Dietary protein and blood pressure

Epidemiological studies

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We dance round in circles and suppose,
But the secret sits in the middle and knows…
(Robert Frost)

Abstract

Background

Elevated blood pressure is a major risk factor for cardiovascular diseases. Diet and lifestyle have a substantial impact on blood pressure, but the role of protein intake is not yet clear. This thesis focuses on total dietary protein, types of protein (i.e. plant and animal), protein from specific sources (i.e. dairy, meat, and grain), and specific amino acids in relation to blood pressure levels and incident hypertension.

Methods

The associations of dietary protein, protein types, and protein from specific sources with population blood pressure levels were cross-sectionally examined in 20,820 Dutch adults aged 25 to 65 y (MORGEN Study). The relation with risk of hypertension was examined in 3,588 of these adults with 15 years of follow-up (Doetinchem Study) and in 2,241 older Dutch adults (≥55y) with 6 years of follow-up (Rotterdam Study). In the latter cohort we also examined the relation of specific amino acids (i.e. glutamic acid, arginine, lysine, cysteine, tyrosine, and essential amino acids) with blood pressure levels and risk of hypertension. As an ancillary Study, a fully controlled randomized cross-over trial with different protein-rich diets was conducted to obtain objective biomarkers for dietary protein types that may be used in future epidemiological studies. Finally, we performed several meta-analyses to summarize our findings for dietary protein and protein types in relation to blood pressure and incident hypertension, combined with data from the literature.

Results

The epidemiological studies presented in this thesis and a meta-analysis of observational studies showed no associations of total protein and animal protein with blood pressure or incident hypertension. A meta-analysis of 14 randomized controlled trials, however, showed a pooled blood pressure effect of protein supplementation (weighed mean contrast in intake of 41 g/d) of -2.1 mmHg systolic (95%-CI: -2.9 to -1.4) when compared to carbohydrate intake. In the epidemiological studies in this thesis plant protein was significantly inversely associated to blood pressure levels (-1.8/-1.0 mmHg with 14 grams higher energy adjusted intake), but not with incident hypertension (all HR per SD ~1.00). Meta-analyses of cross-sectional studies showed a small differential association of plant and animal protein with blood pressure (-0.52 mmHg per SD of dietary plant protein versus +0.03 mmHg per SD of animal protein), but this association was not present in meta-analyses of prospective studies and trials. The epidemiological analyses on meat protein and dairy protein in this thesis revealed no consistent associations with blood pressure or incident hypertension. Grain protein was inversely associated with diastolic (but not systolic) blood pressure, and with borderline significant

lower risk of hypertension in a general Dutch population (HR: 0.75, 95% CI: 0.73 to 1.00), but this association was absent in older adults. No associations with blood pressure or incident hypertension were found for amino acid intakes. Finally, we identified a combination of 3 urinary amino acids as a potential biomarker for meat protein intake and a combination of 7 plasma amino acids as a potential biomarker for grain protein intake

Conclusion

Results from this thesis suggest a small beneficial effect of protein on blood pressure if consumed instead of carbohydrates. Plant protein, e.g. from grain, may be more beneficial to blood pressure than animal protein but data are too limited to draw firm conclusions. After validation, future epidemiological studies could make use of biomarkers as more robust estimates for protein from specific sources and amino acid intakes. Randomized controlled trials are warranted to examine the blood pressure effect of specific types of protein, reflecting habitual intakes in western societies, compared to different types of carbohydrate. At present, a prudent diet for the prevention of hypertension with adequate amounts of dietary protein, preferable from plant sources, is recommended.

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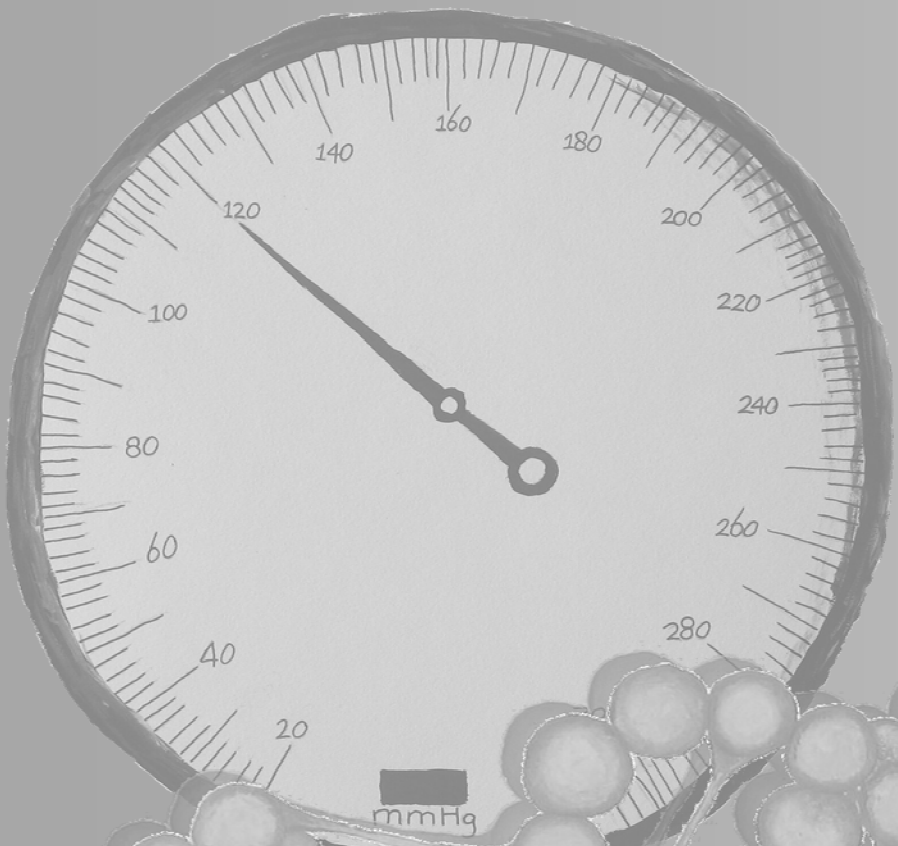
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Chapter 1

General introduction

Elevated blood pressure is a strong, independent and modifiable risk factor for cardiovascular and renal diseases.[1] There is evidence that systolic blood pressure is a better predictor for cardiovascular risk than diastolic blood pressure, especially after the age of 50.[2] People are considered hypertensive when their blood pressure (systolic/diastolic) is ≥140/90 mmHg, or when antihypertensive medication is used. The risk for death from cardiovascular diseases, however, already begins to increase at systolic blood pressure levels above 115 mmHg.[1] Preventive measures to reduce blood pressure in the population can have a large impact on cardiovascular morbidity and mortality.[1,3] It has been estimated that a population-wide reduction in systolic blood pressure of only 2 mmHg results in a 6% reduction in fatal stroke, and a 4% reduction in fatal coronary heart disease.[4]

Well-established measures that contribute to the prevention of hypertension are physical activity, maintenance of normal body weight, and a low intake of alcohol and salt.[1,3,5] In addition, data from the large DASH trial among 459 (pre-) hypertensive adults showed that blood pressure can be substantially reduced by a diet rich in fruits, vegetables, and low-fat dairy products compared to a typical US diet, with reductions in systolic blood pressure being -5.5 mmHg in the total DASH population and -11.4 mmHg in hypertensive participants.[6] More recently, interest has grown in the influence of diet composition and macronutrient intake on blood pressure, but the importance of dietary protein for human blood pressure is not yet clear.

The work presented and discussed in this thesis focuses on the relation between dietary protein and blood pressure. In the present chapter, protein metabolism, the assessment of protein intake, and protein in the Dutch diet are described (PART I). The second part provides a brief overview of protein intake in relation to blood pressure and hypertension, and potential underlying mechanisms for a protein-blood pressure effect (PART II). Finally, an outline is given of the studies presented in this thesis.

Part I – Dietary protein: metabolism and intake

Definition, digestion and absorption

Dietary proteins consist of polypeptides of amino acids, and the order and proportion of amino acids determine the folding and characteristics of the protein.[7] Several amino acids (i.e. leucine, isoleucine, lysine, valine, threonine, methionine, tryptophan, phenylalanine, and histidine) are considered essential, which means that they cannot be synthesized by the body and should be covered by diet (**Table 1.1**).[8,9] Semi-essential amino acids (i.e. cysteine, tyrosine, arginine, proline, and glycine) can only be synthesized from other amino acids and an adequate dietary intake for these amino acids may be required during limited availability of precursors or stress conditions.[8,9] Non-essential amino acids can be synthesized by the human body from a keto-acid or a carbon chain.[9]

***Table 1.1**. Overview of essential, conditionally essential (with precursors), and non-essential amino acids.* [7-9]

Essential	Conditionally essential (precursors)	Non-essential
Leucine	Cysteine (methionine, serine)	Alanine
Isoleucine	Tyrosine (phenylalanine)	Asparagine
Lysine	Arginine (glutamine, glutamate, aspartate, proline)	Aspartic acid
Valine	Proline (glutamate)	Glutamic acid
Threonine	Glycine (serine, choline)	Glycine
Methionine	Glutamine (glutamate, ammonia)	Hydroxyproline
Tryptophan		Serine
Phenylalanine		
Histidine		

After dietary intake, protein is degraded to di- en tri peptides and amino acids which are then absorbed in intestinal cells.[7] In the intestine and splanchnic tissues, the absorbed di- and tripeptides are broken down into amino acids, after which 30 to 50% of essential amino acids and up to 90% of glutamate is used for synthesis of energy (ATP), proteins, and other nitrogen-containing compounds, or metabolized to other amino acids (proline, ornithine, glutamate, alanine, citrulline) that are released in the blood.[7] The remaining amino acids are transported to the liver that takes up about 50% to 65%, except for the branched-chain ami-

no acids which are released from the liver without being metabolized.[7] The blood therefore contains a large pool of amino acids that originate partly from the diet, whereas another part is a result of metabolic processes.

The rate of absorption of dietary protein may differ between sources. Bilsborough et al[10] summarized the results of ten studies on absorption rates of protein from several specific sources. Casein and whey protein isolates were absorbed faster than protein from raw and cooked egg white, pea flour, and milk protein, with absorption rates ranging from 1.3 g/h for raw egg to 8-10 g/h for whey isolate. These differences in absorption rates may be translated into postprandial plasma amino acid levels. In sixteen young healthy adults intake of whey protein, as a model for a "fast" protein, resulted in a short but high peak of plasma amino acids (e.g. peak of leucine lasting for ~220 minutes with a maximum of 350% from baseline), while with casein protein, as a model for a "slow" protein, the peak was lower but prolonged (e.g. peak of leucine lasting for >370 minutes, with a maximum of 190% from baseline).[11] Whether this differential influence of protein types on postprandial plasma amino acid levels could be relevant to blood pressure is not known.

Whether the intake of different types of protein exerts more prolonged effects, reflected in fasting amino acid levels, is currently unknown. In 73 individuals with high cardiovascular risk, different fasting plasma amino acid profiles were found within participants after 4 weeks on a plant protein diet compared to baseline values; e.g. a lower ratio of lysine to arginine (2.7 versus 3.4, $p<0.001$) and increased levels of arginine (72 versus 61 nmol/ml, $p<0.001$) and glycine (281 versus 235 nmol/ml, $p<0.001$).[12] However, no control group was included in this study and observed differences may (partly) be explained by other factors such as increased muscle metabolism during exercise that was part of the intervention program. We could not identify other studies on how dietary protein types affect fasting plasma amino acid levels are available.

Assessment of intake of total protein, protein types and amino acids

Accurate measurement of dietary exposure is a methodological challenge in observational studies. Dietary intake is usually estimated using memory-based methods, such as food frequency questionnaires (FFQ), 24-h recalls or food diaries.[13] These assessment methods, however, are prone to error that may lead to biased estimates for the effect of diet on disease. Random errors, such as recall errors on frequency of consumption and portion sizes usually attenuate associations to the null.[14,15] Systematic errors such as over- or underreporting of intake, or errors because foods in the FFQ are not questioned in sufficient detail for the exposure of interest, might result in differential misclassification and could affect the associations in various directions.[13]

Total protein intake of an individual is relatively constant over time.[16,17] In a study in in 63 men and 58 women to validate the FFQ from the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the reproducibility for energy adjusted total protein intake of a 3 times repeated assessment (6 month intervals) was good with Pearson correlation coefficients of 0.73 in men and 0.70 in women.[18] In addition, the main part of dietary protein is achieved from basic foods that are consumed on a daily basis in the Netherlands, such as meat, dairy and bread.[19] The frequency of consumption and portion sizes of these foods are remembered relatively well leading to adequate ranking of participants for total protein intake. In the validation study of the FFQs of the EPIC study and the Rotterdam Study, both used in this thesis, the correlations with nitrogen or urea as biomarkers of total energy adjusted protein intake were between 0.6 and 0.7.[14,20] The assessment of plant and animal protein intake, and protein types (e.g. from dairy, meat, or grain), faces greater difficulties because most FFQs have not been designed for the estimation of protein from specific sources. Protein rich foods may not have been questioned in sufficient detail to be able to rank participants according to a specific protein type; e.g. meat products that may vary in protein content are questioned in one item. Moreover, a systematic error in Western countries may result from over-reporting of plant food intake because of social desirability, which may bias beneficial associations for plant protein toward the null.

A better estimation of intake may be achieved using reliable biomarkers. Nitrogen is available from all amino acids and a characteristic element of protein. In individuals that are in steady state, the amount of nitrogen in 24-h urine is a useful biomarker of overall protein intake.[21] With regard to protein types no such consensus exists. In several studies the urinary amino acids 3-methylhistidine, 1-methylhistidine or taurine are used as biomarkers of meat- or animal protein intake.[22-24] Also urinary excretion of the amino acid carnosine has been proposed as a biomarker for meat protein.[25] However, none of these potential bio-

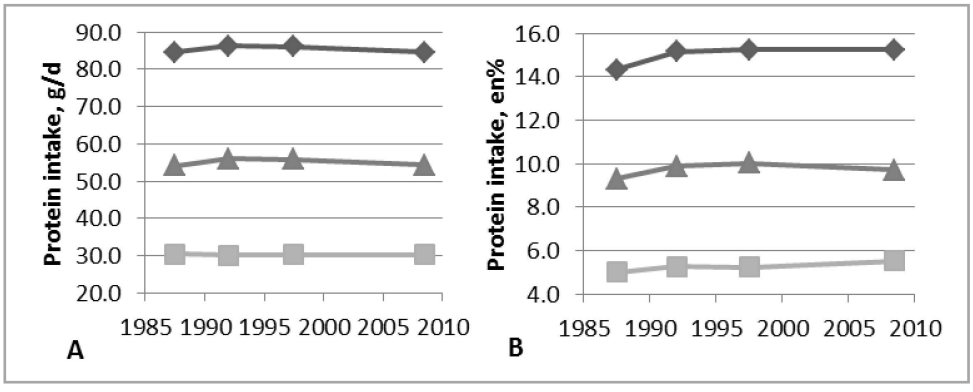

***Figure 1.1**. Protein intake in the Dutch population from 1988 to 2010 expressed in grams per day (A) and as energy percentage (B).[26,27,63]*

=total protein, = animal protein, and = plant protein

markers have sufficiently been validated. Biomarkers for other major protein types, i.e. dairy and grain protein, are lacking.

Habitual protein intake in the Netherlands

Food consumption surveys in the Netherlands have shown a stable protein intake of ~85 g/d in the past two decades (**Figure 1.1**)[26,27], with average total protein intake being ~73 g/d for women and ~96 g/d for men.[27] The contribution of protein to energy, however, showed a small increase over time, i.e. from 14.3 en% in 1987-1988 to 15.2 en% in 2007-2010.[26,27] Approximately two thirds of protein intake in the Dutch diet originates from animal sources, whereas one third originates from plant sources.[27] Results from a standardized, computer-assisted 24-h dietary recall in 3,980 Dutch adults from the EPIC study showed that the main contributors to total protein intake were dairy (23%), meat (38%), and grains (17%) (**Figure 1.2**).[19] No data are available on the habitual amino acid intake in the Netherlands, but chemical analysis of a diet with ~50% of protein from meat, dairy, and eggs, ~40% from cereals (mainly wheat products), and ~10% from vegetables and fruits showed that the most important amino acid was glutamic acid (21% of total protein), followed by proline (8%), leucine (7%), aspartic acid (7%) and lysine (6%).[28]

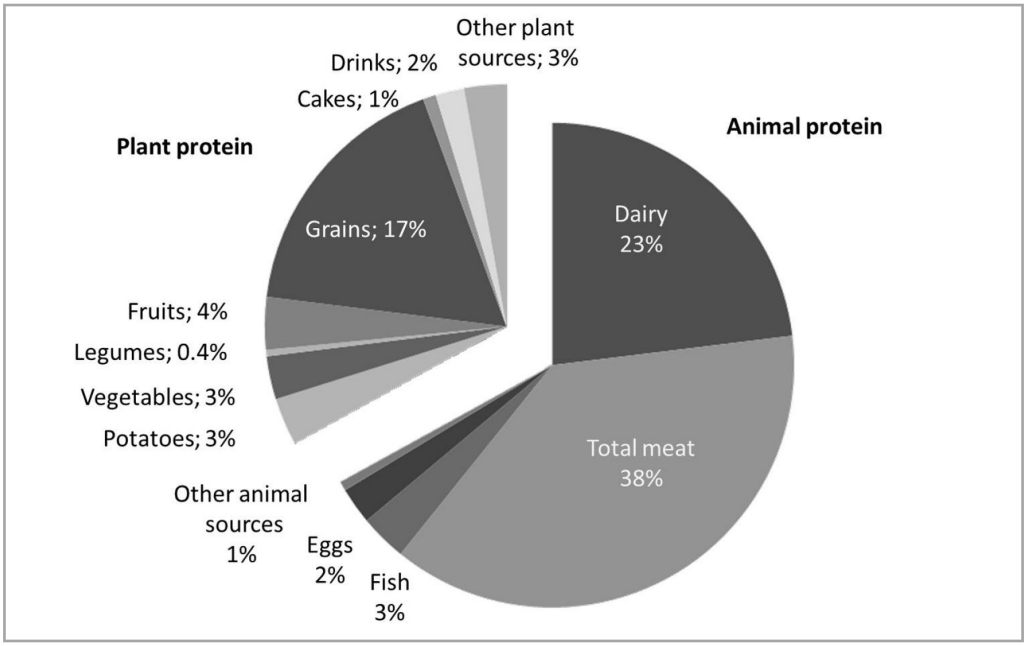

Figure 1.2. *Contribution of plant and animal protein sources to total protein intake in the Netherlands.*[19]

Part II – Protein intake and blood pressure

A systematic overview of existing literature on protein intake and blood pressure is presented in **Chapter 2.** A number of relevant studies in this field is highlighted below to provide a rationale for the present thesis.

Total dietary protein

There is growing evidence for an inverse association of total dietary protein with blood pressure. Observational follow-up data (6-years) of 11,342 normotensive US men from the MRFIT trial showed a significant inverse association between protein intake and blood pressure, but the estimates were small (-0.06 mmHg systolic per en%, p<0.01).[29] In the INTERSALT study among 10,020 normotensive adults from 32 countries, stronger associations were found, i.e. -0.5 mmHg systolic (p<0.01) per g of total 24-h urinary nitrogen (~6 g/d of dietary protein). The association was more pronounced in individuals aged 40-59 y compared to those aged 20-39 y (-0.9 versus -0.2 mmHg per g of total 24-h urinary nitrogen).[30] Also in several randomized trials a beneficial blood pressure effect of dietary protein was demonstrated. In a trial among 99 Dutch untreated (pre-)hypertensive adults a significant blood pressure reduction of 4.9 mmHg was found after 4 weeks supplementation of 60 g protein/d (20% pea, 20% soy, 30% egg, and 30% milk-protein isolate), compared to maltodextrine.[31] The Omniheart randomized crossover trial in 164 US adults included two separate control treatments, namely carbohydrates and monounsaturated fat.[32] In this study systolic blood pressure decreased 1.4 mmHg more after a 6-week high protein diet compared with a diet high in carbohydrates (p=0.002). The blood pressure effect was more pronounced in hypertensives (-3.5 mmHg, p=0.006) than in normotensives (-0.9 mmHg, p=0.05). Compared to a diet high in monounsaturated fat, however, there was no blood pressure effect for protein (-0.1 mmHg, p=0.90).[32]

Taken together, there is evidence, mainly from trials, that a higher protein intake could lower blood pressure. However, data on long term (>5 years) influence of protein on blood pressure is scarce. This needs to be investigated in population-based cohort studies.

Plant versus animal protein

As summarized in 2010 by Craig et al. usually a lower blood pressure is reported in vegetarians compared to omnivores.[33] It is possible that the high proportion of plant protein in these diets partly explains this difference in blood pressure. In the large cross-sectional INTERMAP study among 4,680 adults from China, Japan, UK and USA, systolic blood pressure was 1.01 mmHg lower with 2.8 en% (=2 SD) higher plant protein intake after adjustment for dietary

and lifestyle factors (p<0.01), whereas with 5.8 en% (=2 SD) higher animal protein intake there was no significant blood pressure difference (+0.2 mmHg, p>0.05).[34] The relation of plant and animal protein with hypertension incidence has been addressed in a few prospective studies.[35,36] In the PREMIER study among 810 pre- or mild hypertensives a stronger reduction in hypertension risk was observed for increased intake of plant protein (-21%, p=0.08) compared to animal protein (-1%, p=0.90).[35] Also in the SUN cohort of 5,880 Hispanic university graduates, a 50% reduced hypertension risk was present in the highest quintile compared to the lowest quintile of plant protein intake (95%-CI: 0.2-0.9), whereas for animal protein there was no risk reduction. Confirmation of these findings in other prospective studies would strengthen the evidence for a differential effect of plant and animal protein on blood pressure.

Protein from specific sources

Several protein-rich foods have been associated with blood pressure. In a meta-analysis on dairy, a 16% reduced risk for elevated blood pressure (i.e. ≥130/85 mmHg, or use of antihypertensive medication) was found for low fat dairy (95%-CI: 0.74-0.95).[37] In the CARDIA study an inverse association with hypertension risk was found for plant foods, including fruits, vegetables, nuts, legumes, and whole- and refined-grain products, whereas meat was unfavourably associated.[38] From these studies it cannot be concluded whether protein or other nutrients in these foods accounted for the lower risk of hypertension. We identified two trials on meat protein.[39,40] In a 12-week parallel trial among 64 hospital staff members, a diet with 40% of protein from meat sources (from beef, chicken, lamb, sausage, pork, and prawns) resulted in a non-significant blood pressure effect of -1.8 mmHg systolic and -1.2 mmHg diastolic (p-value not given) compared with a diet in which meat protein was replaced by plant protein (from cereals, vegetables, legumes, and nuts).[39] In a small cross-over trial among 35 men no difference in blood pressure effect was seen (no p-value given) between a 6-week diet including 50% of protein from meat (from pork, beef, and chicken) compared with a diet in which the meat protein was replaced by non-meat protein (from vegetables, eggs, and dairy).[40] The blood pressure effect of dairy and soy protein has been investigated in a large cross-over trial in 352 (pre-)hypertensive adults.[41] For both types of protein 8 weeks of supplementation resulted in approximately the same blood pressure reduction compared to carbohydrates (-2.0 mmHg for soy protein and -2.3 mmHg for dairy protein, both p<0.01). No studies have been conducted on grain protein, the main source of plant protein in the Netherlands, in relation to blood pressure. Taken together, it is not yet known to what extent different sources of protein in the western diet are important in determining population blood pressure levels.

Amino acids

In the INTERMAP study in 4,680 adults it was estimated that individuals with high plant and low animal protein intake consumed greater proportions of glutamic acid, cysteine, proline, phenylalanine, and serine, and smaller proportions of a number of other amino acids (e.g., glycine, alanine, histidine, threonine, methionine, lysine).[34] Possibly, a differential blood pressure effect of protein types is due to the role of specific amino acids, but data on this subject are scarce. In the INTERMAP study, a 2 SD higher intake of glutamic acid (4.7% of total protein) was associated with 1.5 mmHg lower systolic blood pressure and 1.0 mmHg lower diastolic blood pressure ($p<0.05$) after adjustment for several confounders like physical activity, alcohol consumption and dietary factors.[42] In a meta-analysis on 11 arginine supplementation trials the pooled blood pressure effect was -5.4/-2.7 mmHg with arginine doses ranging from 4 to 24 g/d.[43] Another amino acid that has been investigated in a randomized controlled trial is tyrosine, of which a 2 weeks supplementation of 7.5 g/d in 13 mildly hypertensive adults did not affect blood pressure.[44] However, whether dietary intake levels of arginine (on average ~4 g/d) and tyrosine (~3 g/d) are important for human blood pressure is unknown. Epidemiological studies investigating the relation between specific amino acids and population blood pressure are therefore warranted.

Mechanisms for an effect of dietary protein on blood pressure

The underlying mechanisms for a potential blood pressure effect of dietary protein have not yet been clarified. However, several hypotheses have been suggested that involve renal function, the central nervous system, the role of specific amino acids or peptides, and body weight regulation.

Dietary protein intake can induce changes in renal function including an increase in glomerular filtration rate, which may facilitate renal sodium excretion[31,45], and consequently prevents the sodium dependent blood pressure rise. On the other hand, chronic high intake of sulphur-containing amino acids (cysteine, methionine) in protein could influence the acid-base balance in the blood.[46] Compensatory increases in renal acid excretion and ammoniagenesis may lead to impaired renal function on the long term and consequently increase blood pressure. Other mechanisms through which a disturbed acid-base balance have been suggested to influence blood pressure are increased cortisol production[47], increased calcium excretion[48], or decreased citrate excretion[49].

