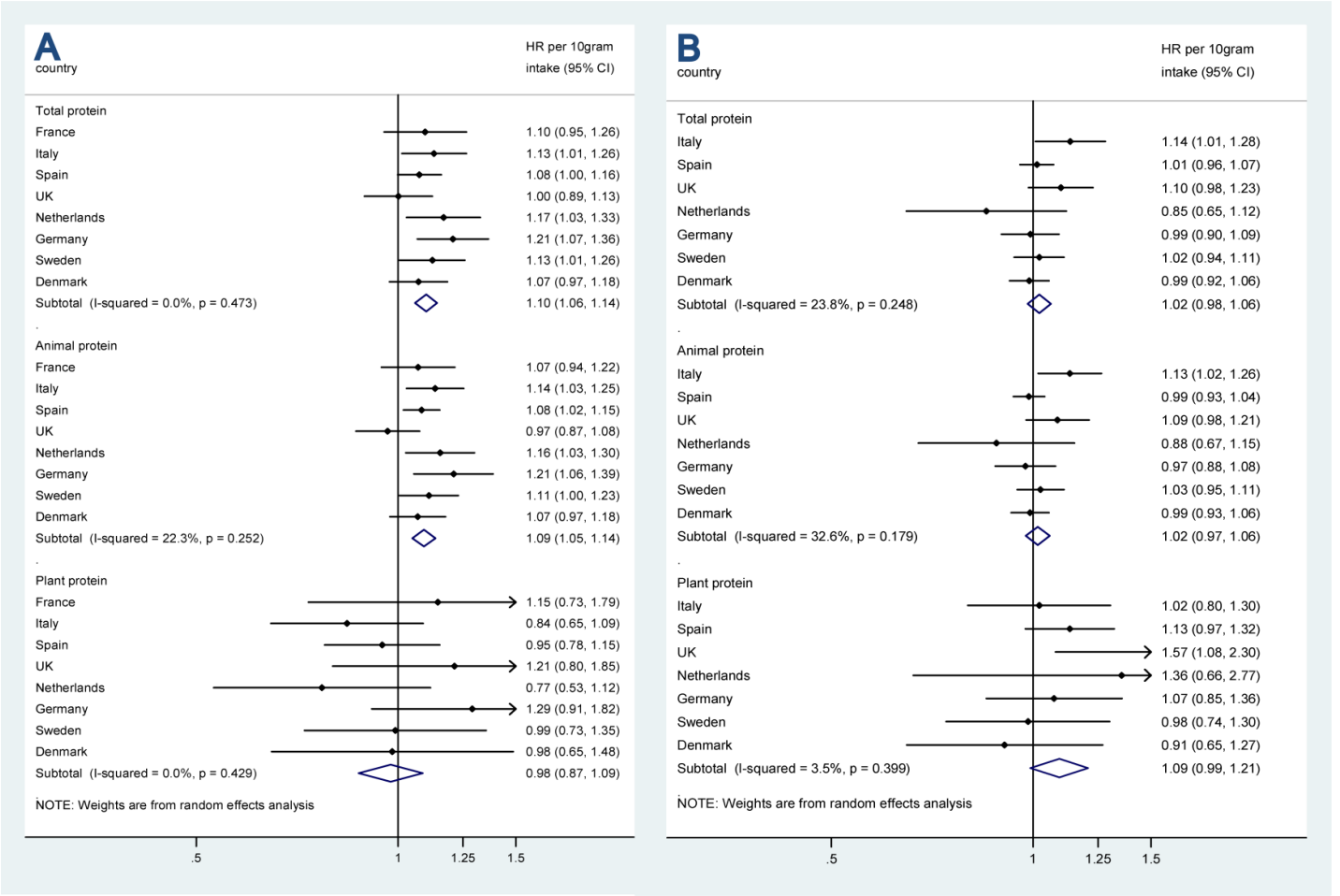

Figure 1 Meta-analyzed HRs of type 2 diabetes associated with 10-g increments of total, animal, and plant protein. Meta-analyzed (pooled country specific) HRs and 95% CI. Protein intake is FFQ-estimated intake, adjusted for energy using the residual method. *A*: Women. *B*: Men. The HRs are adjusted for energy, smoking, education, physical activity, alcohol, fiber, SFA, MUFA, PUFA, cholesterol, soft drinks, tea, coffee, BMI, and waist (not adjusted for carbohydrates; i.e., a substitution model).

Table S1: Sex-specific meta-analysed (pooled) hazard ratios and 95%CI for the association between protein intake and type 2 diabetes in the EPIC-InterAct case-cohort.

	Q1 (low)	Q2	Q3	Q4	Q5 (high)	P_{trend}	Per 10 g
Total protein *N cases/n*	(m=1096/2213, w=896/2754)	(m=1034/2129, w=1122/2966)	(m=1159/2245, w=1147/2973)	(m=1233/2322, w=1251/3083)	(m=1276/2332, w=1423/3236)		(m=5798/11241, w=5839/15012)
Median protein*	(m=69,w=74)	(m=80 , w=83)	(m=89 , w=91)	(m=99 , w=98)	(m=114, w=109)		(m=89 , w=91)
Model 1, men	1	0.93 (0.80–1.10)	1.06 (0.93–1.23)	1.24 (0.97–1.58)	1.43 (1.14–1.81)	<0.001	1.08 (1.03–1.14)
Model 2, men	1	0.95 (0.80–1.13)	1.07 (0.92–1.24)	1.22 (0.95–1.57)	1.34 (1.01–1.78)	<0.001	1.08 (1.03–1.14)
Model 3, men	1	1.00 (0.83–1.19)	1.09 (0.91–1.32)	1.27 (0.96–1.70)	1.36 (0.95–1.95)	0.003	1.09 (1.02–1.16)
Model 4, men	1	0.90 (0.69–1.17)	0.92 (0.74–1.13)	1.02 (0.81–1.29)	1.04 (0.83–1.31)	0.15	1.02 (0.98–1.06)
Model 1, women	1	1.19 (1.02–1.37)	1.25 (1.05–1.49)	1.47 (1.16–1.87)	1.81 (1.42–2.31)	<0.001	1.19 (1.13–1.26)
Model 2, women	1	1.24 (1.04–1.47)	1.28 (1.09–1.50)	1.47 (1.17–1.83)	1.80 (1.53–2.17)	<0.001	1.19 (1.14–1.24)
Model 3, women	1	1.21 (1.00–1.48)	1.22 (1.00–1.50)	1.44 (1.12–1.85)	1.73 (1.38–2.17)	<0.001	1.14 (1.04–1.25)
Model 4, women	1	1.00 (0.83–1.21)	1.03 (0.88–1.20)	1.11 (0.90–1.37)	1.37 (1.06–1.79)	<0.001	1.10 (1.06–1.14)
Animal protein N cases/n	(m=1059/2175, w=904/2765)	(m=961/2069, w=1053/2904)	(m=1216/2294, w=1180/2995)	(m=1196/2281, w=1260/3091)	(m=1366/2422, w=1442/3257)		(m=5798/11241, w=5839/15012)
Median protein*	(m=36,w=39)	(m=47 , w=48)	(m=55 , w=56)	(m=65 , w=64)	(m=80, w=76)		(m=55, w=56)
Model 4, men	1	0.79 (0.61–1.03)	0.97 (0.72–1.32)	0.88 (0.66–1.17)	1.01 (0.71–1.43)	0.221	1.02 (0.98–1.06)
Model 4, women	1	1.05 (0.86–1.29)	1.06 (0.87–1.29)	1.17 (0.97–1.42)	1.38 (1.01–1.90)	0.001	1.09 (1.05–1.14)
Plant protein N cases/n	(m=1344/2440, w=1199/3033)	(m=1171/2255, w=1187/3017)	(m=1107/2198, w=1156/3000)	(m=1093/2184, w=1108/2946)	(m=1083/2164, w=1189/3016)		(m=5798/11241, w=5839/15012)
Median protein*	(m=18,w=19)	(m=23 , w=23)	(m=26 , w=26)	(m=30 , w=29)	(m=38 , w=35)		(m=26 , w=26)
Model 4, men	1	1.03 (0.86–1.23)	1.12 (0.93–1.34)	1.27 (1.04–1.55)	1.50 (1.16–1.93)	0.006	1.09 (0.99–1.21)
Model 4, women	1	1.09 (0.94–1.26)	0.94 (0.76–1.17)	0.99 (0.77–1.28)	1.24 (0.91–1.68)	0.193	0.98 (0.87–1.09)

Model 1 includes age (= time scale) and covariates energy, center, and sex. Model 2, see model 1 plus covariates smoking, education, physical activity, and alcohol. Model 3, see model 2 plus covariates fiber, SFA, MUFA, PUFA, cholesterol, soft drinks, tea, and coffee (not adjusted for carbohydrates; i.e., a substitution model). Model 4, see model 3 plus covariates BMI and waist. Q, quintile.

*FFQ-estimated intake, adjusted for energy by the residual method

Table S2. Sensitivity analyses of the fully adjusted model (model 4[#]); Hazard ratios and 95%CI additionally adjusted for the effect possible lifestyle changes as a result of a medical condition, and models to explore the contribution of various protein sources to the associations of protein type with T2D risk.

Protein sensitivity analyses	Overall	Men	Women
	HR (95% CI)	*HR (95% CI)*	*HR (95% CI)*
Potential lifestyle changes[A]			
Total	1.11 (1.02-1.21)	1.03 (0.94-1.14)	1.15 (1.04-1.27)
Animal	1.09 (1.00-1.17)	1.04 (0.92-1.17)	1.12 (1.03-1.22)
Plant	1.12 (0.98-1.29)	1.18 (0.91-1.54)	1.12 (0.95-1.32)
Excluding sources[B]			
Animal excl. meat	1.04 (0.99-1.08)	1.00 (0.94-1.06)	1.09 (1.02-1.16)
Animal excl. dairy	1.05 (1.02-1.07)	1.03 (0.98-1.08)	1.09 (1.04-1.14)
Animal excl. fish	1.05 (1.01-1.08)	1.02 (0.97-1.06)	1.08 (1.04-1.13)
Plant excl. bread	0.97 (0.89-1.07)	0.99 (0.83-1.17)	0.98 (0.84-1.14)
Plant excl. potatoes	1.02 (0.92-1.13)	1.07 (0.96-1.20)	0.97 (0.87-1.09)
Plant excl. pasta&rice	1.05 (0.94-1.17)	1.13 (1.03-1.25)	1.05 (0.94-1.17)
Animal protein sources[C]			
Red meat	1.00 (1.00-1.01)	1.00 (1.00-1.01)	1.00 (1.00-1.01)
Poultry	1.00 (0.99-1.01)	0.99 (0.98-1.00)	1.01 (1.00-1.02)
Processed meat	1.01 (1.00-1.02)	1.01 (1.00-1.03)	1.02 (1.00-1.03)
Fish	1.01 (1.00-1.01)	1.00 (1.00-1.01)	1.01 (1.00-1.02)
Eggs	1.00 (0.96-1.04)	1.01 (0.95-1.07)	0.98 (0.94-1.02)
Milk and dairy	1.01 (1.00-1.01)	1.00 (0.99-1.01)	1.01 (1.00-1.02)
Cheese	1.00 (0.99-1.01)	1.00 (0.99-1.01)	1.00 (0.99-1.01)
Plant protein sources[C]			
Potatoes	1.01 (0.98-1.04)	1.01 (0.98-1.04)	1.03 (0.97-1.09)
Pasta and rice	0.98 (0.97-1.00)	0.99 (0.96-1.01)	0.99 (0.96-1.02)
Bread	1.01 (1.00-1.02)	0.99 (1.00-1.02)	1.01 (1.00-1.02)
Legumes	1.07 (1.01-1.13)	0.99 (1.00-1.11)	1.07 (0.99-1.16)
Leafy vegetables	1.04 (0.96-1.13)	0.99 (0.78-1.12)	1.13 (1.04-1.22)
Fruiting vegetables	1.02 (0.97-1.09)	0.99 (0.86-1.03)	1.11 (1.03-1.20)
Cabbage	1.01 (0.92-1.12)	0.99 (0.92-1.16)	1.01 (0.91-1.11)
Other vegetables	0.99 (0.96-1.03)	0.99 (0.90-1.03)	1.02 (0.97-1.08)
Nuts	0.99 (0.97-1.02)	0.99 (0.98-1.04)	0.97 (0.93-1.01)

model 4: (age = timescale) energy, centre, sex, smoking, education, physical activity, alcohol, fiber, SFA, MUFA, PUFA, cholesterol, soft drinks, tea, coffee, BMI and waist (not adjusted for carbohydrates, i.e. substitution model, protein at the expense of CHO). Protein intake was defined as FFQ estimated intake, energy-adjusted by the residual method.

[A] Extra analysis excluding individuals who might have made dietary adjustments and/or lifestyle changes because of presence of chronic disease at baseline n=11,043 (i.e. due to history of hypertension, hyperlipidaemia, myocardial infarction and/or stroke); HR per 10 gram of protein.

[B] Extra analysis excluding specific groups of sources from the total amount of protein from animal and plant origin; HR per 10 gram of protein.

[C] Analyses for 1gr increments of protein intake from various sources of protein from animal and plant origin.

Table 3 BMI-specific meta-analyzed (pooled) HRs (95% CI) (per 10 g protein intake) for the association between protein intake and type 2 diabetes: results of the adjusted model (model 4)

	Overall	Men	Women
Animal protein *N* (cases)	26,253 (11,637)	11,241 (5,798)	15,012 (5,839)
BMI (kg/m2)			
<25	1.08 (1.00–1.17); 52.4	1.02 (0.88–1.17); 50.2	1.11 (0.99–1.25); 53.1
25–30	1.04 (1.00–1.07); 57.4	1.07 (0.99–1.15); 56.7	1.07 (1.01–1.14); 58.2
>30	1.06 (1.00–1.11); 60.7	0.96 (0.91–1.02); 62.1	1.19 (1.09–1.32); 60.0
Plant protein *N* (cases)	26,253 (11,637)	11,241 (5,798)	15,012 (5,839)
BMI (kg/m2)			
<25	1.04 (0.89–1.23); 25.9	0.97 (0.76–1.24); 26.1	1.15 (0.90–1.46); 25.8
25–30	1.06 (0.96–1.17); 26.5	1.13 (0.99–1.29); 26.3	1.01 (0.84–1.21); 26.6
>30	1.08 (0.87–1.33); 26.8	1.17 (0.93–1.47); 26.2	1.12 (0.78–1.61); 27.2

Data are HR (95% CI); median protein intake unless otherwise indicated. Median protein intake: FFQ-estimated intake, adjusted for energy using the residual method. Model 4 includes age (= time scale) and covariates energy, center, sex, smoking, education, physical activity, alcohol, fiber, SFA,MUFA, PUFA, cholesterol, soft drinks, tea, coffee, BMI, and waist (not adjusted for carbohydrates; i.e., a substitution model). The pooled HRs per 10 g protein intake did not include risk scores for Dutch men and French women because of the low number of obese subjects in these groups.

Conclusions

Our study, the largest of its kind in terms of sample size, number of cases, and follow-up years, is the first to investigate the association between type 2 diabetes incidence and protein intake at a general European level. We found that high total protein at the exchange of carbohydrates is associated with a small elevated risk of type 2 diabetes. This association was largely explained by animal protein intake. BMI and waist circumference attenuated the associations. Plant protein intake was not associated with type 2 diabetes incidence in our cohort.

In this current study, with low heterogeneity between the eight countries, we observed a positive association for total and animal protein and type 2 diabetes risk, independent of known type 2 diabetes risk factors and dietary factors including fat, saturated fat, and fiber intake. We observed that type 2 diabetes incidence was 17% higher in individuals with the highest total protein intake compared with individuals with the lowest intake and that type 2 diabetes incidence increased 6% per 10-g increment of total protein intake at the expense of carbohydrates. A 10-g increment represents ~50 g meat or fish, a glass of milk, or 50 g nuts. We reviewed the associations with increasing protein intake at the expense of carbohydrates because this is the most suitable source to replace protein, which is reflected by the lower carbohydrate intakes of participants with high-protein intakes. Furthermore, in clinical trials carbohydrates are also the source of choice to replace protein. Analyses of protein intake at the expense of fat intake were comparable with the substitution of carbohydrates. The HRs with increasing intake of protein over the quintiles show a linear

dose-response relation. The association between total protein intake and type 2 diabetes appears to be largely explained by animal protein, with a 22% higher type 2 diabetes incidence when comparing highest versus lowest quintile and 5% higher incidence per 10-g increment of animal protein intake. The magnitude of the increased type 2 diabetes risk associated with high total protein intake is comparable with the results of the earlier Dutch cohort study [3], a biomarker-calibrated cohort in the U.S. [4], two small cross-sectional studies in Asian populations (*n* <150) [8,9], and one small (*n* = 1,190) Greek population [10].
We observed that type 2 diabetes incidence was 38% higher in women with the highest animal protein intake compared with women with the lowest intake and that type 2 diabetes incidence increased 9% per 10-g increment of animal protein intake. In obese women, the association was even stronger, with a risk increase of 19% per 10-g increment of animal protein intake. In men, only a weak nonsignificant association was present. A difference between men and women has been observed in a prior study, though it was most evident in men [30]. In our study, it cannot be explained by differences in total protein intake and/or protein sources. On average, women did have a 20% lower absolute intake of total, animal, and plant protein, but the energy percentage of protein in the diet was equal for men and women. Also, the contribution of protein sources did not differ between sexes. Most dietary and lifestyle factors, associated with protein intake, did not differ substantially between men and women; only women with high protein intake were less likely to be physically active and were more restricted alcohol consumers. Further, the association remained similar after adjustment for BMI and waist in women but was attenuated in men. So, it seems that measures of abdominal obesity largely explain the association of protein intake and type 2 diabetes for men, which is not the case in women. Further research is required to explore why animal protein intake was found to be positively associated with type 2 diabetes risk in women only.

In our study, the association between protein intake and type 2 diabetes was attenuated by measures of body composition, most evident in men. This is in line with earlier research [3,4] and could be explained by the strong independent effect of abdominal obesity on type 2 diabetes risk and the positive correlation of protein intake with overweight and obesity [31]. We found that the association between protein intake and type 2 diabetes was strongest in obese women in contrast to prior research declaring weaker associations with increasing BMI [3]. Our findings may be explained by the fact that higher total protein intake, and/or higher protein intake from animal sources, is associated with weight and weight gain [31,32]. In our data, this effect would be stronger in women than in men. More research is needed to explain these mixed results. Unfortunately, it was not possible to consider weight change as a mediating factor between protein intake and type 2 diabetes incidence because data on weight change in our cohort were ascertained at fixed time intervals after baseline recruitment, so there is the potential for information bias.

In contrast to suggested beneficial short-term effects of dietary protein on glycemic control [5,33], our study found that habitually high intake of protein increases type 2 diabetes risk. This discrepancy between short- and long-term effects of protein intake can be explained by differences in energy content and/or in long-term and acute effects of dietary protein. In energy-restricted diets, high-protein content as a percentage of total energy is found to be beneficial, while absolute protein intake is similar or only modestly increased compared with protein intake in energy balance. We observed that in the general population, in energy balance or positive energy balance, a high absolute protein intake is associated with increased type 2 diabetes risk. The mechanism of the potential harmful effect of high dietary protein intake on type 2 diabetes is largely unknown. It could be driven by high protein sources, such as red or processed meat, and factors associated with protein intake or protein per se, e.g., based on amino acid profiles. Dietary proteins are known to increase glucagon, which could partially explain high blood glucose levels. Dietary proteins also increase insulin secretion, possibly leading to hyperinsulinemia, a risk factor for insulin resistance. A recent study suggested there could be a key role for the plasma amino acid levels of isoleucine, leucine, valine, tyrosine, and phenylalanine in the pathogenesis of type 2 diabetes [34], which have also been found to be associated with type 2 diabetes incidence in EPIC-Potsdam [35]. High levels of these plasma amino acids predicted future diabetes, e.g., as found for single plasma amino acids, such as leucine, with an HR of 3.66 [1.61–8.29], and for combinations of the amino acids isoleucine, tyrosine, and phenylalanine, with an HR of 5.99 [2.34–15.34] comparing the highest versus lowest quartile [34]. This is in line with earlier experimental elevations of plasma amino acids by infusion, which resulted in impaired insulin-stimulated glucose disposal and insulin-mediated suppression of (hepatic) glucose production [36,37]. The above-mentioned branched-chain amino acids and tyrosine and phenylalanine are mainly present in meat and dairy, though they are available in all protein-rich foods.

No specific group of protein sources accounted for the positive association of animal protein and type 2 diabetes incidence. Protein from meat did not explain the association in our cohort, and neither did protein from dairy or fish. So, although the well-established association between meat consumption and type 2 diabetes is suggested to be mainly due to other nutrients, such as iron, nitrites, sodium, or advanced glycation end products [12,38], a direct effect of protein from meat cannot be excluded. In our analyses, protein from dairy and protein from cheese were not accountable for the reported reduced type 2 diabetes risk associated with dairy [14–16] and cheese consumption. Fish consumption is not associated with type 2 diabetes [20–23,39], so possibly our observed association between animal protein and increased type 2 diabetes risk is counterbalanced by potential risk-reducing nutritional components of fish.