The central nervous system is a key regulator of blood pressure by modulating cardiac output and peripheral resistance. Because protein content of the diet modifies availability of amino acid precursors for neurotransmitters, macronutrient composition of the diet is hypothesised to influence blood pressure regulation.[50] Indeed, increased postprandial sympathetic activation has been found after carbohydrate rich meals[51], but specific data for dietary

protein intake are lacking. Tyrosine is a precursor of catecholamines in the brain (dopamine, norepinephrine, epinephrine) and has been hypothesised to reduce cardiovascular sympathetic tone and consequently blood pressure.[52]

Several other amino acids have been hypothesised to influence blood pressure. Arginine is a precursor for the vasodilator nitric oxide. A high intake of arginine or its precursors, such as glutamic acid, could therefore be related to lower blood pressure. Lysine, on the other hand, competes with arginine in the transport system in the gut and could unfavourably affect blood pressure.[53,54] Cysteine binds excess aldehydes, which may be formed in the human body when glucose metabolism is impaired (present in ~50% of essential hypertensives[55]).[56] Because aldehydes are thought to increase peripheral vascular resistance through modulation of calcium channels, intake of this amino acid is hypothesised to beneficially influence blood pressure.[56]

In the past decade there has been increasing interest in peptides encrypted in dietary protein that can be derived from foods like tuna, eggs and milk.[57] Peptides with specific sequences of amino acids, such as lactotripeptides that consist of Isoleucine-Proline-Proline and Valine-Proline-Proline, have been shown to inhibit the angiotensin I-converting enzyme (ACE) *in vitro*.[57,58] Although antihypertensive effects have been reported in human trials with functional foods containing high amounts of promising peptides[59], no evidence for ACE inhibition was found in those trials assessing parameters of the renin-angiotensin system.[60] It is at present unknown to what extent digestion via gastrointestinal enzymes in humans releases antihypertensive peptides after normal protein intake and whether that could exert a physiological response either in the gut or elsewhere after entering the circulation.

Protein has been shown to have a stronger satiating effect than other macronutrients, and may therefore beneficially influence weight.[61] Because a lower body weight has been shown to beneficially affect blood pressure[62], this may be another pathway through which protein could reduce the risk of hypertension.

Objective and outline of the thesis

There is growing evidence for a beneficial effect of dietary protein on blood pressure. This may be attributable to plant protein, but more research on this subject is needed. Whether there is a differential effect of protein from more specific sources, such as dairy, meat, and grain, and whether specific amino acids influence blood pressure is unknown. Also data on subject characteristics that modify the blood pressure response to dietary protein are scarce. The objectives of this thesis were therefore: 1) to examine whether habitual intake of dietary protein is related to blood pressure level or the incidence of hypertension, 2) to examine whether plant and animal protein, protein from specific sources (dairy, meat, and grain), or specific amino acids are related to blood pressure levels or the incidence of hypertension, and 3) to examine whether subject characteristics like age, gender, BMI, and hypertensive status, could modify the association between dietary protein and blood pressure.

A schematic overview of this thesis is given in **Figure 1.3**. We first conducted a systematic literature review on dietary protein in relation to blood pressure, with a focus on specific types of protein and specific population subgroups (**Chapter 2**). Subsequently, we studied the relation between dietary protein and blood pressure levels in the general Dutch population of the MORGEN cohort (**Chapter 3**). The relation between protein intake and incident hypertension was prospectively examined in the population-based Doetinchem cohort (**Chapter 4**), and in the general older population of the Rotterdam Study (**Chapter 5**). In the latter cohort we additionally investigated the relation of several amino acids with blood pressure levels and hypertension incidence (**Chapter 6**). Finally, we conducted the Biomarker study; a fully controlled dietary intervention trial to identify objective biomarkers for dairy, meat, and grain protein that may be used in future epidemiological studies (**Chapter 7**).

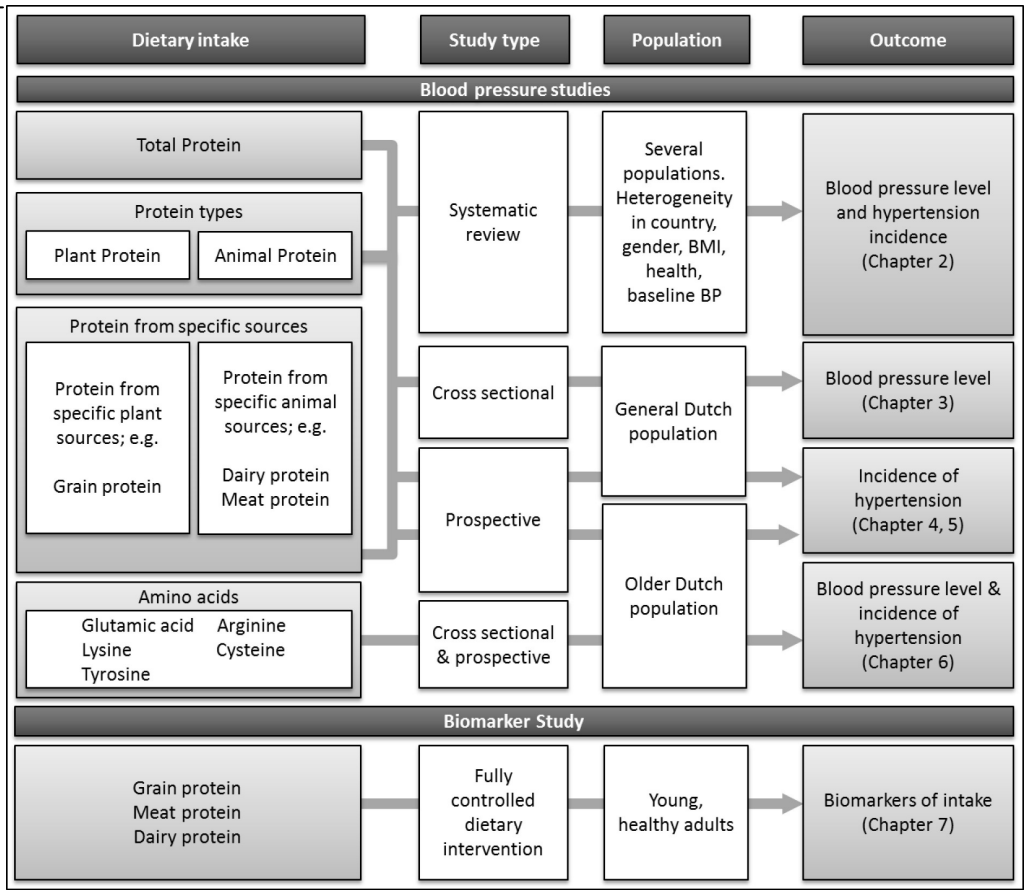

Figure 1.3. *Schematic overview of the studies described in this thesis. Additionally, in each blood pressure study stratified analyses were conducted for the following subgroups: gender, age, overweight status, and blood pressure status.*

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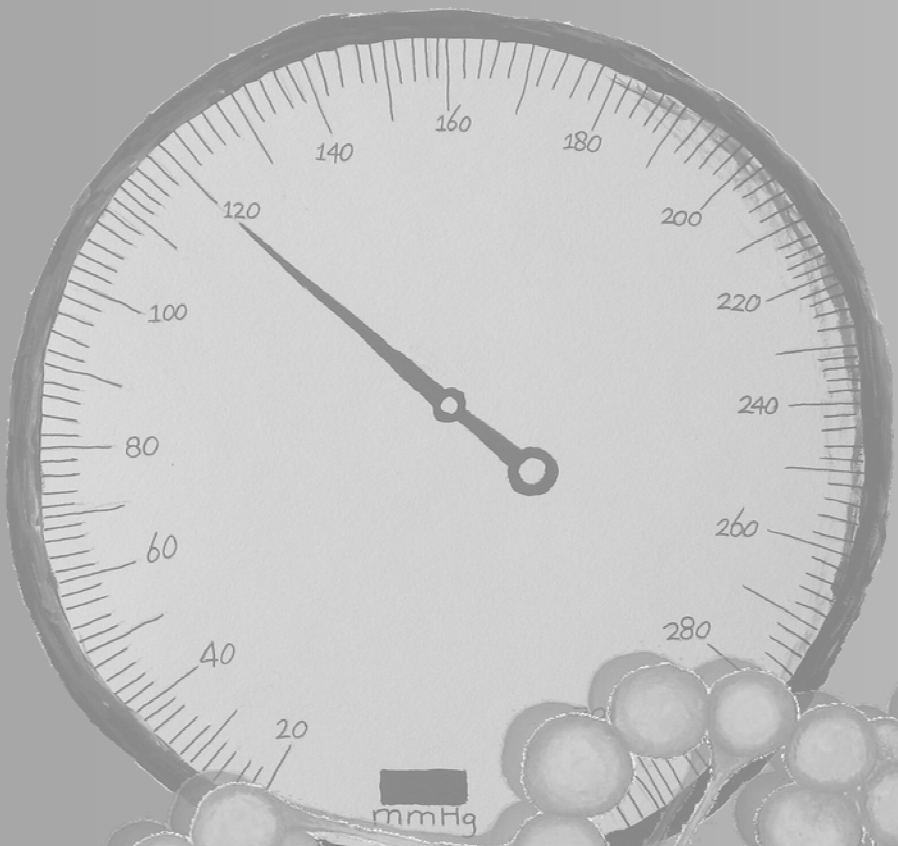
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Chapter 2

Dietary protein and blood pressure: a systematic review

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Abstract

Background

Elevated blood pressure, which is a major risk factor for cardiovascular disease, is highly prevalent worldwide. Recently, interest has grown in the role of dietary protein in human blood pressure. We performed a systematic review of all published scientific literature on dietary protein, including protein from various sources, in relation to human blood pressure.

Methodology/Principal Findings

We performed a MEDLINE search and a manual search to identify English language studies on the association between protein and blood pressure, published before June 2010. A total of 46 papers met the inclusion criteria. Most observational studies showed no association or an inverse association between total dietary protein and blood pressure or incident hypertension. Results of biomarker studies and randomized controlled trials indicated a beneficial effect of protein on blood pressure. This beneficial effect may be mainly driven by plant protein, according to results in observational studies. Data on protein from specific sources (e.g. from fish, dairy, grain, soy, and nut) were scarce. There was some evidence that blood pressure in people with elevated blood pressure and/or older age could be more sensitive to dietary protein.

Conclusions/Significance

In conclusion, evidence suggests a small beneficial effect of protein on blood pressure, especially for plant protein. A blood pressure lowering effect of protein may have important public health implications. However, this warrants further investigation in randomized controlled trials. Furthermore, more data are needed on protein from specific sources in relation to blood pressure, and on the protein-blood pressure relation in population subgroups.

Introduction

Elevated blood pressure is an independent risk factor for cardiovascular diseases (CVD) and renal impairment.[1] There is no evidence for a threshold effect: from systolic blood pressure levels as low as 115 mmHg onward, risk of CVD doubles for each increment of 20 mmHg.[1] It has been estimated that, at population level, a reduction in systolic blood pressure of only 2 mmHg would result in a 6% reduction in fatal stroke, and a 4% reduction fatal coronary heart disease (CHD).[2]

Well-known dietary and lifestyle interventions to prevent hypertension include moderate physical activity, maintenance of normal body weight, low alcohol and salt intake, and a diet rich in fruits, vegetables, and low-fat dairy products.[2,3] More recently, interest has grown into dietary patterns and macronutrient intakes, including protein.[4,5] Whether protein content of the diet or type of protein is important for human blood pressure is, however, unclear. We systematically reviewed all scientific literature, published before June 2010, on dietary protein in relation to human blood pressure, with a focus on specific types of protein and possible interactions with age, gender, blood pressure level, and overweight.

Methods

Ethical approval was not required for this review because only published data were included.

Search strategy

A systematic search was performed in MEDLINE (www.ucbi.ulm.nih.go) to identify studies on the association between dietary protein and blood pressure, published before June 2010. Search terms on dietary protein and blood pressure or hypertension were used to search for words in title or abstract and Medical Subject Headings. The search was limited to studies in human adults and English-language literature. In addition, we performed a manual search using reference lists of original articles and previous reviews.[6-9] For all studies, we retrieved the original publication.

We selected any observational study or trial that examined the relationship between dietary protein and blood pressure in humans. All titles, abstracts, and full papers of potentially relevant studies were assessed for eligibility based on predefined inclusion and exclusion criteria. Papers were excluded: 1) if data on exposure (dietary protein) or outcome (blood pressure, hypertension) was not reported, 2) if no data were reported on the relationship between exposure and outcome, 3) if the exclusive effect of protein could not be calculated

(e.g. blood pressure studies that focused on dietary patterns, or soy combined with isoflavones). Furthermore, review papers were excluded, as were drug trials and studies conducted in patient groups or pregnant women.

Data collection and data synthesis

From each included paper we extracted data on protein intake, source of protein, and blood pressure values or estimated risk of hypertension according to a predefined standard form. In addition, we extracted data on design, place of study, number of participants, population characteristics (including initial blood pressure, sex, and age), dietary assessment method (food frequency questionnaire (FFQ), 24-hour recall, food diary, biomarker), adjustment for confounders, and measures of variation.

To allow better comparison of results from observational studies we expressed associations in these studies by standard units of protein intake that correspond to approximately 1 SD of protein intake in the Dutch population, i.e. 25 g/d (3.5 en%) for total protein, 11 g/d (1.4 en%) for plant protein, and 23 g/d (2.9 en%) for animal protein.[10,11]

Results

The systematic search in MEDLINE resulted in 2,681 titles to be screened. Inclusion criteria were fulfilled by 40 papers, and the hand search yielded another 6 papers (**Figure 2.1**). In total, 15 observational studies, 13 biomarker studies and 20 trials were selected.

Total dietary protein and blood pressure: observational data

Twelve observational studies focused on habitual total protein intake and blood pressure or risk of hypertension (**Table 2.1**). Most of these studies had a cross-sectional design and showed predominantly weak inverse associations.[12-20] However, although hypothesis-generating, a major drawback of a cross-sectional design is that protein intake and blood pressure are assessed at the same moment in time, which makes it difficult to address the temporality of the association. Subjects with elevated blood pressure, or otherwise at increased cardiovascular risk, may have changed their food intake (including protein intake) upon medical advice. Causality can, therefore, be better established in prospective studies.

So far, only three studies prospectively examined the association of total dietary protein with change in blood pressure or incident hypertension. Total protein intake was not clearly associated with change in systolic blood pressure after 8 years of follow up in 1714 US men (+0.16 mmHg per y per 3.5 en% systolic, p=0.04) [21], and after 7 years of follow up in 4146

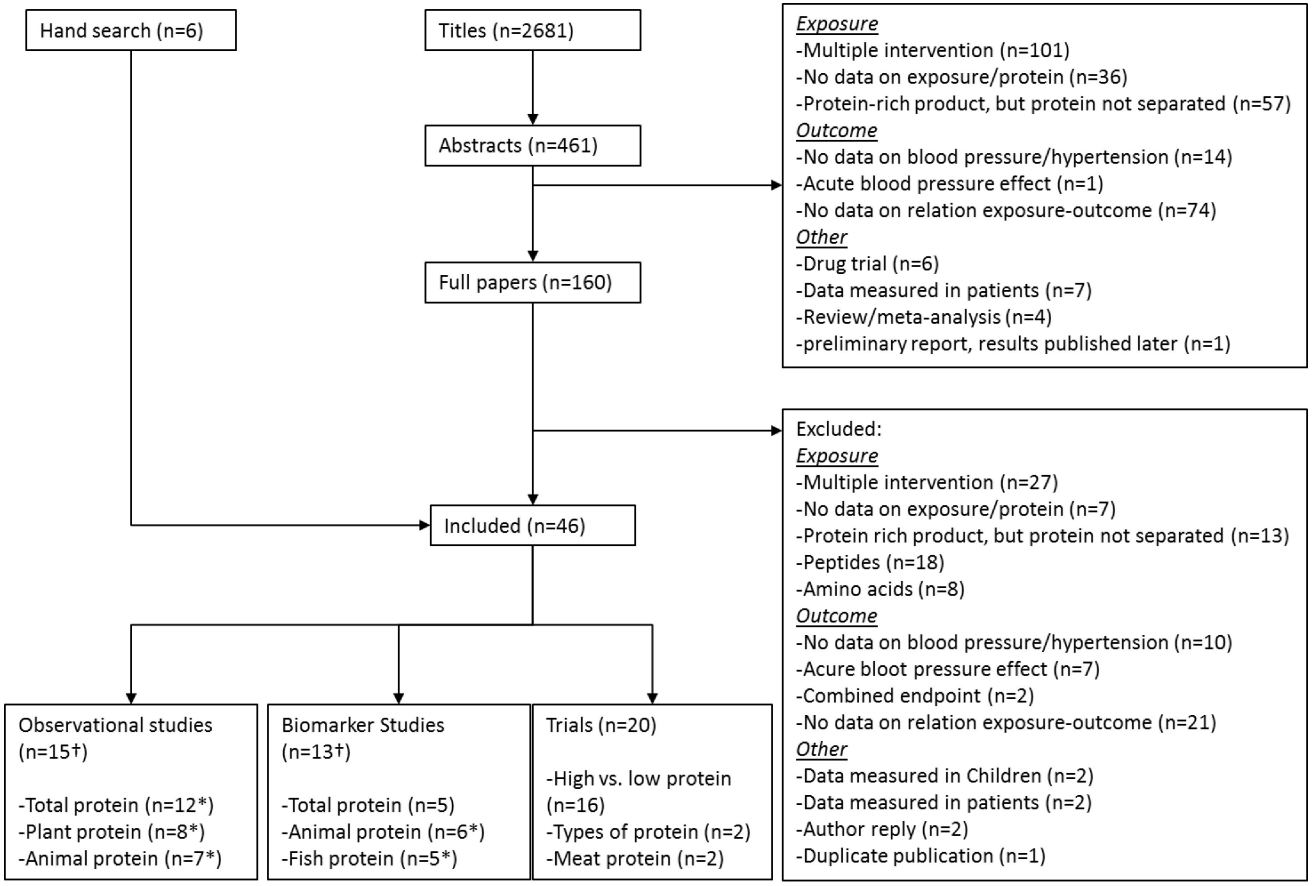

Figure 2.1. *Flow chart of systematic literature search.*
**Numbers overlap because several studies investigated different types of protein.*
†Numbers overlap because two studies investigated protein intake using questionnaires as well as biomarkers.

Table 2.1. *Observational studies of total protein intake and blood pressure.*

Author, year	respondents	Initial BP (mmHg)	Habitual protein intake	Dietary assess-ment	BP outcome (SBP/ DBP)	BP outcome per 25 g/d or 3.5 en% (SBP/DBP)	P-value (SBP/ DBP)	Statistical adjustment
Cross-sectional studies								
Pellum, 1983[16]	61 normotensive US adults mean age ~24	M: 119/73 F: 107/68	M: 101 g/d F: 65 g/d (≈14 en%)	3-d food record	-0.13/ … mmHg per g/d	~ -3.33/ … mmHg per 25 g/d	…/ …	Sex, serum HDL, exercise, fat intake
Havlik, 1990[13]	402 male US twins aged 42-56 y	128/82	15 en% (=75 g/d)	FFQ	… / +0.11 mmHg per g/d of energy adjusted protein	…/ ~ +2.75 mmHg per 25 g/d	NS/ 0.02	Weight, serum cholesterol, triglycerides, total energy intake
Wang, 2008[20] (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	16 en%	2x 24h recall	-0.08/ +0.03 mmHg per en%	~ -0.28/~ +0.11 mmHg per 3.5 en%	0.41/ 0.73	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, energy intake, intake of Ca and K, urinary creatinine, urinary Na
He, 1995[14]	827 Chinese adults mean age ~38[1]	~113/70	~12 en% (≈93 g/d)	3x24-h recall	-3.6/ -2.2 mmHg per SD (=39 g/d)	~ -2.28/ ~ -1.38 mmHg per 25 g/d	<0.05 /NS	Age, BMI, alcohol, urinary Na, energy intake, resident area
Reed, 1985[17]	6496 Japanese men in Hawaii aged 46-69 y	…	…	24-h recall	-3 mmHg/ -1 mmHg for Q5 (≥122) vs. Q1 (<67 g/d)	~ -0.85/ ~ -0.28 mmHg per 25 g/d	<0.001/ 0.03	Age
Umesawa, 2009[19]	7585 Japanese men and women aged 40-69 y[1]	M: 137/83 F: 135/81	M: 83 g/d F: 65 g/d	Single 24h recall	-0.29/ -0.42 mmHg per 25.5 g/d	~ -0.28 / ~ -0.41 mmHg per 25 g/d	NS/ <0.05	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of sodium, potassium, and calcium
					M: +0.07/ -0.31 mmHg per 27.4 g/d F: -0.61/ -0.55 mmHg per 20.6 g/d	M: ~ +0.06/ ~ -0.28 mmHg per 25 g/d F: ~ -0.74/ ~ -0.66 mmHg per 25 g/d	<0.01/ NS NS/ <0.05	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of sodium, potassium, and calcium
Masala, 2008[15] (EPIC)	7601 Italian women aged 35-64 y	123/79	…	FFQ	+0.38 / +0.60 mmHg per log(g/d)	~ +1.22/ ~ +1.93 mmHg per 25 g/d	0.76/ 0.43	Age, BMI, waist circumference, smoking, education, physical activity, energy intake

Table 2.1. *Observational studies of total protein intake and blood pressure (continued).*

Author, year	respondents	Initial BP (mmHg)	Habitual protein intake	Dietary assess-ment	BP outcome (SBP/ DBP)	BP outcome per 25 g/d or 3.5 en% (SBP/DBP)	P-value (SBP/ DBP)	Statistical adjustment
Cross-sectional studies (continued)								
Garcia-Palmieri, 1984[12]	7932 men from Puerto Rico aged 45-64 y	…	…	24-h recall	SBP: between -0.03 and +0.03 mmHg per g/d depending on subgroup[2]; DBP: …	~+0.13/ … mmHg per 25 g/d	NS/ …	Age, smoking, weight, education, serum glucose, heart rate, intake of milk, fat, carbohydrates, coffee, alcohol
Stamler, 1996b[18] (MRFIT)	11342 US men aged 35-57 y[1]	125/84	17 en%	4-5x 24-h recall	-0.06/ -0.06 mmHg per en%	~ -0.20/ ~ -0.21 mmHg per 3.5 en%	<0.01/ <0.001	Age, race, BMI, education, smoking, serum cholesterol, antihypertensive drugs, Na and K intake, alcohol and caffeine intake; corrected for regression dilution bias
Prospective studies								
Stamler, 2002[21]	1714 men, aged 40-55 y[1]	135/87	15 en%	FFQ	+0.05/ -0.02 mmHg per year per en%	~ +0.16/ ~ -0.05 mmHg per 3.5 en%	0.04/ 0.16	Age, height, weight (+ change), education, alcohol, smoking
Liu, 1996[22] (CARDIA)	4146 US blacks and whites aged 18-30 y[1]	~110/69	~15 en%	FFQ	~ -0.16/ ~ -0.34 mmHg per year per 3 en%	~ -0.20/ ~ -0.40 mmHg per year per 3.5 en%	NS/ NS	Age, BMI, education, exercise, smoking, alcohol, hostility score, use of antihypertensive medication, intake of K and Ca
Alonso, 2006[23] (SUN)	5880 Hispanic, university graduates, mean age ~36 y	…	~18 en%	FFQ	HR (95%-CI) = 0.9 (0.6; 1.4) for Q5 vs. Q1 of energy adjusted protein	NA	0.51	Age, sex
					Multivariable HR (95%-CI) = 0.8 (0.4; 1.4) for Q5 vs. Q1 of energy adjusted protein		0.26	Age, sex, BMI, exercise, alcohol, smoking, hypercholesterolemia, intake of total energy, Na , fruit, vegeTables, fiber, caffeine, magnesium, potassium, low-fat dairy, MUFA, SFA

BP=blood pressure, SBP=systolic blood pressure, DBP=diastolic blood pressure, M=men, F=women, en%=energy percentage; 95%-CI=95% confidence interval, MUFA=monounsaturated fat, PUFA=polyunsaturated fat, SFA=saturated fat, Na=sodium, K=potassium, Ca=calcium, BMI=body mass index; NS=not statistically significant (p>0.05); … = value not given.

[1]Users of anti-hypertensive medication were not excluded, [2]urban/rural, middle-aged/old age.

young US adults (-0.20 mmHg per year per 3.5 en% systolic, p>0.05) [22]. It should be noted that in these two studies respondents using antihypertensive medication were not excluded from the analyses, which may have affected the associations. In 5880 university graduates of the prospective SUN cohort, not using antihypertensive medication, a non-significant 20% lower 2-year hypertension risk was found (p=0.26).[23] In this study the population was quite young (mean age ~36 y), and blood pressure may not have been as sensitive to influence from protein intake as in an older population.

Concluding, most cross-sectional studies on total protein intake and blood pressure or incident hypertension showed a weak inverse association, whereas no clear conclusion could be drawn from prospective studies. A small beneficial effect on blood pressure may exist, but well conducted prospective studies and randomized controlled trials may provide better estimates of a protein effect on blood pressure.

Biomarkers of total dietary protein and blood pressure: observational data

Daily urinary nitrogen excretion, about 85% excreted in the form of urea, correlates with dietary protein as calculated from weighed food records (r= 0.4-0.8) and reflects ~80% of total protein intake.[24] As shown in **Table 2.2**, in five cross-sectional studies urinary total nitrogen [25] or urinary urea nitrogen [11,25-28] was used to estimate the association between total protein intake and blood pressure.

In the large INTERSALT-study, including 10,020 adults from 32 countries, an inverse association of -0.5 mmHg systolic (p<0.01) per g of total 24-h urinary nitrogen was observed.[25] Also in 4,680 respondents from the INTERMAP study, 24h urea nitrogen was inversely related to systolic blood pressure (-0.9 mmHg per 5.34 g), although this was not statistically significant.[11] In the remaining studies, summarized in **Table 2.2**, single spot or overnight urines were used to estimate protein intake.[26-28] Although these estimates are less reliable than estimates from 24-h urine, the results were in line with those of the studies mentioned above.

Concluding, in studies among participants that are in nitrogen balance, good agreement has been found between one or two 24-h urine collections and diet-history estimates of protein intake.[24] Findings from biomarker studies, therefore, suggest that protein intake may have a beneficial effect on blood pressure.

Total dietary protein and blood pressure: trial data

In 16 trials the blood pressure effect of a high protein diet was assessed (**Table 2.3**). Most trials were only small (number of participants per intervention group: n=7 to n=30), and the conflicting results may be due to chance findings.[29-39] In one of the larger trials, a parallel

Table 2.2. *Observational studies of biomarkers of total protein intake and blood pressure.*

Author, year	respondents	Initial BP (mmHg)	Habitual protein intake	Dietary assessment	BP outcome (SBP/ DBP)	P-value	Statistical adjustment
cross-sectional studies							
Kihara, 1984[28]	1120 traditional Japanese aged over 30 y	M: 132/79 F: 129/76	…	Urea nitrogen/ Cr in single-spot urine (mol:mol)	M: +0.13/ +0.02 mmHg F: -0.04/ -0.01 mmHg per unit (partial regression coefficients)	<0.05/ NS NS/ NS	…
Iseki, 2003[27]	1299 Japanese adults, mean age ~49 y[1]	~121/74	~1.1 g/kg/ day	Urea nitrogen in single spot urine	-3.0/ -2.4 mmHg per g/kg/day	…/ …	Unadjusted
Cirillo, 2002[26]	3705 Italian adults aged 25-74 y[1]	127/76	…	Urea nitrogen in overnight urine	-5.2/ … mmHg per log(urea) in mmol/h	<0.01/ …	Age, sex, BMI, exercise, alcohol, smoking, antihypertensive drugs, urinary Na, K, Ca, creatinine clearance
Elliott, 2006[11] (INTERMAP)	4680 respondents from China, Japan, UK and USA aged 40-59 y[1]	119/74	China: 12 en%; Other countries: 15-16 en%	Urea nitrogen in 24h urine	M: -0.77/ -0.40 mmHg F: -1.11/ -0.41 mmHg per 5.34 g/24h (2 SD)	NS/ NS NS/ NS	…
Stamler, 1996a[25] (INTERSALT)	10020 adults from 32 countries worldwide aged 20-59 y[1]	119/73	…	Total nitrogen in 24-h urine	-0.50 mmHg per g/ -0.41 mmHg per g Older respondents (40-59 y): -0.92/ -0.48 mmHg per g Younger respondents (20-39 y): -0.20/ -0.38 mmHg per g	<0.01/ <0.01 <0.01/ <0.05 …/ <0.05	Age, sex, BMI, alcohol and 24h urinary Na, K, Ca, Mg; corrected for regression dilution bias
Stamler, 1996a (INTERSALT)	10020 adults from 32 countries worldwide aged 20-59 y[1]	119/73	…	Urea nitrogen in 24-h urine	-0.57/ -0.50 mmHg per g	<0.05/ <0.01	Age, sex, BMI, alcohol and 24h urinary Na, K, Ca, Mg; corrected for regression dilution bias

BP=blood pressure, SBP=systolic blood pressure, DBP=diastolic blood pressure, M=men, F=women, Na=sodium, K=potassium, Ca=calcium, BMI=body mass index; PUFA=polyunsaturated fat, SFA=saturated fat, 3MH=3-methylhistidine; Cr=creatinine; NS=not statistically significant (p>0.05) ; … = value not given.
[1]Users of anti-hypertensive medication were not excluded.

Table 2.3. *Trials of total protein intake and blood pressure.*

Author, year	Blin-ding	Participants	Initial BP (mmHg, Interven-tion vs. control)	Type of intervention	Intake of protein in control group	ΔProtein	ΔCH	ΔFat	Duration of interven -tion	ΔSBP/ ΔDBP due to intervention	P-value
Cross-over trials											
DeHaven, 1980[32]	…	7 healthy obese participants, aged 23-38 y	114/69	Pure prot (=boiled turkey), low caloric (400 Kcal) diet vs. mixed (turkey+grapejuice) low caloric diet	50 en%	"High"	"High"	0	3 to 5.5 weeks for each diet	+5/ +1 mmHg*	…
Daniels, 1990[31]	…	7 normotensive healthy adults (6 M, 1 F), aged 22-49 y	…./…	High prot vs. low prot diet	0.55 g/kg/d	+1.45 g/ kg/d	"high"	"similar"	4 days	+3/ +2 mmHg	NS/ NS
Papakon-stantinou, 2010[38]	sb	17 obese, newly diagnosed type 2 diabetes patients, aged 30-65 y	134/86 vs. 134/80	Low caloric (-700 kcal) high protein low fat diet vs. low caloric low protein high fat diet.	61 g/d ≈15 en%	+56 g/d ≈+15 en%	-4 g/d ≈0 en%	-28 g/d ≈-15 en%	4 weeks per diet, 3 weeks wash out	-9/ -5 mmHg	<0.001/ <0.001
Sacks, 1984[39]	sb	23 US vegans, aged 22-41 y	112/74	High prot supplement (60 g wheat prot : 40 g soy protein) vs. low prot supplement (rice prot)	70 g	M: +60 g/ d; F: +45 g/d	M: +2 g/d; F: +1 g/d	…	6 weeks for each diet	+1/ +0.6 mmHg*	NS/ NS

Table 2.3. *Trials of total protein intake and blood pressure (continued).*

Author, year	Blin-ding	Participants	Initial BP (mmHg, Interven-tion vs. control)	Type of intervention	Intake of protein in control group	ΔProtein	ΔCH	ΔFat	Duration of interven -tion	ΔSBP/ ΔDBP due to intervention	P-value
Cross-over trials (continued)											
Appel, 2005[5] (Omni-Heart)	db	164 US participants (55% African Americans), mean age 64 y	131/77	Prot rich diet (~50% plant prot) vs. CH rich diet	15 en%	+10 en%	-10 en%	0	6 weeks for each diet	-1.4/ -1.2 mmHg prehypertensives: -0.9/ -0.9 mmHg hypertensives: -3.5/ -2.4 mmHg	0.002/ <0.001 0.047/ 0.01 0.006/ 0.008
Appel, 2005 (Omni-Heart)	db	164 US participants (55% African Americans), mean age 54 y	131/77	Prot rich diet (~50% plant prot) vs. fat rich diet	15 en%	+10 en%	0	-10 en%	6 weeks for each diet	-0.1/ -0.4 mmHg prehypertensives: 0.0/ -0.4 mmHg hypertensives: -0.2/ -0.5 mmHg	0.90/ 0.20 0.98/ 0.27 0.79/ 0.51
parallel trials											
Ferrara, 2006[33]	db	15 healthy men in exercise training project, aged 18-36	111/72 vs. 110/76	High vs. normal prot diet	15 en%	+7 en%	-10 en%	+3 en%	6 months	-2.1/ +0.9 mmHg	NS/ NS
Hendler, 1988[34]	…	17 healthy obese participants, mean age ~31	120/79 vs. 121/79	Pure prot, low caloric (440 kcal) diet vs. mixed low caloric diet	41 en%	+54 en%	-53 en%	-1 en%	3 weeks	-2/ -8 mmHg	NS/ NS

Table 2.3. *Trials of total protein intake and blood pressure (continued).*

Author, year	Blin-ding	Participants	Initial BP (mmHg, Interven-tion vs. control)	Type of intervention	Intake of protein in control group	ΔProtein	ΔCH	ΔFat	Duration of interven-tion	ΔSBP/ ΔDBP due to intervention	P-value
Parallel trials (continued)											
Meckling, 2007[37]	…	30 overweight/ obese women (premenopausal), aged 20-62	134/82 vs. 129/82	High prot low caloric (1383 kcal) diet vs. control low caloric (1391 kcal) diet	~16 en%	+8.2 en%	-12.9 en%	+4.8 en%	12 weeks	-3/ -4 mmHg*	…/ …
Meckling, 2007	…	30 overweight/ obese women (premenopausal), aged 20-62	134/82 vs. 129/82	High prot low caloric (1217 kcal) diet+ exercise vs. control low caloric (1260) diet + exercise	~18 en%	+19.0 en%	-14.6 en%	-3.3 en%	12 weeks	0/ 0 mmHg*	…/ …
Burke, 2001[30]	o	41 Australian treated hypertensives, mean age ~57 y[1]	133/75	Soy prot suppl vs. maltodextrin supplement (2x2 RCT with soluble fiber)	12 en%	+11 en%	-13 en%	+2 en%	8 weeks	-5.9/ -2.6 mmHg	<0.01/ <0.01
Leidy, 2007[36]	o	46 obese women (8 drop-outs), aged 28-80	109/69 vs. 113/73	High protein (pork) low caloric (750 kcal) vs. normal protein (milk) low-caloric diet	18 en%	+12 en%	-12 en%	0	12 weeks	-2/ +2 mmHg	NS/ NS
Hodgson, 2006[35]	o	60 Australian participants, mean age 57 y[1]	129/80 vs. 134/77	CH replaced by lean red meat prot vs. maintaining normal diet	18.6 en%	+5.2 en%	-4.3 en%	-0.6 en%	8 weeks	-4.0/ -1.3 mmHg	0.02/ 0.25
Brinkworth, 2004[29]	…	64 obese type 2 diabetes patients, mean age ~62 y[1]	148/83 vs. 140/76	High vs. low prot diet; Both groups 8 weeks energy restricted (~6.7 MJ/ day) and 4 weeks energy balance	15 en%	+15 en%	-15 en%	0	12 weeks	-0.3/ -1.7 mmHg	NS/ NS

Table 2.3. *Trials of total protein intake and blood pressure (continued).*

Author, year	Blin-ding	Participants	Initial BP (mmHg, Interven-tion vs. control)	Type of intervention	Intake of protein in control group	ΔProtein	ΔCH	ΔFat	Duration of interven-tion	ΔSBP/ ΔDBP due to intervention	P-value
Parallel trials (continued)											
Muzio, 2007[42]	sb	100 obese participants with MetS, mean age ~52 y[1]	142/85 vs. 141/82	Low vs. high CH diet. Both diets providing a deficit of ~500 kcal	13 en%	+6 en%	-17 en%	+11 en%	5 months	-6/ -1 mmHg*	<0.05/ …
Pijls, 1999[40]	sb	121 type 2 diabetes patients, mean age ~63 y[1]	138/79 vs. 138/79	Counseling by dietician; reduced SFA alone vs. reduced SFA + reduced prot (isocaloric)	0.95 g/kg/d	+0.19 g/kg	+1 g/d	SFA: +2.9 g/d; UFA: +5 g/d	6 months	+5.4/+4.6 mmHg	0.07/ <0.01
Delbridge, 2009[43]	…	141 obese (≥27 kg/m^2) men and women aged 18-75 y	135/85 vs. 131/83	High protein diet vs. high CH diet after 12 weeks of low caloric diet (≈500-550 kcal/d)	22 en%*	+6 en%*	-12 en%*	+2 en%*	12 months	-4.6/ -1.1 mmHg	0.04/ 0.58
Gardner, 2007[41]	sb	311 obese women (premenopausal), aged 25-50 y	116/75	Comparison of several weight loss diets; Atkins (AT) vs. Zone (ZO)	18 en%	0.6 en%	-10.9 en%	9.8 en%	12 months	-4.3/ -2.3 mmHg	…/…
				Atkins vs. LEARN (LE)	18.5 en%	2.5 en%	-12.7 en%	+11.4 en%		-4.5/ -2.2 mmHg	…/…
				Atkins vs. Ornish (OR)	18.3 en%	+2.3 en%	-17.9 en%	+14.5 en%		-5.7/ -3.7 mmHg	…/…

*o=open; sb=single-blind; db=double-blind; MetS=metabolic syndrome; SBP=systolic blood pressure, DBP=diastolic blood pressure, M=men, F=women, en%=energy percentage; CH=carbohydrates; prot=protein; SFA=saturated fat, UFA=unsaturated fat; NS=not statistically significant (p>0.05); … = value not given; *Best guess on basis of graph/implicit data in paper.*

[1]Users of anti-hypertensive medication were not excluded.

trial in which 121 type 2 diabetes patients received counselling on normal or reduced protein intake, an increase in blood pressure was found (+5.4 mmHg systolic, p=0.07).[40] However, the low range of intake may have influenced the results. Another large parallel trial among 311 obese women, in which different weight loss diets were compared, showed a decrease in systolic blood pressure of -5.7 mmHg systolic (p value not given).[41] However, contrast in protein intake was low (2.3 en%), and blood pressure decrease may be a result of exchange in carbohydrates and fat instead of increase in protein intake. Other large studies showed a decrease in blood pressure on a high protein diet, although no clear dose-response relation could be distinguished.[5,42,43] In 100 obese participants with metabolic syndrome, systolic blood pressure changed -6 mmHg ($p<0.05$) with 6 en% higher protein intake [42], and in 141 obese adults 6 en% higher protein intake resulted in a blood pressure change of -4.6 mmHg (p=0.04) [43].

In almost all trials the high protein diet was compared with a high carbohydrate diet. The only study in which two different control diets were included was the OmniHeart trial.[5] In this 6-week, fully controlled cross-over feeding trial in 164 healthy US adults partial substitution of carbohydrates (10 en%) with protein significantly lowered systolic blood pressure with -1.4 mmHg systolic (p=0.002). No difference in blood pressure response was observed when the protein-rich diet was compared with a diet high in mono-unsaturated fat (-0.1 mmHg systolic, p=0.90). Recently, a trial was conducted in which only a high fat diet was included as control diet.[38] In this trial, however, the number of participants was very low (n=17), and the systolic blood pressure effect of -9 mmHg may be a chance finding.

In conclusion, the results of trials suggest that increased intake of protein may be beneficial to blood pressure, although no clear dose – response association could be distinguished. From the results of the OmniHeart study, the only trial in which two different isocaloric control diets (high in carbohydrates and high in fat) were used, a conclusion can be drawn that both protein and mono-unsaturated fat have blood pressure lowering properties. However, it is also possible that a reduced intake of carbohydrates, rather than a higher intake of mono-unsaturated fat or protein, is responsible for a reduced blood pressure. In a trial on macronutrients and blood pressure it is important to keep energy intake in both treatment groups constant, to rule out blood pressure effects of energy and change in weight. Measurements of blood pressure effects after high intake of one of the macronutrients, therefore, will always be relative to the intake of the other two macronutrients, and the answer to the question whether total protein intake itself influences blood pressure may never be given, unless specific mechanisms are found through which protein intake may affect blood pressure.

Table 2.4. *Observational studies of plant protein intake and blood pressure.*

Author, year	respondents	Initial BP (mmHg)	Habitual plant protein intake	Dietary assess-ment	BP outcome (SBP/ DBP)	BP outcome per 11 g/d or 1.4 en%	P-value	Statistical adjustment
Cross-sectional studies								
Joffres, 1987[44] (Honolulu heart study)	615 men of Japanese ancestry	~…/…	…	24h recall	-4.6/ -1.8 mmHg for Q4 (36-78 g/d) vs. Q1 (4-21 g/d)	~ -1.14/ ~ -0.44 per 11 g/d	0.006/ 0.02	Age, BMI
Wang, 2008[20] (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	5 en%	2x 24h recall	-0.98/ -0.70 mmHg per en%	~ -1.37/ ~ -0.98 mmHg per 1.4 en%	<0.01/ <0.01	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, energy intake, intake of Ca and K, urinary creatinine, urinary Na
He, 1995[14]	827 Chinese adults mean age ~38[1]	~113/70	~9 en% (≈76 g/d)	3x24-h recall	-1.6/ -1.3 mmHg per SD (=44 g/d)	~ -0.41/ ~ -0.32 mmHg per 11 g/d	NS/ NS	age, BMI, alcohol, urinary Na, energy intake, residential area
Elliott, 2006[11] (INTERMAP)	4680 respondents from China, Japan, UK and USA aged 40-59 y[1]	119/74	China: 10 en% Other countries: 5-7 en%	4x24h recall	-1.11/ -0.71 mmHg per 2.8 en% (2 SD) -1.01/ -0.95 mmHg per 2.8 en% (2 SD)	 ~ -0.51/ ~ -0.48 mmHg per 1.4 en%	<0.01/ <0.05 NS/ <0.05	Age, sex, weight, height, exercise, alcohol, sample, history CVD or DM, family history of hypertension, special diet, supplement use, 24h urinary Na, K Additionally adjusted for: intake of Ca, SFA, PUFA, cholesterol, fiber
Umesawa, 2009[19]	7585 Japanese men and women aged 40-69 y[1]	M: 137/83; F: 135/81	M: 40 g/d F: 31 g/d	Single 24h recall	+0.59/ -0.31 mmHg per 13.1 g/d M: +0.64/ -0.26 mmHg per 14.0 g/d F: +0.46/ -0.41 mmHg per 10.8 g/d	~ +0.50/ ~ -0.26 mmHg per 11 g/d M: ~ +0.50/ ~ -0.20 mmHg per 11 g/d F: ~ +0.47/ ~ -0.41 mmHg per 11 g/d	<0.05/ NS NS/ NS NS/ <0.05	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of sodium, potassium, and calcium
Masala, 2008[15] (EPIC)	7601 Italian women aged 35-64 y	123/79	…	FFQ	+1.18/ -0.23 mmHg per log(g/d)	~ +3.79/ ~ -0.74 mmHg per 11 g/d	0.28/ 0.73	Age, BMI, waist circumference, smoking, education, physical activity, energy intake and intake of animal protein

Table 2.4. *Observational studies of plant protein intake and blood pressure (continued).*

Author, year	respondents	Initial BP (mmHg)	Habitual plant protein intake	Dietary assess-ment	BP outcome (SBP/ DBP)	BP outcome per 11 g/d or 1.4 en%	P-value	Statistical adjustment
Prospective studies								
Wang 2008, (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	5 en%	2x 24h recall	Change from baseline to 6 months: -0.53/ -0.37 mmHg per en%	~ -0.74/ ~ -0.52 mmHg per 1.4 en% per 6 months	0.08/ 0.09	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, intake of Ca and K, urinary creatinine, urinary Na + 6-month changes in several variables
Wang 2008, (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	5 en%	2x 24h recall	OR (95%-CI) for hypertension= 0.79 (0.60-1.02) per en%	NA	0.08	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, intake of Ca and K, urinary creatinine, urinary Na
Stamler, 2002[21]	1714 men aged 40-55[1]	135/87	3.5 en%	FFQ	-0.24/ -0.14 mmHg per year per en%	~ -0.34/ ~ -0.19 per 1.4 en%	<0.01/ <0.01	Age, height, weight (+ change), education, alcohol, smoking
Alonso, 2006[23] (SUN)	5880 Hispanic, university graduates, mean age ~36 y	…	…	FFQ	HR (95%-CI) = 0.8 (0.5; 1.2) for Q5 vs. Q1 of energy adjusted protein intake	NA	0.46	Age, sex
					Multivariable HR (95%-CI) = 0.5 (0.2; 0.9) for Q5 vs. Q1 of energy adjusted protein intake		0.06	Age, sex, BMI, exercise, alcohol, smoking, hypercholesterolemia, and intake of total energy, Na, fiber, caffeine, magnesium, potassium, low-fat dairy, MUFA, SFA

BP=blood pressure, SBP=systolic blood pressure, DBP=diastolic blood pressure, M=men, F=women, en%=energy percentage; 95%-CI=95% confidence interval, MUFA=monounsaturated fat, PUFA=polyunsaturated fat, SFA=saturated fat, Na=sodium, K=potassium, Ca=calcium, BMI=body mass index; NS=not statistically significant (p>0.05) ; … = value not given.

[1]*Users of anti-hypertensive medication were not excluded*

Dietary plant protein and blood pressure: observational data

The association between dietary plant protein and blood pressure or hypertension was examined in 8 observational studies (**Table 2.4**). Most cross-sectional studies showed an inverse association [11,14,15,19,20,44], and this was confirmed in prospective studies [20,21,23]. In a prospective study among 1714 men a systolic blood pressure difference of -0.34 mmHg per year per 1.4 en% ($p<0.01$) was found after a follow-up of 8 y.[21] It should be noted, however, that estimates were not adjusted for important potential confounders like sodium and potassium. In two other studies, in which estimates were adjusted for these confounders, a 21% reduction in hypertension risk per en% of plant protein intake ($p=0.08$) was found after 18 months of follow-up in 810 untreated pre- or mild hypertensives of the PREMIER study [20], and a 50% lower 2 year hypertension risk for the highest quintile of plant protein intake versus the lowest quintile ($p=0.06$) was found in 5880 university graduates of the SUN cohort [23].

In conclusion, results from observational studies indicate an inverse association between dietary plant protein and blood pressure. However, despite adjustment for many potential confounders in multivariable models, residual confounding (e.g. by other macronutrients, fiber or flavonoid intake) in observational studies cannot fully be excluded.

Dietary animal protein and blood pressure: observational data

In 7 observational studies the relationship between dietary animal protein and blood pressure was investigated (**Table 2.5**), with results from cross-sectional studies being inconclusive [11,15,19,20,45]. In studies with a prospective design no association or only weak associations were observed, with systolic blood pressure differences of -0.06 mmHg per 2.9 en% ($p=0.84$) after 6 months in 810 untreated pre- or mild hypertensives [20], and +0.16 mmHg per 2.9 en% per year ($p<0.01$) in 1714 men.[21] Furthermore, no difference in hypertension risk with high intake of animal protein was observed in 5880 university graduates of the SUN cohort.[23]

In conclusion, observational studies provide no evidence for an association of animal protein with blood pressure. However, also for these studies, despite inclusion of many potential confounders in their multivariate model, residual confounding (e.g. by intake of other macronutrients or salt) cannot be excluded.

Biomarkers of dietary plant protein or animal protein and blood pressure: observational data

We did not find any studies that used a biomarker specifically for plant protein intake. With regard to animal protein intake, urinary excretion of 3-methylhistidine (3-MH) has been suggested as marker of meat consumption because it is synthesized in the muscle of mammals and released and excreted in urine after intake of muscle protein.[46] Six cross-sectional stu-

Table 2.5. *Observational studies of animal protein intake and blood pressure.*

Author, year	respondents	Initial BP (mmHg)	Habitual animal protein intake	Dietary assessment	BP outcome (SBP/ DBP)	Standardized BP outcome per 23 g/d or 2.9 en% (SBP/DBP)	P-value	Statistical adjustment
Cross-sectional studies								
Zhou, 1994[45]	705 rural Chinese aged 45-59 y[1]	~117/75	0.1 to 5.3 en%	24h recall	Inverse association (only standardized regression coefficients presented in the paper)	…	…	Age, BMI, heart rate, alcohol
Wang, 2008[20] (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	11 en%	2x 24h recall	+0.08/ -0.03 mmHg per en%	~ +0.23/ ~ -0.09 mmHg per 2.9 en%	0.42/ 0.71	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, energy intake, intake of Ca and K, urinary creatinine, urinary Na
Elliott 2006[11] (INTER-MAP)	4680 respondents from China, Japan, UK and USA aged 40-59 y[1]	119/74	China: 2.5 en% Other countries: 9-10 en%	4x24 h recall	+0.20/ -0.02 mmHg per 5.8 en% (2 SD) +0.22/ +0.25 mmHg per 5.8 en% (2 SD)	 ~ +0.11/ ~ +0.12 mmHg per 2.9 en%	NS/ NS NS/ NS	Age, sex, weight, height, exercise, alcohol, sample, history CVD or DM, family history of hypertension, special diet, supplement use, 24h urinary Na, K Additionally adjusted for: intake of Ca, SFA, PUFA, cholesterol, fiber
Umesa-wa, 2009 [19]	7585 Japanese men and women aged 40-69 y[1]	M: 137/83 F: 135/81	M: 43 g/d F: 35 g/d	Single 24h recall	-0.56/ -0.17 mmHg per 19.9 g/d M: -0.29/ -0.11 mmHg per 22.3 g/d F: -0.80/ -0.23 mmHg per 16.4 g/d	~ -0.64/ ~ -0.20 mmHg per 23 g/d M: ~ -0.30/ ~ -0.11 mmHg per 23 g/d F: ~ -1.12/ ~ -0.32 mmHg per 23 g/d	<0.05/ NS NS/ NS <0.001/ NS	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of sodium, potassium, and calcium
Masala, 2008 [15] (EPIC)	7601 Italian women aged 35-64 y	123/79	…	FFQ	+0.99/ +0.58 mmHg per log(g/d)	~ +3.18/ ~ +1.87 mmHg per 23 g/d	0.21/ 0.23	Age, BMI, waist circumference, smoking, education, physical activity, energy intake and intake of plant protein

Table 2.5. *Observational studies of animal protein intake and blood pressure (continued).*

Author, year	respondents	Initial BP (mmHg)	Habitual animal protein intake	Dietary assessment	BP outcome (SBP/ DBP)	Standardized BP outcome per 23 g/d or 2.9 en% (SBP/DBP)	P-value	Statistical adjustment
Prospective studies								
Wang 2008 (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	11 en%	2x 24h recall	Change from baseline to 6 months: -0.02/ +0.01 mmHg per en%	~ -0.06/ ~ +0.03 mmHg per 2.9 en% per 6 months	0.84/ 0.97	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, intake of Ca and K, urinary creatinine, urinary Na + 6-month changes in several variables
Wang 2008 (PREMIER)	810 untreated pre- or mild hypertensives aged 25-79 y	135/85	11 en%	2x 24h recall	OR (95%-CI) for hypertension= 0.99 (0.93-1.07) per en%	NA	0.90	Age, sex, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline blood pressure, alcohol, intake of Ca and K, urinary creatinine, urinary Na
Stamler 2002[21]	1714 men, aged 40-55[1]	135/87	11.5 en%	FFQ	+0.06/ -0.002 mmHg per year per en%	~ +0.16/ ~ -0.01 mmHg per 2.9 en%	<0.01/ 0.44	Age, height, weight (+ change), education, alcohol, smoking
Alonso 2006[23] (SUN)	5880 Hispanic, university graduates, mean age ~36 y	…	…	FFQ	HR for hypertension (95%-CI) = 1.1 (0.7; 1.6) for Q5 vs. Q1 of energy adjusted protein intake Multivariate HR (95%-CI) = 1.0 (0.6; 1.8)	NA	0.84 0.70	Age, sex Age, sex, BMI, exercise, alcohol, smoking, hypercholesterolemia, and intake of total energy, Na, fruit, vegeTables, fiber, caffeine, magnesium, potassium, low-fat dairy, MUFA, SFA

BP=blood pressure; SBP=systolic blood pressure; DBP=diastolic blood pressure; en%=energy percentage; 95%-CI=95% confidence interval; MUFA=monounsaturated fat; PUFA=polyunsaturated fat; SFA=saturated fat; Na=sodium; K=potassium; Ca=calcium; BMI=body mass index; CVD=cardiovascular disease; DM=diabetes mellitus; Q=quintile; NS=not statistically significant (p>0.05) ; … = value not given.