The findings of this study did not confirm the suggested reduced type 2 diabetes risk associated with protein intake from plant products (especially legumes [18] and nuts [19]). This could be related to the large proportion of bread, pasta and rice, and potatoes among the plant protein sources and relatively low intake of vegetables, legumes, and nuts, although no indication for a risk-reducing effect of protein from vegetables, legumes, and nuts was present in our analyses. To estimate protein intake, we used uncalibrated results of FFQs, so the intake is not equal to the 24-h protein intake in EPIC reported by Halkjaer et al. [40].

Our study with a large sample size from eight European countries and long follow-up had several strengths. The prospective design, with data collection before the occurrence of type 2 diabetes, and the use of validated FFQs at baseline reduce possible biased recall of diet, although it is possible that diets have changed during follow-up, which could influence the results. The strict validation of diabetes cases reduced the probability of misclassifying noncases as cases. In contrast, it cannot be ruled out that incident and prevalent type 2 diabetes cases have remained undiagnosed, which may lead to an underestimation of main effects and reduced power. Further, we were able to adjust our associations for a wide range of potential risk factors for type 2 diabetes and dietary factors, so the observed positive association between protein intake and type 2 diabetes is likely to be explained by proteins per se. The possibility of unmeasured or residual confounding cannot be ruled out, though. Information on *trans*-fatty acids was, for example, not available, but in Europe intake in non–margarine using, low–dairy using countries intake was probably low, and in margarine-using diary countries such as the Netherlands, *trans*-fatty acids correlate with PUFA intake. Because of the observational design, conclusions regarding causality cannot be drawn. The associations could for example relate to a less healthy diet and/or lifestyle, even though total and animal protein intake was not associated with known risk factors such as higher SFAs or lower fiber intake.

Overall, we conclude that a greater intake of total protein intake is associated with a higher type 2 diabetes incidence in European populations, but the effect of protein intake is small and known type 2 diabetes risk factors are also important. Our results show that protein of animal origin is largely responsible for the association - not plant protein. The association is confirmed in women, not in men, and is strongest in obese women. The association cannot be explained by a single food source. In view of the rapidly increasing prevalence of type 2 diabetes, limiting iso-energetic diets high in dietary proteins, particularly from animal sources, should be considered.

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Duality of interest

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Abstract

Increasing protein intake and soy consumption appear to be promising approaches to prevent metabolic syndrome (MetS). However, the effect of soy consumption on insulin resistance, glucose homeostasis, and other characteristics of MetS is not frequently studied in humans. We aimed to investigate the effects of a 4-wk, strictly controlled, weight-maintaining, moderately high-protein diet rich in soy on insulin sensitivity and other cardiometabolic risk factors. We performed a randomized crossover trial of 2 4-wk diet periods in 15 postmenopausal women with abdominal obesity to test diets with 22 energy percent (En%) protein, 27 En% fat, and 50 En% carbohydrate. One diet contained protein of mixed origin (mainly meat, dairy, and bread), and the other diet partly replaced meat with soy meat analogues and soy nuts containing 30 g/d soy protein. For our primary outcome, a frequently sampled intravenous glucose tolerance test (FSIGT) was performed at the end of both periods. Plasma total, LDL, and HDL cholesterol, triglycerides, glucose, insulin, and C-reactive protein were assessed, and blood pressure, arterial stiffness, and intrahepatic lipid content were measured at the start and end of both periods. Compared with the mixed-protein diet, the soy-protein diet resulted in greater insulin sensitivity [FSIGT: insulin sensitivity, 34 ± 29 vs. 22 ± 17 $(mU/L)^{-1} \cdot min^{-1}$, P = 0.048; disposition index, 4974 ± 2543 vs. 2899 ± 1878, P = 0.038; n = 11]. Total cholesterol was 4% lower after the soy-protein diet than after the mixed-protein diet (4.9 ± 0.7 vs. 5.1 ± 0.6 mmol/L, P = 0.001), and LDL cholesterol was 9% lower (2.9 ± 0.7 vs. 3.2 ± 0.6 mmol/L, P = 0.004; n = 15). Thus, partly replacing meat with soy in a moderately high-protein diet has clear advantages regarding insulin sensitivity and total and LDL cholesterol. Therefore, partly replacing meat products with soy products could be important in preventing MetS.

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Chapter 3

Partly replacing meat protein with soy protein alters insulin resistance and blood lipids in postmenopausal women with abdominal obesity

3

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INTRODUCTION

Metabolic syndrome (MetS) is a cluster of clinical features associated with an increased risk of type 2 diabetes (T2D) and cardiovascular disease. It includes disturbed glucose homeostasis, increased blood lipids, abdominal obesity, and increased blood pressure (BP). Diet can play an important role in the prevention of MetS, especially a high-protein diet [1]. High-protein diets can be useful because dietary proteins may reduce obesity via thermogenesis and increased satiety, improve glucose homeostasis and blood lipids via increased insulin secretion and a direct effect on insulin resistance, and improve insulin sensitivity (S_I) and body composition via skeletal muscle protein synthesis [2]. Besides the total protein content of the diet, the dietary protein source could be of relevance.

Soy has a high biologic value and contains several potential health-related nutritional factors, e.g., essential amino acids, biologic active peptides, and nonprotein compounds, such as isoflavones. Increasing soy protein intake is known to improve blood lipids and is inversely associated with obesity [3, 4]. Increased intake of legumes, soybeans in particular, is associated with a lower incidence of T2D [5], and intake of tofu and other soy products is associated with a lower risk of glycosuria in postmenopausal women [6]. However, some epidemiologic studies found no clear inverse association between soy intake and incidence of T2D [7, 8]. Also, soy protein tended to be associated with a reduced risk of MetS in women but with an elevated risk in men [9]. The estrogen-like activity of isoflavones could be involved in this sex-specific effect. However, current insights into the molecular mechanisms of the action of soy protein suggest that it is a promising approach to improve insulin action and glycemic control [10]. Although numerous studies evaluated the health benefits of soy protein, only a few studies addressed insulin action, glycemic control, and/or prevention of MetS in individuals without diabetes. There is limited human data directly examining insulin action and glucose homeostasis, especially dynamic and postprandial glycemic variables. Most human research on fasting glucose and insulin found no improvements [11], although animal studies do seem promising [12].

The primary objective of this trial was to evaluate the effect of a high-protein diet rich in soy on insulin resistance and glycemic control in participants with abdominal obesity. Second, we assessed whether soy protein had beneficial effects on cardiometabolic health, such as blood lipid profile, fat storage in the liver, BP, and endothelial function. We hypothesized that soy intake would result in better glycemic variables, insulin action, and blood lipid profile compared with a diet without soy.

STUDY DESIGN

Participants

In total, 15 postmenopausal women participated in this trial. Inclusion criteria were as follows: *1*) aged 45–70 y; *2*) a waist circumference of >80 cm; *3*) stable body weight for at least 6 mo; *4*) stable exercise habits during the past 6 mo; and *5*) not participating in any vigorous exercise program. Exclusion criteria were as follows: *1*) (undiagnosed) diabetes but not impaired fasting glucose and/or impaired glucose tolerance as evaluated by an oral glucose tolerance test at screening; *2*) active heart disease, i.e., history of myocardial infarction or angina pectoris; *3*) following, or recently followed, a diet plan or supplement or medication use known to affect this trial, such as hormone replacement therapy or antibiotics; and *4*) habitual use of >1 soy product per week. A preliminary screening, including an oral glucose tolerance test, and a medical history and physical questionnaire were used to check eligibility of participants.

On average, the 15 women were 61 ± 5 y old and had a waist circumference of 90 ± 10 cm. Eight women had ≥1 additional cardiometabolic risk factors, such as impaired fasting glucose (n = 1), impaired glucose tolerance (n = 1), low HDL (n = 2), increased systolic blood pressure (SBP) (n = 4), and/or increased diastolic blood pressure (DBP) (n = 2).

The experimental protocol and procedures were approved by the Medical Ethical Committee of Wageningen University. All participants gave their written consent before participation. The trial was registered at clinicaltrials.gov as NCT01694056.

Design

This was a randomized, crossover, strictly controlled dietary intervention trial that was conducted from August 2012 until December 2012. After a 1-wk run-in period on a control diet, participants were randomly assigned to 1 of the 2 experimental diets for 4 wk. At the end of the first experimental period, participants returned to their usual diet for a free-living washout period of 4 wk. Subsequently, participants were assigned to the other experimental diet for 4 wk. Participants were asked to keep lifestyle factors known to modify cardiometabolic risk factors stable during these 12 wk. At the start and end of each experimental period, blood was drawn and measurements were performed. On the last day of each experimental period, a 24-h urine sample was collected. The trial was single blinded, i.e., investigators performing measurements were unaware of the dietary regimen.

Diets

A run-in diet familiarized participants with trial procedures and was used to establish an accurate energy intake to keep body weight stable. The control diet was similar to the diet habitually consumed in The Netherlands ("traditional Dutch diet"). Two isocaloric experimental diets were considered: *1*) a high-protein diet of mixed, non-soy sources

(HP_{mix}); or 2) a high-protein diet of mixed sources including soy (HP_{soy}). Main protein sources were milk and milk products, yogurt and cheese, and meat and meat snacks (mainly pork and chicken). For the HP_{soy} to include 30 g/d soy protein, meat and meat snacks were partly replaced with soy meat analogues (Alpro) and soy nuts (Dietisnack). Commonly available foods were used to compose the diets, and no protein supplements were given. Both isocaloric experimental diets aimed to have a moderately increased protein fraction of 21 energy percent (En%) and a reduced fat content (~30 En%). Both experimental diets were controlled for equivalent amounts of dietary fibers and MUFAs, PUFAs, SFAs, and cholesterol. For example, extra SFAs were added to the HP_{soy} because meat products contain more SFAs than soy products.

Menus were designed for 8 levels of energy intake, ranging from 7 to 14 MJ/d, with increments of 1 MJ. To maintain stable body weight, the participants were allocated to an energy intake level close to their habitual energy intake, which was estimated before the start of the trial using an FFQ [13] or the Schofield equation. Approximately 90% of each participant's total daily energy requirements was provided to the participants during the intervention periods and run-in. The remaining 10% was free for participants to choose from a provided list of limited products with protein amounts of <0.7 g/portion. Compliance with the diet was ensured because most foods and drinks were provided to the participants, and hot meals were consumed under supervision of dieticians from the division. In practice, every Monday through Friday, participants came to the university building, where they consumed their hot meals. All foods were weighed to the nearest gram for each participant. After lunch, participants received a take-home package with foods and drinks for their evening meal, breakfast, and snacks. On Fridays, participants received a take-home package with foods and beverages for the weekend, plus instructions for preparing these foods.
Body weight was measured twice every week on a digital balance accurate to 0.1 kg with participants wearing indoor clothing with empty pockets, without shoes. Caloric intake was adjusted, when necessary, to maintain a stable body weight throughout the trial period (±1.0 kg from initial weight). When participants had incidentally increased energy requirements, e.g., because of sports, a bread roll was provided with the same relative macronutrient composition as the intervention diet. Participants kept a diary in which consumption of free-choice products was recorded along with illnesses, use of medication, deviations from the diet, and other remarks.
Duplicate portions of the 2 experimental diets with an energy level of 11 MJ were collected daily, and the final 70-d samples were analyzed for energy, fat, protein, ash, and dry matter by standard methods of analysis (Wageningen University Laboratories). Carbohydrate content (including fiber) of the diets was calculated by the difference. Nutrients in the free-choice items were calculated [14] and then added to the analyzed values. Isoflavones were determined using reversed-phase HPLC with electrochemical detection [15].

Data collection and outcome measures

At the start and end of each dietary period, fasting blood samples were taken, body composition was determined, intrahepatic lipid (IHL) content was quantified, and BP and vascular measurements were performed. S_I and glucose homeostasis were only evaluated at the end of both experimental periods to restrict the physical burden on participants.

Insulin sensitivity

After a 12-h overnight fast, a modified frequently sampled intravenous glucose tolerance test (FSIGT) was performed [16]. A Teflon cannula was inserted into both antecubital veins, 1 for infusion of glucose and insulin and 1 for blood draws. After taking fasting blood samples (t = 0), a glucose bolus (300 mg/kg) was given intravenously within 1 min. At t = 20, an additional bolus of insulin (20 mU/kg; Novo Nordisk Pharmaceuticals) was given. Blood was sampled at frequent intervals over a 3-h period (0, 2, 4, 8, 19, 22, 30, 40, 50, 70, 100, and 180 min after the start of glucose injection) for analyses of plasma glucose and insulin. At regular time points, an additional small volume of blood was sampled to monitor blood glucose.

Cardiovascular measures

Macrovascular regional arterial stiffness was assessed by applanation tonometry [17]. This non-invasive reproducible measurement was performed with a validated commercially available system (SphygmoCor; AtCor Medical) at the start and end of each experimental period. After a 10 min rest in a supine position, a pressure sensor (applanation tonometer) was applied on the right-hand radialis to record peripheral arterial pressure pulse waveforms. These waveforms were calibrated using SBP and DBP values from conventional brachial cuff measurements on the left arm and then converted to an aortic pressure waveform and pulse wave analysis. This pulse wave analysis provided the augmentation index (AIx), which is the amount of pressure added to the systolic pressure peak based on the reflected wave, expressed as a percentage of pulse pressure (SBP–DBP) [17]. Additionally, it provided central SBP, central pulse pressure, the ratio of myocardial blood flow-to-oxygen demand (subendocardial viability ratio), and the ejection duration index.

Body composition and intra hepatic lipids

Body composition—fat mass and fat free mass—was measured using DXA. IHL content was measured by image-guided single-voxel spectroscopy, a quantitative version of ^{1}H-magnetic resonance spectroscopy, on a 3.0 Tesla magnetic resonance (MR) scanner (Syngo MR B17; Siemens) [18]. Analyses for IHLs included 10 participants because of missing data (n = 5) due to contraindications for MR.

Plasma analyses

Glucose, insulin, blood lipids, and C-reactive protein (CRP) were measured in fasting plasma samples obtained at the start and end of each period. Glucose and insulin were additionally measured in plasma samples of the FSIGT. Blood was collected into vacutainers containing EDTA (BD Biosciences), and plasma was immediately separated by centrifugation at 2000 × *g* for 20 min at 4°C and then frozen and stored at −80°C until analyses. Commercial ELISA kits were used to measure insulin (Human Insulin ELISA; Mercodia) and CRP (CRPL3; Cobas, Roche), following the procedures of the manufacturers. Routine biochemical spectrophotometric procedures were used to measure glucose (Cobas, Roche), circulating TGs, total cholesterol, and HDL (Cobas, Roche). LDL was calculated according to the method of Friedewald et al. [19]. Isoflavones were determined using reversed-phase HPLC with electrochemical detection [15].

Urinary sampling and analyses

Twenty-four–hour urine was collected at the end of the 2 experimental periods in 2.7 L urine bottles with boric acid added as a preservative. Collection started after the first voiding in the morning on collection days and continued until and including the first voiding on the next day at the same time. Total 24-h urine volume was logged. After gently mixing, urine samples were taken and frozen at −20°C until analysis for urea by a routine biochemical spectrophotometric procedure (Cobas, Roche).

Calculations

Glucose and insulin responses were analyzed using the minimal model technique and MINMOD Millennium software [16]. With the MINMOD software, we modeled the following: *1*) S_I, which quantifies the capacity of insulin to promote glucose disposal; *2*) glucose effectiveness, which is the capacity of glucose to mediate its own disposal; *3*) the acute insulin response to glucose (AIRg), which addresses the adequacy of insulin secretion; and *4*) the disposition index (DI), calculated as AIRg × S_I, to add balance to these indices, ensuring that both insulin concentration and action are factored in to disposition (DI).

FSIGT analyses included 11 participants because of missing data for 1 person due to problems placing the cannulas and exclusion of 3 participants because of difficulties fitting their data in the MINMOD software. Steady-state S_I was calculated by HOMA-IR based on fasting glucose and insulin values.

Statistical analyses

Differences in baseline values before the 2 intervention diets, differences between baseline values and values after 4 wk, and mean results of the FSIGT were compared with repeated-measures ANCOVA, adjusting for period. All other results were compared with ANCOVA, adjusting for baseline values and period. Data are presented as means ± SDs, and $P \leq 0.05$ was considered to be statistically significant. Statistical analyses were performed using SAS version 9.2 software. A sensitivity analysis was performed for IHLs, excluding 1 participant with an elevated IHL content 12 SDs greater than the group mean (12.4% vs. 1.4 ± 10.9%),
The sample size was based on a power calculation with S_I measured by the FSIGT as outcome variable, with a power of 80% and a significance level of 0.05. Because of an expected increase in S_I of 20% and a 12–22% within-subject variation in the FSIGT, 13 participants were required to be enrolled in this study.

RESULTS

Participant characteristics and diets

All 15 participants completed the trial. Baseline values for participant characteristics are shown in **table 1**. No significant differences in participant characteristics at the start of the 2 intervention diets were seen, except for body weight, which was somewhat lower at the start of the HP_{mix} compared with the HP_{soy}. Based on FFQs, the background diet of participants was estimated to contain 18%, 45%, and 34% of energy from protein, carbohydrate, and fat, respectively.

Analyses of duplicate portions revealed that the high-protein diets contained on average 22%, 50%, and 27% of energy from protein, carbohydrate, and fat, respectively (**Table 2**). Total protein (+9 g/d), fat (+4 g/d), and cholesterol intake (+29 mg/d) were slightly greater during the HP_{soy} than during the HP_{mix} ($P < 0.01$). Isoflavone content of the diets was 48 mg/d for the HP_{soy} and 0 mg/d for the HP_{mix}. No carryover or period effects were identified, and compliance with the diets was high. Diaries of participants did not show deviations from the provided diets that could have affected the results.

Urea excretion was similar at the end of the 2 experimental periods (mixed compared with soy, 87 ± 12 vs. 81 ± 23 g/d, $P = 0.95$), which indicates that participants had an equivalent amount of protein intake at the end of the 2 experimental periods. Furthermore, the difference in soy protein consumption was affirmed by the difference in plasma isoflavones after the 2 diets (HP_{soy}, 1.5 ± 1.0 vs. HP_{mix}, 0.0 ± 0.0 μmol/L, $P < 0.001$).

Table 1 Plasma biochemistry and other metabolic characteristics of postmenopausal women at the start and end of the 4-wk high-protein diet of mixed, non-soy sources and high-protein diet including soy[1]

Characteristic	Before intervention		After intervention		Pvalue (ANCOVA)	
	Soy diet	Mixed diet	Soy diet	Mixed diet	Soy vs mixed[2]	Time effect[3]
Anthropometric measures						
Weight, *kg*	69.7 ± 12.9	69.1 ± 12.3	69.2 ± 12.7	68.6 ± 12.3	0.61	0.042
Body fat, *%*	35 ± 7	35 ± 7	34 ± 7	34 ± 7	0.10	<0.001
Abdominal fat, *%*	40 ± 12	42 ± 10	39 ± 11	39 ± 11	0.13	<0.001
Intra hepatic lipids[4], *% of H_2O peak*	3.3 ± 5.2	3.2 ± 3.4	2.9 ± 4.2	2.3 ± 3.3	0.20	0.07
Glycemic control						
Fasting glucose, *mmol/L*	5.6 ± 0.7	5.7 ± 0.6	5.4 ± 0.5	5.4 ± 0.4	0.90	0.003
Fasting insulin, *mU/L*	3.8 ± 1.8	4.0 ± 2.2	3.4 ± 1.7	3.0 ± 1.4	0.12	0.017
HOMA-IR	1.0 ± 0.5	1.0 ± 0.6	0.5 ± 0.2	0.4 ± 0.2	0.12	0.014
Blood lipid profile, *mmol/L*						
Triglyceride	1.3 ± 0.4	1.2 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	0.53	<0.001
Total cholesterol	5.8 ± 0.7	5.9 ± 0.7	4.9 ± 0.7	5.1 ± 0.6	0.001	<0.001
LDL cholesterol	3.5 ± 0.6	3.6 ± 0.7	2.9 ± 0.7	3.2 ± 0.6	0.004	<0.001
HDL cholesterol	1.8 ± 0.4	1.8 ± 0.4	1.5 ± 0.3	1.6 ± 0.3	0.77	<0.001
Total cholesterol:HDL ratio	3.4 ± 0.9	3.4 ± 1.0	3.3 ± 1.0	3.5 ± 0.9	0.12	0.62
Inflammation						
C-Reactive protein, *mg/L*	2.8 ± 2.5	2.6 ± 2.3	1.4 ± 1.3	2.1 ± 1.7	0.11	0.010
Cardiovascular measures						
Systolic blood pressure, *mmHg*	121 ± 19	123 ± 15	121 ± 13	120 ± 16	0.97	0.37
Diastolic blood pressure, *mmHg*	72 ± 10	70 ± 10	72 ± 8	70 ± 10	0.11	0.77
central Systolic blood pressure, *mmHg*	115 ± 19	115 ± 15	114 ± 14	114 ± 16	0.71	0.50
central Diastolic blood pressure, *mmHg*	73 ± 9	71 ± 10	73 ± 8	71 ± 10	0.14	0.76
Aumentation index[5], *%*	29 ± 7	28 ± 5	32 ± 5	30 ± 5	0.16	0.025
HR, *pulses/s*	65 ± 10	62 ± 9	66 ± 10	67 ± 10	0.19	0.022
ED, *s*	342 ± 17	343 ± 16	337 ± 15	335 ± 14	0.29	0.021
SEVR, *%*	144 ± 23	153 ± 25	145 ± 23	142 ± 24	0.12	0.10

[1]All values are means ± SD

[2]Pvalues are based on repeated measures ANCOVA, adjusted for baseline values and period

[3]Pvalues are based on repeated measures ANCOVA, adjusted for period

[4]Data missing for 5 participants due to contraindications to undergoing an MRI scan (n = 10)

[5]Adjusted for heart rate

Table 2 Intakes of total energy, macronutrients, and other relevant nutrients from the high-protein diet of mixed, non-soy sources and high-protein diet including soy consumed by postmenopausal women[1]

	Soy protein diet[1]	**Mixed protein diet[1]**
Energy intake, *MJ/d*	9.1 ± 1.8	9.0 ± 1.7
Protein, *g/d*	120 ± 24*	111 ± 21
Protein, *g/kg/d*	1.7 ± 0.3*	1.6 ± 0.3
Fat, *g/d*	67 ± 14*	63 ± 12
Saturated fat	29	26
Mono unsaturated fat	24	23
Poly unsaturated fat	16	23
n-3	3	3
n-6	13	20
Carbohydrates[2], *g/d*	263 ± 57	274 ± 55
Alcohol, *g/d*	4 ± 3	4 ± 4
Cholesterol[3], *mg/d*	233 ± 46*	204 ± 38
Soy isoflavones[4], *mg/d*	48	0

[1]All values are means ± SD (or means when only duplicate portions were available) *Different from mixed , P<0.05

Macronutrient composition of energy from protein, carbohydrate, and fat, respectively:
for high-protein diet of mixed sources including soy, 22%, 49%, and 27%;
for high-protein diet of mixed, non-soy sources, 21%, 52%, and 26%

[2] Including fiber.