[1]Users of anti-hypertensive medication were not excluded.

Table 2.6. *Observational studies of biomarkers of animal protein intake and blood pressure.*

Author, year	Respondents	Initial BP (mmHg)	Initial biomarker level	Biomarker assessment	BP outcome (SBP/DBP)	P-value	Statistical adjustment
Cross-sectional studies							
Liu, 2000b[76] (CARDIAC)	619 Chinese subjects aged 48-56 y	120/70	3MH=211 µmol/d	24h urinary 3MH	-3.25/ -2.86 mmHg/ 88 µmol/d BMI<26 kg/m^2 (n=497): -2.39/ -2.24 mmHg/88 µmol/d BMI ≥ 26 kg/m^2 (n=30): -6.75/ -4.82 mmHg/88 µmol/d	<0.01/ <0.01 <0.01/ <0.01 0.03/ 0.04	Age, sex, BMI, alcohol urinary Na, urinary K
Zhou, 1994[45]	705 rural Chinese aged 45-55 y	~115/73	…	Overnight urinary 1MH	Inverse association with SBP (standardized regression coefficients)	…	Age, BMI, heart rate, ethnic groep
Liu, 2002[77] (CARDIAC)	1135 Chinese subjects aged 48-56 y	122/73	3MH=198 µmol/d	24h urinary 3MH	-0.02/ -0.02 mmHg/µmol/d	0.048/ 0.01	Age, sex, BMI, urinary Na/K, urinary Ca, urinary Mg
Liu, 2002 (CARDIAC)	1135 Chinese subjects aged 48-56 y	122/73	3MH:Cr=191 µmol/mg	24h urinary 3MH:Cr (µmol/mg)	-0.02/ -0.02 mmHg/unit	0.02/ 0.01	Age, sex, BMI, urinary Na/K, urinary Ca, urinary Mg
Liu, 2000a (CARDIAC)	1151 Chinese subjects aged 48-56 y	120/71	3MH:Cr=216 µmol/g	24h urinary 3MH:Cr ratio (µmol/g)	-0.046/ -0.039 mmHg/unit	0.001/ <0.001	Age, sex
Liu, 2001[78] (CARDIAC)	1614 Chinese subjects from 4 different ethnic groups, aged 48-56 y	~129/79	3MH:Cr= 142 to 258 µmol/mg	24h urinary 3MH:Cr ratio (µmol/mg)	-0.04 to -0.25/ -0.10 to -0.36 (Partial correlation coefficients)	<0.01/ <0.01	Age, sex, urinary Na
Liu, 2000a[79] (CARDIAC)	1681 Japanese subjects aged 48-56 y	124/75	3MH:Cr=206 (µmol/g)	24h urinary 3MH:Cr ratio (µmol/g)	…/ -0.008 mmHg/unit	NS/ 0.012	Age, sex
Liu, 2002[77] (CARDIAC)	1991 Chinese subjects aged 48-56 y	123/73	…	24h urinary 3MH	OR for hypertension(95%-CI) = 0.60 (0.40; 0.90) for ≥253 vs. <253 µmol/d	0.01	Age, sex, BMI, urinary Na/K, urinary Ca, urinary Mg

Table 2.6. *Observational studies of biomarkers of animal protein intake and blood pressure (continued).*

Author, year	Respondents	Initial BP (mmHg)	Initial biomarker level	Biomarker assessment	BP outcome (SBP/DBP)	P-value	Statistical adjustment
Cross-sectional studies (continued)							
Liu, 2002 (CARDIAC)	1991 Chinese subjects aged 48-56 y	122/73	…	24h urinary 3MH:Cr (µmol/mg)	OR for hypertension(95%-CI) = 0.38 (0.24; 0.59) for ratio ≥224 vs. <224	<0.01	Age, sex, BMI, urinary Na/K, urinary Ca, urinary Mg
Yamori, 1990[80] (CARDIAC)	7334 subjects from 20 different countries aged 50-54 y	…	…	24h urinary 3MH:Cr ratio (mol/mol)	-568/ -339 mmHg/unit	<0.05/ <0.05	Unadjusted

cs=cross-sectional; BP=blood pressure; SBP=systolic blood pressure; DBP=diastolic blood pressure; Na=sodium; K=potassium; Ca=calcium; Mg=magnesium; BMI=body mass index; 1MH=1-methylhistidine; 3MH=3-methylhistidine; Cr=creatinine; NS=not statistically significant (p>0.05).

dies included in this review used urinary 3-MH excretion to estimate animal protein intake in predominantly Asian populations (**Table 2.6**). Overlap between studies may exist, since all populations formed part of the study population of the World Health Organization Cardiovascular Disease and Alimentary Comparison (CARDIAC) study, which is an international population-based cross-sectional study in more than 20 countries, among which are China and Japan. All studies showed inverse associations with blood pressure. However, because studies were conducted mainly in Asian populations, results may not be generalizable to other populations. Furthermore, urinary 3-MH may partly reflect muscle catabolism in the human body itself, i.e. during starvation, cachexia, or heavy physical activity.[47] This phenomenon was not taken into account in the various studies, and overestimation of associations between animal protein and blood pressure could have occurred. The findings of these biomarker studies, therefore, should not be overemphasized. A challenge for future protein research will be to find reliable biomarkers for plant and animal protein and intake of protein from specific dietary sources.

Dietary plant protein or animal protein and blood pressure: trial data

The blood pressure response after protein intake from plant and animal sources was investigated in only 2 randomized controlled trials (**Table 2.7**). A systolic blood pressure effect of +1 mmHg systolic (p=0.90) was seen in 23 type 2 diabetics after a diet containing protein only from plant sources (from soy, vegetables, and legumes) compared to a diet in which 60% of the plant protein was replaced by animal protein (from beef, poultry, fish, and milk).[48] However, the number of 23 participants is low, and this blood pressure effect was not significant. Furthermore, these participants suffered from albuminuria, which may have influenced the results on blood pressure. In 49 healthy students a soy protein isolate resulted in a non significant systolic blood pressure response of +0.6 mmHg (p-value unknown) compared to a casein protein isolate.[49] However, because in this trial only soy protein and casein protein were investigated, we cannot extrapolate these findings to plant protein and animal protein from a mix of sources.

In summary, only 2 small trials evaluated the blood pressure effect of plant protein versus animal protein. More evidence on the blood pressure effect of plant and animal protein is needed from large randomized controlled blood pressure trials.

Dietary protein from specific sources and blood pressure

Only few observational studies addressed the relation of protein from specific sources (e.g. fish, meat) to blood pressure. In five studies the association with blood pressure was examined for urinary taurine [50-52] or serum taurine [45,53] which the authors regarded as a bi-

Table 2.7. *Trials on intake of types of protein and blood pressure.*

Author, year	Study design	Participants	Initial BP (plant protein intervention vs. animal protein intervention)	Type of intervention	ΔProtein	ΔCH	ΔFat	Duration of intervention	ΔBP due to intervention (SBP/ DBP)	P-value
Wheeler, 2002[48]	x, …	23 type 2 diabetes patients, with albuminuria[1]	151/85 mmHg	Meals with only plant prot vs. meals with 60% animal and 40% plant prot	0	0	0	6 weeks for each diet	+1/ +1 mmHg*	0.90/ 0.75
Brussaard, 1981[49]	p, …	49 healthy normotensive students	123/69 mmHg vs. 124/69 mmHg	Soy prot isolate vs. casein prot isolate (2:1)	0	0	0	4 weeks	+0.6/ +0.3 mmHg*	NS/ NS

*x=cross-over, p=parallel; SBP=systolic blood pressure, DBP=diastolic blood pressure, M=men, F=women, en%=energy percentage; CH=carbohydrates; prot=protein; NS=not statistically significant (p>0.05); … = value not given; *Best guess based on graph/implicit data in paper.*
[1]Users of anti-hypertensive medication were not excluded.

omarker of seafood protein intake (data not in Table). Three of these studies were conducted among Asian populations (n=705 to n=1,681) [45,51,52], whereas the others were conducted in Brazil (n=57) and USA (n=168).[50,53] In all these studies inverse associations with blood pressure were observed, but no information about the strength of the associations was given.

The blood pressure effect of meat protein was only investigated in two trials (data not in Table).[54,55] In a parallel trial among 64 hospital staff members, a diet with 40% of protein from meat sources (from beef, chicken, lamb, sausage, pork, and prawns) resulted in a non-significant blood pressure effect of -1.8 mmHg systolic and -1.2 mmHg diastolic (p-value not given) compared with a diet in which the meat protein was replaced by plant protein (from cereals, vegetables, legumes, and nuts).[54] In a small cross-over trial among 35 men no difference in blood pressure effect was seen (no p-value given) between a diet including 50% of protein from meat (from pork, beef, and chicken) compared with a diet in which the meat protein was replaced by non-meat protein (from vegetables, eggs, and dairy).[55]

Because isoflavones may influence blood pressure [56], several studies on soy could not be taken into account because observational data were not adjusted for isoflavone intake [57-61], or because, in trials, soy protein contained isoflavones [62-66]. To the best of our knowledge, there are at present no other studies on specific protein sources and blood pressure. Epidemiological studies and randomized controlled trials in this field are, therefore, warranted.

Dietary protein and blood pressure in subgroups of the population

In several studies specific subgroup analyses were conducted to identify subgroups whose blood pressure is more sensitive for protein intake. We explored, furthermore, whether differences in protein-blood pressure associations could be identified in the results of studies among specific populations.

In the OmniHeart trial the effect of total dietary protein was more pronounced in hypertensives than in prehypertensives (-3.5 mmHg versus -0.9 mmHg for systolic blood pressure). This difference of protein effect in subgroups of blood pressure could not be recognized in observational studies. In trials, however, populations with, on average, elevated blood pressure were more sensitive to the blood pressure lowering effect of protein than populations with, on average, normal blood pressure (Out of 9 trials in populations with elevated blood pressure [5,29,30,35,37,38,40,42,43] 7 trials showed a decrease in blood pressure with high protein intake [5,30,35,37,38,42,43], whereas out of 7 trials in populations with normal blood pressure [31-34,36,39,41] only 2 trials [34,41] showed a decrease).

With regard to age, in the INTERSALT study a stronger inverse association of urinary nitrogen with blood pressure was observed in respondents aged 40-59 y than in respondents aged 20

-39 y (systolic blood pressure: -0.9 mmHg/g versus -0.2 g/d).[25] Furthermore, inverse associations were found more often in studies conducted in participants aged over 50 (out of 5 studies [5,29,30,35,40], in 3 studies an inverse association or a blood pressure lowering effect was found [5,30,35]) than in studies conducted in younger participants (out of 9 studies [16,22,23,31-34,39,41], in 4 studies an inverse association was found [16,23,34,41]). However, the number of studies that were conducted among these specific populations was small, and solid conclusions cannot be drawn.

In a study on urinary 3-MH and blood pressure, the inverse association was more pronounced in respondents with a BMI higher than 26 kg/m^2 than in respondents with a normal BMI (Δ systolic blood pressure=-6.8 mmHg versus -2.39 mmHg per 88 µmol urinary 3-MH/d).[67] Among the other studies, however, only one study was explicitly conducted among normal weight respondents[14], so no conclusion can be drawn on difference in sensitivity related to weight, although studies in overweight/obese participants often showed inverse associations (Out of 11 studies [5,18,20,29,32,34-37,41,42], 7 studies showed an inverse association or a decrease in blood pressure with high protein intake [5,29,34,35,37,41,42]).

Finally, in two studies subgroup-analyses were conducted for men and women, but no effect modification was shown.[19,28] Also in studies that were specifically conducted in men [12,13,17,21,25,33] or women [36,37,41], no difference in sensitivity was seen.

In conclusion, the possible beneficial effect of protein intake on blood pressure seems stronger in people with higher initial blood pressure and, possibly, in older people. Additional predefined subgroup analyses in future epidemiologic studies and trials in which subgroups are compared, may provide better insight into the role of dietary protein in blood pressure.

Discussion

A reduction in systolic blood pressure of only 2 mmHg may already result in a 6% reduction in fatal stroke, and a 4% reduction fatal coronary heart disease (CHD).[2] Knowledge on the effect of dietary protein, therefore, may have an important public health impact. A substantial body of evidence suggests a, possibly weak, beneficial effect of total dietary protein on blood pressure, which may be most apparent in populations with elevated blood pressure and possibly older populations. We cannot exclude, however, that this effect is due to a lower carbohydrate intake. In observational studies more often an inverse association was found for plant protein than for animal protein. The beneficial effect of protein, therefore, may be mainly due to protein from plant sources. Data on protein from specific sources are too scarce to draw any conclusions.

The aim of the current systematic review was to give a comprehensive overview of the evidence on dietary protein and human blood pressure, published until June 2010. Papers were independently screened by 2 reviewers, and data of 46 studies were extracted using a predefined procedure. Several other reviews on protein and blood pressure have already been conducted in the past.[6-9] However, the most comprehensive review of these is already 14 years old.[9] Furthermore, the present review is the first to focus on possible blood pressure effects of different protein types and on sensitivity of population subgroups.

Several methodological issues of studies need to be addressed. First, in observational studies, even after extensive adjustment for potential confounders, residual confounding may exist from other nutrients associated with protein intake, or from energy, which is not only correlated to protein, but also to several other blood pressure-determinants like exercise, BMI, and dietary pattern. It is difficult to say how much the remaining confounding from known or unknown nutrients that are correlated to plant or animal protein have influenced the estimates in observational studies. Randomized controlled trials in which the effects of plant protein and animal protein are compared, keeping other nutrients constant, are needed. Second, a diet high in one type of protein (animal protein or plant protein) does not necessarily mean that the other protein type is replaced, as a diet may be high or low in both types of protein. Most of the observational studies investigating types of protein did not adjust their estimates for intakes of other protein types. In randomized trials these factors are more standardized.[68] Third, respondents in observational studies may be misclassified according to their self-reported protein intake, which may dilute the protein-blood pressure association.[69] Fourth, for investigation of long-term effects of protein on blood pressure, an observational study is the most suitable type of study, because of the costs of a trial. However, contrasts between high and low protein intake are often larger in trials than in observational studies. Short term effects of protein on blood pressure can, therefore, be more easily detected in trials. Finally, all observational studies were conducted in the general population, whereas trials were more often conducted in selected populations that are possibly more sensitive to blood pressure interventions. However, in several trials blood pressure was the secondary outcome [29,31-34,36,37,40-42,48]. If participants in these studies were not blinded for the results of the blood pressure-measurements, bias may have been introduced, because awareness of blood pressure may influence participants' lifestyle or other behaviour.

The underlying mechanism for a potential beneficial effect of protein on blood pressure has not yet been clarified. Several hypotheses have been put forward. First, dietary protein has been related to synthesis of cellular ion channels, which may indirectly influence the pathways in blood pressure regulation.[25] High protein intake may induce natriuresis, leading to lower blood pressure.[26,62,70] Second, experiments suggest that dietary protein or protein fractions could improve insulin sensitivity and thereby blood pressure.[71-73] Third, dietary protein supplementation may result in a higher concentration of the amino acids tyrosine and

tryptophan in regions of the brain or blood vessel wall, triggering a vasodilatory response.[7] The amino acid arginine, which is a substrate for nitric oxide, may play a role in vasodilatation, although it is unclear whether dietary intake of arginine is relevant in this respect.[73,75] Finally, as has already been stated in this review we cannot exclude that a lower blood pressure is related to a lower carbohydrate intake instead of a higher protein intake.

In conclusion, evidence suggests a small beneficial effect of protein on blood pressure, especially for plant protein. More data on protein from specific sources like dairy, grain or nuts and data in population subgroups should be obtained from epidemiological studies. Furthermore, there is a need for blood pressure trials that focus on plant and animal protein and protein from specific sources. Preferably, these trials should be conducted in untreated (pre) hypertensive people. Finally, studies aimed at potential blood pressure lowering mechanisms related to protein intake are warranted.

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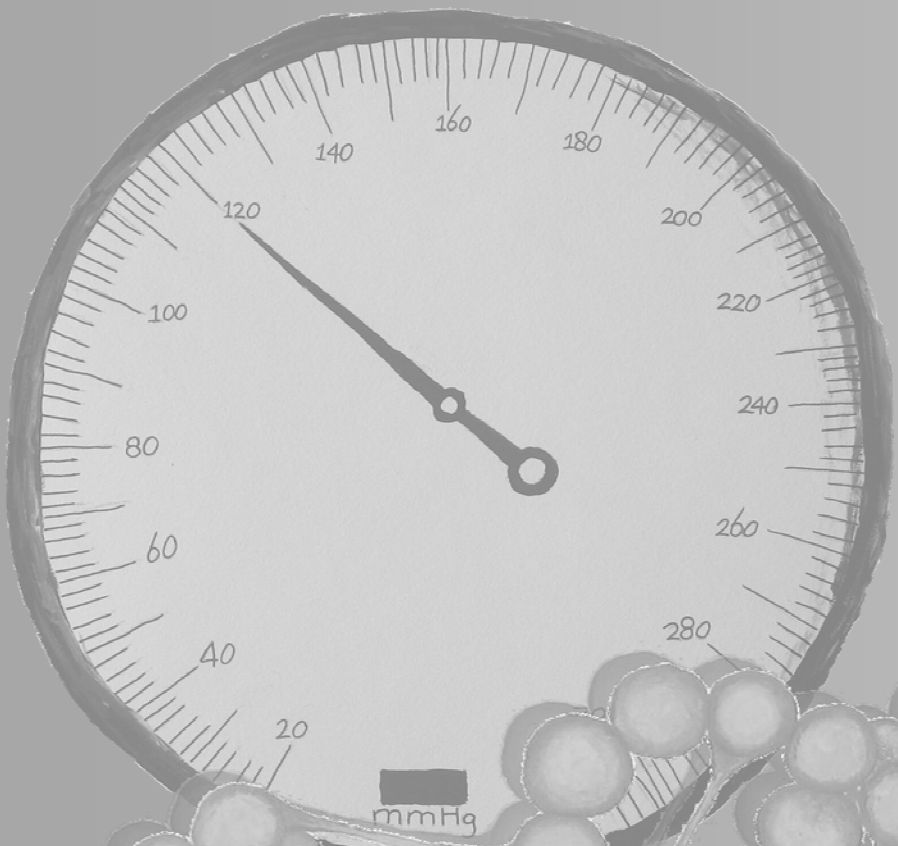
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Chapter 3

Sources of dietary protein in relation to blood pressure in a general Dutch population

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Abstract

Background

Little is known about the relation of different dietary protein types with blood pressure**.** We examined whether intake of total, plant, animal, dairy, meat, and grain protein was related to blood pressure in a cross-sectional cohort of 20,820 Dutch adults, aged 20-65 y and not using antihypertensive medication.

Design

Mean blood pressure levels were calculated in quintiles of energy-adjusted protein with adjustment for age, sex, BMI, education, smoking, and intake of energy, alcohol, and other nutrients including protein from other sources. In addition, mean blood pressure difference after substitution of 3 en% carbohydrates or MUFA with protein was calculated.

Results

Total protein and animal protein were not associated with blood pressure (p_{trend}=0.62 and 0.71 respectively), both at the expense of carbohydrates and MUFA. Systolic blood pressure was 1.8 mmHg lower (p_{trend}<0.01) in the highest (>36 g/d) than in the lowest (<27 g/d) quintile of plant protein. This inverse association was present both at the expense of carbohydrates and MUFA and more pronounced in individuals with untreated hypertension (-3.6 mmHg) than in those with normal (+0.1 mmHg) or prehypertensive blood pressure (-0.3 mmHg; $p_{interaction}$<0.01). Meat and grain protein were not related to blood pressure. Dairy protein was directly associated with systolic blood pressure (+1.6 mmHg, p_{trend}<0.01), but not with diastolic blood pressure (p_{trend}=0.24).

Conclusions

Total protein and animal protein were not associated with blood pressure in this general untreated Dutch population. Plant protein may be beneficial to blood pressure, especially in people with elevated blood pressure. However, because high intake of plant protein may be a marker of a healthy diet and lifestyle in general, confirmation from randomized controlled trials is warranted.

Introduction

Elevated blood pressure is a major risk factor for cardiovascular disease and renal impairment. It has been estimated that already from systolic blood pressure levels as low as 115 mmHg onward, risk of cardiovascular disease increases linearly with increasing blood pressure.[1] Therefore, health authorities emphasize the importance of dietary and lifestyle interventions beneficially influencing blood pressure including physical activity, obtaining a healthy body weight, moderate alcohol consumption, reduced salt intake, and increased potassium intake.[2,3] More recently, interest has grown into dietary patterns and macronutrient intakes, including dietary protein.[4,5] A substantial body of evidence suggests a, possibly weak, beneficial effect of protein on blood pressure, although findings are not conclusive.[6,7]

Protein intake is a rather heterogeneous exposure and types of protein (i.e. animal and plant protein and protein from specific sources like dairy, meat, grain) might differentially influence blood pressure. In several observational studies [8-14] the association with blood pressure was investigated separately for plant protein and animal protein. Results were inconclusive, although there is a trend towards a slightly more beneficial effect of plant protein than of animal protein on blood pressure. Data on specific protein sources in relation to blood pressure are scarce. We observed no association between intake of dairy, meat, and grain protein with 6-year incidence of hypertension in a previous analysis including 2241 adults (≥55 y) from the population based Rotterdam Study.[12] He et al. recently published findings of a randomized, double-blind cross-over trial among 352 prehypertensive and hypertensive participants in which blood pressure effects of supplementation with soy protein, milk protein and complex carbohydrates was investigated.[15] Compared with carbohydrate, soy protein and milk protein (40g/d) resulted in a -2.0 mmHg and -2.3 mmHg net change in systolic blood pressure, respectively, but the achieved blood pressure reductions did not differ between soy and milk protein supplementation.

blood pressure response to protein intake may differ between population subgroups, which may be an important issue because of public health recommendations.[5,16] In the INTERSALT study among 10,020 adults from 32 countries the inverse association between protein intake and blood pressure was more pronounced in participants aged >40 y than in younger participants.[16] Furthermore, in the OmniHeart trial in 164 adults, blood pressure reductions during a high-protein diet were larger in hypertensive participants than in prehypertensive participants.[5] However, more research is needed to be able to draw firm conclusions on potentially sensitive population subgroups.

In the present analysis, we examined whether intake of total protein, plant protein, animal protein, and protein from specific sources was related to blood pressure level in a general Dutch population of 20,820 adults. With respect to protein sources our main focus was on protein from dairy, meat, and grain, as these are the main sources of animal and plant pro-

tein in the Netherlands.[17] Additionally, we assessed whether protein-blood pressure associations were modified by gender, age, BMI, and blood pressure level.

Methods

Study population

We used data from the population-based Monitoring Project on Risk Factors for Chronic Diseases (MORGEN project), which is part of the Dutch EPIC cohort. Details of the study have been described elsewhere.[18] In brief, between 1993 and 1997 22,606 men and women aged 20-65 y completed questionnaires on diet, lifestyle, and health and underwent a physical examination. The Medical Ethics Committee of the Netherlands Organization for Applied Scientific Research (TNO) approved the study protocol and all participants signed informed consent form. We excluded 16 participants with missing data on blood pressure and 1,093 participants who used antihypertensive medication. Additionally, we excluded 677 participants who were diabetic, had a history of myocardial infarction or stroke, or were pregnant, leaving 20,820 men and women for the present analyses.

Dietary assessment and exposure categories

Dietary intake was assessed using a self-administered semi-quantitative food frequency questionnaire (FFQ) on 178 foods and beverages consumed during the preceding year.[19] Colored photographs were used to facilitate estimation of portion sizes, and seasonal variation in food intake was taken into account. Total energy and nutrient intakes were calculated using an extended version of the Dutch Food Composition Table of 1996.[20]

Animal protein was defined as protein from dairy, meat, fish, eggs, and animal protein from mixed dishes. Plant protein included protein from grain, potatoes, fruits, vegetables, nuts, legumes, soy, and plant protein from mixed dishes. Dairy protein was calculated as protein from all kind of milk, yogurt, coffee creamer, curd, pudding, porridge, custard, ice-cream, whipped cream, and cheese. Meat protein included protein from all meat and meat based products, and grain protein was calculated as plant protein from rice, bread, pasta and bakery products. In addition, we calculated protein from potatoes (including fries), vegetables, fruits, and legumes (without green beans and peas).

In a validation study among 63 men and 58 women good reproducibility was shown for energy adjusted total protein intake with Pearson correlation coefficients of 0.73 in men and 0.70 in women.[21] The relative validity of the FFQ was assessed against 12 monthly 24-h recalls over a 1-year period. Pearson correlation coefficients for energy adjusted total protein intake after correction for intra-individual variation were 0.71 for men and 0.67 for

women.[21] Energy adjusted total protein intake as assessed from the FFQ also correlated well with urinary nitrogen excretion in four 24h urine samples at 3-month intervals, i.e. Pearson correlation coefficients of 0.56 for men and 0.69 for women. [21] For types of protein the FFQ was not validated, but correlations for milk and milk products and bread, as surrogate markers for dairy and grain protein, were good (all r>0.65), whereas correlations for meat were lower, especially for men (r_{men}=0.39; r_{women}=0.59).[19]

Blood pressure

Systolic and diastolic blood pressure (first and fifth Korotkoff sounds, respectively) was measured by trained nurses using a random zero sphygmomanometer on the left arm in supine position, after a 5-minute rest. Blood pressure was measured twice, 30 seconds apart, and the mean of the two readings was used. During physical examination, regular audits were performed to check adherence to the blood pressure measuring protocol (e.g. resting time, adequate cuff size). Normotension was defined as systolic blood pressure ≤120 mmHg and diastolic blood pressure ≤80 mmHg. Hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg (participants using antihypertensive medication were excluded). All other participants were considered to be prehypertensive.

Lifestyle factors

Body weight (to nearest 0.1 kg) and height (to nearest 0.5 cm) were measured with participants wearing light indoor clothing without shoes and body mass index (BMI) was calculated (kg/m^2). Data on age, gender, education, lifestyle factors, history of major diseases, medication use, and any prescribed diets were collected by questionnaires. A questionnaire on physical activity pattern in the preceding year was introduced in 1994 and was completed by 16,073 participants (77%) of our cohort. Participants were classified in categories of alcohol intake (none, moderate, high), smoking status (current smoker/non-smoker), educational level (3 categories), and physical activity (4 categories, ranging from inactive to very active[18]).

Statistical analysis

Data analysis was performed using SAS version 9.1 (SAS Institute Inc.). Protein intake was first adjusted for total energy intake according to the residual method.[22] Baseline characteristics of the study population were calculated in quintiles of energy-adjusted total protein intake, and are presented as means ± standard deviation, percentages, or medians with interquartile range.

We used general linear models to calculate average blood pressure levels with 95% confidence intervals (CI) in quintiles of energy-adjusted protein intake (total, animal, plant, dairy, meat and grain). The basic model (model 1) included age and gender. In model 2, further adjustment was made for BMI, education, smoking, and alcohol consumption. The fully adjusted model (model 3) additionally included daily intake of total energy, saturated fatty acids, carbohydrates, fiber, calcium, magnesium, potassium, and protein intake from other sources than the one under study. Because grain comprised only 48% of plant protein intake we conducted post hoc analyses in which we calculated fully adjusted mean blood pressure in tertiles of dietary protein intake from potatoes, vegetables, fruits, and legumes.

To investigate whether physical activity confounded the protein-blood pressure associations, post hoc analyses were conducted per 5 grams of total, animal and plant protein in the subgroup with data on physical activity using the full model (model 3) with or without additional adjustment for physical activity. In addition we performed substitution analyses to investigate the blood pressure difference with exchange of nutrients. By including total protein and carbohydrate as continuous variables in the same multivariable model (model 3) we investigated the blood pressure difference with 3 energy percentage (en%) higher total protein intake at the expense of carbohydrates. The difference in the coefficients of total protein and carbohydrates plus their covariance was used to estimate blood pressure difference and 95% confidence interval for the substitution. Similarly we investigated the blood pressure difference of 3 en% higher total protein at the expense of mono-unsaturated fat. The same substitution analyses were performed for animal protein and plant protein.

Finally, for total, plant and animal protein, pre-defined subgroup analyses were performed in strata of gender, age (<50 y and ≥50 y), BMI (<25 kg/m^2 and ≥25 kg/m^2), and blood pressure level (normotensives, prehypertensives and untreated hypertensives), using the full model (model 3).

Results

Descriptive statistics

The mean age of the population was 42 ± 11 y and 45% were men. Average blood pressure was 120.0 ± 15.6 mmHg systolic and 76.1 ± 10.4 mmHg diastolic, and 15% of the population had untreated hypertension. The mean energy-adjusted total protein intake of the study population was 84 ± 12 g/d (~15 energy%), with 52 ± 13 g/d derived from animal sources. After energy adjustment of dietary protein, age and sex adjusted Pearson partial correlation coefficients were 0.89 for total protein with animal protein, 0.07 for total with plant protein, and -0.39 for plant protein with animal protein. Major sources of animal protein intake were

Table 3.1. *Characteristics by quintiles of energy adjusted total protein intake of 20,820 Dutch adults.*

	Quintiles of energy adjusted total protein intake (g/d)				
	<74 (n=4173)	**74 to 81 (n=4166)**	**81 to 86 (n=4159)**	**86-93 (n=4166)**	**>93 (n=4156)**
Median intake, g/d	70	78	83	89	98
Age, y	41 ± 11	42 ± 11	42 ± 11	43 ± 11	43 ± 11
Gender, % male	49	43	42	43	50
High education, %	22	26	26	26	22
Systolic BP, mmHg	119.5 ± 15.6	119.5 ± 15.8	119.6 ± 15.9	120.8 ± 15.7	120.7 ± 15.3
Diastolic BP, mmHg	75.8 ± 10.3	75.7 ± 10.3	76.0 ± 10.4	76.7 ± 10.5	76.5 ± 10.3
Hypertension, %[1]	13.6	14.1	14.5	15.8	15.6
Body mass index, kg/m^2	24.0 ± 3.6	24.5 ± 3.8	24.9 ± 3.7	25.2 ± 3.8	25.8 ± 4.1
Overweight, %	35	38	43	48	53
High physical activity, %[2]	9 ± 12	8 ± 11	8 ± 11	9 ± 11	10 ± 13
Alcohol among consumers, glass/d[3, 4]	2.0 (1.0-3.6)	1.4 (0.7-2.9)	1.4 (0.7-2.4)	1.3 (0.7-2.1)	1.3 (0.7-2.1)
Current smoking, %	46	38	36	30	32
Dietary intake					
Total energy, kJ/day	10186 ± 3282	9204 ± 2799	9054 ± 2634	9157 ± 2589	10131 ± 3157
Total protein, g/d (en%)	72 ± 23 (12)	75 ± 21 (14)	80 ± 20 (15)	86 ± 20 (16)	105 ± 27 (18)
Animal protein, g/d (en%)	40 ± 15 (7)	44 ± 14 (8)	49 ± 13 (9)	55 ± 12 (11)	71 ± 19 (12)
Plant protein, g/d (en%)	33 ± 12 (5)	31 ± 10 (6)	31 ± 10 (6)	31 ± 10 (6)	34 ± 12 (6)
Dairy protein, g/d (en%)[5]	14 ± 9 (2)	17 ± 9 (3)	20 ± 10 (4)	24 ± 10 (6)	33 ± 16 (6)
Meat protein, g/d (en%)[6]	16 ± 10 (3)	18 ± 10 (3)	20 ± 9 (4)	22 ± 10 (5)	27 ± 12 (5)
Grain protein, g/d (en%)[7]	15 ± 7 (2)	15 ± 7 (3)	15 ± 6 (3)	15 ± 7 (3)	17 ± 8 (3)
Total fat, g/d (en%)	95 ± 37 (35)	87 ± 31 (36)	87 ± 30 (36)	88 ± 29 (36)	97 ± 35 (36)
Saturated fat, g/d (en%)	38 ± 15 (14)	35 ± 13 (15)	36 ± 13 (15)	36 ± 12 (15)	41 ± 16 (15)
Carbohydrates, g/d (en%)	288 ± 96 (48)	254 ± 80 (47)	245 ± 77 (46)	245 ± 75 (44)	263 ± 92 (44)
Fiber, g/d	24 ± 8	24 ± 7	24 ± 7	25 ± 7	27 ± 8
Calcium, mg/d	849 ± 340	918 ± 342	1014 ± 357	1145 ± 382	1498 ± 581
Magnesium, mg/d	350 ± 110	345 ± 97	354 ± 92	370 ± 94	423 ± 117
Potassium, mg/d	3534 ± 1037	3493 ± 927	3587 ± 867	3762 ± 885	4294 ± 1090

Unless indicated otherwise, data are presented as mean ± SD or %.
[1]Hypertension is defined as systolic blood pressure≥140 mmHg or diastolic blood pressure≥90 mmHg (participants using antihypertensive medication were excluded); [2]Data from a subgroup (n=16,073). In consecutive quintiles n=3,255, n=3,229, n=3,190, n=3,184, and n=3,215. High physical activity was defined as ≥3.5 hours moderate activity (4.0>MET≥6.5) and ≥2 h/wk vigorous activity (MET ≥6.5) ; [3]Percentage of alcohol consumers in consecutive quintiles 63%, 63%, 63%, 60% and 58%; [4]Presented as median with interquartile range because of skewed distribution; [5]Protein intake from all kind of milk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, and cheese; [6]Protein intake from all kind of meats, meat products and poultry; [7]Plant protein intake from all kinds of breads, cake and cookies, grains and grain products.

dairy (42%) and meat (40%). Plant protein intake mainly comprised grain protein (48%), whereas other sources were potatoes (10%), vegetables (7%), fruits (4%), and legumes (2%).

Participants with a higher intake of total protein had a somewhat higher blood pressure and were more likely to be overweight or obese, whereas they were less likely to be a current smoker than participants with a low intake (**Table 3.1**). Fat intake and carbohydrate intake did not differ between quintiles, whereas higher intake of protein was accompanied with higher intake of minerals (i.e. calcium, magnesium, and potassium).

Protein intake and blood pressure

Intake of total and animal protein was not clearly associated with blood pressure (**Table 3.2)**, whereas in the highest quintile of dietary plant protein mean blood pressure was -1.8/ -1.0 mmHg lower than in the lowest quintile (p_{trend}<0.01). Sensitivity analysis within the subgroup of 16,073 participants for whom data on physical activity were available, showed essentially similar estimates when physical activity was additionally included in the multivariable model. Betas for systolic blood pressure per 5 grams of total protein was

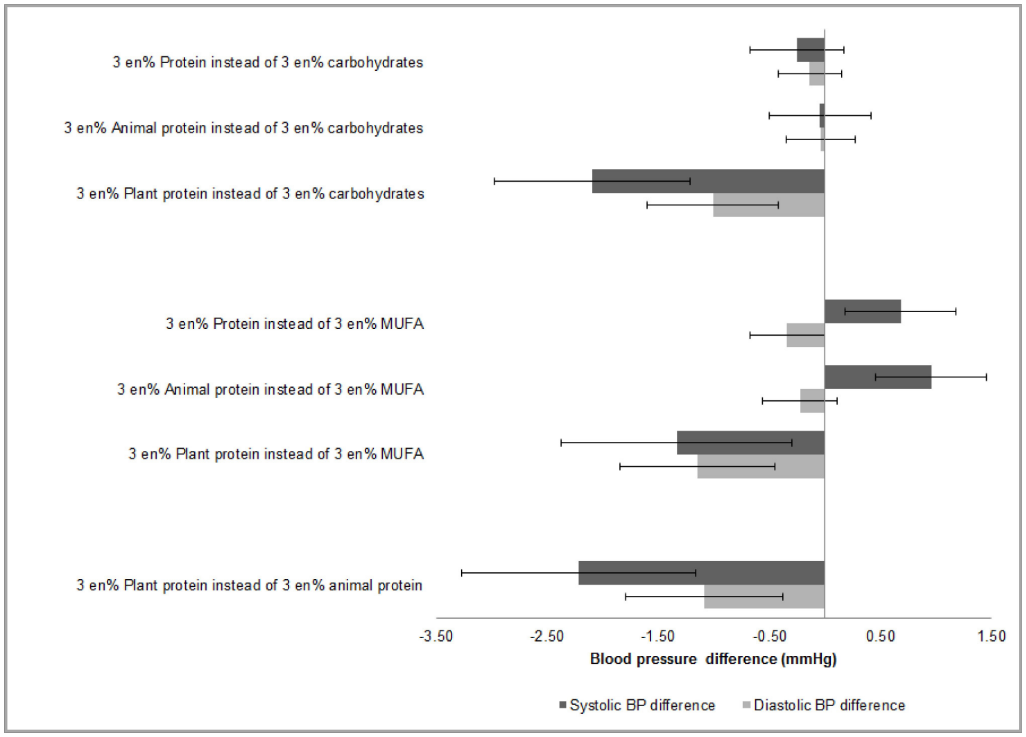

Figure 3.1. *Fully adjusted systolic blood pressure difference (mmHg) associated with replacement of 3 en% of carbohydrates or fat by total, plant or animal protein and by replacement of animal protein by plant protein.*

Table 3.2. *Fully adjusted systolic and diastolic blood pressure levels in 20,820 untreated Dutch adults in quintiles of energy adjusted total, animal and plant protein intake.*

	Median intake(g)	Model 1		Model 2		Model 3	
		SBP	SBP	SBP	SBP	SBP	SBP
Total protein							
Q1	69	119.6 (119.2 - 120.0)	75.9 (75.6 - 76.2)	120.2 (119.8 - 120.6)	76.5 (76.2 - 76.8)	120.1 (119.6 - 120.6)	76.2 (75.9 - 76.5)
Q2	78	119.8 (119.4 - 120.2)	75.9 (75.6 - 76.2)	120.1 (119.7 - 120.5)	76.2 (75.9 - 76.4)	120.2 (119.7 - 120.6)	76.1 (75.8 - 76.3)
Q3	83	119.8 (119.4 - 120.3)	76.1 (75.8 - 76.4)	119.8 (119.4 - 120.2)	76.1 (75.8 - 76.4)	119.9 (119.5 - 120.3)	76.1 (75.8 - 76.4)
Q4	89	120.7 (120.2 - 121.1)	76.6 (76.3 - 76.9)	120.5 (120.0 - 120.9)	76.4 (76.1 - 76.7)	120.5 (120.1 - 120.9)	76.5 (76.2 - 76.8)
Q5	98	120.1 (119.7 - 120.5)	76.2 (75.9 - 76.5)	119.4 (118.9 - 119.8)	75.6 (75.3 - 75.9)	119.3 (118.8 - 119.8)	75.9 (75.5 - 76.2)
p_{trend}		0.01	0.02	0.03	<0.01	0.14	0.62
Animal protein							
Q1	36	119.0 (118.5 - 119.4)	75.4 (75.1 - 75.7)	119.8 (119.4 - 120.3)	76.1 (75.8 - 76.4)	119.9 (119.4 - 120.4)	76.0 (75.7 - 76.4)
Q2	45	119.7 (119.3 - 120.2)	76.0 (75.8 - 76.3)	120.0 (119.6 - 120.5)	76.3 (76.0 - 76.6)	120.1 (119.7 - 120.5)	76.2 (75.9 - 76.5)
Q3	51	120.3 (119.9 - 120.7)	76.4 (76.1 - 76.7)	120.3 (119.9 - 120.7)	76.4 (76.1 - 76.7)	120.3 (119.9 - 120.7)	76.3 (76.1 - 76.6)
Q4	58	120.6 (120.1 - 121.0)	76.4 (76.1 - 76.7)	120.4 (119.9 - 120.8)	76.2 (76.0 - 76.5)	120.4 (119.9 - 120.8)	76.3 (76.0 - 76.5)
Q5	68	120.4 (120.0 - 120.8)	76.4 (76.1 - 76.7)	119.4 (119.0 - 119.9)	75.7 (75.4 - 76.0)	119.3 (118.8 - 119.8)	75.9 (75.5 - 76.2)
p_{trend}		<.001	<.001	0.32	0.04	0.39	0.71
Plant protein							
Q1	25	120.9 (120.5 - 121.3)	76.6 (76.3 - 76.9)	120.6 (120.2 - 121.0)	76.5 (76.2 - 76.8)	121.0 (120.4 - 121.5)	76.6 (76.2 - 76.9)
Q2	29	120.4 (119.9 - 120.8)	76.5 (76.2 - 76.8)	120.0 (119.6 - 120.4)	76.3 (76.0 - 76.6)	120.2 (119.8 - 120.7)	76.3 (76.0 - 76.6)
Q3	32	120.3 (119.9 - 120.7)	76.5 (76.2 - 76.8)	120.1 (119.7 - 120.6)	76.3 (76.0 - 76.6)	120.2 (119.8 - 120.6)	76.3 (76.0 - 76.6)
Q4	34	119.4 (118.9 - 119.8)	75.9 (75.6 - 76.2)	119.6 (119.1 - 120.0)	75.9 (75.6 - 76.2)	119.4 (119.0 - 119.9)	75.9 (75.6 - 76.2)
Q5	39	119.0 (118.6 - 119.4)	75.3 (75.0 - 75.6)	119.6 (119.2 - 120.1)	75.6 (75.3 - 75.9)	119.2 (118.6 - 119.7)	75.6 (75.2 - 76.0)
P_{trend}		<.001	<.001	<0.01	<.001	<.001	<0.01

Values are average blood pressure and 95% confidence interval
Model 1: Adjusted for age and gender; Model 2: Additionally adjusted for BMI, educational level, smoking, and alcohol consumption; Model 3: Additionally adjusted for total energy, saturated fatty acids, carbohydrates, fiber, calcium, magnesium, potassium, and protein intake from other sources than the one under study, if applicable.

Table 3.3. *Fully adjusted systolic and diastolic blood pressure in 20,820 untreated Dutch adults in quintiles of dairy, meat and grain protein intake.*

	Median intake(g)	SBP	SBP
Dairy protein			
Q1	9	119.0 (118.4 - 119.7)	76.0 (75.5 - 76.4)
Q2	15	119.8 (119.4 - 120.3)	76.0 (75.7 - 76.3)
Q3	21	119.9 (119.5 - 120.3)	76.1 (75.8 - 76.4)
Q4	26	120.6 (120.2 - 121.1)	76.2 (75.9 - 76.5)
Q5	36	120.6 (119.9 - 121.3)	76.4 (75.9 - 76.9)
p_{trend}		<0.01	0.24
Meat protein			
Q1	9	119.5 (119.0 - 120.0)	75.8 (75.5 - 76.1)
Q2	16	120.3 (119.9 - 120.8)	76.2 (75.9 - 76.5)
Q3	21	120.4 (120.0 - 120.8)	76.7 (76.4 - 77.0)
Q4	25	120.2 (119.8 - 120.6)	76.0 (75.7 - 76.3)
Q5	32	119.5 (119.1 - 120.0)	76.0 (75.6 - 76.3)
p_{trend}		1.00	0.83
Grain protein			
Q1	9	119.9 (119.5 - 120.4)	76.3 (76.0 - 76.6)
Q2	13	120.5 (120.0 - 120.9)	76.4 (76.1 - 76.7)
Q3	15	119.7 (119.3 - 120.1)	76.0 (75.7 - 76.3)
Q4	18	120.2 (119.7 - 120.6)	76.3 (76.0 - 76.6)
Q5	22	119.7 (119.2 - 120.2)	75.7 (75.4 - 76.0)
p_{trend}		0.42	0.03

Values are average blood pressure and 95% confidence interval, adjusted for age, gender, BMI, educational level, smoking, alcohol consumption, total energy, saturated fatty acids, carbohydrates, fiber, calcium, magnesium, potassium, and protein intake from other sources than the one under study, if applicable.

0.13±0.06 mmHg with physical activity in the model versus 0.14±0.05 without physical activity. For animal and plant protein betas per 5 grams were 0.15±0.03 versus 0.16±0.02 mmHg and -0.43±0.005 versus -0.41±0.006 respectively.

Substitution analysis in which 3 energy% of carbohydrates or MUFA was substituted by total or animal protein did not show a difference in blood pressure (**Figure 3.1**). However, when 3 en% of carbohydrates was substituted by plant protein, blood pressure was -2.1/ -1.0 mmHg lower (p<0.01). Also substitution of 3 en% of mono-unsaturated fatty acids by plant protein resulted in a lower blood pressure (-1.3/-1.2 mmHg, p<0.05)

With respect to protein from specific sources, systolic blood pressure in the highest quintile of dairy protein intake was 1.6 mmHg higher than in the lowest quintile (p_{trend}<0.01), which we did not observe for diastolic blood pressure **(Table 3.3)**. Intake of meat protein or grain protein was not associated with blood pressure. With respect to plant protein from other sources than grain, systolic blood pressure was +0.8 mmHg higher in the highest (median intake=5.2 g/d) than in the lowest (1.4 g/d) tertile of potato protein. (p_{trend}=0.01). For protein intake from vegetables (2.9 g/d in highest vs. 1.3 g/d in lowest tertile), fruits (2.0 vs. 0.4 g/d), and legumes (1.2 vs. 0.1 g/d) this difference in systolic blood pressure was -0.9 mmHg (p_{trend}<0.01), +0.1 mmHg (p_{trend}=0.50), and +0.8 mmHg (p_{trend}<0.01), respectively.

Age, gender, and BMI did not independently modify the associations between protein intake and blood pressure (data not shown). The association between total protein intake and blood pressure was not significantly modified by blood pressure level ($p_{interaction}$=0.14, **Figure 3.2**). With regard to protein types we observed no effect modification of blood pressure level on the relation between animal protein and blood pressure ($p_{interaction}$=0.16), whereas plant protein was inversely associated with systolic blood pressure in untreated hypertensives (-3.6 mmHg, p_{trend}<0.01) but not in normotensives (-0.1 mmHg, p_{trend}=0.39) and prehypertensives (+0.2 mmHg, p_{trend}=0.97, $p_{interaction}$<0.01).

Discussion

In this cross-sectional study in 20,820 Dutch adults aged 20-65 years, total dietary protein and animal protein were not related to blood pressure. High intake of plant protein was associated with lower blood pressure, which was most pronounced in untreated hypertensive individuals. Protein from meat and grain were not related to blood pressure, whereas dairy protein was directly associated with systolic, but not diastolic blood pressure.

We conducted the current study among a large population of 20,820 Dutch adults. Protein intake is usually tightly regulated [23] and we consider it likely that protein intake measured in this study gives a good estimate of lifelong exposure. Nevertheless, due to the cross-sectional design of the study it is possible that participants at increased cardiovascular risk, changed their diet upon medical advice. For this reason, we excluded individuals with diabetes, prevalent cardiovascular diseases, and clinically diagnosed hypertension (i.e. using antihypertensive medication). Because elevated blood pressure is often asymptomatic we consider intentional dietary changes unlikely in participants that are not aware that they have a high blood pressure. However, a total of 3,999 participants (19%) reported that high blood pressure had ever been observed. Intakes of protein types of this group were not different from those in other participants (total protein: 15±2 en% for both groups; animal protein: 9.3±2.5 en% vs. 9.7±2.5 en%; plant protein 6±1 en% for both groups). Also intake of nutrients that are indicators of a healthy lifestyle were similar between the groups; fiber

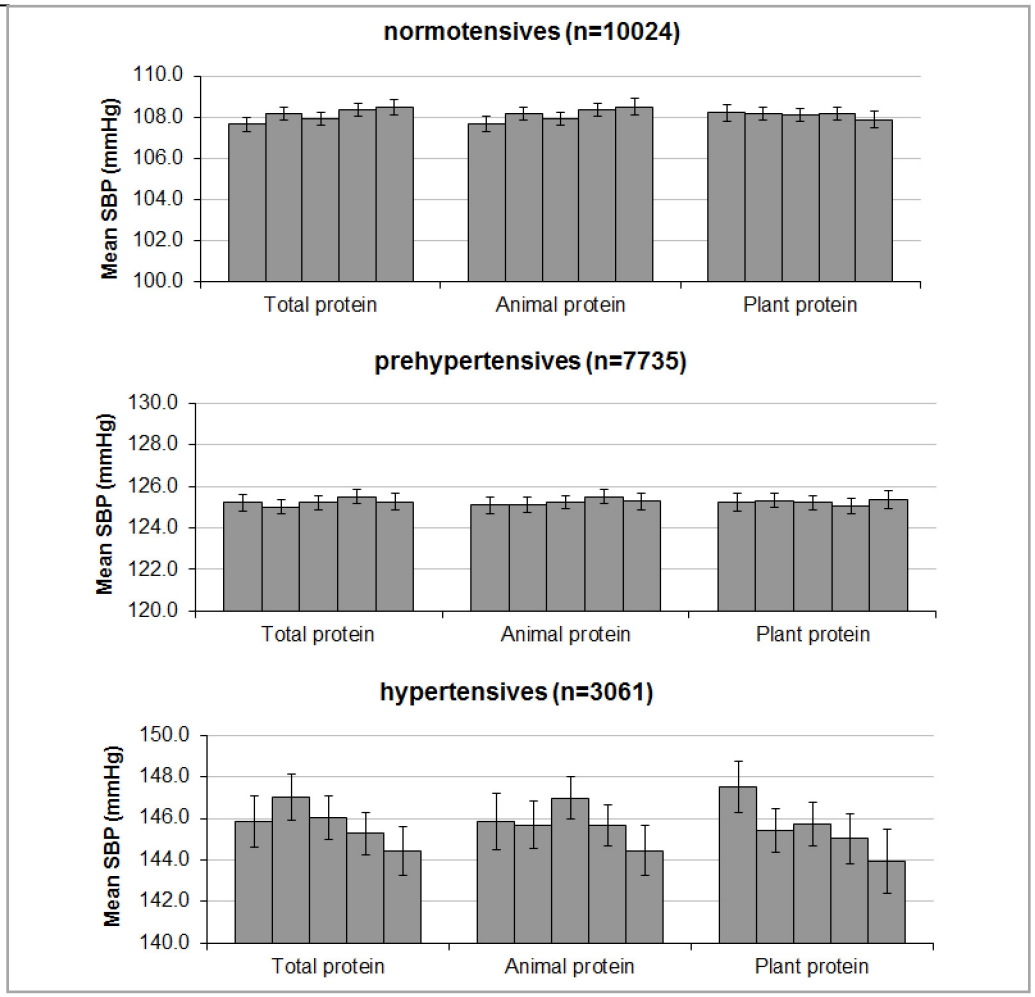

Figure 3.2. *Systolic blood pressure in quintiles of protein intake, stratified by hypertension status*

SBP=systolic blood pressure, Values are average blood pressure and 95% confidence interval, adjusted for age, gender, BMI, education, smoking, alcohol consumption, total energy, saturated fatty acids, carbohydrates, fiber, calcium, magnesium, potassium, and protein intake from other sources than the one under study, if applicable.