[3] Intake from free-choice products reported via food dairy plus the estimated amount of intake based on calculations from food tables, no analyzed data from duplicates was available.

[4] No intake was allowed via free-choice items, values only report analyzed data of duplicate portions.

Metabolic and cardiovascular measures

The metabolic and cardiovascular measurements before and after the 2 experimental diets are presented in Table 1. Including soy protein in a moderately high-protein diet had beneficial effects on S_I as evaluated by the FSIGT (**Figure 1**), independent of body weight. The S_I index and the DI were greater after the HP_{soy} than after the HP_{mix} (Figure 1), without differently affecting fasting glucose and insulin (Table 1). However, glucose effectiveness and the acute insulin response were not different at the end of either diet (Figure 1).

Total cholesterol after the HP_{soy} was lower than after the HP_{mix}, as was LDL cholesterol. Last, CRP was nonsignificantly lower after the HP_{soy} than after the HP_{mix}. No diet effects were observed for TGs, HDL cholesterol, the total-to-HDL cholesterol ratio, IHLs, BP, other cardiovascular measures, or body composition.

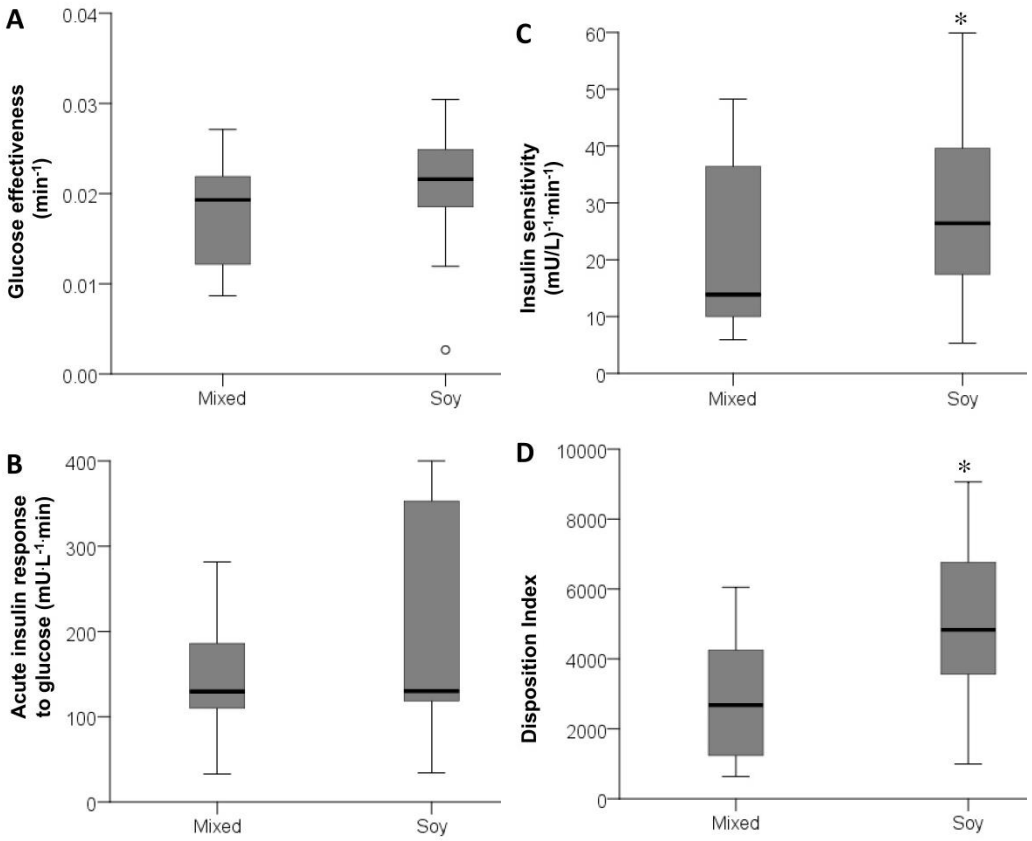

Figure 1 Glucose effectiveness (A), the acute insulin response to glucose (B), insulin sensitivity (C), and the disposition index (D), derived from the frequently sampled intravenous glucose tolerance test after 4 wk of the high-protein diet of mixed, non-soy sources and high-protein diet including soy consumed by postmenopausal women. Values are means ± SDs, n = 11. The open circle indicates an outlying individual data point. *Different from mixed, P < 0.05.

After 4 wk, both diets lowered fasting glucose, insulin, and steady-state HOMA-IR (all $P < 0.02$) (Table 1). Also, plasma concentrations of TGs, total cholesterol, LDL and HDL cholesterol, and CRP decreased after 4 wk of both diets (all $P < 0.01$) (Table 1). Liver fat tended to be lower after 4 wk of both diets ($P = 0.07$) (Table 1). A sensitivity analysis excluding 1 participant with an elevated IHL content only slightly altered these statistics ($P = 0.05$). Both central and peripheral SBP and DBP were unaltered by the experimental diets. Arterial stiffness, defined as AIx, increased, whereas heart rate and ED decreased after 4 wk. Although energy intake and body weight were frequently monitored, participants lost a small amount of weight during both intervention periods (~0.5 kg, $P = 0.042$). Total fat percentage and abdominal fat percentage were also decreased (both $P < 0.001$).

DISCUSSION

To our knowledge, this study is the first to show benefits of partly replacing meat with soy in a moderately high-protein diet on S_I in individuals without diabetes. S_I and DI, measured with the FSIGT, were greater after 4 wk of the HP_{soy} than after the HP_{mix}. We further confirmed the beneficial effect of soy on total and LDL cholesterol. Therefore, partly replacing meat with soy products could be important in preventing or treating MetS. Our results further suggest that moderately high-protein reduced-fat diets may have the ability to improve several cardiometabolic risk factors.

The 2 experimental diets differed only in protein source; meat was partly replaced with soy while the contribution of other protein sources (dairy, legumes) was kept comparable, as were other dietary factors known to affect glucose metabolism, such as dietary fiber and SFAs. A "whole-food approach" was used instead of providing supplements, because we wanted to study the effect of increased soy protein intake via soy food consumption on glucose homeostasis and insulin action. Moreover, such a diet approach reflects general practice. Finally, the intervention was strictly controlled; all participants completed the trial, and compliance to both diets was good. Previous studies indicated that the intake of 30 g soy protein was feasible and adequate in achieving lower total and LDL cholesterol and improved glucose metabolism in postmenopausal women [20, 21]. This was confirmed by the results of the present study: blood lipids were lower and S_I was higher after the soy protein diet compared with a diet with an equal amount of protein but from mixed, nonsoy protein sources. A 4-wk period was chosen to address short-term adaptations to changes in dietary intake and to reduce the risk of alterations in other lifestyle factors known to modify cardiometabolic risk factors. Previous work showed adaptations to protein diets lasting 2–5 wk [22, 23], and soy protein diets improved cholesterol within 3–6 wk [24]. Nonetheless, expected improvements were small because overall our population was fairly metabolically healthy, despite all women having a waist circumference of ≥80 cm.

Based on previous research, we expected improvements in postprandial glycemic control rather than fasting glucose and insulin [11]. Indeed, our trial found benefits of the HP_{soy} compared with the HP_{mix} on the capacity of insulin to promote glucose disposal, i.e., S_I and DI (55% and 72% higher, respectively), as measured by an FSIGT, without differences in fasting measures of glycemic control, i.e., fasting glucose and insulin, and HOMA index. Adequacy of insulin secretion and the capacity of glucose to mediate its own disposal were not improved. It was shown that genistein, the most abundant isoflavone in soy, has direct antidiabetic effects on β-cells [25]. However, this is not the most likely explanation for our findings, because we observed no differences in AIRg and plasma insulin. Also, other studies concluded that isoflavones alone do not improve glycemic control, so either the soy protein component alone or a synergistic effect between protein, amino acids, and isoflavones may

be attributable [26]. Arginine, which is found in high absolute amounts in soy protein, is an interesting candidate, because it was shown to have the potential to lower the postprandial insulin-to-glucagon ratio [27].

We confirmed the cholesterol-lowering effect of soy protein intake [4, 28]. The HP_{soy} resulted in lower total and LDL cholesterol than the HP_{mix} (4% and 9%, respectively). The effects of partly replacing meat with soy in a real-life setting may even be larger, because total fat and cholesterol intake were somewhat overcompensated in the HP_{soy} compared with the HP_{mix}. The effect may also be stronger in individuals with greater LDL cholesterol concentrations than our participants with only mildly increased LDL cholesterol (3.5 mmol/L), because studies with higher baseline LDL cholesterol result in greater improvements [28]. The lipid-lowering mechanism may be driven by changes in important transcription factors involved in lipid metabolism [29], because soy protein is known to regulate sterol regulatory element-binding protein (SREBP)-1 and PPARα [10, 30]. A downregulation of SREBP-1 results in a reduced expression of lipogenic genes, whereas upregulation of PPARα increases expression of genes involved in lipid oxidation. Besides lowering circulating lipids, this could also reduce hepatic TGs [30]. Increased hepatic lipid accumulation can result in so-called "lipotoxicity" when lipid accumulation exceeds innate storage capacity, which is associated with hepatic insulin resistance, contributing not only to hyperglycemia but also to hypertriglyceridemia [31]. Indeed, animal studies revealed that soy protein can reduce hepatic lipotoxicity and attenuate MetS via effects on PPAR, liver X receptor, and SREBP signaling [29, 32–34]. However, in our study, the reduction in total and LDL cholesterol and the improved S_I were not accompanied by a lower IHL content. In addition, the consequences of our soy protein diet on regulation of hepatic lipid metabolism could not be assessed.

Low-grade inflammation is associated with insulin resistance and diabetes [35]. Although nonsignificant, we found a decrease in CRP (−60%) by partly replacing meat with soy products. Previous research found that soy protein can lower CRP [36, 37]. Isoflavones also seem to be related to lower CRP [38], although a meta-analysis provided insufficient evidence of CRP-lowering capacity, except for individuals with elevated CRP at baseline [39]. Our population did not have elevated baseline CRP, and this might partly explain the lack of effect for soy on low-grade inflammation, but this warrants more research.

Although soy isoflavones were shown to improve endothelial function and lower BP after 6–8 wk [40, 41] and soy protein improved BP after 12 wk [42], we found no benefits of the HP_{soy} on BP and arterial stiffness within 4 wk. Probably no improvements were seen because adaptations in vascular function may take >4 wk and our population was quite healthy, with relatively normal BP and arterial stiffness. Also, effects of isolated soy protein and/or isoflavones may be incomparable with whole foods.

To date, there is little research on soy and body composition, but protein-sparing effects with meat vs. soy were suggested [43]. Genistein and daidzein reduced adiposity in animal studies [44, 45]. Animal protein is suggested to induce greater diet-induced thermogenesis than vegetable protein [2]. After a 12-wk randomized controlled trial of soy intake in elderly women, adiposity was mildly decreased and lean mass increased [46]. This is not confirmed by our findings. Our trial was probably too short to expect differences.

In addition to the soy protein effect, our data suggest that increasing protein intake at the expense of fat, without lowering carbohydrates and fiber, could be a healthy dietary strategy for women at risk of MetS. Both moderately high-protein reduced-fat diets—which increased habitual protein intake to 22 En% (equivalent to 1.7 g/kg)—decreased fasting glucose (−4%), fasting insulin (−18%), and HOMA-IR (−55%). Furthermore, following either diet reduced fasting TGs (−20%), total cholesterol (−15%), and LDL and HDL cholesterol (both −14%), without altering the total-to-HDL ratio. CRP decreased by 35%, total body fat percentage by 2%, and abdominal fat percentage by 5%. IHLs were nonsignificantly decreased by 20% after both diets. Although BP was unaltered, both diets slightly increased the AIx (7%). After both diets, participants lost <1% of their initial body weight, so we suggest that a major part of the improvements are a result of the trial diets. Previous research on this is inconclusive, but >4% weight loss seems necessary to improve S_I [47]. However, our results warrant more research because we did not have a normal-protein control diet.

In conclusion, we found clear benefits of partly replacing meat with soy in a moderately high protein diet on S_I and total and LDL cholesterol in individuals with increased risk of MetS. Thus, partly replacing meat products with soy products could be an option to treat or prevent MetS. Our trial gives important new insights in the continuing discussion about the effects of macronutrient composition of diets on glycemic control. It suggests that glycemic control and several cardiometabolic risk factors could be improved by increased protein intake at the expense of fat, without lowering carbohydrates and fiber. Nonetheless, generalization of these results will depend on longer-term studies in which both men and women are included.

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Abstract

Background Increasing protein intake and soy consumption appear promising approaches to prevent the metabolic syndrome (MetS). A beneficial effect of soy consumption on inflammation could play a role, but has not been studied frequently. We therefore aimed to investigate the effects of a 4-week strictly controlled weight-maintaining moderate high-protein diet, rich in soy, on markers of inflammation.

Methods We performed a randomized cross-over trial of two 4-week diet periods in 15 postmenopausal women with abdominal overweight to test diets with 22 E% protein, 27 E% fat and 50 E% carbohydrate. One diet contained protein of mixed origin, mainly meat, dairy and bread ($HP_{mix)}$, the other diet partly replaced meat with soy meat analogues and soy nuts, containing 30 g soy protein per day (HP_{soy}). Plasma biomarkers of inflammation, IFN-γ, IL-6, IL-8, IL-10, TNF-α, CRP, SAA, sICAM-1 and sVCAM-1, were assessed at the start and end of both periods and were compiled in a summary score.

Results Compared to HP_{mix}, HP_{soy} resulted in a significantly lower summary score for low-grade inflammation after excluding participants with CRP>6mg/L and extreme outliers (Z-score: -0.2 ± 0.3 vs. -0.1 ± 0.2, P=0.04; n=7). The individual markers we assessed were not significantly different after HP_{soy} than after HP_{mix}, although CRP and SAA tended to be lower.

Conclusions Partly replacing meat with soy in a moderate high-protein diet for 4-weeks did not have clear advantages on individual inflammatory markers. Our results do suggest it may improve the total level of low-grade inflammation.

Submitted for publication

Chapter 4

Effects of partly replacing meat protein with soy protein on the inflammatory state in postmenopausal women with abdominal obesity

4

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INTRODUCTION

A low grade inflammatory state, characterized by increased concentrations of circulatory C-reactive protein (CRP), interleukins, and tumor necrosis factor (TNF)-α, is associated with heart disease, type 2 diabetes and the metabolic syndrome (MetS) [1]. Inflammation might be one of the pathways through which diet affects insulin resistance [1, 2].

Dietary choices to lower pro-inflammatory markers can play an important role in the prevention of MetS. It is known that diets rich in fiber, fruits and vegetables, polyphenols and low in refined grains and saturated fatty acids (SFA) positively affect the pro-inflammatory state [1]. It can be suggested that reducing fat content of a diet is able to lower inflammation, because of suggested potential positive associations of total fat [3] and/or SFA [4] with inflammation. If increasing protein intake at the expense of fat is also able to improve the inflammatory state is not known.

Partly replacing meat with soy could have additional benefits. Lowering meat intake could be beneficial, as meat intake seems to be associated with inflammatory markers [5]. Also, whole soy food intake is found to reduce some markers of inflammation [6-8], however a brief review of clinical data concluded that neither soy foods nor isoflavones affect Interleukin (IL)-6 and TNF-α [9]. Exact mechanisms through which soy possibly affects inflammation are largely unknown. Soy may be beneficial because of fiber, polyunsaturated fat, and polyphenol phytoestrogens content, which are all individually associated with lower levels of inflammation. However, up to now it seems that the effects are small or not evident.

Recently, we reported that partly replacing meat products for soy products in a moderate high-protein resulted in greater insulin sensitivity in postmenopausal women with characteristics of MetS [10]. Additionally, after the soy-protein diet (HP_{soy}) total cholesterol was 4% lower than after the mixed-protein diet (HP_{mix}) and low density lipoprotein (LDL) cholesterol was 9% lower. Also we found nonsignificant lower CRP concentrations after HP_{soy} compared to HP_{mix}.

In the present study we report on results after measuring additional markers of inflammation. The primary objective of this research was to evaluate the effect of a moderate high-protein diet rich in soy on inflammation markers in women with abdominal overweight. We hypothesized that partly replacing meat with soy would result in an improved low-grade inflammatory state.

PARTICIPANTS AND METHODS

Participants

In total 15 postmenopausal women participated in this trial. On average they were 61 $\pm$ 5 years old and had a waist circumference of 90 $\pm$ 10 cm. Eight women had one or more additional cardio-metabolic risk factors, i.e. impaired fasting glucose (n=1), impaired glucose

tolerance (n=1), low high density lipoprotein (n=2), increased systolic blood pressure (n=4), and/or increased diastolic blood pressure (n=2). The experimental protocol and procedures were approved by the Medical Ethical Committee of Wageningen University. All participants gave their written consent before participation. The trial was registered in the NIH clinical trial database at clinicaltrials.gov as NCT01694056. Further details on participants and methods have previously been published [10].

Design

This trial was a randomized, cross-over, strictly-controlled dietary intervention. After a one-week run-in period on a control diet, participants were randomly assigned to one of both experimental diets for 4 weeks. At the end of the first experimental period, participants returned to their usual diet for a free-living wash-out period of 4 weeks. Subsequently participants were assigned to the other experimental diet for again 4 weeks. Participants were asked to keep lifestyle factors, known to modify cardio-metabolic risk factors, stable in these 12 weeks. At the start and end of each experimental period fasting blood samples were drawn.

Diets

This trial examined two isocaloric experimental diets: HP_{mix}: with mixed, non-soy protein sources and HP_{soy}: with a major contribution of soy protein. Main protein sources for both diets were milk and milk products, yoghurt and cheese, meat and meat snacks (mainly pork and chicken). For HP_{soy} to include 30 g soy protein per day, meat and meat snacks were partly replaced with soy meat analogues (Alpro) and soy nuts (Dietisnack). Commonly available foods were used to compose both diets, no protein supplements were given. Both isocaloric experimental diets aimed to have a moderately increased protein fraction of 21 energy percent (E%), and a reduced fat content (~30 E%). Analyses of duplicate portions revealed that the moderate high-protein diets contained on average 22 E%, 50 E%, and 27 E% from protein, carbohydrate, and fat, respectively. Both experimental diets were controlled for equivalent amounts of dietary fibers, cholesterol, monounsaturated and polyunsaturated fatty acids and SFA. For example, extra SFA was added to HP_{soy}, because meat products contain more SFA than soy products. Further details on these diets have previously been published [10].

No carryover or period effects were identified and compliance to the diets was high. To maintain stable bodyweight the participants were allocated to an energy intake level close to their habitual energy intake. Closely 90% of participant's total daily energy requirement was provided to the participants. The remaining 10% was free for participants to choose from a provided list of limited products, with an amount of protein of less than 0.7 g/portion. Compliance to the diet was ensured as most foods and drinks were provided

to participants, and hot meals were consumed under supervision of dieticians of the division.

Body weight was measured twice every week. To maintain a stable body weight throughout the trial period (±1.0 kg from initial weight) caloric intake was adjusted, when necessary. Participants kept a diary in which consumption of free-choice products was recorded along with illnesses, use of medication, deviations from the diet and other remarks. Diaries of participants did not show deviations from the provided diets that could have affected the results.

Data collection and outcome measures

Plasma analyses.

Inflammatory markers were measured in fasting plasma samples obtained at the start and end of each dietary period. Blood was collected into vacutainers containing EDTA (Becton Dickinson), plasma was immediately separated by centrifugation at 2000 g for 20 minutes at 4 °C and then frozen and stored at -80 °C until analyses. Plasma pro-inflammatory and anti-inflammatory cytokines and vascular injury markers were assessed in our lab (Wageningen University) by 4-plex multi-array biomarker assay developed by MesoScaleDiscovery (MSD) on the basis of electrochemiluminescence detection. We measured Interferon (IFN)-y, IL-10, IL-12p70, IL-13, IL-1B, IL-2, IL-4, IL-6, IL-8, TNF-α, CRP, Serum Amyloid A (SAA), soluble intercellular cell adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1s (sVCAM-1). As plasma samples from 3 women were missing, data were available for 12 subjects. The so-called 80% rule [11] was applied to retain only those markers which have 80% or more values above the detection limit for at least one of the two experimental periods, resulting in retention of 9 out of the 14 pro-inflammatory (n=8) and anti-inflammatory (n=1) variables. Values below the detection limit that remained in the truncated data set were replaced by a value set at half of the detection limit.

Summary score.

A summary score for low-grade inflammation was calculated to cluster conceptually related pro-inflammation markers and improve statistical efficiency. A *z*-score for each pro-inflammatory marker was calculated because these markers were expressed on different scale units. The pro-inflammatory *z*-scores were averaged to obtain a summary score for low-grade inflammation for each participant, as follows:

$$\text{Summary score} = [z\text{-score}(\log_e CRP) + z\text{-score}(\log_e IFN\text{-}\gamma) + z\text{-score}(\log_e IL\text{-}6) + z\text{-score}(\log_e IL\text{-}8) + z\text{-score}(TNF\text{-}\alpha) + z\text{-score}(\log_e SAA) + z\text{-score}(sICAM\text{-}1) + z\text{-score}(sVCAM\text{-}1)] : 8$$

IL-10 was not included in the total *z*-score, because it is an anti-inflammatory marker. A comparable summary score for low-grade inflammation has been used in previous investigations, but then without IFN-γ and sVCAM-1 [2].

Statistical analysis.

Differences in baseline values before both intervention diets were compared with repeated-measures analysis of covariance (ANCOVA) adjusting for period. All other results were adjusted for baseline values and period. Sensitivity analyses were performed excluding participants with CRP>6mg/L (n=4), exceeding the cutoff for normal CRP values. For both SAA and IFNγ one participant with an extreme outlier (outlier>10SD from mean±SD without this observation; SAA=62.1mg/L and IFNγ=109.3 pg/L) was excluded for sensitivity analyses. Data are presented as mean±SD and Pvalues ≤0.05 were considered to be statistically significant. Statistical analyses were performed using SAS 9.2 software.

RESULTS

Values of inflammatory markers before intervention were not significantly different between HP_{soy} and HP_{mix}. In **Table 1** inflammatory markers before and after both experimental diets are presented. Intake of total energy, protein, fat and carbohydrates in HP_{soy} and HP_{mix} were shown previously [10].

Plasma CRP and SAA were lower after HP_{soy} than after HP_{mix}, albeit not significant (CRP: 2.1 ± 2.0 vs. 2.8 ± 2.5 mg/L, P=0.40; SAA: 2.9 ± 2.2 vs. 9.4 ± 17.5 mg/L, P=0.30). Sensitivity analyses excluding participants with CRP>6mg/L slightly increased the difference (CRP: 1.3 ± 2.1 vs. 2.2 ± 1.0, P=0.08; SAA: 2.8 ± 2.1 vs. 12.2 ± 21.1, P=0.25; n=8).

In the total study population partly replacing meat protein with soy protein in a moderate high-protein diet had no beneficial effects tested by the summary score of inflammatory markers. (z-score: -0.3 ± 0.6 vs. -0.2 ± 0.4, P=0.76). After exclusion of one participant with high plasma IFN-γ at the end of one diet the z-score tended to be lower after HP_{soy} compared with HP_{mix} (z-score: -0.3 ± 0.5 vs. -0.1 ± 0.4, P=0.09, n=11). The summary score was significantly lower after HP_{soy} compared with HP_{mix} in a model excluding participants with CRP>6mg/L and participants with extreme outliers for SAA and IFN-γ (z-score: -0.2 ± 0.3 vs. -0.1 ± 0.2, P=0.04, n=7).

After 4 weeks, both diets lowered fasting IL-6, TNF-α, sICAM-1, sVCAM-1 and the total z-score (all $P < 0.03$, Table 1). Borderline significant reductions were found for IL-8, CRP (both P=0.06) and SAA (P=0.09), which were significant after sensitivity analyses (CRP:P=0.033, excluding participants with CRP>6mg/L, n=8, SAA:P=0.002, excluding an outlier for SAA, n=11, and IFN-γ: P=0.009, excluding an outlier for IFN-γ, n=11).

Table 1 Inflammatory measures at the start and end of 4-wk diet periods with mixed and soy protein diets (n=12)

Characteristic	Before intervention		After intervention		Pvalue (ANCOVA)	
	Soy diet	Mixed diet	Soy diet	Mixed diet	Soy vs. mixed[1]	Time effect[2]
Inflammatory markers (pg/L)						
Interferon-γ	8.5 ± 1.7	9.4 ± 5.8	14.4 ± 30.0[4]	7.1 ± 4.1	0.66	0.20
Interleukin-10	0.15 ± 0.08	0.18 ± 0.08	0.14 ± 0.07	0.16 ± 0.13	0.99	0.15
Interleukin-6	0.65 ± 0.38	0.53 ± 0.26	0.56 ± 0.54	0.41 ± 0.24	0.32	0.012
Interleukin-8	5.2 ± 2.9	4.4 ± 1.7	3.9 ± 1.0	3.9 ± 1.0	0.91	0.06
Tumor necrosis factor-α	2.0 ± 0.4	2.0 ± 0.3	1.7 ± 0.4	1.7 ± 0.3	0.73	<0.001
Inflammatory markers (mg/L)						
C-Reactive protein	3.4 ± 2.8	3.3 ± 2.9	2.1 ± 2.0	2.8 ± 2.5	0.40	0.06
SAA	5.1 ± 3.9	5.1 ± 3.1	2.9 ± 2.2	9.4 ± 17.5[5]	0.30	0.09
sICAM-1	0.37 ± 0.06	0.37 ± 0.06	0.34 ± 0.05	0.35 ± 0.07	0.60	0.003
sVCAM-1	0.42 ± 0.09	0.42 ± 0.08	0.39 ± 0.09	0.40 ± 0.08	0.37	0.023
Z-score[6]	0.23 ± 0.37	0.20 ± 0.41	-0.25 ± 0.56	-0.19 ± 0.44	0.76	<0.001

All values are means ± SD

[1]Pvalues are based on repeated measures ANCOVA adjusted for baseline values and period. For IFN-y,IL-10, IL-6, IL-8, CRP and SAA the loge was used.

[2]Pvalues are based on repeated measures ANCOVA adjusted for period

[3]Z-score is the total score based on all pro-inflammatory markers, thus excluding IL-10.

[4]Results mean plasma IFN-γ excluding 1 outlier - before intervention: soy 8.5 ±1.7, mixed 9.7 ± 6.0 and after intervention soy 5.7 ± 2.1, mixed 7.2 ± 4.3

[5]Results mean plasma SAA excluding 1 outlier - before intervention: soy 5.2 ± 4.1, mixed 5.2 ± 3.2 and after intervention soy 3.0 ± 2.3, mixed 4.6 ± 5.8

[6]*Z-score=[Z-score (log_eCRP) + Z-score (log_eIFN-γ) + Z-score (log_eIL-6)+ Z-score (log_eIL-8) + Z-score (TNF-α)+ Z-score (log_eSAA) + Z-score (sICAM-1) + Z-score (sVCAM-1)]:8*

SAA=Serum Amyloid A, sICAM-1= soluble intercellular cell adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1

DISCUSSION

Partly replacing meat with soy in a moderate-high-protein diet for 4 weeks tended to decrease CRP and SAA, but did not significantly alter individual inflammatory markers. The summary score of inflammation was lower after HP_{soy} than after HP_{mix}, after excluding participants with CRP> 6mg/L and outliers for SAA and IFN-γ. Our results also suggest that moderate-high-protein reduced-fat diets may have the ability to improve several inflammatory markers.

Partly replacing meat with soy products did tend to decrease CRP and SAA (38% and 43% reductions, respectively), and the summary score of low-grade inflammation was significantly lower after sensitivity analysis, however our findings were not as pronounced as expected. Although animal studies seem promising, prior human research on soy foods or specific constituents of soy on inflammation reported mixed results.

Thus the fact that we did not find significant alterations but did find some tendencies in improvements is in line with prior research. In recent observational studies [12, 13] and intervention trials [14, 15] soy protein and/or isoflavones were, or tended to be, related to lower IL-6, TNF-α and/or CRP. However an earlier meta-analysis provided insufficient evidence of a CRP lowering capacity by soy isoflavones, except for people with elevated CRP at baseline [16]. An earlier brief review of clinical data concluded that neither soy foods nor isoflavones affect IL-6 and TNF-α [9]. Finally, another review [17] reported that soy protein does not affect markers of inflammation, that soybean processing may affect anti-inflammatory ability and that health status of participants might determine anti-inflammatory efficacy. Further, it was concluded that the majority of trials with soy-derived isoflavones did not observe a significant effect on inflammatory processes in humans [17]. One trial even found that a high isoflavone soy diet increased IL-6 in women [8]. So possibly, the small nonsignificant reductions in individual inflammatory markers in our trial could be explained by low power of our trial, because of a short period of exposure, and/or relatively large within-subject variation in inflammatory markers [18]. Yet we did find changes in LDL cholesterol, while also blood cholesterol is known to vary with large temporal fluctuations. Though, LDL cholesterol is observed to have a within-subject biologic variation (CVi) of 7.8, while biological variation in most inflammatory markers we measured is much higher [19]. Possibly trials with more participants or participants with metabolic problems, such as elevated inflammatory markers, would find partly replacing meat with soy foods to be effective in lowering markers of low-grade inflammation. As many trials did find nonsignificant reductions in various markers of inflammation by introducing soy foods, partly replacing meat with soy warrants more research.

The results of CRP in this report are slightly different from the results reported earlier, where we reported a nonsignificant reduction of 60% [10]. This is likely due to missing data of 3 participants in the present analysis, as the measurements by routine commercial ELISA kit (CRPL3; Cobas, Roche) used earlier and multiplex used now were highly correlated (r=0.99). Both experimental diets differed only in protein source, meat was partly replaced with soy while keeping the contribution of other protein sources (dairy, legumes) comparable, as well as other dietary factors, e.g. dietary fiber and SFA. Possibly the effects of partly replacing meat with soy in a real-life setting are stronger, because in our soy diet total fat and cholesterol intake were controlled for and were even somewhat overcompensated. A 'whole food approach' was used instead of providing supplements, as we wanted to study the effect of increased soy protein intake via soy food consumption and to reflect general practice. Finally, the intervention was strictly controlled, all participants completed the trial and compliance to both diets was good. A 4-week period was chosen to address short-term adaptations to changes in dietary intake and to reduce risk of alterations in other lifestyle factors known to modify cardio-metabolic risk factors.

Our data additionally suggest that increasing protein intake at the expense of fat, without lowering carbohydrates and fiber, could affect the inflammatory state. Both moderate-high-protein reduced-fat diets, increasing habitual protein intake to 22 E% (=1.7 g/kg), decreased total z-score (-0.5 points). The decrease in total z-score reflects reductions in fasting IL-6 (-18%), IL-8 (-19%), TNF-α (-15%), sICAM-1 (-7%), sVCAM-1 (-6%), CRP (-27%), SAA (-27%), IFN-γ (-30%) and a tendency of reduced fasting IL-10 (-9%). Although participants lost a small amount of body weight (<1%) and weight loss is associated with improvements in these markers of inflammation, we suggest that the major part of these improvements are likely a result of the trial diets. In trials with improvements in inflammation via diet weight loss is usually much greater, with significant reductions in adipose tissue, which is known to release many mediators of inflammation. Further, prior research showed improvements in inflammation by a healthy diet, independent of weight loss [17]. A reduction of total fat [3] and/or SFA [4] content of a diet may be capable to lower inflammation. Further, it is possible that increased protein intake in our trial was beneficial, as anti-inflammatory effects have been found in trials using high-protein diets. A progressive resistance training combined with a protein-enriched diet equivalent to ~1.3 g/kg compared with 1.1 g/kg reduced circulating IL-6 concentrations in elderly women [20]. Another trial observed that a 6-month hypocaloric high-protein diet versus hypocaloric high-carbohydrate diet improved TNF-α, IL-6 and CRP in normal, obese females without diabetes [21]. However, as far as we know, no data is available about this small amount of weight loss and markers of inflammation. Further, our results warrant more research as we did not have a normal-protein control diet.

In conclusion, we did not find clear benefits on individual markers of inflammation by partly replacing meat protein with soy protein in a moderate-high protein diet in women with increased risk of MetS. Nevertheless, total low-grade inflammation, as calculated by the summary z-score, may reduce when meat is partly replaced by soy. In addition to our prior observation of improved insulin sensitivity and lower circulating lipids [10], partly replacing meat with soy products could also be beneficial regarding low-grade inflammation and thus important in preventing or treating MetS. Our trial provides another important new insight, namely that inflammatory markers may be improved by increased protein intake at the expense of fat, without lowering carbohydrates and fiber. Larger and longer-term studies in which both men and women are included could help to get more insight in if and how low-grade inflammation can be improved with increased intake of protein and/or partly replacing meat with soy.

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The authors' responsibilities were as follows: MvN, MM and EF designed research; MvN conducted research; MvN and MM collected data; MvN analyzed data, performed statistical analyses and wrote the paper. All authors have read and approved the final manuscript.

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Abstract

Background Arginine is a potentially interesting amino acid for people with characteristics of the metabolic syndrome. It is found to have beneficial effects on postprandial metabolism by increasing insulin secretion and decreasing hyperlipidemia and hyperglycemia; especially when metabolism is challenged, e.g. in diabetic patients or after a high-fat meal. However, whether arginine-rich proteins are equally effective is not known.

Methods In this double-blind randomized crossover trial, 18 men with characteristics of the metabolic syndrome, received a high-fat liquid meal (95 g fat) without (control) or with 30 g added protein: pea protein (arginine-rich), wheat protein (low in arginine) and their hydrolysates. Metabolic factors (insulin, glucose, triglycerides) were measured at baseline and hourly until 6h after meal consumption. Circulating inflammation markers and arterial stiffness were measured at baseline and at regular time points.

Results Meals with intact pea and wheat protein resulted in higher postprandial insulin concentrations compared with the high-fat liquid meal $P<0.001$; hydrolysates did not further increase plasma insulin. All meals resulted in a postprandial increase in plasma triglycerides, and a decrease in plasma glucose, blood pressure and augmentation index (arterial stiffness); no effect of added protein was seen. For inflammation markers no differences in postprandial responses were seen.

Conclusions Adding protein to a high-fat liquid meal increased circulating insulin. However, arginine-rich protein (pea) was not superior to a protein low in arginine (wheat). Both added proteins did not affect vascular function and postprandial inflammatory markers. No additional benefits of protein hydrolysates were seen.

Submitted for publication

Chapter 5

Postprandial effect of arginine-rich protein added to a high-fat liquid meal in men with characteristics of the metabolic syndrome

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INTRODUCTION

Postprandial dysmetabolism, i.e. hyperglycemia, hyperinsulinism, dyslipidemia and inflammation, is suggested to be an important cardio-metabolic risk factor [1-3]. The postprandial inflammatory response is associated with an increased risk of cardiovascular events as it is characterized by an increase in plasma acute phase proteins, the activation of white blood cells and an increased arterial stiffness, i.e. diminished elasticity of the arterial wall. Thus strategies to improve postprandial metabolism are important, especially while people spend most of their waking hours in the postprandial state.

The postprandial inflammatory response, mainly a result of postprandial rises in lipids, i.e. plasma triglyceride and FFA, and blood glucose, is particularly provoked by consumption of a high-fat diet [4-6]. Furthermore, the postprandial response can be worsened by presence of the metabolic syndrome (MetS), obesity and T2DM [5]. Obese individuals have stronger postprandial inflammatory responses compared with healthy lean people (6). People with T2DM show high postprandial inflammatory responses increasing with their degree of insulin resistance [7], together with higher postprandial concentrations of triglycerides, FFA and glucose, and with delayed clearance.

Dietary proteins, and more specific the amino acid arginine, are suggested to have anti-inflammatory properties by positively affecting postprandial metabolism [6, 8, 9]. Supplementation of arginine is known to improve postprandial metabolism by increasing insulin secretion and decreasing lipidemia and glycemia; especially when metabolism is challenged, e.g. in diabetic patients, the metabolic syndrome or after a high-fat meal. Long-term L-arginine supplementation enhanced insulin sensitivity and endothelial function and reduced inflammation in non-diabetic patients [10]. However, whether arginine-rich proteins are equally effective is currently unknown.

We therefore examined whether protein, and more specific arginine-rich protein, added to a high-fat liquid meal (HFLmeal) improves postprandial metabolism and cardiovascular risk factors. Additionally we examined whether increased speed of absorption is advantageous, by the use of protein hydrolysates [11].

MATERIALS AND METHODS

Participants

In total 18 adult men, aged 57–70 years, with characteristics of MetS were recruited from the general community. Eligibility was initially assessed by a medical questionnaire. Potential participants were invited for a screening to measure blood pressure (BP), fasting plasma HDL, triglycerides, and glucose by an oral glucose tolerance test (OGTT). Participants had a waist larger than 94cm and 1 or more other characteristics of MetS according to criteria defined by the international diabetes federation [12]. All participants were non-diabetic, non-smokers, non-allergic to milk or wheat, without a history of

cardiovascular, liver or renal disease, and with stable weight and exercise habits in the past 6 months. None of the participants used lipid lowering medication or corticosteroids. Five participants used BP medication, but not on test days. The procedures followed were in accordance with the ethical standards of the Human Investigation Review Committee of Wageningen University. All participants gave their written consent before participation. The trial was registered in the NIH clinical trial database at clinicaltrials.gov as NCT01215370.

Design

In this double-blind crossover challenge trial, participants randomly received a HFLmeal without (control) or with 30g added protein: pea protein (PP; arginine-rich), wheat protein (WP; low in arginine) or their hydrolysates (PPH, WPH), on 5 separate days with a washout period of at least 1week (average 10days). The evening before each test-day participants consumed a standardized low-fat meal and refrained from exercise and alcohol. After an overnight fast (12h) baseline plasma and vascular measurements took place. Then, the HFLmeal was consumed within 10min, thereafter circulating metabolic factors were measured hourly until 6h after the meal, inflammatory markers were measured after 2h, 4h and 6h and vascular function was measured 3h and 6h after HFLmeal ingestion.

Test Meal

The control HFLmeal (550 ml) consisted of 49% whipped cream and 3% sugar (~4 MJ, 94 g fat, 55 g saturated fat, 11 g carbohydrate and 6 g protein). Additional protein was added for 4 test meals: 30 g PP (Roquette, Nutralys S85F) or PPH (both equivalent to ~2.9 g arginine, an amount found to be effective in prior research [13]), or an equal amount WP (Roquette, Nutralys W) or WPH. To obtain hydrolysates both proteins were enzymatically predigested for 3h by Umamizyme, followed by 3h by peptidaseR. The degree of hydrolysis (DH) of pea and wheat hydrolysates was 24.3% and 19.0% respectively. **Table 1** describes the composition of meals and added proteins.

Table 1. Composition of the high-fat liquid meal without (control) or with 30g added protein

	Control	Pea Protein	Wheat Protein	Pea Protein Hydrolysate	Wheat Protein Hydrolysate
Energy (MJ)	3.7	4.3	4.3	4.3	4.3
Fat (g)	94	94	94	94	94
Saturated fat (g)	55	55	55	55	55
Carbohydrate (g)	16	16	16	16	16
Protein (g)	6	36	36	36	36
Added protein (g)	-	30	30	30	30
Arginine (g)	-	2.94	0.78	2.94	0.78
Degree of Hydrolysis (%)	-	1.8	3.2	24.3	19.0

Measurements

Participants' height and weight were measured, body composition was assessed by BodPod. Blood was collected via a catheter in an antecubital vein using vacutainers containing EDTA (Becton Dickinson), plasma was immediately separated by centrifugation at 2000g for 20min at 4°C and then frozen and stored at -80°C until analyses. Macro vascular regional arterial stiffness was assessed by applanation tonometry (SphygmoCor, AtCor Medical) [14]. After 10min rest in supine position, a pressure sensor was applied on the right-hand radialis to record peripheral arterial pressure pulse waveforms. These waveforms were calibrated using SBP and DBP from brachial cuff measurements in the left arm and then converted to an aortic pressure waveform and Pulse Wave Analysis (PWA). This PWA provides the augmentation index (Aix), which is the amount of pressure added to the systolic pressure peak based on the reflected wave, expressed as a percentage of pulse pressure (PP=SBP-DBP) [14]. Additionally, it provides central SBP (cSBP), central PP (cPP), myocardial blood flow and oxygen demand ratio (subendocardial viability ratio, SEVR%) and ejection duration index (ED%).

Blood sample analyses

Routine biochemical spectrophotometric procedures were used to measure circulating glucose, triglycerides, total cholesterol and HDL (Cobas, Roche). LDL cholesterol was calculated by the Friedewald equation [15]. Commercial ELISA kits were used to measure insulin (LDN, Nordhorn, Germany), acute phase protein C-Reactive Protein (CRP) (CRPL3, Cobas, Roche), intercellular Adhesion Molecule 1 (ICAM-1) (Human ICAM-1 ELISA, R&D Systems), Von Willebrand factor (vWF) (Nunc Maxisorp), and cytokines Interleukin 6 (IL-6) (Human IL-6 ELISA, R&D Systems) and Monocyte Chemotactic Protein-1 (MCP-1) (Human MCP-1 ELISA, R&D Systems).