$p_{interaction}$ for total protein=0.14, $p_{interaction}$ for animal protein=0.16, $p_{interaction}$ for plant protein=<0.01

intake in the group with a history of high blood pressure was 24±7 g/d versus 25±8 g/d in the other group, and potassium intake was 3675±961 mg/d versus 3748±1019 mg/d. Therefore, we do not expect that reverse causality has influenced our findings.

Extensive data collection in this large population based cohort allowed adjustment for many potential confounders. Nevertheless, physical activity, which is an important blood pressure determinant, was not assessed until 1994 and data were available for only 77% of our cohort. In this subgroup physical activity appeared not to confound the association between

dietary protein and blood pressure. We therefore consider it unlikely that lack of adjustment for physical activity has affected our findings.

Protein intake in the present study was assessed using a self-administered semi-quantitative FFQ. Validation against 24-hour dietary recalls and 24-hour urine samples showed good correlations for total dietary protein (all correlation coefficients >0.55), indicating that participants could be adequately ranked according to their protein intake.[21] However, the FFQ was not validated for protein types. Although correlations with 24-h recalls were good for milk and bread, as surrogate markers for protein from dairy and grain, correlations for meat, as surrogate marker for meat protein, were lower, especially in men (r=0.39).[19] Misclassification of participants, especially for meat protein, may have led to attenuated associations with blood pressure, and these findings should therefore be interpreted with caution.

The lack of significant association between total protein and blood pressure in our study is in agreement with previous observational studies showing inconclusive results.[6] Results of trials, however, suggest that protein may have a small beneficial effect on blood pressure.[5,6,24,25] Most of these trials had a carbohydrate-rich control diet. The fully controlled Omniheart trial in 164 US adults additionally compared a protein rich diet with an isocaloric diet that was rich in mono-unsaturated fat.[5] blood pressure was similar during these diets, and the authors therefore argued that reduced carbohydrate rather than increased protein intake lowers blood pressure. We could not confirm this hypothesis with our substitution analysis that yielded no association of dietary protein with blood pressure, irrespective of whether protein was exchanged with carbohydrates or monounsaturated fat. This discrepancy may be explained by contrast in protein intake, which was only 4 en% between extreme quintiles in the present study whereas it was 10 en% in Omniheart. Moreover, blood pressure in our cohort was low (120/76 mmHg) compared to that of (pre)hypertensive trial participants.

In our analysis plant protein was inversely associated with blood pressure, whereas we observed no association for animal protein. In OmniHeart [5], blood pressure reductions may have been due to extra intake of plant protein, which accounted for two thirds of the difference in protein intake between the diets. A differential effect of dietary plant and animal protein on blood pressure might be explained by differences in amino acid composition. In the INTERMAP study in 4,680 adults, individuals with a high intake of plant protein also had a relatively high intake of glutamic acid.[26] With a 2 SD higher intake of glutamic acid (4.7% of total protein) the authors observed 1.5 mmHg lower systolic and 1.0 mmHg lower diastolic blood pressure levels. On the other hand, although we adjusted our estimates for many potential confounders including potassium and fiber as healthy diet indicators, we cannot exclude the possibility that unmeasured beneficial nutrients that are closely correlated to plant protein (e.g. polyphenols) or healthy lifestyle in general have contributed to the observed associations between plant protein and blood pressure.

The inverse association of plant protein with blood pressure could not be explained by grain protein, which comprised 48% of plant protein intake. Therefore we performed post-hoc analysis to explore whether other sources of plant protein could explain the observed inverse association for plant protein. This was not the case for protein intake from potatoes, legumes, and fruits, which was either directly or not associated with blood pressure. However, intake of vegetable protein, which contributed 7% to plant protein intake in our population, showed a small inverse relationship with blood pressure and could possibly (partly) explain a beneficial association of plant protein with blood pressure. On the other hand, a high vegetable protein intake may also be a marker for a healthy diet and lifestyle, which may have contributed to the observed inverse associations.

With respect to protein from animal sources, meat protein (40% of animal protein intake) was not associated with blood pressure. This is in line with results from previous analysis in 2241 older Dutch adults of the Rotterdam cohort, where intake of meat protein was not related to hypertension risk.[12] Moreover, protein from several meat sources did not affect blood pressure compared to plant protein or non-meat protein in a randomized controlled trial among 64 hospital staff members and a randomized controlled cross-over trial among 35 men respectively.[27,28] For dairy protein (42% of animal protein intake) we found a direct association with systolic, but not with diastolic blood pressure. In the Rotterdam cohort dairy protein was not associated with incident hypertension.[12] Also, in a fully controlled weight loss trial including 65 adults, a diet containing 15 en% milk protein did not affect blood pressure compared to a diet in which the milk protein was exchanged for fat.[29] Moreover, in a double-blind randomized cross-over trial including 352 (pre)hypertensive participants milk protein supplementation (40 g/d) resulted in a blood pressure reduction of -2.3 mmHg compared to carbohydrate supplementation.[15] Therefore, the direct association between dairy protein and systolic blood pressure that we observed in the current study may well be a chance finding.

Our results suggest that untreated hypertensive individuals could be more sensitive to a beneficial effect of plant protein than normotensive or prehypertensive individuals. This is in line with findings from the OmniHeart study [5], in which larger blood pressure reductions were found for increased protein intake (largely from plant sources) in untreated hypertensives than in prehypertensives. Because over 30% of the global adult population is estimated to be hypertensive, this finding could have important public health implications and warrants further investigation.

In conclusion, intake of total protein and animal protein was not associated with blood pressure in this general Dutch population not using antihypertensive medication. Our results suggest that plant protein may lower population blood pressure level by ~2 mmHg, especially in those with elevated blood pressure levels. This may have important public health implications because a downward shift in population blood pressure by 2 mmHg may reduce cardiovascular mortality by ~5%.[3] However, due to the cross-sectional design a definitive

conclusion on causality cannot be drawn. Moreover, we cannot exclude that high plant protein is a marker for a healthy lifestyle in general. Therefore, confirmation from randomized controlled trials is warranted.

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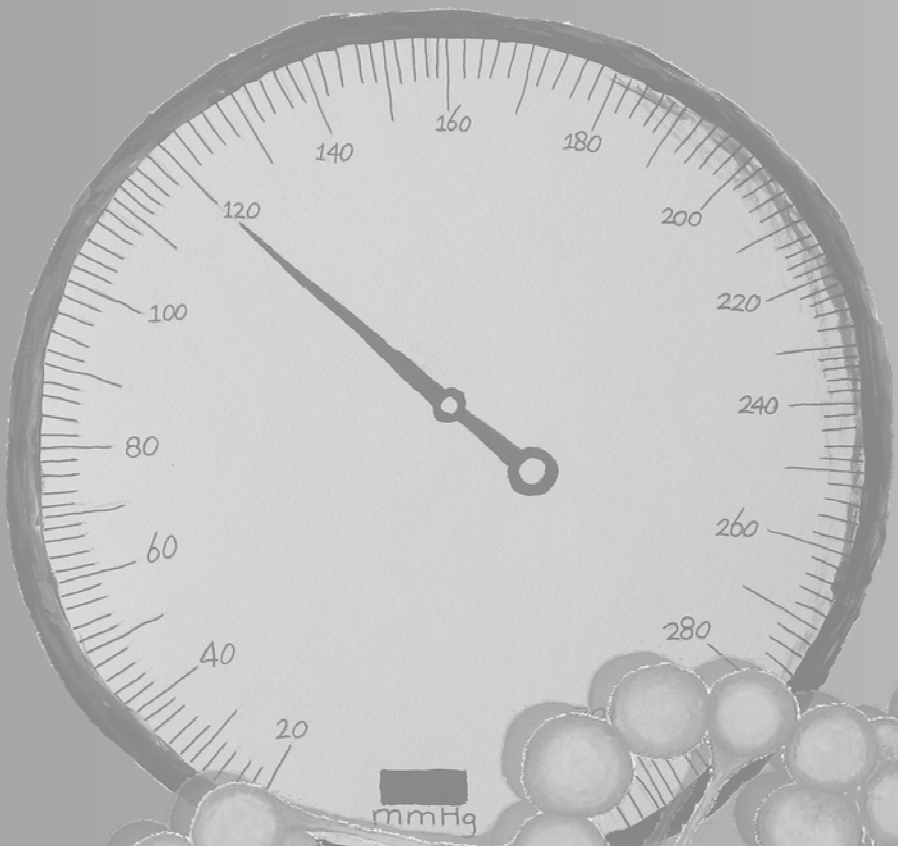
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Chapter 4

Sources of dietary protein and risk of hypertension in a general Dutch population

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Johanna M. Geleijnse, Jolanda M.A. Boer,
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Abstract

Background

Evidence suggests a small beneficial effect of dietary protein on blood pressure, especially for plant protein. We examined the relation between several types of dietary protein (total, plant, animal, dairy, meat, and grain) and risk of hypertension in a general population of 3588 Dutch adults, aged 26-65 y, who were free of hypertension at baseline.

Methods

Measurements were done at baseline and after 5 and 10 years of follow-up. Hazard ratios (HRs), with 95%-confidence intervals (95%-CI) for incident hypertension were obtained in tertiles of energy-adjusted protein, using time dependent Cox regression models. Models were adjusted for age, sex, BMI, education, smoking, baseline systolic blood pressure, dietary confounders, and protein from other sources (if applicable).

Results

Mean blood pressure was 118/76 mmHg at baseline. Protein intake was 85±22 g/day (~15 en%) with 62% originating from animal sources. The main sources of protein were dairy (28%), meat (24%), and grain (19%). During follow-up 1568 new cases of hypertension were identified (44% of participants). Energy-adjusted intake of total protein, plant protein, and animal protein was not significantly associated with hypertension risk (all HRs ~1.00, p>0.60). Protein from grain showed a significant inverse association with incident hypertension, with a HR of 0.85 (95% CI: 0.73-1.00, p_{trend}=0.04) for the upper tertile (≥ 18 g/d) vs. lower tertile (<14 g/d), whereas protein from dairy and meat were not associated with incident hypertension.

Conclusions

higher intake of grain protein may contribute to the prevention of hypertension, which warrants confirmation in other population-based studies and randomized controlled trials.

Introduction

Health authorities emphasize the importance of dietary and lifestyle factors for the prevention of hypertension, which is a strong risk factor for cardiovascular disease.[1] Even small effects of these dietary and lifestyle factors on blood pressure can have great public health impact. It has been estimated that a reduction in systolic blood pressure of only 2 mmHg may already result in a 6% reduction in fatal stroke, and a 4% reduction in fatal coronary heart disease (CHD).[2] Dietary and lifestyle recommendations include physical activity, maintenance of a healthy body weight, reduced salt intake and moderation of alcohol consumption.[2,3] More recently, interest has grown into the influence of dietary patterns and macronutrient intakes on blood pressure.[4,5]

A substantial body of evidence suggests a, possibly weak, beneficial effect of protein on blood pressure, although findings are not conclusive.[6,7] An important study in this respect is the large INTERSALT study in 10 020 adults from 32 countries, in which a significant 0.5 mmHg lower systolic blood pressure was observed with each gram of 24-h urinary nitrogen (mean nitrogen excretion of 9.95 ± 3.11), as a biomarker for total protein intake.[8] This inverse association was confirmed by results of the OmniHeart randomized cross-over trial, in which systolic blood pressure of 164 healthy US adults decreased 1.4 mmHg more after a 6-week high protein diet compared with a diet high in carbohydrates.[5] However, no difference in blood pressure change was found compared with a diet high in mono-unsaturated fat.

Protein intake is a rather heterogeneous exposure and specific types of protein (i.e. animal, plant) or protein from specific sources (e.g. dairy, meat, grain) may differentially influence blood pressure. In several observational studies [9-15] the association with blood pressure was investigated separately for plant protein and animal protein. Results were inconclusive, although there was a trend to a slightly more beneficial effect of plant protein on blood pressure. In a prospective cohort study among 810 untreated pre- or mild hypertensives aged 25 -79 y (PREMIER), risk for developing hypertension was 21% lower per en% of plant protein intake, whereas for animal protein no association was observed.[11] Also in a prospective cohort study among 5880 Hispanics (SUN cohort), a 50% reduction in hypertension risk with high intake of plant protein was observed in the highest quintile compared to the lowest quintile, whereas intake of animal protein did not influence hypertension risk.[10] So far, data on specific protein sources in relation to blood pressure is scarce. In a previous analysis in the Rotterdam Study, including 2241 Dutch adults aged ≥55y, we found no clear associations between protein from different dietary sources and 6-year incidence of hypertension.[13]

In the present analysis, we examined whether total protein intake and intake of plant and animal protein was associated with risk of hypertension during 10 years of follow-up in a more general Dutch population-based cohort of 3,588 adults, aged 26 to 65 y. In the Netherlands approximately two thirds of dietary protein is from animal origin with the main

sources being dairy and meat, whereas plant protein is mainly obtained from grains.[16] We also analysed the associations for these protein sources.

METHODS

Design and study population

We used data from the ongoing prospective Doetinchem cohort study, which has been described in detail elsewhere.[17] In brief, 12 405 volunteers, aged 26-65 years, were examined between 1987 and 1991. A sample of these respondents (n=6386) was invited for follow-up examination in 1993-1997, in 1998-2002 and in 2003-2007. An extensive food frequency questionnaire (FFQ) was implemented from 1993 onwards.

In 1993 (subsequently referred to as 'baseline') 6113 participants underwent physical examination, and blood pressure measurements were obtained in 6100 participants. We excluded 1652 participants (27%) with prevalent hypertension, defined as blood pressure ≥140/90 mmHg and/or use of antihypertensive medication. Furthermore, we excluded 732 participants without information on hypertension status during both follow-up measurements, Finally, we excluded 128 participants with a history of cardiovascular disease, self-reported diabetes at baseline, because of pregnancy at baseline or during follow-up, or because missing dietary data, leaving 3588 participants for the present analysis.

Dietary assessment

Dietary intake was assessed at baseline and during both follow-up measurements using a self-administered semi-quantitative FFQ, developed for the international EPIC study (European Prospective Investigation into Cancer and Nutrition), on 178 foods and beverages consumed during the preceding year.[18] Colored photographs were used to facilitate estimation of portion sizes, and seasonal variation in food intake was taken into account. Total energy and nutrient intakes were calculated using an extended version of the Dutch Food Composition Table of 1996.[19]

Animal protein was defined as protein from dairy, meat, fish, eggs, and animal protein from mixed dishes. Plant protein included protein from soy, nut, grain, fruits, vegetables, legumes, and plant protein from mixed dishes. Dairy protein was calculated as protein from milk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, and cheese, meat protein included protein from all meat, meat products and poultry. Grain protein was defined as protein from rice, bread, pasta and plant protein in grain-containing bakery products.

The FFQ was validated in 63 men and 58 women and Pearson correlation coefficients of 0.73 in men and 0.70 in women were found for reproducibility of energy adjusted total protein intake.[20] Additionally, the relative validity of the FFQ was assessed against 12 monthly 24-h recalls over a 1-year period. Pearson correlation coefficients for energy adjusted protein intake after correction for intra-individual variation were 0.71 for men and 0.67 for women.[20] The correlation coefficients with urinary nitrogen excretion in four 24h urine samples at 3-month intervals were 0.56 for men and 0.69 for women, although data suggested slight underestimation of protein intake by the FFQ (mean percentage of underestimation: 7% for men and 12% for women).[20]For types and sources of protein (e.g. from plant, animal, dairy, grain) the FFQ was not validated. However, correlations with 24-h recalls were good for milk and milk products (r_{men}=0.69; r_{women}=0.77) and bread (r_{men}=0.76; r_{women}=0.78), whereas correlations for meat were lower, especially for men (r_{men}=0.39; r_{women}=0.59).[18]

Blood pressure

blood pressure was measured by a trained technician using a random-zero sphygmomanometer, with the participant in sitting position. Systolic blood pressure was recorded at the appearance of sounds (first-phase Korotkoff) and diastolic blood pressure was recorded at the disappearance of sounds (fifth-phase Korotkoff). Blood pressure was measured twice, separated by a pulse count. The mean of two measurements was used for data-analysis. During physical examination, regular audits were performed to check adherence to the blood pressure measuring protocol (e.g. resting time, adequate cuff size). Hypertension was defined as systolic blood pressure at least 140 mmHg or diastolic blood pressure at least 90 mmHg or use of antihypertensive medication.

Assessment of potential confounders

Information on potential confounders was collected at baseline and during both follow-up examinations. Body weight (to nearest 0.1 kg) and height (to nearest 0.5 cm) were measured with participants wearing light indoor clothing without shoes and body mass index (BMI) was calculated (kg/m^2). Data on age, gender, education, lifestyle factors, history of major diseases, medication use, and any prescribed diets were collected by questionnaires. An extensive questionnaire on physical activity was introduced in 1994 and was completed by 2936 participants (81%). Questionnaire data were used to create variables on alcohol intake (none, moderate, high), smoking status (current smoker/non-smoker), educational level (3 categories), and physical activity (4 categories, ranging from inactive to very active [21]).

Statistical analysis

Intake of total protein and different types of protein was first adjusted for total energy intake according to the residual method.[22] Baseline characteristics of the study population across tertiles of energy-adjusted total protein intake, are presented as means ± standard deviation, percentages, or medians with interquartile range.

We used time dependent Cox regression models to calculate hazard ratios (HR) with 95%-confidence intervals (95% CI) for the association between dietary protein intake and 10-y incidence of hypertension. We defined the exposure as the cumulative average energy adjusted protein intake to reduce measurement error and to estimate long-term intake. P for trend was estimated by modelling median intake of baseline tertiles.

For participants who did not develop hypertension during follow-up we computed survival time as years from baseline to the end of the study period (i.e. 10-y examination visit) or until end of follow-up. For participants who developed hypertension, we attributed 2.5 y of follow-up if hypertension was present at the 5-y examination visit, and 7.5 y of follow-up if hypertension was present at the 10-y examination visit.

The basic model (model 1) included age and gender. In model 2, we further adjusted for BMI, educational level, smoking, alcohol use, and baseline systolic blood pressure. The full model (model 3) additionally included daily intake of total energy, saturated fatty acids, poly -unsaturated fatty acids, carbohydrates, fiber, calcium, magnesium, and potassium, and protein intake from other sources than the one under study, if applicable. Age, gender, and lifestyle covariates were updated each measurement round. For dietary covariates the cumulative average intake was calculated up to each measurement round. Dietary calcium was strongly correlated to dairy protein intake (r=0.82). Therefore we conducted an additional analysis without calcium in the model to check for multicollinearity.

To mimic a situation in which dietary protein was exchanged for dietary carbohydrates, we performed an additional analysis using the full model (model 3) with mono-unsaturated fatty acids as additional covariate instead of carbohydrates. To investigate whether physical activity confounded the protein-blood pressure associations, we performed a sensitivity analysis per 5 grams of total, plant, and animal protein in the subgroup of 2892 participants (81%) with complete data on physical activity, using the full model with and without additional adjustment for physical activity.

Finally, we performed a number of pre-defined subgroup analyses for total, plant and animal protein, in strata of age, (< 45 y and ≥45 y), gender, overweight status (<25 kg/m^2 and ≥25 kg/m^2), and baseline systolic blood pressure (<130 mmHg and ≥130 mmHg), using the full model. Data analysis was performed using SAS version 9.1 (SAS Institute Inc.) and a two-sided p-value of <0.05 was considered statistically significant.

Table 4.1. *Baseline characteristics by baseline tertiles of energy adjusted total protein intake of 3,588 Dutch adults (26-65 y), without hypertension or use of antihypertensive medication at baseline.*

	Tertile of energy adjusted total protein intake			
	<81 g/d (n=1184)	81 to 89 g/d (n=1184)	>89 g/d (n=1220)	P_{trend}
Median intake (g/d)	75	85	95	
Age, y	44±10	44±9	45±10	0.03
Gender, % men	52	56	57	0.02
Body mass index, kg/m^2	24.4±3.3	24.7±3.1	25.3±3.3	<0.01
Overweight,%[1]	38	43	48	<0.01
Education, % high	19	23	20	0.21
Systolic blood pressure, mmHg	118.2±10.6	117.6±10.8	117.8±10.3	0.39
Diastolic blood pressure, mmHg	75.6±7.7	75.7±7.7	75.8±7.6	0.58
Alcohol among consumers, glasses/d[2]	1.4±(0.7-2.9)	1.1±(0.7-2.1)	1.0±(0.6-2.0)	<0.01
Current smokers, %	38	28	28	<0.01
Dietary intake				
Total energy, kJ/day	9752±2802	9198±2399	9627±2690	0.27
Total protein, g/d (en%)	75±20 (13)	82±18 (15)	98±23 (18)	<0.01
Plant protein, g/d (en%)	32±10 (6)	31±9 (6)	32±10 (6)	0.94
Grain protein[5] g/d (en%)	16±7 (3)	16±6 (3)	17±7 (3)	<0.01
Animal protein, g/d (en%)	43±13 (8)	51±12 (10)	65±16 (12)	<0.01
Dairy protein[3], g/d (en%)	18±8 (3)	22±9 (4)	32±13 (6)	<0.01
Meat protein[4] g/d (en%)	17±9 (3)	20±8 (4)	24±9 (4)	<0.01
Total fat, g/d (en%)	92±31 (35)	89±28 (36)	93±31 (36)	0.57
Saturated fat, g/d (en%)	38±13 (14)	37±12 (15)	40±13 (15)	<0.01
Mono-Unsaturated fat, g/d (en%)	35±12 (13)	34±11(14)	35±12 (13)	0.70
Poly-unsaturated fat, g/d (en%)	19±7 (7)	17±6 (7)	17±7 (7)	<0.01
Carbohydrates, g/d (en%)	274±80 (48)	248±69 (46)	251±77 (44)	<0.01
Potassium, mg/d	3638±908	3739±796	4171±939	<0.01
Magnesium, mg/d	358±99	367±84	409±103	<0.01
Calcium, mg/d	936±313	1083±320	1409±471	<0.01
Fiber, g/d	25±7	25±6	26±7	<0.01

Data are presented as mean±SD or %, unless stated otherwise.
[1]BMI ≥25 kg/m^2; [2]Percentage of alcohol consumers in all tertiles ~62%; alcohol consumption is presented as median with interquartile range because of skewed distribution; [3]Protein intake from milk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, and cheese; [4]Protein intake from meat, meat products and poultry; [5]Protein intake from rice, bread, pasta and plant protein in grain-containing bakery products.