Data analyses and statistical considerations

Data were analyzed by using SAS9.2. All variables were expressed as mean±SD. Whether the added protein altered the response compared with the control HFLmeal was tested by planned comparisons, separately it was tested whether hydrolysates were different compared with intact proteins. Linear mixed models were used to examine overall effects and differences in time-responses between control, PP and WP, and between intact proteins and hydrolysates using 'meal', 'time' and 'meal by time' as fixed effects and participant as random effect. Because of planned comparisons we have not corrected Pvalues or confidence intervals for multiple comparisons. For variables with different postprandial time-responses between meals, total area under the curve (AUC) and positive incremental AUC (pos-iAUC) were calculated using the trapezoidal rule. Pos-iAUC accurately describes postprandial responses whereas total AUC is highly correlated with fasting values [16].

One-way ANOVA was used to test differences between AUC and pos-iAUC of control vs. PP and WP, and intact proteins vs. hydrolysates; PP vs. PPH, and WP vs. WPH. Analyses were adjusted for baseline measurements, if they were of significant influence. Statistical significance was set at $P<0.05$.

RESULTS

Participants

Eighteen men participated in this trial. All participants had a waist circumference of >94cm and 1 or more additional characteristic of MetS. Seven men had 2 MetS characteristics; 5 men had 3; 5 men had 4 and 1 man had 5; i.e. SBP ≥130 mmHg (n=14), DBP≥85 mmHg (n=6), fasting glucose ≥6.2 but <7.8 mmol/L (n=4), HDL<1.03 mmol/L (n=5) and fasting triglyceride>1.7 mmol/L (n=7). Group characteristics are listed in **Table 2**.

Table 2. Characteristics of the participants (n=18)

Variable	Mean ± SD	Range
Age, *years*	64 ± 4	(56-70)
Height, *m*	180 ± 5	(173-191)
Weight, *kg*	92 ± 15	(75-123)
Body mass index, kg/m^2	28.6 ± 4.5	(24.1-39.4)
Body fat, *%*	32 ± 8	(18-49)
Metabolic syndrome, *n (criteria out of 5)*	2.8 ± 1.0	(2-5)
Waist, *cm*	105 ± 11	(94-130)
Fasted glucose, *mmol/L*	5.7 ± 0.5	(4.9-6.7)
2Hrs glucose, *mmol/L*	6.6 ± 1.7	(3.9-10.2)
Triglycerides, *mmol/L*	1.6 ± 0.8	(0.6-3.7)
HDL cholesterol, *mmol/L*	1.4 ± 0.4	(0.9-2.3)
Systolic blood pressure, *mmHg*	138 ± 15	(109-172)
Diastolic blood pressure, *mmHg*	81 ± 7	(68-95)
Total cholesterol, *mmol/L*	5.9 ± 1.0	(4.5-7.9)

All values are means ± standard deviation and range.

Metabolic parameters

Fasting values of plasma metabolic variables were not significantly different between test days. Plasma values of insulin, triglycerides and glucose before and hourly after all meals are depicted in **Figure 1 and Table 3.** Consumption of the HFLmeal increased mean plasma insulin concentrations at 1h ($P_{time}<0.001$), followed by a gradual return to baseline the next hours. Adding protein to the HFLmeal further increased this insulin-response, with a pos-iAUC for intact proteins of 116% for PP and 119% for WP compared with the control HFLmeal (both $P<0.001$). Adding PPH resulted in a similar response as PP, however the insulin-response was lower after WPH compared with WP ($P_{time*meal}=0.004$).

Consumption of the HFLmeal increased triglycerides with a peak concentration at 4h and it slightly reduced circulating glucose levels (Figure 1 and Table 3, both P_{time}<0.001). Adding protein or protein hydrolysates to the HFLmeal did not affect postprandial triglyceride and glucose responses.

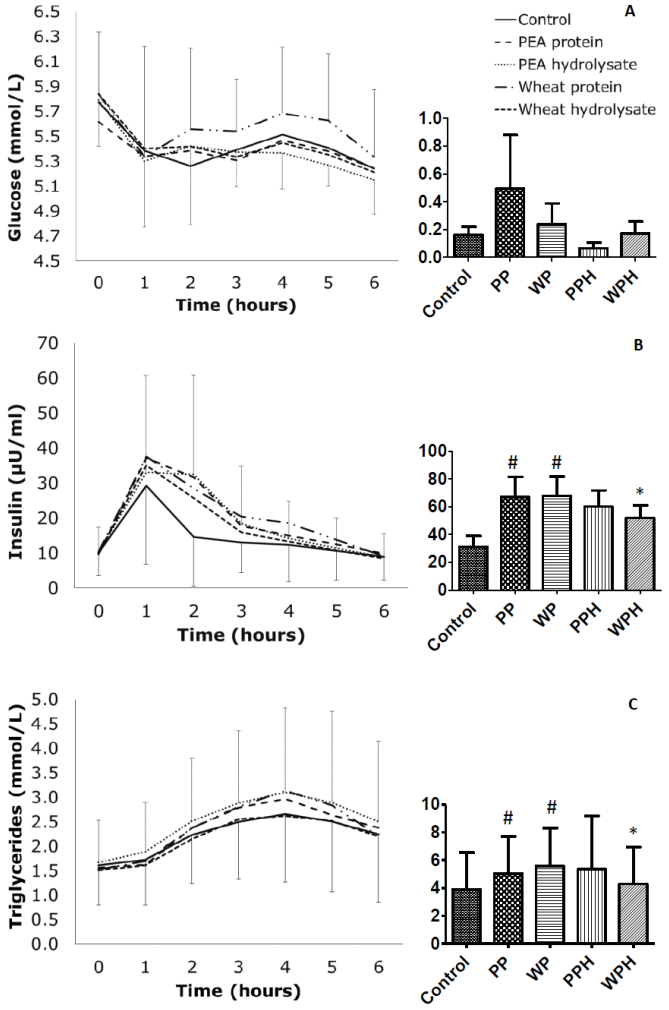

Figure 1 Postprandial responses on glucose (A), Insulin (B) and Triglycerides (C) after a high-fat liquid meal without or with 30g added protein, n=18. Test results for the model comparing control vs. PP and WP are for Glucose P_{meal}=0.001, P_{time}<0.001, $P_{meal*time}$=0.920. Insulin P_{meal}=<0.001, P_{time} <0.001, $P_{meal*time}$=<0.001, and Triglycerides P_{meal}=0.005, P_{time}<0.001, $P_{meal*time}$=0.866. All values±SD and test results are shown in Tables 3-4. Boxplots depict pos-iAUC, with # = significantly different from control, and * = WP different from WPH.

Table 3. Baseline and hourly postprandial metabolic plasma values after a high-fat liquid meal without (control) and with 30g added protein

	Liquid meal	Time	Delta						Pvalue			
		Baseline	1hr	2hr	3hr	4hr	5hr	6hr	meal	time	interaction meal*time	
Glucose	Control	5.8 ± 0.6	-0.4 ± 1.1	-0.5 ± 0.7	-0.4 ± 0.5	-0.3 ± 0.5	-0.4 ± 0.5	-0.5 ± 0.4	0.001	<0.001	0.920	#
(mmol/l)	Pea	5.6 ± 0.5	-0.3 ± 0.7	-0.2 ± 0.6	-0.3 ± 0.3	-0.2 ± 0.3	-0.2 ± 0.2	-0.4 ± 0.2				
	Wheat	5.8 ± 0.5	-0.5 ± 0.9	-0.3 ± 0.7	-0.3 ± 0.4	-0.2 ± 0.5	-0.2 ± 0.5	-0.5 ± 0.5				
	Pea Hydrolysate	5.8 ± 0.4	-0.5 ± 0.5	-0.4 ± 0.6	-0.4 ± 0.3	-0.4 ± 0.3	-0.5 ± 0.2	-0.6 ± 0.3	0.812	<0.001	0.619	$
	Wheat Hydrolysate	5.8 ± 0.6	-0.4 ± 0.8	-0.4 ± 0.8	-0.5 ± 0.4	-0.4 ± 0.4	-0.5 ± 0.4	-0.6 ± 0.3	0.997	<0.001	0.837	*
Insulin	Control	10.0 ± 6.5	19.2 ± 22.4	4.6 ± 14.1	3.0 ± 8.7	2.3 ± 10.5	0.6 ± 8.4	-1.1 ± 6.7	<0.001	<0.001	<0.001	#
(μU/ml)	Pea	9.6 ± 6.1	27.7 ± 35.2	22.1 ± 28.9	8.2 ± 14.4	5.5 ± 9.5	2.9 ± 7.9	0.3 ± 6.7				
	Wheat	10.2 ± 6.3	27.3 ± 36.2	18.3 ± 25.1	10.2 ± 16.2	8.4 ± 11.5	3.6 ± 8.7	-1.1 ± 6.2				
	Pea Hydrolysate	10.1 ± 7.3	23.0 ± 27.7	22.3 ± 28.4	8.3 ± 16.4	4.1 ± 10.6	1.4 ± 8.5	-1.4 ± 6.7	0.211	<0.001	0.848	$
	Wheat Hydrolysate	10.0 ± 8.8	25.0 ± 26.5	15.7 ± 26.1	6.0 ± 11.6	3.3 ± 11.0	0.8 ± 8.8	-1.6 ± 6.7	0.001	<0.001	0.004	*
Triglyceride	Control	1.6 ± 0.8	0.1 ± 0.9	0.6 ± 1.0	0.9 ± 1.2	1.1 ± 1.4	0.9 ± 1.4	0.6 ± 1.4	0.005	<0.001	0.866	#
(mmol/l)	Pea	1.6 ± 0.7	0.1 ± 0.6	0.8 ± 0.8	1.2 ± 1.0	1.4 ± 1.3	1.1 ± 1.2	0.8 ± 1.4				
	Wheat	1.5 ± 0.6	0.2 ± 0.7	0.9 ± 0.9	1.3 ± 1.1	1.6 ± 1.3	1.3 ± 1.3	0.7 ± 1.1				
	Pea Hydrolysate	1.7 ± 0.9	0.2 ± 1.0	0.8 ± 1.3	1.2 ± 1.5	1.4 ± 1.7	1.2 ± 1.9	0.8 ± 1.7	0.518	<0.001	0.989	$
	Wheat Hydrolysate	1.5 ± 0.7	0.1 ± 0.8	0.6 ± 0.9	1.1 ± 1.2	1.1 ± 1.4	1.0 ± 1.5	0.7 ± 1.2	0.873	<0.001	0.956	*

All values are means ± standard deviation; n=18. # Model comparing control vs. PP and WP, $ Model comparing PP vs. PPH, * Model comparing WP vs. WPH

Inflammation

Plasma values of CRP, ICAM-1, IL-6 and MCP-1 before and 2h, 4h and 6h after all HFLmeals are shown in **Table 4.** After consumption of the control HFLmeal IL-6 increased (P_{time}<0.001) and MCP-1 decreased (P_{time}<0.001), while CRP and ICAM-1 were unaffected. Overall, adding intact protein and protein hydrolysates to the HFLmeal did not alter these postprandial responses.

Table 4. Baseline and postprandial plasma inflammatory cytokines after a high-fat liquid meal without (control) and with 30g added protein

	Liquid meal	Time	Delta			Pvalue			
		Baseline	2hr	4hr	6hr	meal	time	interaction meal*time	
CRP	Control	1.8 ± 1.8	0.1 ± 2.0	0.0 ± 1.9	0.0 ± 1.9	0.004	0.974	1.000	#
(mmol/l)	Pea	1.8 ± 1.7	0.1 ± 1.9	0.1 ± 1.9	0.0 ± 1.8				
	Wheat	2.2 ± 1.8	0.2 ± 2.0	0.2 ± 2.1	0.2 ± 2.2				
	Pea Hydrolysate	1.8 ± 2.0	0.1 ± 2.1	0.1 ± 2.1	0.0 ± 2.0	0.275	0.992	0.995	$
	Wheat Hydrolysate	2.1 ± 2.1	0.1 ± 2.3	0.1 ± 2.3	0.1 ± 2.3	0.172	0.979	0.994	*
ICAM-1	Control	119.3 ± 29.4	5.4 ± 39.6	-1.2 ± 24.4	0.2 ± 24.5	0.060	0.101	0.761	#
(mmol/l)	Pea	119.6 ± 27.6	6.7 ± 28.2	0.4 ± 29.1	1.5 ± 27.0				
	Wheat	120.4 ± 29.8	9.5 ± 26.0	10.6 ± 31.1	3.6 ± 25.7				
	Pea Hydrolysate	119.3 ± 26.6	-2.1 ± 23.9	-0.5 ± 23.7	0.2 ± 25.7	0.160	0.857	0.437	$
	Wheat Hydrolysate	119.8 ± 24.7	3.4 ± 26.4	1.8 ± 29.1	-0.4 ± 25.8	0.782	0.471	0.897	*
IL-6	Control	2.1 ± 1.4	-0.6 ± 1.1	0.2 ± 1.4	0.9 ± 2.1	0.004	<0.001	0.977	#
(mmol/l)	Pea	2.6 ± 2.4	-0.5 ± 2.1	0.5 ± 2.1	1.0 ± 2.2				
	Wheat	4.8 ± 8.1	-1.4 ± 5.0	-0.7 ± 4.7	0.4 ± 4.5				
	Pea Hydrolysate	2.3 ± 2.1	-0.7 ± 1.1	-0.2 ± 1.1	0.8 ± 1.6	0.114	<0.001	0.772	$
	Wheat Hydrolysate	2.0 ± 2.2	-0.1 ± 1.9	0.9 ± 2.1	1.0 ± 2.2	0.661	<0.001	0.493	*
MCP-1	Control	121.1 ± 76.4	-11.2 ± 57.4	-19.3 ± 52.4	-15.3 ± 50.0	0.389	<0.001	0.998	#
(mmol/l)	Pea	117.3 ± 42.1	-7.0 ± 42.0	-18.0 ± 39.8	-12.2 ± 35.2				
	Wheat	117.7 ± 53.1	-11.8 ± 46.7	-23.0 ± 42.1	-18.8 ± 38.9				
	Pea Hydrolysate	122.4 ± 48.1	-8.1 ± 48.4	-18.5 ± 41.0	-10.9 ± 42.5	0.086	<0.001	0.992	$
	Wheat Hydrolysate	119.6 ± 40.9	-4.5 ± 36.3	-22.9 ± 26.7	-13.3 ± 33.1	0.961	0.019	0.898	*

All values are means ± standard deviation; n=18. CRP, C-reactive Protein; ICAM-1, Intercellular Adhesion Molecule-1; IL-6, Interleukin-6; MCP-1, Monocyte chemoattractant protein-1. # Model comparing control vs. PP and WP, $ Model comparing PP vs. PPH, * Model comparing WP vs. WPH

Vascular function

Vascular measures and plasma values of vWF (time=0,3,6) are shown in **Table 5**. After the HFLmeal Aix75, cSBP, cPP and ED decreased (24%, 6%, 7% and 2% respectively), Aix75 remained that low up to 6h, while cSBP, cPP and ED returned to baseline (all P_{time}<0.001). SEVR and vWF were unaltered after the HFLmeal. All meals with added protein and protein hydrolysates gave similar responses as the HFLmeal. Only PPH gave a different response compared with intact protein, as it slightly increased vWF, whereas PP resulted in a small decrease.

Table 5. Baseline and postprandial vascular function after a high-fat liquid meal without (control) or with 30g added protein

	Liquid meal		**Delta**		**Pvalue**			
		Baseline	3hr	6hr	meal	time	interaction meal*time	
Aix	Control	22.4 ± 4.9	-5.4 ± 5.4	-5.2 ± 4.9	0.024	<.0001	0.654	#
(%)	Pea	22.7 ± 5.0	-5.7 ± 6.1	-6.4 ± 5.6				
	Wheat	22.0 ± 3.9	-6.9 ± 3.7	-6.4 ± 4.6				
	Pea Hydrolysate	22.4 ± 4.2	-6.5 ± 5.2	-5.9 ± 5.9	0.302	<.0001	0.558	$
	Wheat Hydrolysate	22.6 ± 4.8	-6.4 ± 5.1	-5.6 ± 5.2	0.663	<.0001	0.704	*
Central SBP	Control	123.9 ± 14.3	-6.5 ± 10.8	-3.3 ± 11.3	0.052	<.0001	0.740	#
(mmHG)	Pea	123.9 ± 15.1	-10.0 ± 13.7	-6.2 ± 13.2				
	Wheat	122.9 ± 12.7	-11.2 ± 12.1	-6.4 ± 13.8				
	Pea Hydrolysate	123.4 ± 13.4	-8.8 ± 12.7	-3.3 ± 12.2	0.934	<.0001	0.653	$
	Wheat Hydrolysate	122.5 ± 13.8	-7.6 ± 14.1	-1.9 ± 15.8	0.312	<.0001	0.736	*
Central PP	Control	43.0 ± 9.9	-3.0 ± 10.1	-0.7 ± 9.9	0.128	<.0001	0.500	#
(mmHG)	Pea	44.3 ± 11.8	-5.6 ± 10.5	-3.9 ± 10.6				
	Wheat	43.4 ± 12.0	-6.5 ± 10.8	-3.8 ± 11.4				
	Pea Hydrolysate	43.3 ± 11.3	-4.4 ± 9.8	-1.1 ± 9.4	0.971	<.0.001	0.409	$
	Wheat Hydrolysate	42.8 ± 9.9	-3.4 ± 10.5	-0.8 ± 12.5	0.627	0.014	0.978	*
SEVR	Control	159.2 ± 25.2	4.8 ± 27.1	4.0 ± 30.1	0.277	0.141	0.429	#
(%)	Pea	160.5 ± 27.6	-3.9 ± 29.3	1.6 ± 30.2				
	Wheat	157.0 ± 23.4	0.6 ± 27.2	5.5 ± 27.8				
	Pea Hydrolysate	162.8 ± 26.3	-5.6 ± 26.6	-3.6 ± 29.7	0.726	0.050	0.423	$
	Wheat Hydrolysate	156.6 ± 27.9	-1.0 ± 25.1	2.5 ± 29.8	0.008	0.375	0.420	*
ED	Control	335.7 ± 13.1	-5.3 ± 18.6	-1.1 ± 17.4	<.0001	<0.001	0.368	#
(%)	Pea	337.2 ± 13.2	-10.2 ± 17.5	-5.0 ± 17.2				
	Wheat	332.5 ± 16.8	-12.5 ± 17.8	-3.0 ± 18.3				
	Pea Hydrolysate	338.6 ± 17.3	-10.5 ± 18.4	-4.5 ± 16.5	0.470	<.0001	0.976	$
	Wheat Hydrolysate	334.5 ± 16.0	-9.7 ± 15.9	0.6 ± 16.3	0.199	<.0001	0.300	*
vWF	Control	10.5 ± 3.6	-0.2 ± 4.1	0.5 ± 5.2	0.596	0.366	0.743	#
(mmol/l)	Pea	11.4 ± 4.0	-1.1 ± 3.7	-0.8 ± 4.0				
	Wheat	11.3 ± 3.8	-0.5 ± 3.8	-0.4 ± 4.5				
	Pea Hydrolysate	9.8 ± 3.0	0.9 ± 3.5	0.5 ± 2.8	0.057	0.928	0.032	$
	Wheat Hydrolysate	10.3 ± 3.5	-0.3 ± 3.5	-0.2 ± 3.3	0.226	0.688	0.775	*

All values are means ± SD; n=18. #Model comparing control vs.PP and WP, $Model comparing PP vs.PPH, *Model comparing WP vs.WPH Aix, Augmentation index; ED, Ejection Duration; PP, Pulse Pressure; SBP systolic blood pressure; SEVR, Subendocardial Viability Ratio; vWF, Von Willebrand Factor

DISCUSSION

In this trial we investigated postprandial responses after a HFLmeal with and without added, arginine-rich, protein. Adding protein to the HFLmeal increased the insulin response, but did not alter other metabolic parameters we measured. No additional effect of high-arginine content of protein was seen. The postprandial inflammatory response after the control HFLmeal was rather modest, with only a small rise in IL-6 at 6h. Adding protein, either with low- or high-arginine content, did not affect this, neither did it affect postprandial changes in vascular function. Generally, similar responses were seen after protein hydrolysates and intact proteins.

Consumption of the HFLmeal resulted in a postprandial increase in triglycerides and insulin. Addition of protein to this HFLmeal caused an extra increase of insulin, as was expected based on insulin secreting properties of dietary proteins and amino acids, a known effect on β-cells [17]. We did not find a reduced increase in glucose and triglycerides by adding protein to the HFLmeal, which we did expect because of the known effect of insulin on postprandial glucose and fat metabolism (6). The plasma glucose concentrations in the control condition were very low due to the nature of our HFLmeal (e.g. high-fat, low glucose), therefore probably the extra rise in insulin did not further lower glucose levels. Our unexpected finding of a, non-significant, increase in triglycerides after addition of protein resembles findings by van Meijl et al. [18]. They observed a significant increase of triglycerides after addition of milk protein to a high-fat meal [18]. As they suggested, such an increase is not likely to be a direct effect of added protein per se, as the nature of protein does not affect postprandial lipidemia, nor of higher energy intake.

The HFLmeal did not affect all inflammatory markers we assessed, consequently it could not be fully assessed if arginine-rich protein could restrict or counteract postprandial inflammation. Maybe other inflammatory markers, such as IL-1, IL-18, and/or adipokine adiponectin might have changed upon the HFLmeal, however we measured the most likely candidates based on prior research. In our trial, increases in plasma triglycerides and insulin after the HFLmeal apparently did not cause a clear inflammatory response, only plasma IL-6 after 6h slightly increased. Previous studies did find inflammatory responses after high-fat meals in healthy subjects and especially in people with obesity, T2DM or MetS [4, 19-22]

The inflammatory response may be not as evident as assumed, as it was not so clear in our population of middle aged, abdominally overweight men, characterized with at least two components of MetS. Especially because a nearly similar HFLmeal challenge as currently used previously resulted in a more pronounced rise in IL-6 in a healthy young population [4]. Why we and several other high-energy high-fat meal trials [21, 23-26] did not, or only partly, find a clear inflammatory response - characterized by increased levels of adhesion molecules, cytokines, oxidative stress and leukocyte activation - is unclear. Current knowledge on postprandial hyperlipidemia and hyperglycemia shows that both conditions

induce several processes involved in inflammation. Our HFLmeal contained a very low amount of carbohydrates (14E%) and did not result in a postprandial increase in glucose. We consider the absence of glycemia to be probably responsible for the lack of effect on TNF-α, as in vivo studies have demonstrated increased production of TNF-α by PBMC's as an effect of glucose consumption [27]. However, carbohydrate content of high-energy high-fat meals with none or only partly inflammatory responses varies greatly - i.e. 27E% [21], 34E% [26], 36E% [25], 49E% [23, 24] - so low carbohydrate content does not seem the only explanation.
Hyperlipidemia stimulates IL-6 and VCAM-1, decreases endothelial function and may increase TNF-α and MCP-1 via nuclear factor kappaB [27]. However, based on our results, only a rise in triglycerides seems not enough to trigger inflammation, which is in line with Lundman 2007 [21] who concluded that the magnitude of postprandial triglyceridemia appears not to influence increased levels of plasma IL-6. The reduction in MCP-1 we observed after the HFLmeal was unexpected, and we do not have a clear explanation for this.

An important determinant for the postprandial response is the fatty acid composition of the meal. Triglyceride responses after meals rich in MUFA seem higher than after meals rich in SFA [28]. However, the meal-induced effect was largely independent of the type of fat consumed or of being lean or obese, despite the clear differences in postprandial triglycerides. MUFA meals may be metabolically more challenging for the body than SFA meals with a more pronounced decrease in arterial stiffness, which indicates that MUFA may be most potent to provoke physiologic vascular responses [28]. Nonetheless, consumption of an 8wk SFA diet resulted in expression of genes involved in pro-inflammatory processes in adipose tissue, while a more anti-inflammatory profile was seen after a MUFA diet [29]. Our HFLmeal mainly consisted of SFA, while MUFA in some trials seems more metabolically challenging. However, MUFA may reduce IL-6 and MCP-1, without activating NF-kB [27]. Another possible explanation for the absence of an inflammatory response in some trials is suggested protective capacity of young and fit individuals, however this seems not applicable in our trial of men with characteristics of MetS.
Overall, it is quite difficult to compare results of postprandial trials, because of the variety of compositions of high-energy high-fat meals, liquid and solid, diverse age and health status of the populations, and because the definition and markers used for the inflammatory response are not well defined. This highlights the need to further study effects of high-fat and/or high-energy induced hyperlipidemia, hyperglycemia and hyperinsulinemia on inflammation. Absence of a clear inflammatory response in our trial did not allow us to draw conclusions about possible anti-inflammatory effects of proteins in general and more specific arginine-rich proteins.

Besides effects on inflammation, high-fat high-energy meals are known to have a worsening effect on endothelial function [30-34], as they usually provoke endothelial stress markers [35] and decline flow mediated dilatation (FMD) [23]. This effect on endothelial function seems to be mainly driven by oxidative stress, through direct inactivation of nitric oxide (NO), and to be correlated with the magnitude of postprandial hyperlipidemia [33]. Free fatty acid elevation impairs insulin-mediated vasodilation and NO production, both main determinants of postprandial arterial stiffness [36]. Because of the insulinotropic effect of proteins we expected a favorable effect on the endothelium when added to a HFLmeal. We observed a postprandial decrease of Aix and ED up to 6h after the HFLmeal, which is in line with previous findings [37, 38]. Phillips et al. found a delay in return to baseline Aix in T2DM patients, a delay we also found in our population without impaired glucose metabolism, but with characteristics of MetS [37]. We did not observe an altered response after adding protein to the HFLmeal. Based on previous research, it was not completely clear what to expect, as the mechanism of the postprandial decrease of Aix is not very well understood. Nutrient delivery could possibly affect arterial smooth muscle relaxation in the general circulation [4, 38], and probably arterial stiffness and wave reflections change caused by neutrally or hormone-mediated peripheral vasodilation [28]. A postprandial decrease in Aix has been proposed to be favorable because of lowering central SBP. Indeed we found reduced central SBP, PP and ED, while none of the meals altered SEVR. Adding protein resulted in similar responses, with non-significantly lower BP. It needs further exploration how reduced stiffening and improved cardiac functioning relate to the known worsening effect of a high-fat meal on endothelial function [30-34]. The increase in insulin could have resulted in vasodilatation, which then lowered BP. The non-significant extra decrease in BP with added protein may be due to higher postprandial insulin concentrations. Because added protein did not significantly alter postprandial vascular responses, the changes upon our HFLmeal could be solely meal and/or circadian effects.

We did not observe different responses after protein hydrolysates compared to intact proteins. Protein hydrolysates should be faster available and because we did not observe an effect of hydrolysates on the postprandial response, we may conclude that speed of absorption and digestion of proteins could not have been a limiting factor explaining absence of an effect of adding protein to the HFLmeal. Alternatively, we could have missed an early effect of hydrolysates within the first hour. Unfortunately, we do not have data on plasma amino acid concentrations to further confirm these suggestions.

In conclusion, we did not find beneficial effects of arginine-rich protein (pea) on the postprandial response after a HFLmeal in men with abdominal overweight. Arginine-rich protein was not superior compared to protein low in arginine (wheat). Moreover, addition of protein to the HFLmeal only resulted in increased insulin secretion, with no effect on other metabolic and inflammatory parameters or vascular function. No additional benefits of protein hydrolysates over intact proteins were seen. In our population of men with abdominal overweight the postprandial increase in triglycerides alone did not provoke a clear inflammatory response. Due to the lack of this response, we could not fully test anti-inflammatory effects of arginine-rich protein.

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Chapter 6

General discussion

With increasing obesity and sedentary lifestyles the prevalence of type 2 diabetes (T2D) and cardiovascular disease (CVD) is rising worldwide. This increased risk of T2D and CVD can be defined by a cluster of clinical features known as the metabolic syndrome (MetS). Mets includes abdominal obesity, disturbed glucose homeostasis, dyslipidaemia, and increased blood pressure (BP), which can all be improved by diet and potentially by protein intake. Defining recommendations on protein intake to improve metabolic health are challenging, as current evidence by systematic reviews and meta-analyses of high-protein diets on T2D risk and fatal and non-fatal CVD outcomes is unconvincing [1, 2]. Overall, vegetable proteins seem preferable over animal proteins regarding fatal and non-fatal CVD outcomes, with special emphasis on soy protein intake and low density lipoprotein (LDL) cholesterol [1]. Complexity grows because studies report different findings on the short-term and long-term, strengthened by our research where contradictory effects of protein on short-term glucose metabolism and T2D incidence were reported. The influence of dietary proteins and amino acids on blood glucose, lipid metabolism, fat storage in the liver, blood pressure, arterial stiffness and inflammation is complex and involves various mechanisms. Effects may differ by type of protein, amino acid content and the matrix in which it is ingested. Additionally, effects on the long-term may not be as expected by what happens on the short-term. Therefore, the main aim of this thesis was to clarify the impact of protein intake on the risk of developing T2D, aspects of MetS and other cardio-metabolic risk factors on the short-term and long-term; first of protein intake in general, then specified by protein type, more specifically soy protein, and arginine-rich protein. We made use of data of a large-scale European case-cohort study, we performed a well-controlled dietary intervention trial in which meat was partly replaced with soy, and a crossover postprandial trial in which arginine-rich protein was added to a high-fat meal.

In this chapter the main results of these three projects will be recapped, starting with epidemiological findings on dietary protein intake and T2D incidence, then effects after 4 weeks soy protein intake, and finally postprandial effects of arginine-rich protein. This is followed by a discussion of the used methodology, interpretation of the results in perspective to other research, an overview of current research on protein intake in relation to MetS, finished by implications and suggestions for future research and conclusions.

MAIN FINDINGS

Dietary protein intake and type 2 diabetes incidence

To verify if long-term high-protein intake is truly positively associated with T2D incidence, we investigated the association between total, animal, and plant protein intake and the incidence of T2D. High total and animal protein intake was associated with modestly elevated T2D incidence in a large cohort of European adults (**chapter 2**). No association was found for plant protein. The association was clear in women, not in men, and was strongest

in obese women. The association could not be explained by a single food source. We concluded that a greater intake of total protein is associated with a higher T2D incidence in European populations, but that the effect of protein intake is small and known T2D risk factors, such as body mass index (BMI), are also important. In view of the rapidly increasing prevalence of T2D, limiting iso-energetic diets high in dietary proteins, particularly from animal sources, should be considered.

Dietary protein intake and cardio-metabolic risk factors

Increasing protein intake and soy consumption appear promising approaches to prevent MetS. However, effects on insulin resistance, glucose homeostasis, and other aspects of MetS are not frequently studied in humans. In contrast to the fact that high protein intake was associated with a higher T2D incidence on the long-term, a weight-maintaining moderate-high-protein diet for 4 weeks improved many cardio-metabolic risk factors (**chapter 3**). It reduced fasting glucose, insulin, homeostasis model assessment-estimated insulin resistance (HOMA-IR), triglycerides (TGs), total-, LDL - and high density lipoprotein (HDL)-cholesterol, fasting IL-6, TNF-α, sICAM-1, sVCAM-1 and the total z-score for inflammation, total body fat percentage, abdominal fat percentage and nonsignificantly decreased intrahepatic lipids (IHLs) and C-reactive protein (CRP) in a strictly controlled dietary crossover trial in postmenopausal women with abdominal overweight.
In addition we showed that partly replacing meat protein with soy protein resulted in greater insulin sensitivity, lower plasma total and LDL cholesterol in healthy postmenopausal women with abdominal overweight. We concluded that partly replacing meat products with soy products could be a successful strategy to treat and prevent MetS. Nonetheless, generalization of these results will depend on longer-term studies in which both men and women are included.

Dietary choices to lower pro-inflammatory markers can also play an important role in the prevention of MetS. Healthy eating patterns such as the Mediterranean diet, vegetarian diets and adherence to the food guide pyramid are associated with lower concentrations of inflammatory markers, also diets rich in whole grains, fruits and vegetables and fish positively affect the pro-inflammatory state, and relative to saturated fatty acids (SFA), polyunsaturated fatty acids are anti-inflammatory [3]. Partly replacing meat with soy may be beneficial because of the fiber, polyunsaturated fat, and polyphenol phytoestrogens content of soy and because meat seems to be associated with low-grade inflammation [4]. Our results showed that a moderate-high-protein reduced-fat diet has the ability to improve several inflammatory markers and that partly replacing meat with soy can improve the overall inflammatory state, with no clear benefits for individual inflammation markers (**chapter 4**). We concluded that it seems that the effects of protein and soy protein intake on the inflammatory state are small.

Arginine-rich protein intake and postprandial cardio-metabolic risk factors

Postprandial dysmetabolism, i.e. hyperglycemia, hyperinsulinemia, dyslipidemia and inflammation, is suggested to be an important cardio-metabolic risk factor [5-7]. Supplementation of arginine is known to improve postprandial metabolism by increasing insulin secretion and decreasing lipidemia and glycemia, especially when metabolism is challenged, for example in diabetic patients, the metabolic syndrome or after a high-fat meal. However, whether arginine-rich proteins are equally effective is unknown. To evaluate whether arginine-rich protein is capable to improve postprandial cardio-metabolic risk factors, a high-fat liquid meal (HFLmeal) challenge was performed in a population of men with abdominal overweight (**chapter 5**). We concluded that addition of protein to the HFLmeal only resulted in increased insulin secretion, with no effect on other metabolic and inflammatory parameters or vascular function, with no benefits of arginine-rich pea protein over protein low in arginine. No additional benefits of protein hydrolysates over intact proteins were seen. In our population of men with abdominal overweight the postprandial increase in triglycerides by the HFLmeal alone did not provoke a clear inflammatory response. Due to the lack of an inflammatory response, we could not properly test anti-inflammatory effects of arginine-rich protein.

METHODOLOGICAL CONSIDERATIONS

In this thesis we were able to study long-term, short-term and post-prandial effects of dietary protein. To do this we used various study designs. Based on observational data we described long-term associations of dietary protein intake and T2D incidence from a large European cohort (**chapter 2**), and we performed two intervention trials: 1) with the aim to describe short-term effects of a 4-week moderately high-protein diet on cardio-metabolic risk factors (**chapter 3 and 4**), and 2) with the aim to study postprandial effects of arginine-rich protein (**chapter 5**). Because of the different designs our outcome measures were also different.

Outcome measures and design

The observational data allowed us to study T2D incidence, the intervention trials gave us the opportunity to look in more detail at a broad range of cardio-metabolic risk factors, including glucose metabolism in a strictly controlled setting. Although our 4-week intervention trial indicated that increasing protein intake (22En%), while reducing fat, had beneficial effects on fasting glucose, insulin, HOMA-IR (**Chapter 3**), and the postprandial study revealed that insulin secretion indeed increased by high-protein intake (**Chapter 5**), our observational data from the European cohort indicated that long-term high-protein intake, particularly from animal sources, was associated with an increased T2D incidence

(**Chapter 2**). Our 4-week intervention trial indicated that partly replacing meat with soy in a moderate-high-protein diet has clear advantages regarding insulin sensitivity and total and LDL cholesterol and could thus partly be a successful strategy to treat and prevent MetS. Unfortunately we could not assess the association between soy intake and T2D incidence, because intake of soy in Europe is very low.

Different findings between short-term and long-term high-protein intake may be partly explained by differences in the used study designs. Both short-term intervention trials were strictly controlled randomized crossover trials, with participants with abdominal overweight (a waist circumference of >94cm for men, >80cm for women) and 1 or more additional characteristic of MetS, who were in energy balance and without dietary restrictions. All participants received each intervention and thus were their own reference. The 4-week intervention trial was strictly controlled, which means that approximately 90% of each participant's total daily energy requirement was provided to participants during the intervention and run-in periods. The remaining 10% was free for participants to choose from a provided list of limited products with protein amounts of <0.7g/portion. Compliance with the diet was ensured because most foods and drinks were provided to the participants, and hot meals were consumed under supervision of dieticians in our research facility. For the postprandial intervention trial, participants consumed a standardized meal the evening before the test days. In both trials the highly committed participants stayed at our research facility on the test day, where they followed a strictly controlled test protocol.

In contrast to these strictly controlled settings, the observational data, obtained from people in Europe without any dietary restrictions in a real-life setting, may be more prone to measurement error and uncontrolled confounding. Validity of the food frequency questionnaires (FFQs) was assessed comparing intake reported by FFQ with intake reported by 24-hour recalls, and energy adjusted dietary protein intake was verified in a subsample with urinary nitrogen (r=0.63) [8] and thus ranking according to intake was fairly good, however no conclusions about absolute intake can be drawn by FFQ's. In addition, the association between dietary protein intake and T2D may be affected by misreporting, because high-risk groups, e.g. obese, tend to underreport total energy which is associated with underreporting of nutrient intake [9]. However, we adjusted for waist and BMI, which makes it unlikely that this affected our results to a great extent. In our cohort underreporting was greater for fat and alcohol than for protein and carbohydrates [10], mainly introducing error in confounders. By adjustment for total energy intake we may have at least partly resolved measurement errors in our intake data [11]. As our study was observational in design residual confounding can still be present. For evidence on causality we need long-term clinical trials following participants long enough to have sufficient incident cases of T2D, which is not really feasible. An advantage of observational data is that

it can be generalized to the general population, which is not possible for our trial data. In addition, the population used in our intervention trials, i.e. middle aged with characteristics of MetS, differs from the general population which was considered in the observational study.

Protein intake

Effects of proteins in whole foods may differ from those of isolated proteins, protein hydrolysates and/or amino acids and consequences may be different on the short-term and long-term. In this thesis we studied protein intake from whole foods, as well as intact proteins and protein hydrolysates at an epidemiological level and in short-term intervention trials. For both intervention trials we chose an acceptable and realistic level of protein intake per meal or per day, sufficient to affect our main outcome based on prior research. For 4 weeks we provided 22En% of protein, a moderately high-protein intake. A realistic amount, as this amount of protein intake is approximately comparable to the intake of people in the 5^{th} and highest quintile of protein intake in the European cohort. A control diet, with normal protein, fat and carbohydrate intake, could additionally have been useful to be able to make firm conclusions on the effects of high-protein intake. A diet where protein was increased at the expense of carbohydrates, would give even more insight in the sole effects of increasing protein intake. In our 4-week intervention we replaced 30g meat protein per day with 30g soy protein, which is quite a high amount to incorporate in a diet, but feasible when using soy nuts in addition to soy meat analogues. We choose to investigate a low-fat moderate-high-protein diet, in which we replaced 30g meat protein per day with 30g soy protein using whole food items. We tried to keep all other nutrients in the diets comparable, because we wanted to test the sole effect of replacing meat protein with soy protein. However, we did not add isoflavones to the meat protein diet. We overcompensated total protein (+9 g/d), fat (+4 g/d), and cholesterol intake (+29 mg/d), which were slightly greater during the high-protein diet with soy (HP_{soy}) than the mixed high-protein diet (HP_{mix}). This possibly diluted our results, when in a real life setting meat is partly replaced with soy it would possibly be even more beneficial than in our trial, due to lower cholesterol and fat intake and other changes in dietary composition. In the meal challenge 30g protein was added to a HFLmeal, which would be regarded as being at the high end of normal intake in a meal.

We selected intact protein and not isolated amino acids to resemble a whole-food approach. Although we used protein sources low and rich in arginine that were highly comparable based on amino-acid content, possibly the digestion rate of the type of protein could have diluted our results, as wheat protein Nutralis W is a fast and pea protein Nutralis S85F is an intermediate fast protein. Absorption and digestion kinetics are important factors modulating the effects of proteins on metabolic processes [12]. Fast proteins have been

found to give a greater insulin response than slow proteins, which can be beneficial on the short-term, but detrimental on the long term. However, addition of protein to the HFLmeal did not affect the postprandial response, so differences in absorption are not expected to lead to different conclusions.

Measurements of outcome measures

Extended verification of T2D in our cohort made it very likely that all prevalent cases of T2D were identified at baseline. However, no test to identify T2D cases was taken, i.e. oral glucose tolerance test (OGTT), so there is a possibility for undiagnosed T2D at baseline. However, it is not likely that this could have affected our results as it is unlikely that people, having undiagnosed T2D, would make adjustments in dietary intake.

In our trials we tried to reduce measurement errors in risk factors associated with T2D, i.e. fasting glucose and insulin and additional measures of glucose disposal by a frequently sampled intravenous glucose tolerance test (FSIGT). We standardized all test circumstances as far as possible, mainly because of known within-person variation in glucose and insulin concentrations and only little effects may be expected in short-term nutritional interventions. We used a strict protocol and asked participants to standardize activities and personal circumstances. Besides trying to test participants at the same time each test day, thereby excluding effects of circadian rhythm, and standardizing the meal consumed on the evening before measurements, all participants assured us that they kept lifestyle factors in both periods comparable.

To minimize time and carry over effects we planned minimal 4 weeks between both diet periods and at least 6 days between the testing days of our postprandial tests. We did not register physical activity with questionnaires or devices, but because of randomization, changes in activity could only be an issue if participants were more active before a specific test condition, which is not likely to be the case.

Equol-producer status

Dietary intervention studies examining the health effects of soy have raised suggestions that equol-producer status may influence the health effects of soy consumption [13]. The daidzein-metabolite equol has been found to enhance adipocyte differentiation through nuclear peroxisome proliferator activated receptor-gamma (PPARγ) activation and thereby insulin-stimulated glucose uptake in adipocytes by increasing Glucose transporter type 4 (GLUT4) and Insulin receptor substrate 1 (IRS-1) expression [14]. Inability to stratify participants by equol-status in previous clinical studies can possibly partly explain mixed findings on health benefits of soy consumption. In people who are good equol-producers clinical responses to soy protein diets are observed to be optimal, which may explain differences in findings between studies, as in western countries only 25% to 30% of the adult population has the capacity to produce equol vs. 50% to 60% of adults from China, Japan,

and Korea [15]. Recently a trial found that soy nut consumption (25g soy protein) reduced diastolic BP, TGs, CRP and Soluble intercellular adhesion molecule (sICAM) in equol-producing women with MetS (n=4) but not in equol-nonproducers (n=7) [16]. In women without MetS, reductions in diastolic BP and CRP were also only significant in equol-producers (n=28) and not in equol-nonproducers (n=21). In our crossover trial on partly replacing meat with soy protein (**Chapter 3**) we previously did not stratify our analyses by equol-status because only 4 women were equol-producers**.** For this general discussion we additionally did investigate this issue, as Acharjee et al. [16] also published results in such a small subsample. Stratified analyses of our data did not support the findings of Acharjee, as we did not see differences in metabolic markers and vascular measures by equol-producing status. We could not investigate FSIGT and inflammatory markers, because data was only available for 2 equol-producers. However, based on our other results, we find no reason to assume that the responses were different for equol-producers, and we still doubt that n=4 provides enough data to draw firm conclusions on possible differences by equol-status.