Table 4.2. *Cumulative average protein intake in relation to 10 incidence of hypertension in 3,588 Dutch adults (25-65 y).*

	Hazard ratio of hypertension (95%CI)		
	Model 1	**Model 2**	**Model 3**
Total protein (g/d)			
<81	1.00 (ref)	1.00 (ref)	1.00 (ref)
81-89	1.05 (0.93- 1.18)	1.06 (0.93- 1.19)	1.00 (0.88- 1.15)
≥89	1.16 (1.02- 1.31)	1.11 (0.98- 1.25)	1.01 (0.85- 1.19)
p_{trend}[1]	0.02	0.11	0.93
Plant protein (g/d)			
<30	1.00 (ref)	1.00 (ref)	1.00 (ref)
30-34	0.87 (0.77- 0.98)	0.91 (0.81- 1.03)	0.92 (0.80- 1.06)
≥34	0.80 (0.71- 0.90)	0.91 (0.80- 1.03)	0.96 (0.80- 1.16)
p_{trend}[1]	<0.01	0.12	0.65
Animal protein (g/d)			
<48	1.00 (ref)	1.00 (ref)	1.00 (ref)
48-57	1.01 (0.89- 1.14)	0.97 (0.85- 1.10)	0.90 (0.79- 1.03)
≥57	1.23 (1.09- 1.39)	1.11 (0.98- 1.26)	0.97 (0.81- 1.15)
p_{trend}[1]	<0.01	0.08	0.70
Dairy protein (g/d)			
<19	1.00 (ref)	1.00 (ref)	1.00 (ref)
19-27	0.89 (0.79- 1.01)	0.94 (0.83- 1.06)	0.91 (0.78- 1.05)
≥27	1.01 (0.89- 1.14)	1.07 (0.94- 1.21)	1.00 (0.81- 1.25)
p_{trend}[1]	0.77	0.28	0.97
Meat protein (g/d)			
<17	1.00 (ref)	1.00 (ref)	1.00 (ref)
17-24	1.12 (0.99- 1.26)	1.01 (0.89- 1.14)	0.97 (0.85- 1.10)
≥24	1.29 (1.14- 1.46)	1.09 (0.95- 1.23)	0.99 (0.85- 1.16)
p_{trend}[1]	<0.01	0.22	0.92
Grain protein (g/d)			
<14	1.00 (ref)	1.00 (ref)	1.00 (ref)
14-18	0.88 (0.79- 0.99)	0.91 (0.81- 1.03)	0.91 (0.80- 1.03)
≥18	0.76 (0.68- 0.87)	0.82 (0.72- 0.93)	0.85 (0.73- 1.00)
p_{trend}[1]	<0.01	<0.01	0.04

All types of protein were energy adjusted according to the residuals method[22]
Model 1: Adjusted for age and gender; Model 2: Additionally adjusted for BMI, educational level, smoking, alcohol use and baseline systolic blood pressure; Model 3: additionally adjusted for intake of total energy, saturated fatty acids, poly-unsaturated fatty acids, carbohydrates, fiber, calcium, magnesium, potassium, and (in analyses of protein types) for other protein types.
[1] *P for trend was estimated by modeling median intake of baseline tertiles.*

RESULTS

Descriptive statistics

The mean age of the total study population was 44±10 years and 44% was male. Mean BMI was 25±3 kg/m^2 and 43% of participants was overweight or obese (BMI ≥25 kg/m^2). Baseline blood pressure was 118/76 mmHg. Mean protein intake was 85±22 g/d (~15 en%), of which 63% originated from animal sources. Major sources of animal protein intake were dairy (45% of animal protein intake) and meat (38%). Plant protein intake mainly comprised grain protein (51%), whereas the next main sources were potatoes (11%), vegetables (7%), fruits (4%), and legumes (2%).

Baseline characteristics and dietary intake of the study population according to tertiles of energy adjusted total protein intake are shown in **Table 4.1**. The percentage males increased significantly across tertiles of energy adjusted protein intake as well as the number of overweight participants. With regard to dietary intake, the higher intake of total dietary protein in the highest tertiles was mainly reflected in differences in animal protein intake, whereas the intake of plant protein intake was relatively constant over tertiles of energy adjusted total protein intake. Also, intake of fat and carbohydrates did not differ significantly across consecutive tertiles, although carbohydrate intake was somewhat higher in the lowest category of total protein. The intake of potassium, magnesium, and calcium increased significantly across tertiles of energy adjusted total protein intake.

Protein intake and incident hypertension

After a mean follow-up time of 7.5±2.9 years (26 500 person years), 1568 new cases of hypertension were identified. The number of incident hypertension cases in increasing baseline tertiles of energy adjusted total protein were respectively 57, 58, and 63 per 1000 person years. Associations between protein intake and incident hypertension are shown in **Table 4.2**. Intake of total, plant and animal protein intake was not clearly associated with incident hypertension, with all fully adjusted HRs being close to 1.00 (All p_{trend}>0.60). When the full model was adjusted for mono-unsaturated fatty acids instead of carbohydrates, the HRs of upper tertile versus lower tertile were 1.04 (95%-CI: 0.89-1.23) for total protein (p_{trend}= 0.62), 0.96 (0.79- 1.15) for plant protein (p_{trend}= 0.59), and 1.00 (0.84- 1.19) for animal protein (p_{trend}= 0.98).

Within the subgroup of 2892 participants for whom data on physical activity was available (21 566 person years) 1217 new cases of hypertension were identified. In this subgroup we found identical HRs per 5 grams of total, plant and animal protein both with and without additional adjustment for physical activity (respectively 1.02, 0.97-1.06; 1.01, 0.90-1.13; 1.02,0.97-1.06). Predefined subgroup analyses showed that the association between protein

and hypertension risk did not vary among strata of age, gender, BMI, or baseline blood pressure (all $p_{interaction}>0.15$)

When focusing on the main protein sources, intake of dairy protein and meat protein was not associated with incident hypertension. (Table 4.2) Sensitivity analysis excluding dietary calcium from the multivariable analysis on dairy protein indicated some degree of multicollinearity (i.e. the width of the confidence intervals slightly decreased). Leaving calcium out of the model, however, yielded essentially similar results: HR of the third tertile compared to the lowest tertile: 0.99, 0.84-1.17. Intake of grain protein showed a significant 15% lower risk of hypertension in the upper tertile compared to the lowest tertile. (Multivariate HR 0.85, 95%CI 0.73-1.00; $p_{trend}=0.04$). Other sources of plant protein (i.e. potatoes, legumes, vegetables, and fruits) were not related to hypertension risk (all p>0.30, data not shown)

Discussion

In this prospective cohort study among 3588 participants without hypertension at baseline, intake of total, plant, and animal protein was not associated with 10-year incidence of hypertension. Also, intake of protein from dairy and meat, the main sources of animal protein, was not associated with hypertension risk. A high intake of grain protein, was significantly associated with a 15% lower risk for hypertension.

The present analyses were conducted in a population based cohort with repeated measurements of dietary intake and lifestyle over 10 years of follow-up.[17] Because dietary intake was assessed 3 times during follow-up, we were able to reduce measurement error and estimate long-term protein intake by using the cumulative average in time dependent Cox models. Extensive data were available on potential confounders, although baseline assessment of physical activity was not performed in participants who were enrolled before 1994. However, similar protein-blood pressure associations were obtained with and without adjustment for physical activity in participants with complete data.

The self-administered FFQ of the current study has been validated against 24-hour dietary recalls and 24-hour urine samples.[20] Correlations were good with correlation coefficients for total protein, plant protein and animal protein being >0.60, indicating that participants could be adequately ranked according to their protein intake. The FFQ was not validated for protein from specific sources, but correlations for milk and bread, as surrogate markers for dairy and grain protein, where good (>0.65). However, correlations for meat were lower, especially for men (r=0.39).[18] This may have caused misclassification of participants according to meat protein intake and, as a consequence, the results for this type of protein may have been biased towards no association. To explore the potential influence of protein sources on

blood pressure in future epidemiological studies, identification of biological markers for intake of protein from specific sources like meat could be useful.

A substantial body of evidence suggests a, possibly weak, beneficial effect of protein on blood pressure, as previously summarized.[6] We adjusted our estimates among others for energy, carbohydrates, saturated fatty acids and poly-unsaturated fatty acids, and in this way we mimicked a situation in which only intake of protein and mono-unsaturated fatty acids do vary. However, in the large OmniHeart cross-over feeding trial among 164 participants, no difference in blood pressure effect was found after a high protein diet compared to a high MUFA diet, which may explain our lack of result for total protein and hypertension risk. In contrast, in the OmniHeart study, a beneficial blood pressure effect was observed after the high protein diet compared to a diet high in carbohydrates.[5] Therefore, to mimic exchange of protein with carbohydrates, we performed an additional analysis using the full model, with adjustment for MUFA instead of carbohydrates. However, this did not essentially change our results. Further research is needed to investigate the blood pressure effect after exchange of different macronutrients.

Several observational studies have been conducted that investigated the association with blood pressure separately for plant and animal protein, showing inconclusive results, although in some studies plant protein seemed to be more beneficial than animal protein. In our study we did not see a difference between these two types of protein in our study. The discrepancy of our findings with those in the Premier Study in which risk for developing hypertension was 21% lower per en% of plant protein intake[11] may be found in the fact that only individuals with elevated blood pressure were included. Possibly these adults were more sensitive to blood pressure lowering effects of plant protein. In the Spanish SUN cohort a 50% risk reduction for hypertension was found for plant protein.[10] However, possibly the distribution of protein sources between the current study and the SUN cohort was different. In Spain, on average more legumes are eaten, and residual confounding from isoflavones in soy may play a role.

Evidence on specific sources of protein in relation to blood pressure is scarce.[6] A few observational studies have been conducted in which urinary taurine was used as a biomarker of dietary seafood protein, showing inverse associations.[23-25] In the Netherlands, the intake of seafood protein is very low (~3% of total protein intake [16]), so we could not investigate this association in the current study. Furthermore, in two trials the effect of meat protein on blood pressure was investigated, but no significant effect was observed. However, in a previous analysis in the Rotterdam Study, including 2241 Dutch adults aged ≥55y, we observed a direct association of meat protein with incidence of hypertension in those aged ≥70 y.[13] In the current analysis we did not observe an association between meat protein and hypertension. However, because of ageing kidney function in the elderly of the Rotterdam Study may have been declined [26], which affects handling of high protein intake, and, consequently, in-

crease risk of hypertension. The difference with the results of the Rotterdam Study may, therefore, be explained by the younger age of the current population.

With high grain protein intake, we observed a significant 15% reduced hypertension risk. Although the mechanisms via which protein (sources) may reduce blood pressure are largely unknown, amino acid composition may play a role. In the INTERMAP study a 2 SD higher intake of glutamic acid (4.7% of total protein) was associated with 1.5 mmHg lower systolic blood pressure and 1.0 mmHg lower diastolic blood pressure.[27] A major contributor to grain protein intake in the Netherlands is wheat from bread [28], which contains high levels of glutamic acid (31.4% [29]). However, we can also not exclude that residual confounding by healthy dietary and lifestyle factors, associated with high grain protein intake, are responsible for the observed associations in this study.

In conclusion, higher intake of grain protein may contribute to the prevention of hypertension, which warrants confirmation in other population-based studies and randomized controlled trials.

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W.M.M.V., J.M.A.B., M.F.E., J.M.G., and W.A. designed the research; W.M.M.V., and J.M.A.B. conducted research; W.A. analysed data; W.A., M.F.E., and M.G. wrote paper; W.A. has the

primary responsibility for final content of the paper; All authors have read and approved the final manuscript.

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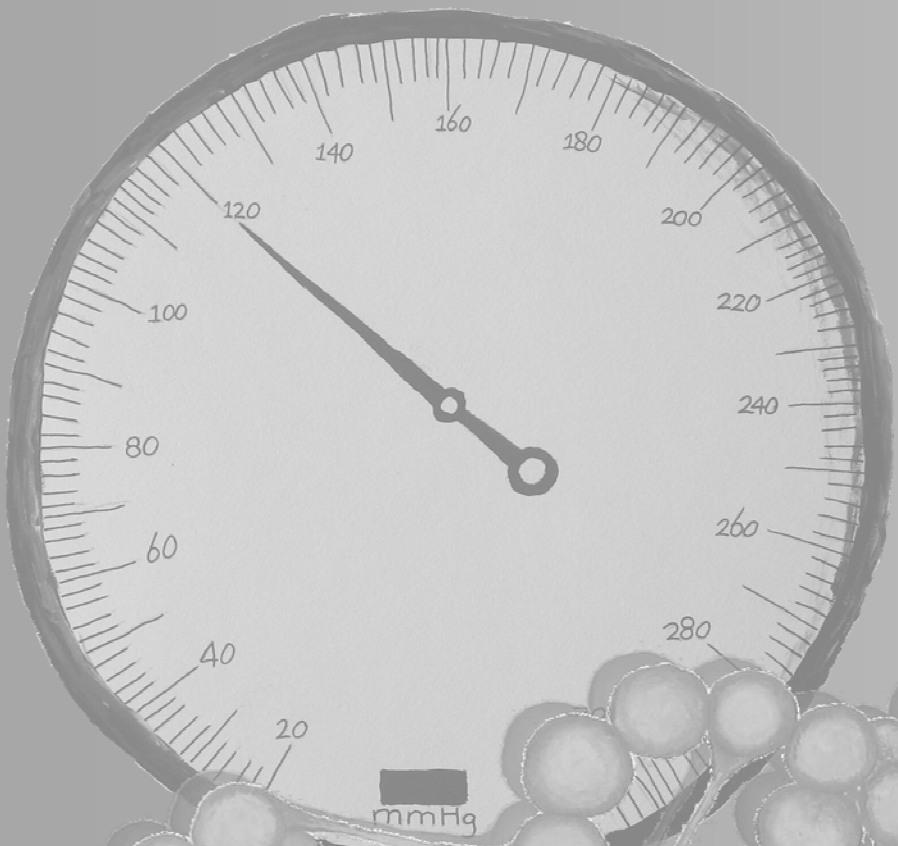
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mmHg

Chapter 5

Dietary protein and risk of hypertension in a Dutch older population: the Rotterdam Study

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Abstract

Background

Several observational studies suggest an inverse association of protein with blood pressure. However, little is known about the role of dietary protein from specific sources in blood pressure.

Method

We examined the relation between several types of dietary protein (total, plant, animal, dairy, meat, grain, fish, soy, and nut) and incident hypertension in 2241 participants from the Rotterdam Study, aged at least 55 years, who were free of hypertension at baseline. Hazard ratios, with 95% confidence intervals (CIs), for incident hypertension during 6 years of follow-up were obtained per standard deviation (SD) of energy-adjusted intake of protein. Hazard ratios were adjusted for age, gender, body mass index (BMI), baseline systolic blood pressure (SBP) smoking, educational level, alcohol, intake of carbohydrates, other nutrients, and other types of protein (if applicable). We conducted stratified analyses by age (cut-off 70 years), gender, and BMI (cut-off 25 kg/m2).

Results

The risk of hypertension in the total cohort (1113 cases) was not related to intake of total protein or types of protein (all hazard ratios ~1.00 per SD). Gender and BMI did not significantly modify the associations of dietary protein with hypertension. In 559 participants aged at least 70 years, the intake of animal protein was positively related to risk of hypertension (hazard ratio 1.37 per SD, 95% CI 1.09–1.72). For participants aged below 70 years no association was found (hazard ratio 0.92, 95% CI 0.81– 1.06).

Conclusion

Total dietary protein or types of protein are not related to incident hypertension in this older population. In the more aged, however, high intake of animal protein may increase the risk of hypertension, which warrants further investigation.

Introduction

Elevated blood pressure, a major risk factor for cardiovascular disease, is highly prevalent worldwide.[1] In the year 2000, 25% of the adult population had hypertension, defined as average systolic blood pressure (SBP) at least 140mmHg, diastolic blood pressure (SBP) at least 90 mmHg, or use of antihypertensive medication. This proportion is likely to increase to 29% in 2025.[1]

Several observational studies and trials have shown an inverse relation between protein intake and blood pressure.[2-5] Observational follow-up data (6 years) from the large MRFIT trial among 11 342 normotensive US men with a mean protein intake of 17 energy percentage, showed a 0.06mmHg lower SBP per energy percentage protein intake.[2] Furthermore, a 20% reduced risk of hypertension for high versus low total protein intake was reported in 5880 Hispanic university graduates, although these findings were not statistically significant.[3] In the INTERSALT study among 10 020 normotensive adults from 32 countries, 24 h urinary total nitrogen and urinary urea nitrogen, as biomarkers for total protein intake, were inversely related with blood pressure.[4] The blood pressure of 164 healthy US adults in the OmniHeart randomized cross-over trial decreased more after a 6-week high protein diet compared with a diet high in carbohydrates[5], whereas no difference in blood pressure was found with a diet high in monounsaturated fat.

Specific types of protein may have different effects on blood pressure. In several observational studies animal protein intake was not associated with blood pressure, whereas an inverse association was observed for plant protein.[3,6-8] Although the relation between blood pressure and protein-rich foods such as dairy[9-11], fish[12], soy[13], and nuts[14] has been examined, data on the association between protein from these foods and blood pressure is scarce.

Finally, there may be subgroups in which blood pressure is differentially affected by protein intake. A stronger inverse association between urinary 3-methylhistidine, a marker for animal protein intake, and blood pressure was found for overweight and obese people in the cross-sectional CARDIAC study among 669 Chinese participants aged 48–56 years[15]. In the OmniHeart trial blood pressure effects were more pronounced in hypertensive than in prehypertensive participants[5]. The sensitivity of blood pressure to dietary influences, including protein intake, may furthermore increase with age as the cardiovascular system becomes less resilient during ageing. Indeed, in the INTERSALT study the inverse association was stronger for participants aged 40–59 years, than for participants aged 20–39 years.[4]

To clarify the role of different types of protein in the development of hypertension, we examined the intake of total protein, types of protein (plant and animal), and protein from specific sources (dairy, meat, fish, soy, nuts) in relation to incident hypertension in the general older population of the Rotterdam study. Additionally, we examined these associations by gender, age and body mass index (BMI), to identify potentially sensitive subgroups.

METHODS

Rotterdam study

The present analyses formed part of the Rotterdam study, a population-based cohort study on the occurrence and progression of chronic diseases and their risk factors in people aged at least 55 years.[16] A schematic design of the Rotterdam study is given in **Table 5.1**. In brief, between 1990 and mid-1993 all residents of a suburb of Rotterdam in this age category were invited to participate and 7983 people (78%) responded. Participants were interviewed at home and 89% was physically examined at the research center. The cohort was re-examined during follow-up in 1993–1995 and 1997–1999. Written informed consent was obtained from all participants. The medical ethics committee of Erasmus University approved the study protocol.

Table 5.1. *Schematic design of the Rotterdam study.*

	Baseline	**2 y follow-up**	**6 y follow-up**
Period	1989-1993	1993-1995	1997-1999
N	7,983	6,315	4,797
Measurements	· Clinical examination (Including blood pressure measurement) · Interview on education, health status, behavior and diet (FFQ)	· Clinical examination (including blood pressure measurement) · Interview on education, health status, and behavior	· Clinical examination (including blood pressure measurement) · Interview on education, health status, and behavior

blood pressure=blood pressure; FFQ=food frequency questionnaire.

Dietary assessment

At baseline, participants completed a checklist at home about foods and drinks they had consumed at least twice a month during the preceding year, as well as dietary habits, use of alimentary supplements, and prescribed diets. Next, during their visit to the research center, they underwent a standardized interview with a trained dietician based on the checklist, using a computerized 170- item semi-quantitative Food Frequency Questionnaire (FFQ), taking into account seasonal variations in fruit, vegetable and fish intake.[17] For each item the frequency was recorded in times per day, week, or month. The number of servings per frequency was expressed in natural units (for example, slice of bread or apple), household measures (for example, cup or spoon), or grams (cooked vegetables or mixed dishes). These dietary data were converted into total energy intake and nutrient intakes per day using the Dutch Food Composition Table of 1993.[18]

In a validation study the FFQ was compared with fifteen 24-h food records, collected over 1 year in six collection periods of 2 or 3 consecutive days, and with 24 h urinary urea excretion during 4 non-consecutive days.[19] The Pearson correlations with the food records, adjusted for age, gender, energy, and within-person variation, were 0.69 for energy intake, 0.50 for fat intake, 0.79 for carbohydrate intake, 0.66 for total protein intake, and 0.59 for plant protein intake. The Spearman correlation with urinary urea was 0.67 for total protein intake.[19]

For the present analyses we assessed protein intake from several specific sources next to total, animal and plant protein. Dairy protein was calculated from various types of milk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, and cheese. Meat protein was calculated from all kinds of meat (including poultry) and meat products, and fish protein included protein from all kinds of fish, crustacean, and shellfish. Grain protein was calculated from bread, cake, cookies, grains and other grain products. Soy protein included protein from tofu and other soy-containing meat substitutes, and nut protein was calculated from nuts and peanut butter. The FFQ was not specifically validated for protein from these sources. However, correlations for nutrients that are known to be associated with several types of protein intake were good with 0.52 for potassium, 0.72 for calcium, 0.71 for magnesium, and 0.52 for saturated fat.[19]

Blood pressure measurements

Blood pressure measurements were taken at the research center by a trained research assistant at baseline and during follow-up examinations after 2 years and after 6 years.[16,20] blood pressure was measured in duplicate at the right upper arm using a random-zero sphygmomanometer with a 32 x17 cm cuff, after the participant had been sitting quietly for at least 5 min. SBP was recorded at the appearance of sounds (first-phase Korotkoff) and SBP at the disappearance of sounds (fifth-phase Korotkoff). SBP and SBP were calculated as the average of the two measurements. Hypertension was defined as SBP at least 140mmHg or SBP at least 90mmHg or the use of antihypertensive medication. At the research center a physician ascertained the indication for which the medication had been prescribed.

Collection of risk factor data

Information on current health status, medical history, medication use, smoking behaviour, and education was obtained by trained research assistants. Participants were classified as current smokers, former smokers, or never smokers. Education was defined as low (primary education), intermediate (secondary general or vocational education), and high (higher vocational education or university). Height and body weight were measured while the participants wore indoor clothing without shoes. BMI was calculated as weight in kilograms divi-

ded by the square of height in meters. Alcohol intake was assessed based on self-reported number of beverages consumed weekly, and converted into grams of ethanol per day. Information on prevalent cardiovascular disease, defined as a history of myocardial infarction or stroke, was assessed during a home interview and verified in medical records at the GP office. Participants who did not take antidiabetic medication received a 37.5% oral glucose solution (75 g of glucose) while in a non-fasting state. Venous glucose levels were then measured before and after 2 h. Diabetes mellitus was defined as the use of antidiabetic medication or a random or post load serum glucose level at least 11.1 mmol/l.

Population for analysis

At baseline 7129 participants underwent physical examination, and reliable blood pressure measurements were obtained in 6985 participants. For the present analyses we excluded 3872 participants (55%) who had hypertension at baseline, 469 participants without information on hypertension status at both follow-up measurements and 403 without data on dietary intake, leaving 2241 participants for the present analyses.

Data analysis

Intake of total protein, types of protein, and protein from specific sources was first adjusted for total energy intake according to the residual method[21], except for protein from fish, soy, and nuts for which consumption was low. Baseline characteristics of the study population were calculated across tertiles of energy-adjusted total protein intake. Data in text and tables are presented as mean ±standard deviation (SD), unless stated otherwise.

We used Cox proportional hazard modelling to estimate hazard ratios with 95% confidence intervals (95% CIs) for 6-year incidence of hypertension and dietary protein intake. We first calculated hazard ratios per SD of energy-adjusted protein intake (total, plant, animal, dairy, meat and grain protein, in g/day) or, because of low intakes and skewed distributions, across two categories indicating use or non-user (protein from fish, soy and nuts). To allow better comparison between types of protein we repeated the analyses per 5 g of energy-adjusted protein intake.

For participants who did not develop hypertension during follow-up we computed survival time as years from baseline to the end of study period (i.e. 6-year examination visit). For participants who developed hypertension, we attributed 1 year of follow-up if hypertension was identified during the 2-year examination visit, and 4 years of follow-up if hypertension was identified during the 6-year examination visit. The basic model (model 1) included adjustment for age (continuous) and gender. Subsequently, we performed multivariable analysis (model 2) with adjustment for age, gender, BMI (continuous, kg/m2), baseline SBP

(continuous, mmHg), smoking status (current/past/never), alcohol consumption (tertiles), and educational level (three categories). In model 3 further adjustment was performed for intake of total energy (continuous, kJ/day), potassium, sodium (only from foods), calcium, magnesium, fiber, carbohydrates, saturated fatty acids, polyunsaturated fatty acids (all continuous, g/day) and other types of protein (if applicable). Because dairy protein and calcium were strongly correlated (r=0.87), hazard ratios for dairy protein were calculated without and with adjustment for calcium. A questionnaire on physical activity was implemented in the Rotterdam study in 1997, and data are available for 27% of our participants (n=616). Post-hoc analyses were conducted in this subgroup using the full model with and without adjustment for physical activity to investigate whether this variable confounded the protein –blood pressure associations.

A number of predefined subgroup analyses were performed for all types of protein that were regularly eaten (total, plant, animal, dairy, meat, grain), in strata of gender, age (cut-off 70 years), and overweight status (cut-off 25 kg/m2), using the full model (model 3). Data analysis was performed using SAS software (SAS Institute, Cary, North Carolina, USA) version 9.1 and a two-sided P value of less than 0.05 was considered statistically significant.

Results

Descriptive data

The mean age of the study population was 65±7 years and 43% was male. The mean BMI was 25.7±3.4 kg/m2, with 54% of the participants being overweight. Because hypertensive participants were excluded from the analysis, mean blood pressure at baseline was rather low for this older population, that is 122±12mmHg systolic and 68±9mmHg diastolic. The diet contained 81±7 g/day of energy-adjusted protein (range 37–150), and the ratio of animal-to-plant protein was approximately 2 : 1. Dairy (30%) and meat (27%) provided most of the protein intake, whereas 3.6% of total protein intake came from fish, 19% from grain, 2.1% from nuts, 0.3% from soy, and 19% from other sources (e.g. potatoes, vegetables, fruits and eggs).

Baseline characteristics of the population by tertiles of energy-adjusted total protein intake are presented in **Table 5.2**. Participants with a high protein intake were younger and were more likely to be overweight or obese. The highest tertile of protein intake comprised less current smokers. Furthermore, with a higher intake of energy-adjusted protein participants had a higher intake of fiber and minerals (potassium, magnesium, calcium, and sodium from foods), and a lower intake of fat and carbohydrates.