DIETARY PROTEIN INTAKE AND THE METABOLIC SYNDROME

The balance between muscle protein breakdown and synthesis, of which the last is directly stimulated by protein intake, preserves skeletal muscle mass which is important for metabolic health. High-protein diets preserve lean body mass during weight loss, with associated health benefits, and have been found to help maintain weight after weight loss. However in energy balance, mid- and long-term metabolic effects of moderately-high amounts of dietary protein intake are unsure, as well as specific health effects of various types of dietary protein.

Total protein intake and cardio-metabolic risk factors and type 2 diabetes incidence

Long-term associations of total protein intake and type 2 diabetes incidence and cardiovascular outcomes

High total protein intake seems to be associated with increased T2D incidence in healthy adults, as concluded in a meta-analysis from recent literature [1]. Indeed we found a positive association of high total protein intake with T2D incidence in our large cohort of European adults. It is uncertain if there is also a relation between total protein intake and CVD [1]. A positive association of high total protein intake and all-cause mortality was found in a Mediterranean population at high cardiovascular risk, both when protein was replaced by carbohydrates and fat [17]. However, a meta analyses of 15 randomized controlled trials (RCTs) showed no effect on both glycemic control and outcome markers of cardiovascular disease by low-fat high-protein diets for more than 12 months compared to diets low in protein in healthy and insulin-resistant participants [2]. Most of these RCTs started with a

short-term energy restriction after which the greatest part of follow up was controlled for energy balance or free living. Based on these results it was concluded that it seems too early to recommend high-protein diets in management of overweight and obesity, and that potential risks cannot be ruled out [2]. This is in line with research that concluded that dietary proteins have little beneficial effects for prevention of CVD, at least as the lipid content of protein sources is taken into account [18].

Short-term effects of total protein intake on glucose metabolism

Long-term associations of high-protein diets and T2D incidence are not in line with reported beneficial effects on the short-term [19-21]. Indeed, in contrast to higher T2D incidence with increasing protein intake, we found that increasing protein intake (22En%) at the exchange of fat for 4 weeks had beneficial effects on fasting glucose, insulin and HOMA-IR. However, caution should be taken interpreting these results, because we did not have a normal-protein control diet. Partly replacing carbohydrates with protein (25En%) for 6 weeks was not found to improve insulin sensitivity as measured by QUICKI [22]. Also, increasing protein intake (20&30 En%) by reducing fat for 4 weeks did not affect insulin sensitivity as measured by FSIGT [23]. Another trial even found that increased protein intake (28En%) for 6 weeks reduced insulin sensitivity as measured by euglycemic-hyperinsulinemic clamps [24]. Thus, with regard to glucose metabolism it seems that there is an upper-limit of protein intake, because a high-protein (30En%) vs moderate-high-protein (20En%) diet resulted in increased fasting glucose, at least when SFA intake is low (7En%) [23].

Both short-term and long-term effects may be related to the fact that high-protein intake increases postprandial insulin secretion, which is in line with known insulinotropic properties of amino acids [25]. We found that adding wheat and pea protein to a high-fat meal challenge increased postprandial insulin, without affecting the glucose response. Possibly the increase in insulin helps controlling blood glucose on the short-term, although it may be negative on the long-term. Based on our postprandial and 4-wk results in context of other publications on glycemic control [26, 27], moderate-high-protein but not too high-protein intake, e.g. 20-25En%, seems beneficial for people with characteristics of MetS and T2D patients, at least on the short-term. However, evidence on high-protein intake in energy balance is limited, since many trials investigate protein intake in weight loss diets.

Short-term effects of total protein intake on cardio-metabolic risk factors

It can further be suggested that increasing protein intake improves cardio-metabolic risk factors. Dietary protein intake is associated with the blood lipid profile [28], reduces IHL [29, 30], and is possibly related to inflammation via changes in microbiota [31]. We indeed found that increasing protein intake (22En%) at the expense of fat for 4 weeks had beneficial effects on the blood lipid profile (fasting TG, total, LDL, HDL cholesterol), IL-6, TNF-α, sICAM-1, sVCAM-1 and the total z-score for inflammation, total body and abdominal fat

percentage and that it tended to reduce CRP and IHL. Even a hypercaloric diet with high-protein intake (23-26En%) tends to lower TG and IHL [29, 30]. Partial substitution of carbohydrate with protein has also been found to improve lipid levels [32]. In contrast increasing protein intake (20&30 En%) by reducing fat for 4 weeks did not affect plasma lipids and lipoproteins[23]. Proteins may further have BP lowering capacity, and high-protein intake, compared with carbohydrate, was associated with lower BP in a meta-analysis [33]. Indeed substitution of carbohydrate with protein was shown to lower BP and reduce estimated CVD risk [32], but in our trial we did not find improvements in BP. Our participants even had higher augmentation index (Aix), which is unfavorable, with lower heart rate and ejection duration after 4 weeks moderate-high-protein diet. In overweight children, protein intake has also been found to be associated with increased arterial stiffness [34]. So, although high total protein intake and CVD on the long-term seem unrelated, on the short-term protein intake seems to improve the blood lipid profile, IHL, BP and may affect other cardiovascular risk factors.

Various protein types and cardio-metabolic risk factors and type 2 diabetes incidence

Long-term associations of various protein types and type 2 diabetes incidence and cardiovascular outcomes

We found that protein of animal origin is largely responsible for positive association of high total protein intake with T2D incidence in our large cohort of European adults. This is in line with other epidemiological findings [1]. Most studies report a positive association, while animal protein was inversely associated with T2D in Japanese women [35]. Different findings may occur because not all animal protein may be T2D risk increasing, since protein from dairy [36, 37] and fish may reduce T2D risk [38]. The association found in our cohort could not be explained by one or more specific types of animal protein, though. A higher animal protein intake is also found to be associated with an increased risk of fatal and non-fatal cardiovascular outcomes [17].

No association between plant protein and T2D incidence was found in our study, which is in line with other epidemiological research [35, 39]. Although total plant protein intake seems unrelated to T2D, some proteins of plant origin have been suggested to be inversely associated with T2D, i.e. soy. However, protein from various plant sources seemed unrelated to T2D incidence in our cohort, while soy protein intake was too low to investigate. As of yet, current research on the long-term association between soy and T2D is inconclusive. In Chinese men soy protein is found to be positively associated with hyperglycemia, but not in women [40]. In Chinese women consumption of soybeans was inversely associated with T2D incidence, but the association between soy products (other than soy milk) and soy protein intake with T2D was not significant [41]. Based on current evidence plant protein intake seems inversely associated with CVD mortality in healthy

adults [1] and lower BP [42, 43]. There is no clear evidence of a possible anti-atherosclerotic effect of diets containing specific plant proteins in humans. So, high animal protein intake might increase T2D incidence and risk of fatal and non-fatal cardiovascular outcomes, while plant protein might be beneficial for CVD risk, without affecting T2D risk.

Short-term effects of various protein types on glucose metabolism

In contrast to the association of animal protein with increased T2D incidence, beneficial effects of protein from various sources on insulin secretion and fasting glucose are seen in many short-term trials. Results of some trials suggest that daily ingestion of at least one high-protein meal increases insulin secretion and glucose uptake, thus improves insulin sensitivity, which seems particularly the case for animal protein, more specific whey protein [44]. Whey protein [45, 46] but also protein from fish [47, 48] may indeed be superior over other animal protein types. Specific proteins and/or amino acids might reduce second meal postprandial glycaemia when taken directly or 30min before a meal, e.g. whey and soy protein, possibly mediated by an early phase insulinemic response [49, 50]. Evidence on effects of plant protein types, other than soy, on glucose metabolism is scarce. We found that partly replacing meat with soy protein improved the capacity of insulin to promote glucose disposal, i.e. insulin sensitivity and disposition index, as measured by a FSIGT, although it did not alter fasting glucose and insulin. Replacing one serving of red meat by soy protein in a DASH diet for 8 weeks did improve fasting insulin and HOMA-IR [51]. So, possibly more time is needed to improve fasting values. However, a 6 month RCT did not find favorable effects of 15g soy protein supplements per day on glycemic control and insulin sensitivity (fasting and post-load) [52], neither did a 20g soy protein supplement for 3 months [53]. But it should be noted that, milk/casein protein was used as control in these both trials, which may have masked an otherwise more clear effect of soy, because also milk protein is found to be beneficial. However, casein has a higher insulin-to-glucagon ratio compared to soy and is a slow protein while soy is a fast protein [48, 54].

Short-term effects of various protein types on cardio-metabolic risk factors

Soy protein is known to reduce total and LDL cholesterol [55, 56], with an U.S. Food and Drug Administration label on foods containing soy protein to be cardiovascular protective, based on lower total and LDL cholesterol using 25g soy protein per day. However, a RCT (n=180) supplementing soy protein (15g) and isoflavones (15mg) or isoflavones alone showed no beneficial effects on cardiovascular risk factors (HDL, LDL, total cholesterol, TG, and CRP) at 3 and 6 months in postmenopausal Chinese women with early hyperglycaemia.[57]. In a 4 year RCT, partly replacing meat protein (70% meat protein of total protein) with soy protein (35%) did improve total, LDL cholesterol and TG in T2D patients with nephropathy [58]. In our trial partly replacing meat protein with soy protein reduced LDL cholesterol in overweight postmenopausal women. Non-soy legume

consumption has also been found to lower circulating total and LDL cholesterol in a meta-analysis [59]. Not much research has been performed on other plant protein types than soy protein. We found no reduction in postprandial lipemia by pea and wheat protein. In contrast to long-term associations of animal protein with CVD risk, on the short-term it reduces postprandial lipemia after a high fat meal. Whey protein reduces postprandial lipemia to a greater extent than casein [60], cod and gluten protein [46]. Also, protein from fish seems able to lower cholesterol and improve the blood lipid profile [61, 62].
Inflammation seems unrelated to specific types of protein. We indeed found no differences between pea and wheat protein on postprandial inflammatory markers. However, partly replacing meat protein with soy protein reduced the total inflammatory score, while not significantly altering individual markers of inflammation. Improvements in CRP were reported in a trial after 4 year partly replacing meat protein with soy protein in T2D patients with nephropathy [58]. Soy nuts have been found to stronger reduce CRP than soy protein, and lower Tumor necrosis factor-α (TNF-α) and interleukin-18 (IL-18), while soy protein did not affect TNF-α and IL-18 [63]. Higher poly unsaturated fat content could possibly be responsible for these different results between soy nuts and soy protein. In our trial soy nuts and soy meat analogues were used as soy protein sources, so possible benefits of soy nuts may also be present in our trial.

Besides effects on lipids and inflammation, both observational and trial data show inverse associations of plant protein and BP [1, 42, 43]. Specifically soy protein seems hypotensive [64], possibly because it is rich in arginine, which also seems able to lower BP [65]. In most observational data animal protein does not seem to be associated to BP [43], but on the short-term, compared with carbohydrate, both animal and plant protein are associated with lower BP as concluded by a meta-analysis of RCTs [33]. Of animal proteins mainly dairy protein is suggested to have hypotensive effects [43]. That we did not find an effect of partly replacing meat with soy protein on BP, is in line with the fact that differences between plant and animal protein on BP are less clear in trials than in observational research, however trials comparing animal and plant protein are required [43]. Another explanation why we did not observe differences in BP might be that our participants did not all have elevated BP, because effects of soy protein are greater with higher pre-treatment BP levels. Additionally, although soy protein has been found to be potentially beneficial for vascular health [66-68], we did not observe improvements in arterial stiffness and plasma concentrations of biomarkers reflecting endothelial dysfunction. It may thus be concluded that partly replacing meat protein with plant protein could reduce CVD risk, by beneficially affecting the blood lipid profile and slightly improving low-grade inflammation. In addition, increasing dietary protein intake, irrespective of the type, by decreasing carbohydrate intake may be beneficial for BP.

Understanding the divergent findings in short- and long-term studies

It seems that moderate-high-protein intake, predominantly of plant origin, is beneficial in an overall healthy diet, in healthy people, as well as in those at risk for T2D and CVD, at least on the short-term. On the long-term, short-term benefits do not seem to persist, because a moderate-high-protein diet is associated with higher T2D incidence and elevated risk of CVD. This positive association between protein intake and T2D and CVD in observational studies is likely to be due to proteins per se, mainly by protein of animal origin, however, the possibility of unmeasured or residual confounding cannot be ruled out. For example, protein intake is positively associated with BMI, a strong risk factor for T2D and CVD. Furthermore, high-protein intake is associated with dietary patterns rich in total and saturated fat, cholesterol and low in fiber. Lastly, intervention trials, including ours, are mostly performed in individuals in whom metabolic alterations are already present, while data of observational studies are from the general population. It is not unlikely that people with a challenged metabolism, e.g. insulin resistant people, respond differently to dietary changes compared to healthy people. In our short-term trials we might have even seen more pronounced outcomes in obese, people with more characteristics of MetS, and/or more metabolically disturbed individuals. On the other hand, we should consider the hypothesis that a short-term benefit, i.e. glucose and lipid metabolism, does not necessarily translate into a long-term advantage, i.e. insulin sensitivity.

On the long-term, high-protein diets could have adverse effects on glycemic control by increased insulin demand, due to higher fasting glucose, by diminished suppression of hepatic glucose output and enhanced conversion of proteins in the liver into glucose or glycogen through gluconeogenesis [69]. It has been suggested that over-activation of the mammalian target of rapamycin/S6 kinase 1 (mTOR/S6K1) pathway and inhibitory phosphorylation of insulin receptor substrate-1 (IRS-1) underlie impairments of insulin action seen in humans when circulating amino acids are increased via intravenous infusion [70]. It could be speculated that amino acid induced stimulation of insulin secretion temporarily overrules direct – detrimental - effects of amino acid on glucose metabolism. People that are less insulin sensitive may thus have short-term benefits of a high-protein intake, as protein stimulates insulin secretion and this extra insulin response helps controlling blood glucose. On the long-term, however, it may induce hyperinsulinemia, and prolonged hyperinsulinemia may drive insulin resistance [71].

Moreover, it is likely that the amount and source of protein are relevant in the possible explanation for differences between short-term and long-term effects of protein intake. Besides differences between fast- and slow-proteins, the amino acid composition of proteins may influence cardio-metabolic risk factors. Capacity of dietary proteins to stimulate insulin and insulin signaling secretion varies, possibly by differences in early

release of incretin hormones and amino acid composition [72]. Most amino acids stimulate insulin secretion, of which arginine, lysine, phenylalanine, leucine, isoleucine and valine seem most insulinogenic [12, 25, 73, 74]. Arginine was found to increase insulin most compared to the essential amino acids when given intravenously in high amounts (30g) [25, 75], however orally ingested arginine, in an amount likely to be in a high-protein meal (~10g), did not stimulate insulin secretion while it did reduce the increase in glucose [76]. In our trial pea protein (arginine-rich, intermediate fast protein), was not found to differently affect postprandial insulin and glucose compared to wheat protein (low in arginine, fast protein). In a trial where the postprandial rise in insulin did not improve the glucose response it was concluded that fast- proteins, such as whey and soy, reduce insulin action to a greater extent than slow-proteins, i.e. casein, and therefore may not be recommended for glycemic control in T2D patients [77].

Additional to the potential adverse effects by increased insulin secretion, a potential deleterious effect of specific amino acids on insulin signaling is suggested, particularly for branched-chain amino acids (BCAA), i.e. leucine, isoleucine and valine. Positive associations between elevated blood BCAA concentrations and T2D incidence [34,35] and altered insulin signaling [36,37] have been reported. BCAAs, and specifically leucine, may induce insulin resistance via the mTORC1 and S6k1 associated pathways [78]. However, the association between diet, circulating BCAAs, and insulin resistance might not be mediated by high-protein or BCAA intake, and is suggested to be linked to a defect of protein or BCAA metabolism in insulin resistant individuals. Indeed, it is not clear whether plasma BCAA concentrations accurately reflect long-term dietary protein intake [20]. Furthermore, glucose and insulin responses by individual amino acids do not seem to fully explain different glucose and insulin concentrations by various protein types [12]. Possibly also the amount of protein is important, because it has been recently found that high-protein intake (30En%) for 4-weeks did increase plasma BCAAs while moderate-high-protein intake (20En%) did not [23]. In this trial new evidence for an association of BCAAs with higher fasting glucose, insulin and HOMA-IR was found. Additionally, increased BCAA by higher protein intake was associated with a higher acute insulin response to glucose (AIRg) and lower metabolic clearance rate of insulin (MCRi) but was not associated with fasting glucose and insulin.

Finally, associations between dietary protein intake and T2D and CVD risk could also be related to nutrients associated with protein intake, e.g. total and saturated fat, cholesterol and fiber. Differences between plant and animal protein might be explained by carbohydrates, fiber and polyunsaturated fat which are associated with higher plant protein intake and total fat and cholesterol that are associated with higher animal protein intake. However, differences may also be explained by specific sources of protein, such as dairy,

fish, legumes, whole grains and soy or wheat meat analogues, based on their high quality amino acid profile. In both our long-term and short-term studies it is difficult to determine if the increase in protein intake as such is responsible for the associated health effects. In our cohort we used a substitution model exchanging carbohydrates for proteins, however exchanging total fat for proteins gave similar results. In our 4 week intervention trial the fat content of the diet was reduced to increase protein intake, so it remains to be determined if increasing protein intake per se results in all beneficial effects we found, and if replacing carbohydrates with protein intake would be equally effective.

Potential mechanism of improved insulin sensitivity by soy protein

Beneficial effects of soy protein, and non-protein compounds in soy foods such as isoflavones, on insulin action and glycemic control may be mediated by changes in lipotoxicity (**Figure 1**). Long-term soy protein intake results in a relatively low insulin-to-glucagon ratio, which leads to a hypolipidemic effect via sterol regulatory element-binding protein-1 (SREBP-1) [79]. Soy protein and isoflavones can reduce hepatic accumulation of triglycerides and cholesterol by regulating important transcription factors involved in lipid metabolism, i.e. SREBP-1 and PPARα [79, 80]. Furthermore soy protein associated isoflavones can activate PPARγ, a crucial factor controlling adipocyte differentiation [81]. In animal studies effects of soy protein on PPAR, LXR and SREBP signaling have been related to reduced hepatic lipotoxicity, inflammation, adipocyte hypertrophy and attenuation of MetS [82-87]. We could not investigate underlying mechanisms in our trial, but we did find a non-significant reduction in IHL and lower total inflammation score by partly replacing meat protein with soy protein. Effects of soy protein on inflammatory markers may be small as most trials found no association [3]. We found a non-significant reduction of CRP in women without low-grade inflammation, maybe participants' health status influences anti-inflammatory capacity of soy protein.

The amino acid arginine, which is found in high absolute amounts in soy protein, is an interesting candidate by which soy protein improves lipotoxicity. A direct role of arginine in insulin sensitivity is not very clear, [88, 89]. Arginine has the potential to lower the postprandial insulin/glucagon ratio, which is associated with reduced lipotoxicity. Further, enhanced expression of key genes responsible for glucose and fatty acid oxidation has been found after L-arginine supplementation [90]. Effects of arginine are most likely mediated via the Nitric Oxide (NO) pathway. Besides effects of NO on vasodilatation, which can improve glucose homeostasis, NO can have a variety of metabolic effects which reduce lipotoxicity, glucose homeostasis and inflammation. Its role in lipid metabolism may involve changes in lipogenesis, lipolysis and hypocholesterolemic effects. Glucose metabolism is suggested to be affected by inhibition of hepatic gluconeogenesis and glycogen synthesis, and stimulation of glucose transport and glucose oxidation [90].

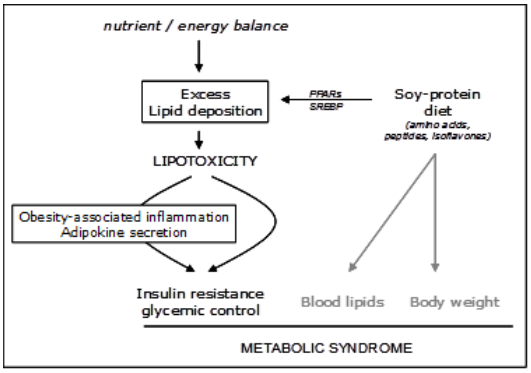

Figure 1. Proposed mechanism of action of a soy protein diet on insulin resistance, glycemic control and MetS. An increased nutrient or energy balance will increase lipid deposition in adipose and non-adipose tissue. When accumulation exceeds innate storage capacity so-called 'lipotoxicity' will develop. Lipotoxicity can drive the obesity-associated low-grade inflammatory state, alter adipokine secretion and affect insulin action and glycemic control. Soy protein is suggested to reduce lipotoxicity, probably via an effect on SREBP-1 and PPARα and γ, improving insulin and glucose metabolism. Besides, soy protein reduces blood lipids and via thermogenic and satiating mechanisms controls body weight; all together explaining the beneficial effect of soy protein on MetS.

IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Potential risk of long-term high-protein intake

It seems too early to generally recommend high-protein diets, because high total protein intake seems to be associated with increased T2D incidence in healthy adults, as presented in this thesis, and possibly also with CVD. Conversely, a moderate-high-protein intake does seem beneficial for people with characteristics of MetS and T2D patients, at least on the short-term. Evidence to support a moderate-high-protein intake, when being in energy balance, is limited, since many trials investigate protein intake in weight loss diets. Results from mainly prospective cohort studies suggest a safe intake of up to at least 1.2-1.5 g protein/kg BW/day or approximately 15-20En% for healthy elderly populations. Potential adverse effects of a protein intake exceeding 20-23En% remain to be investigated [1], it seems that a much higher intake (30En%) can increase fasting glucose [23].

To get more insight in the exact role of high-protein intake in the development of T2D, future studies on the effect of various protein types and specific amino acids on glucose metabolism are warranted. Focus should be on insulinogenic properties of various types of protein and on the role of absorption kinetics of slow and fast proteins [12]. Also, BCAAs in relation to T2D risk factors should be further explored, because up to know it is unclear how plasma circulating BCAAs relate to BCAA intake and insulin resistance. Preferably, long-term intervention trials will be executed, in which both healthy and metabolically challenged participants consume a moderate-high-protein diet, for a year or longer. In such a trial, ideally intervention arms should differentiate plant and animal protein, more specifically

meat, whey and cod protein and soy, non-soy legume, and wheat protein. Data should then be collected on circulating BCAAs, dynamic measures of insulin sensitivity, and if possible T2D incidence, to help understand different associations between protein types.

Differences between plant and animal protein

Differences between animal and plant protein both on the short- and long-term have not been intensively studied. Protein intake of animal origin is associated with increased T2D incidence and risk of fatal and non-fatal cardiovascular outcomes, although cod and whey protein could be protective. On the short-tem, postprandial glucose homeostasis and lipemia can be improved by animal protein, particularly whey protein, by increasing insulin secretion and glucose uptake after a high-fat meal. In contrast to animal protein, plant protein is inversely associated with CVD mortality in healthy adults, while it is unrelated to T2D incidence. On the short-term soy protein is known to reduce total and LDL cholesterol and it seems to be hypotensive, further it improved the capacity of insulin to promote glucose disposal in our trial. Not much is known about associations of plant protein types other than soy.

Most short-term trials comparing various protein types studied effects of dairy proteins (casein, whey), cod, wheat and soy protein. To increase understanding of different effects by plant and animal protein, it would be interesting to compare various plant and animal protein types within one trial, e.g. comparing effects of protein content of a meat based diet, a dairy and fish based diet and a plant based diet. Including the impact on gastric emptying, and possible changes in the gut microbiome by proteins, that possibly impact glucose homeostasis, would also be of interest. Testing differences between amino acids, isolated protein, and whole foods would provide more information on how absorption rates and the food matrix influences potential health effects.

Based on our results and in the context to previous research it is now too early to recommend to partly replace meat with soy foods, but it seems promising. For public health relevance, a whole food approach would be more realistic than exchanging proteins, so it would be relevant to prepare an additional trial where meat is partly replaced with soy products, without compensating for nutrients other than protein, e.g. the lesser fat and cholesterol content of the diet. This would represent the advantages of partly replacing meat with soy in a real life setting, which many health conscious and environmental conscious people nowadays to some extent do. To get more knowledge about plant protein as such, an extra diet group where meat is partly replaced with non-soy legumes, and/or nuts, or other plant proteins would be informative. If health benefits are indeed pronounced, that could be an encouragement for people to reduce meat intake. It would be recommended to include enough participants for stratified analyses, to be able to make conclusions on possible differences between men and women, and possible differential effects of isoflavones by equol-producer status.

Timing of protein intake

Although we did not investigate this topic, a new issue in dietary protein research is timing of protein over the day. In recent literature a concept of a meal-based protein threshold is suggested, as it is assumed that equally dividing protein intake over meals would be optimal for protein synthesis. Generally people ingest the greatest part of their daily protein intake at diner while protein intake at breakfast is low. New research highlights the importance of protein intake in grams per meal, besides the current guidelines of protein per kg bodyweight, or as a percentage of total energy, further the importance of essential amino acid intake should be taken into account when suggesting guidelines for optimal health outcomes [91]. A recent trial found that daily addition of a high-protein breakfast, containing 35g of high quality protein, has better efficacy at improving free-living glycemic control compared to a normal-protein breakfast in healthy overweight/obese 'breakfast skipping' adolescents [92]. Investigating postprandial effects of varying protein contents per meal and longer term effects on both glucose and lipid metabolism would be valuable to get more insight if guidelines would improve by advising protein intake in grams per meal instead of grams per day.

FINAL CONCLUSIONS

In view of the rapidly increasing prevalence of MetS and T2D, limiting iso-energetic diets high in dietary proteins, particularly from animal sources, should be considered, as on the long-term they seem to increase T2D and CVD risk. A moderately increased dietary protein intake, predominantly from plant sources, could conversely be a good choice, at least on short-term and mainly in people with impaired metabolism, i.e. insulin resistance and lipid abnormalities. It could be speculated that a high-protein intake is only beneficial for those that are metabolically challenged and only for a short period. However, our research cannot fill the knowledge gap between what happens on the short-term and long-term.

Partly replacing meat with soy could be preventive for MetS by improvements in insulin sensitivity and total and LDL cholesterol. Based on prior research we argued that either soy protein as such or a synergistic effect between protein, isoflavones and amino acids may be accountable for these effects. Arginine, which is found in high absolute amounts in soy protein, is an interesting candidate. Unfortunately, our postprandial tests with arginine-rich protein did not provide new evidence on this topic.

Overall, this research gives important new insights in the continuing discussion about effects of macronutrient composition of diets, more specifically dietary protein intake, on glycemic control and several cardio-metabolic risk factors. Nonetheless, for generalization of these results longer-term intervention trials are needed in which both men and women are included, in a real life setting. We then expect results of partly replacing meat with soy to be even stronger, because of lower fat and cholesterol intake.

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Samenvatting

SAMENVATTING

Het metabool syndroom (MetS) is een cluster van klinische factoren die geassocieerd zijn met een verhoogd risico op diabetes type 2 en hart- en vaatziekten. Mensen hebben MetS als ze een grote buikomvang hebben (>94cm voor mannen; >80cm voor vrouwen) en daarnaast voldoen aan tenminste 2 van de volgende criteria: verhoogde nuchtere bloedglucose waarden, een laag HDL cholesterol gehalte in het bloed en/of een verhoogde bloeddruk of medische behandeling krijgen voor de genoemde condities. Volgens schattingen voldoen ongeveer 1 miljoen Nederlandse volwassenen volgens deze criteria aan MetS. Omdat MetS geassocieerd is met een hoog risico op diabetes type 2 en hart- en vaatziekten, en dus op de daarmee gepaard gaande morbiditeit en mortaliteit, is dit een grote kostenpost voor het zorgstelsel. Kosten die in de toekomst verder zullen toenemen, omdat het aantal mensen met overgewicht en MetS stijgt.

Diabetes is een conditie waarbij het lichaam niet in staat is om de gevaste glucose waarde binnen de normale range te houden (4 tot 7 mmol/L), of niet in staat is om 2 uur na een maaltijd de glucose waarde terug te brengen tot 11 mmol/L. Bij mensen met diabetes type 2 is het lichaam over het algemeen ongevoelig geworden voor insuline, het hormoon dat de bloedsuikerspiegel regelt. Mensen met overgewicht en MetS hebben een verhoogd risico op deze insuline ongevoeligheid. Daarnaast hebben zij een 2-3 keer verhoogd risico te overlijden aan hart- en vaatziekten.

Om te voorkomen dat mensen MetS ontwikkelen is het belangrijk om te weten welke risicofactoren geassocieerd zijn met de criteria. Naast andere factoren is het bekend dat voeding een belangrijke rol kan spelen in het ontstaan, de behandeling en preventie van MetS en mogelijk ook een hoog-eiwit dieet. Hoog-eiwit diëten zijn bijvoorbeeld erg populair voor gewichtsverlies en gewichtsbehoud nadat men is afgevallen. Een bijkomend voordeel van hoog-eiwit diëten kan zijn dat het mogelijk de regulatie van bloedglucose verbetert, doordat bekend is dat eiwitten zorgen voor hogere insuline productie. Verder zijn er ook aanwijzingen dat het kan zorgen voor verbeterde vetwaarden in het bloed, minder vetopslag in de lever en mogelijk ook verminderde ontstekingswaarden in het bloed en een lagere bloeddruk. Naast de totale hoeveelheid eiwit in de voeding zijn ook de eiwitbronnen mogelijk relevant. Zo is dierlijk eiwit geassocieerd met een verhoogd diabetes en hart- en vaatziekten risico, waarbij zuivel en vis mogelijk een uitzondering zijn met een verlaagd risico op diabetes type 2. Plantaardig eiwit, waaronder ook eiwit van soja zou gunstig kunnen zijn voor de bloeddruk en het risico op hart- en vaatziekten. Er zijn aanwijzingen dat arginine, een aminozuur dat in hoge hoeveelheden in soja eiwit voorkomt, een rol zou kunnen spelen in deze gunstige effecten.

Er is nog veel onduidelijk over wat nu precies de effecten zijn van een grote hoeveelheid eiwit in de voeding op korte en lange termijn. Vooral wanneer men niet het doel heeft om af te vallen, dus in energiebalans. Korte termijn onderzoeken vinden vaak gunstige effecten van eiwit, terwijl hoge eiwit inname in lange termijn onderzoeken juist vaak in verband wordt gebracht met een hoger diabetes en hart- en vaatziekten risico. Dit maakt het extra moeilijk om algemene aanbevelingen over eiwit inname te doen. Daarom had dit proefschrift als doel om meer duidelijkheid te krijgen over de mogelijke impact van een hoger aandeel eiwit in de voeding op het ontstaan van diabetes type 2, aspecten van MetS en andere cardio-metabole risico factoren.

Hiervoor werden 3 onderzoeksvragen geformuleerd:

- Is hoge eiwit inname op de lange termijn geassocieerd met het vaker voorkomen van diabetes, terwijl het op de korte termijn juist gunstige effecten heeft op aspecten van MetS, waaronder insuline gevoeligheid?
- Is voor de aspecten van MetS inname van eiwit van soja gunstiger dan eiwit van vlees?
- Heeft arginine, als een typisch aminozuur uit soja eiwit, een mogelijke rol in deze effecten door verbeteringen in de metabole respons direct na een maaltijd?

Om deze vragen te beantwoorden zijn gegevens uit een lange termijn onderzoek gebruikt en zijn 2 korte termijn interventies uitgevoerd.
In **hoofdstuk 2** staat beschreven hoe hoge eiwit inname op de lange termijn samenhangt met het risico op diabetes type 2. Hiervoor zijn de gegevens gebruikt uit een prospectief cohort, waar gedurende 12 jaar mensen uit 10 landen zijn gevolgd zijn. Van deze mensen zijn vele gegevens verzameld bij aanvang van het onderzoek, waaronder ook hoe hun voedingspatroon eruit ziet. In de 12 jaar dat zij zijn gevolgd is vervolgens geregistreerd wie diabetes heeft gekregen. Zo kon worden onderzocht of mensen met een hoge eiwit inname vaker diabetes ontwikkelden. Dit bleek inderdaad het geval en dit verband leek hoofdzakelijk gedreven te worden door eiwit van dierlijke bronnen. Er was geen associatie met plantaardig eiwit. Ondanks dat hoge eiwit inname geassocieerd is met het vaker voorkomen van diabetes in deze Europese populatie, is het effect klein en bekende risico factoren voor diabetes, zoals body mass index (BMI), zijn ook zeer belangrijk.

Hoofdstuk 3 had tot doel de eerste en tweede vraag te beantwoorden. Het beschrijft een gerandomiseerd onderzoek waarbij 15 vrouwen (gemiddeld 61 jaar oud met kenmerken van MetS) 2 periodes van 4 weken een volledig gecontroleerde voeding hebben gebruikt. Dit wil zeggen dat de vrouwen al hun eten en drinken van ons kregen en zelfs iedere middag de warme maaltijd op de universiteit kwamen eten, waarna zij o.a. brood en snacks voor de avond en volgende ochtend meekregen.

Zo kregen zij 2 verschillende voedingspatronen 1) een voeding met verhoogde eiwit inname uit diverse bronnen (voornamelijk vlees, zuivel en brood) en 2) dezelfde voeding met het verschil dat eiwit uit vlees gedeeltelijk werd vervangen voor eiwit uit soja vleesvervangers en soja noten (30 gram/dag). Het was daarbij belangrijk dat ze geen gewicht zouden verliezen, dus werden ze iedere week gewogen en werd indien nodig de hoeveelheid energie bijgesteld. Na beide periodes werden diverse metingen verricht. De resultaten lieten zien dat de vrouwen insulinegevoeliger waren na de 4 weken waarin zij soja eiwit aten vergeleken met dezelfde periode voeding waarin zij meer eiwit aten uit vlees. Ook was het totale en LDL cholesterol gehalte in het bloed lager na de periode met soja eiwit. Verder leek dit onderzoek aanwijzingen te geven dat het verhogen van eiwit inname gunstig zou kunnen zijn voor glucose en insuline waarden, vetwaarden en cholesterol in het bloed, lichaamsvetpercentage en mogelijk ook voor vetopslag in de lever.

Hoofdstuk 4 beschrijft aanvullende resultaten van het in hoofdstuk 3 genoemde onderzoek op ontstekingsreacties in het bloed. De resultaten lieten zien dat de totaalscore voor inflammatie, een maat voor de totale hoeveelheid ontstekingswaarden in het bloed, lager was na de 4 weken waarin zij soja eiwit aten vergeleken met dezelfde periode voeding waarin zij meer eiwit aten uit vlees. Wanneer gekeken werd naar individuele markers voor inflammatie werd geen verschil gevonden tussen de beide voedingspatronen.

Effecten van arginine-rijk eiwit op de reactie van het lichaam direct na een maaltijd zijn beschreven in **hoofdstuk 5**. Hiervoor werd een interventie uitgevoerd met 18 mannen van 57-70 jaar oud met kenmerken van MetS. Deze mannen werden 5 keer uitgenodigd op de universiteit om een vetrijke maaltijd met toegevoegd eiwit te consumeren. Nadat ze deze vloeibare maaltijd in de vorm van een milkshake op hadden bleven ze nog 6 uur in de onderzoeksruimte waar ieder uur diverse metingen werden gedaan. Zo kregen zij 5 verschillende milkshakes zonder (controle) of met 30 gram toegevoegd eiwit: erwt eiwit (arginine-rijk), gluten eiwit (met van nature weinig arginine) en erwt en gluten eiwit dat gehydrolyseerd (door enzymen al een beetje voor-verteerd) was. Uit de resultaten bleek dat het toevoegen van intact erwt en gluten eiwit aan de controle milkshake zorgde voor hogere insuline waarden in het bloed in de uren na de maaltijd. De gehydrolyseerde eiwitten zorgde niet voor een snellere of verdere stijging van deze waarden. Na alle 5 de milkshakes werden stijgingen in triglyceriden gezien en dalingen in bloedglucose, bloeddruk en vaatstijfheid, dus er was geen effect van het toevoegen van eiwit. Ook vonden we geen verschillen in ontstekingsreacties in het bloed. Er waren geen voordelen van een eiwit rijk in arginine op de metabole reactie direct na een maaltijd.

Tot besluit worden in **hoofdstuk 6** de onderzoeken die zijn beschreven in de hoofdstukken 2 tot en met 6 bediscussieerd. Op basis van deze discussie hebben we geconcludeerd dat vanwege de snelle toename in het voorkomen van diabetes type 2 en MetS, het overwogen zou moeten worden om voedingspatronen in energiebalans met hoge eiwit inname, voornamelijk uit dierlijke bronnen, te beperken. Omdat dit op lange termijn het risico lijkt te verhogen op diabetes en hart- en vaatziekten. In contrast kan een voeding met licht verhoogde eiwit inname, hoofdzakelijk uit plantaardige bronnen, een goede keuze zijn, tenminste op korte termijn en vooral voor mensen met verminderde insulinegevoeligheid en problemen met de circulatie van vetten in het bloed.

Verder zou het gedeeltelijk vervangen van vlees met soja in een eiwitrijk voedingspatroon kunnen helpen in de preventie van MetS, door verbeteringen in insuline gevoeligheid en totaal en LDL cholesterol. Wij hebben geen nieuwe aanwijzingen gevonden dat het aminozuur arginine, dat in hoge hoeveelheden in soja eiwit voorkomt, een rol speelt in deze effecten.

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If you feel that I forgot to thank you personally:
My apologies and THANK YOU!!

About the author

RESUME (CURRICULUM VITAE)

Monique van Nielen was born in Eindhoven (NL) in 1981. In 2000 she finished secondary school Gymnasium Beekvliet, Sint-Michielsgestel, after which she studied Architecture, Building and Planning at the Department of the Built Environment, Eindhoven University of Technology (2000-2003). She then went to Maastricht University and completed the bachelor Bioregulation and Health (2004-2008) and later the master Sports and Physical Activity Interventions (2008-2009) at Faculty of Health, Medicine and Life Sciences with honors. She received the top 3% scholarship of Maastricht University.

During her studies she held several functions at diverse companies, she was franchise manager and owned a child-care facility up to 2011. After obtaining her Master's degree Monique was appointed as a research assistant at the School for Public Health and Primary Care CAPHRI, Maastricht University. In 2010 she started her PhD project at the division of Human Nutrition and the division of Epidemiology, Wageningen University under supervision of Prof. Edith JM Feskens and Marco Mensink, MD, PhD, which resulted in this thesis 'Dietary protein intake and aspects of the metabolic syndrome'. During her PhD project she performed two randomized crossover trials and analyzed data of a large-scale European case-cohort study. One crossover trial was performed in the framework of the project 'Protein-rich food against obesity', program 'Pieken in de Delta Oost-Nederland' and partly subsidized by the Ministry of Economic Affairs, Agriculture and Innovation (EL&I), and the provinces of Gelderland and Overijssel. All other research activities were performed with financial support by Alpro Foundation. During her PhD, Monique presented results at several national and international scientific meetings, joined diverse courses, was involved in teaching BSc and MSc courses and supervised 6 MSc students. Next to her PhD project she supervised an intervention on the glycemic index of camel milk and organized a PhDtour to south-west USA and Mexico.

LIST OF SCIENTIFIC PUBLICATIONS

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. J Nutr. 2014;144(9):1423-9.

van Nielen M, Feskens EJM, Mensink M, Sluijs I, Molina E, Amiano P, Ardanaz E, Balkau B, Beulens JW, et al. Dietary protein intake and incidence of type 2 diabetes in Europe: The EPIC-InterAct case-cohort study. Diabetes Care. 2014;37(7):1854-62.

van der Velpen V, Hollman PC, van Nielen M, Schouten EG, Mensink M, Van't Veer P, Geelen A. Large inter-individual variation in isoflavone plasma concentration limits use of isoflavone intake data for risk assessment. Eur J Clin Nutr. 2014;68(10):1141-7.

van Nielen M, Feskens EJM, and Mensink M. Partly replacing meat protein with soy protein tends to influence the inflammatory state in postmenopausal women with abdominal obesity. *Submitted for publication.*

van Nielen M, Feskens EJM, Bünger M, van Amerongen A, van den Heuvel B and Mensink M. Postprandial effect of arginine-rich protein added to a high-fat meal in men with characteristics of the metabolic syndrome. *Submitted for publication.*

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

With the educational activities listed below Monique van Nielen has complied with the educational requirements set by the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Description	Institute, location	Year
Discipline specific activities		
EPIC Workshop 'Practical approaches to dealing with measurement error in dietary exposures'	Cambridge University (UK)	2010
Nutrition Science days	NWO, Deurne	2010
Meeting InterAct consortium	Cambridge (UK)	2010
Nutrition Science days	NWO, Deurne	2011
European Congress on Obesity	ECO, Lyon (France)	2012
Nutrition Science days	NWO, Deurne	2012
20th International congress of Nutrition	IUNS, Granada (Spain)	2013
Nutrition Science days	NWO, Deurne	2013
General courses		
Master class 'Regression'	Graduate School VLAG	2010
PhD retreat	Graduate School VLAG, Baarlo	2010
Workshop 'Successful Presenting'	Mennen Training & Consultancy	2011
Master class 'R'	Graduate School VLAG	2012
Master class 'Longitudinal data analysis'	Graduate School VLAG	2013
How to present a world-class paper	Graduate School Courses	2013
Optional courses and activities		
Staff Seminars and presentations	Wageningen University	2010-2014
Preparation of research proposal	Graduate School VLAG	2010-2014
Epi Research meetings	Wageningen University	2010-2014
Methodology club meetings	Wageningen University	2010-2014
PhD Tour organizing committee member	Wageningen University	2010-2011
Analytical Epidemiology	Wageningen University	2010
PhD Tour to south-west USA and Mexico	Graduate School VLAG	2011

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Colophon

The research described in this thesis was financially supported by the Alpro Foundation. One trial was performed in the framework of the project 'Protein-rich food against obesity', program 'Pieken in de Delta Oost-Nederland' and partly subsidized by the Ministry of Economic Affairs, Agriculture and Innovation (EL&I), and the provinces of Gelderland and Overijssel. Funding for the InterAct project was provided by the EU FP6 program (grant no. LSHM_CT_2006_037197).

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