Table 5.2. *Baseline characteristics by tertiles of energy adjusted total protein intake of 2,241 participants from the Rotterdam Study who were free of hypertension at baseline.*

	Energy-adjusted tertile of total protein intake (g/d)		
	<75 (n=747)	**75-85 (n=747)**	**>85 (n=747**
Age, y	67 ± 7	65 ± 7	64 ± 7
Males, %	45	41	43
Body mass index, kg/m^2	25.1 ± 3.2	25.7 ± 3.2	26.2 ± 3.5
Overweight or obese,%	46	54	63
High educational level, %	11	13	12
Alcohol consumers, %	81	84	81
Physical activity, MET hours/week[1]	104 ± 46	104 ± 53	109 ± 48
Current smokers, %	29	24	23
Systolic blood pressure, mmHg	122.4 ± 11.9	121.8 ± 11.7	121.3 ± 12.2
Diastolic blood pressure, mmHg	68.2 ± 8.6	68.3 ± 8.2	68.6 ± 8.8
Diabetes mellitus, %	5.0	5.1	6.6
Prevalent coronary heart disease, %	10.0	10.2	10.2
Dietary intakes			
Total energy, kJ/day	8611 ± 2259	8303 ± 1970	8575 ± 2207
Total protein, g/d	70 ± 15	81 ± 14	97 ± 19
Animal protein, g/d	42 ± 10	52 ± 10	67 ± 16
Plant protein, g/d	28 ± 8	29 ± 8	30 ± 9
Dairy protein[2], g/d	18 ± 8	23 ± 9	33 ± 13
Meat protein[3], g/d	18 ± 8	22 ± 8	26 ± 11
Grain protein[4], g/d	15 ± 6	16 ± 5	16 ± 6
Fish protein[5], % users	62	70	77
Soy protein[6], % users	1	3	5
Nut protein[7], % users	52	53	58
Total fat, g/d	86 ± 29	81 ± 26	82 ± 29
Saturated fat, g/d	34 ± 12	32 ± 11	32 ± 13
Mono-unsaturated fat, g/d	29 ± 11	27 ± 10	28 ± 11
Poly-unsaturated fat, g/d	17 ± 8	16 ± 8	15 ± 7
Total carbohydrates, g/d	230 ± 69	214 ± 58	214 ± 59
Sodium[8], mg/d	2006 ± 592	2234 ± 588	2518 ± 724
Potassium, mg/d	3400 ± 716	3690 ± 695	4172 ± 834
Magnesium, mg/d	285 ± 70	311 ± 65	351 ± 74
Calcium, g/d	899 ± 264	1099 ± 307	1416 ± 438
Fiber, g/d	16 ± 5	17 ± 4	19 ± 5

Data are presented as mean ± SD or %, unless stated otherwise.
[1]n=616; [2]Includes protein from milk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, and cheese; [3]Includes protein from meat, meat products and poultry; [4]Includes protein from bread, cake and cookies, grains and grain products; [5]Includes protein from fish, crustacean, and shellfish; [6]Includes protein tofu and meat substitutes consisting of protein; [7]Includes protein from nuts, cocktail nuts and peanut butter; [8]Only from foods, discretionary salt intake not measured.

Protein intake and incidence of hypertension

During 6 years of follow-up a total of 1113 cases of hypertension were identified. Incident hypertension was not associated with intake of total protein, plant protein, or animal protein (all hazard ratios ~1.00 per SD; **Table 5.3**). When analysing the association per 5 g of daily protein intake fully adjusted hazard ratios were similar to each other; that is total protein 1.01 (0.97– 1.05), plant protein 1.02 (0.94–1.11), and animal protein 1.01 (0.97–1.05).

Within the subgroup of 616 participants for whom data on physical activity were available, 207 new cases of hypertension developed (3465 person-years). In this subgroup, inclusion of physical activity in the full model did not change the estimates (hazard ratio 1.10 per SD of total protein intake, with and without adjustment).

We observed no clear association between protein intake and hypertension when we focused on protein from specific sources (**Tables 5.3 and 5.4**). All hazard ratios were close to 1.00, with a possible exception for protein from dairy foods, which showed a non-significant hazard ratio of 0.91 (0.82–1.01) without adjustment for calcium. Additional adjustment for calcium resulted in a hazard ratio of 1.00 per SD with a relatively wide 95% CI (0.78– 1.28). Analyses per 5 g of protein intake resulted in fully adjusted hazard ratios of 0.96 (0.92–1.01) for protein from dairy without adjustment for calcium, 1.01 (0.96–1.06) for protein from meat, and 0.98 (0.87–1.10) for protein from grain.

After we stratified by age using the full model (**Figure 5.1**), we observed an increased risk of developing hypertension in participants aged at least 70 years with higher intake of animal protein (hazard ratio 1.37 per SD, 95% CI 1.09– 1.72, $p_{interaction}$=0.22) and protein intake from

Table 5.3. *Hazard ratio for hypertension per SD of energy adjusted protein intake after 6 years of follow-up.*

		Hazard ratio of hypertension (95% CI)		
	SD (g/d)	Model 1	Model 2	Model 3
Total protein	13.2	1.01 (0.96- 1.08)	1.00 (0.94- 1.07)	1.03 (0.92- 1.15)
Plant protein	5.8	0.99 (0.94- 1.05)	1.02 (0.95- 1.08)	1.03 (0.93- 1.13)
Animal protein	13.4	1.02 (0.96- 1.08)	1.00 (0.94- 1.06)	1.02 (0.91- 1.15)
Dairy protein	11.2	0.95 (0.90- 1.01)	0.94 (0.89- 1.00)	0.91[a] (0.82- 1.01)
Meat protein	9.1	1.06 (1.00- 1.13)	1.06 (1.00- 1.13)	1.02 (0.93- 1.10)
Grain protein	3.4	1.03 (0.97- 1.09)	1.02 (0.96- 1.08)	1.02 (0.95- 1.08)

Number of cases: 1,113, for 8,707 person-years
Model 1: adjusted for age (continuous) and gender; Model 2: additionally adjusted for BMI (continuous, kg/m^2), baseline SBP (continuous, mmHg), smoking status (current/past/never), alcohol consumption (tertiles) and educational level (3 categories); Model 3: additionally adjusted for intake of total energy (continuous, kJ/d), potassium, sodium (only from foods), calcium, magnesium, fiber, carbohydrates, saturated fatty acids, polyunsaturated fatty acids and, if applicable, other types of protein (all continuous, g/d)
[a]*Not adjusted for calcium due to multicollinearity*

Table 5.4. *Hazard ratio for hypertension after 6 years of follow-up in categories of fish, soy and nut protein intake.*

	Median intake (g/d)	Cases	Person-years	HR (95% CI)	
Fish protein					
No (n=677)	0.0	331	2669	1.00	(ref)
Yes (n=1545)	3.3	782	6037	1.06	(0.93- 1.21)
Soy protein					
No (n=2153)	0.0	1083	8415	1.00	(ref)
Yes (n=69)	5.2	30	292	0.95	(0.65- 1.39)
Nut protein					
No (n=1018)	0.0	529	3782	1.00	(ref)
Yes (n=1204)	2.4	584	4924	1.05	(0.92- 1.19)

Model 3: adjusted for age (continuous), gender, BMI (continuous, kg/m^2), baseline SBP (continuous, mmHg), smoking status (current/past/never), alcohol consumption (tertiles), educational level (3 categories), intake of total energy (continuous, kJ/d), potassium, sodium (only from foods), calcium, magnesium, fiber, carbohydrates, saturated fatty acids, poly-unsaturated fatty acids and other types of protein (all continuous, g/d).

meat (hazard ratio 1.29 per SD, 95% CI 1.09–1.51, $P_{interaction}$=0.03). No such association with animal protein intake was observed in participants aged 55–69 years (hazard ratio 0.92 per SD, 95% CI 0.81–1.06).

Gender and overweight did not significantly modify the association between protein intake and hypertension risk. Stratification by gender resulted in a hazard ratio of 1.02 (0.88–1.19) per SD of total protein intake for men and 1.14 (0.95–1.36) for women. When we examined risk of hypertension by overweight status, hazard ratios were 1.09 (0.90–1.31) for normal-weight and 0.99 (0.86–1.14) for overweight and obese participants.

Discussion

In a general older Dutch population we found no association of total dietary protein or several types of protein with 6-year risk of hypertension. In those aged at least 70 years, however, a high intake of animal protein, especially from meat, was associated with 37% increased risk of hypertension.

Protein intake was assessed by self-report, which can cause misclassification because of errors in dietary recall. The FFQ that we used was validated against fifteen 24-h food records in 80 participants from the Rotterdam study.[19] Cross-classification into quintiles resulted in correct classification of 83% of the participants in the same or adjacent quintile for energy-adjusted total protein intake, whereas 0% was classified in the most distinct quintile. For

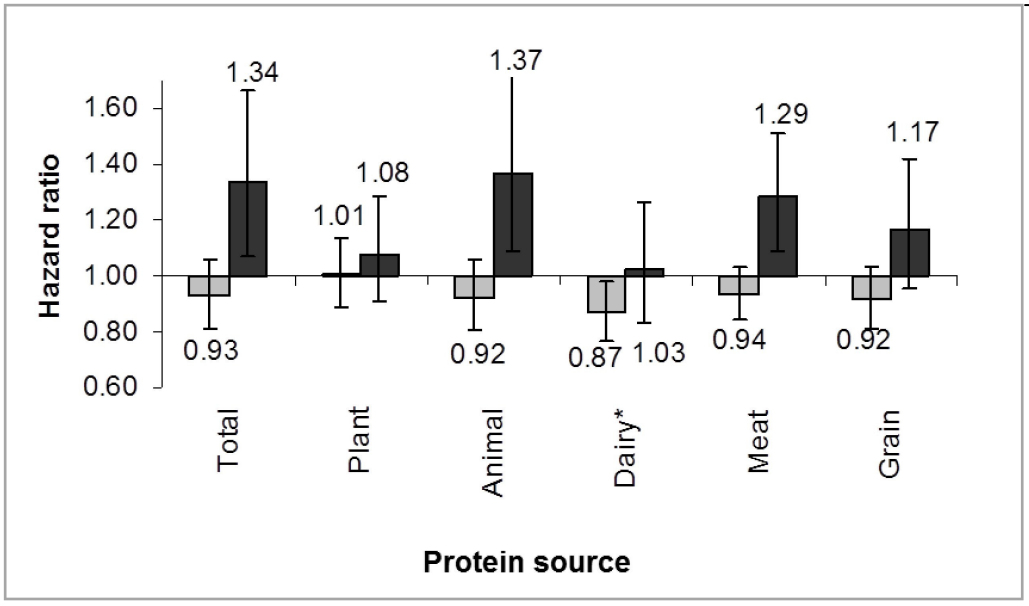

Figure 5.1. *Hazard ratios for incident hypertension per SD of protein intake, by age.*

Age <70y Age ≥70y

All estimates are adjusted for age (continuous), gender, BMI (continuous, kg/m^2), baseline SBP (continuous, mmHg), smoking status (current/past/never), alcohol consumption (tertiles), educational level (3 categories), intake of total energy (continuous, kJ/d), potassium, sodium (only from foods), calcium, magnesium, fiber, carbohydrates, saturated fatty acids, poly-unsaturated fatty acids and other types of protein (all continuous, g/d).
*Not adjusted for calcium, due to multicollinearity.

energy-adjusted plant protein these percentages were 73 and 1.3%, respectively. For total, plant and animal protein, therefore, we do not expect much bias from misclassification. For protein from specific sources (dairy, fish, grain, soy, nut) the FFQ was not validated. However, correlations for nutrients that are known to be associated with (types of) protein intake were good. Also for these types of protein, therefore, we do not expect much misclassification.

In general, the range of protein intake was relatively small, which may have resulted from the homogeneous eating habits of this older population. The SD of the unadjusted mean total protein intake in our population was 20 g/day, which is smaller than the SD of 27 g/day in a

big sample of the Dutch population with a larger age range (18–65 years).[22] Due to the small contrasts in protein intake an existing association between dietary protein and blood pressure may have been missed in the present study. However, repeating our multivariate analysis in quartiles instead of per SD, forcing more contrast in exposure, did not reveal different risk estimates.

Extensive data collection in the Rotterdam study made it possible to control for many potential confounders. Data on physical activity, however, were only available for part of our participants (27%). Additional adjustment for physical activity within this subgroup did not change the conclusions, probably because the multivariable model already included total energy intake and BMI, which are known markers of energy expenditure. An important blood pressure determinant for which the analyses were not fully controlled is sodium intake, as our FFQ did not measure salt use during cooking and at the table. If salt intake is correlated with dietary protein, residual confounding from added salt may have biased inverse associations towards the null, whereas positive associations would be amplified.

We excluded participants who were hypertensive at baseline from our analyses. Because the greatest risk factor for developing hypertension is ageing, the remaining population might have been intrinsically resistant to high blood pressure. However, the percentage of participants who developed hypertension during 6 years of follow-up was similar to the percentage of hypertensive participant excluded at baseline (both ~50%). Furthermore, significant associations between dietary factors and hypertension have been demonstrated in the same study population.[11] We do, therefore, not expect that the null association we found is due to hypertension resistance in the selected population.

Previous observational studies suggested an inverse relation between protein intake and blood pressure or incident hypertension[2-4,23-25], although not consistently[6,8,26]. Several randomized controlled trials confirmed a beneficial effect of dietary protein on blood pressure[5,27-29], but this may also be attributable to a lower intake of carbohydrates. In the OmniHeart trial[5], a randomized fully controlled feeding trial, a stronger decrease in blood pressure was shown after 6 weeks on a high-protein diet as compared with an isocaloric high-carbohydrate diet. This difference was not seen with a diet rich in monounsaturated fat. Therefore, we adjusted the hazard ratios for intake of carbohydrates. However, omitting this adjustment from the full model did not essentially change our results (hazard ratio per SD of total protein intake 1.02, 95% CI 0.93–1.13). In observational studies on types of protein (plant, animal) and change in blood pressure or incident hypertension, inverse associations were found for plant protein but not for animal protein [3,6-8]. However, a 6-week plant protein diet was not superior to an isocaloric mixed protein diet in a blood pressure trial in 23 diabetic patients[30], which was in agreement with earlier findings in normotensive people, when blood pressure was similarly affected by soy protein and casein protein[31], and non-meat and meat protein[32,33]. The discrepancy in findings for plant or animal protein intake between observational studies and trials may be explained by the limitations of observational studies in separating the effects of several nutrients on blood pressure.[24] In our study, hazard ratios for dairy protein were not adjusted for calcium due to a high correlation between dairy protein and calcium, which resulted in a hazard ratio of 0.91 (0.82–1.01) per SD. However, it is not possible to know whether this risk reduction is due to the intake of dairy protein or calcium. When calcium was included in the full model the overall hazard ratio of pro-

tein intake from dairy changed to 1.00 per SD with a relatively wide 95% CI (0.78–1.28), suggesting collinearity between dairy protein and calcium intake. Similarly, the intake of soy protein could not be disentangled for concomitant intake of isoflavones.

We found a 37% increased risk of developing hypertension for higher animal protein intake in a subgroup of older-aged participants. It has been suggested that a high renal acid load, which could result from a diet rich in animal protein, has adverse effects on blood pressure.[34] During ageing, kidney function declines[35,36] which could affect handling of high protein intake and, consequently, increase the risk of hypertension. Alternatively, we cannot exclude residual confounding or effect modification by discretionary salt use (see above). A more unfavourable dietary pattern with a high amount of meat protein could be associated with a higher salt intake. Salt sensitivity increases with age[37], and added salt may amplify an adverse effect of animal or meat protein on blood pressure, especially in the elderly.

In conclusion, we found little evidence for an overall association of dietary protein with incident hypertension in our general older population. People aged at least 70 years who had a high intake of animal protein, however, were at increased risk of developing hypertension. These findings need to be confirmed in other population-based studies, preferably with a sufficiently large range of protein intake and adjustment for use of added salt.

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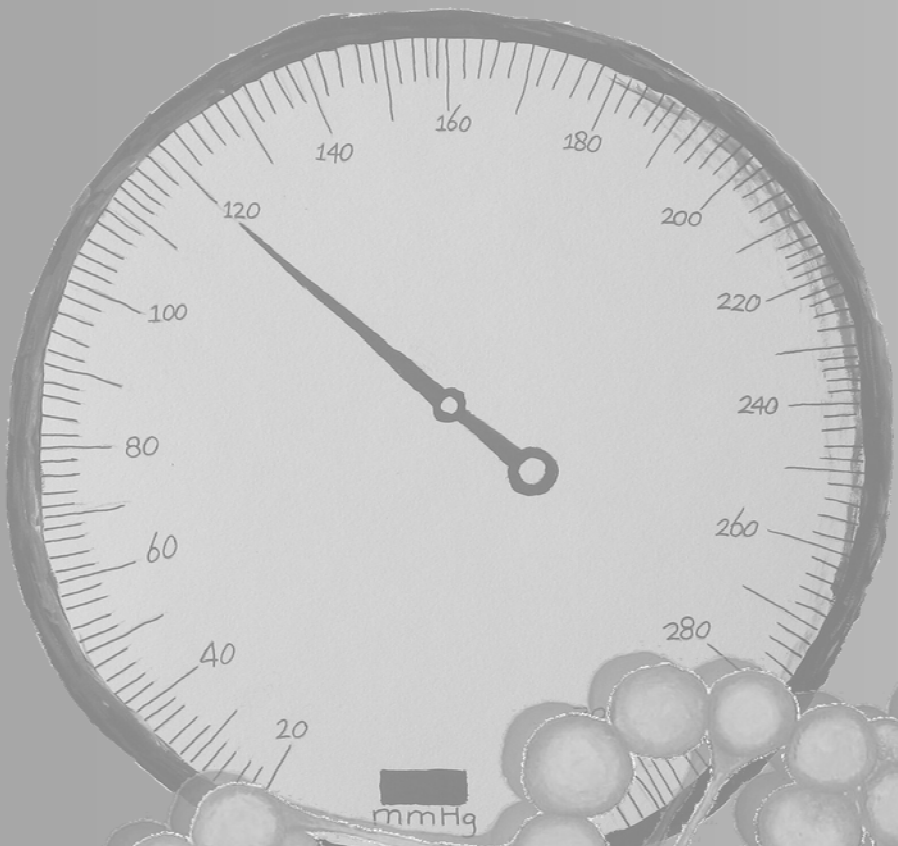
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Chapter 6

Dietary amino acids and risk of hypertension in a Dutch older population: the Rotterdam Study

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Submitted

Abstract

Background

Inverse associations between dietary protein and hypertension have been reported, which may be attributed to specific amino acids.

Objective

We examined whether intake of glutamic acid, arginine, cysteine, lysine, and tyrosine was associated with blood pressure levels (n=3,086) and incident hypertension (n=1,810) in the Rotterdam Study.

Methods

We calculated blood pressure levels in quartiles of amino acid intake as percentage of total protein intake (protein%) with adjustment for age, gender, BMI, smoking, alcohol intake, education, and dietary factors. Subsequently, we used Cox proportional models with the same adjustments to evaluate the associations between specific amino acid intake and hypertension incidence.

Results

Glutamic acid contributed most to protein intake (21 protein%), whereas lysine provided 7%, arginine 5%, tyrosine 4% and cysteine 1.5%. A difference of ~0.3 protein% in tyrosine intake was borderline significantly related to a 2.4 mmHg lower systolic blood pressure (p_{trend}=0.05), but not to diastolic blood pressure (p=0.35). None of the other amino acids was associated to blood pressure. During 6 years of follow-up (7,292 person years) 873 cases of hypertension developed. None of the amino acids were significantly associated with incident hypertension (Hazard ratios ranging from 0.81 to 1.18; all p_{trend}>0.2).

Conclusion

Our data do not support the hypothesis that dietary intakes of the individual amino acids glutamic acid, arginine, lysine, tyrosine, or cysteine as percentage of total protein intake are associated with blood pressure or hypertension incidence. Further evaluations are needed to confirm our findings and to find out whether absolute intake of these amino acids is relevant for the prevention of hypertension.

Introduction

There is a wide consensus that blood pressure can be modified by means of diet and lifestyle modifications such as weight loss, a reduction in salt intake and a dietary pattern rich in fruits and vegetables, such as the DASH diet.[1-3] There is also evidence for a beneficial association between dietary protein and blood pressure.[4] In the well-controlled OmniHeart cross-over trial, systolic blood pressure of 164 healthy US adults consuming a high protein diet for six weeks decreased 1.4 mmHg more compared with a diet high in carbohydrates.[5] In several observational studies the association between protein intake and blood pressure has been studied in more detail suggesting a beneficial association for plant protein whereas no association was observed for animal protein.[6-9]

The mechanisms via which types of dietary protein may differentially influence blood pressure are largely unknown, but amino acid composition may play a role. In the INTERMAP study among 4,680 adults from China, Japan, USA, and UK, it was observed that among those consuming predominantly plant protein compared with animal protein, intake of glutamic acid made up a higher percentage of total protein. In that population a 2 SD higher intake of glutamic acid (4.7% of total protein) was after adjustment for several lifestyle and dietary factors associated with 1.5 mmHg lower systolic blood pressure and 1.0 mmHg lower diastolic blood pressure ($p<0.05$).[10] The hypothesised mechanism for this association was that glutamic acid is a precursor for arginine, which is in turn a precursor for the vasodilator nitric oxide.[11,12] Also several other amino acids have been hypothesized to be involved in blood pressure regulation. Lysine may compete with arginine in the transport system in the gut and herewith unfavourably affect blood pressure.[11,13] Binding of cysteine with excess aldehydes is suggested to beneficially influence blood pressure.[14] Finally, tyrosine may influence catecholamine mechanism by acting as precursor of norepinephrine in the brain, which may reduce cardiovascular sympathetic tone.[15] Essential amino acids (i.e. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine[16]) cannot be synthesized by the body, it could therefore be hypothesised that especially levels of these amino acids in the body can be modified by diet. However, except for lysine, no mechanisms have been described through which these amino acids could influence blood pressure.

Although dietary protein has been associated with blood pressure, it remains unclear whether specific amino acids are associated with blood pressure levels or hypertension incidence. Hence, in the present study we examined whether dietary intakes of the individual amino acids glutamic acid, arginine, lysine, cysteine, and tyrosine were associated with blood pressure levels and incidence of hypertension in the population of the Rotterdam Study.

METHODS

The Rotterdam Study

The present analyses formed part of the Rotterdam Study, a population-based cohort study evaluating the occurrence and progression of chronic diseases and their risk factors in people aged≥55 y.[17] In brief, between 1990 and mid 1993 all residents of a suburb of Rotterdam in this age category were invited to participate and 7,983 people (78%) responded. Participants were interviewed at home and 89% was physically examined at the research centre. Written informed consent was obtained from all participants. The medical ethics committee of Erasmus University approved the study protocol.

For the cross-sectional analysis on amino acid intake and blood pressure levels we excluded 2,602 out of all 7,983 participants because of antihypertensive medication use and 601 participants because of incomplete blood pressure data. In addition we excluded 1,135 participants because of a history of diabetes mellitus, myocardial infarction, or stroke and 559 participants because of incomplete dietary data, leaving 3,086 participants.

Out of the original cohort, 6,418 participants (79%) were re-examined in 1993-1995 and 1997-1999. For the analysis on amino acid intake and incident hypertension we excluded 637 participants with incomplete blood pressure data at baseline or both follow-up periods, 3,135 participants with hypertension at baseline, 581 participants because of a history of diabetes mellitus, myocardial infarction, or stroke, and 255 participants because of incomplete dietary data or incomplete data on survival time, leaving 1,810 participants.

Dietary assessment

At baseline, participants completed a checklist at home about foods and drinks they had consumed at least twice a month during the preceding year, as well as dietary habits, use of alimentary supplements, and prescribed diets. Next, during their visit to the research centre, they underwent a standardized interview with a trained dietician based on the checklist, using a computerized 170-item semi-quantitative food frequency questionnaire (FFQ), taking seasonal variations in fruit, vegetable and fish intake into account.[18] For each item the frequency was recorded in times per day, week, or month. The normal serving for each item was expressed in natural units (for example, slice of bread or apple), household measures (for example, cup or spoon) or grams (cooked vegetables or mixed dishes). These dietary data were converted into total energy intake and nutrient intakes per day using the Dutch Food Composition Table of 1993.[19]

In a validation study the FFQ was compared with fifteen 24h food records, collected over one year in six collection periods of 2 or 3 consecutive days, and with 24h urinary urea ex-

cretion during four non-consecutive days.[20] In short, correlation coefficients between the FFQ and multiple food records were at least 0.52 for the following nutrients: total protein, plant protein, polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), total carbohydrates, polysaccharides, potassium, calcium, fibre, and magnesium.[20] Moreover, 83% of participants were categorised in the same or adjacent quintile for energy adjusted total protein intake. None of the participants were classified in the extreme quintile. For energy adjusted plant protein intake, 73% of participants were categorised in the same or adjacent quintile and 1.3% in the extreme quintile.[20]

We extended the Dutch Food Composition Table of 1996 with data on amino acid content. For this we used data from an existing supplemental table for arginine[21] and data from McCance and Widdowson's that chemically analysed amino acid composition of 150 foods from the food groups grains, milk, eggs, meat, fish, vegetables, fruits, nuts and miscellaneous.[22] We converted amino acid contents of these foods to the Dutch situation according to total protein content of these foods from the Dutch Food Composition Table. Subsequently, we estimated amino acid composition of remaining foods based on those of the analysed foods using predefined assumptions. Finally the amino acid data were linked to the Dutch Food Composition Table[23], which in turn was linked to the data of the Rotterdam Study. In this way, we were able to cover the content of 18 different amino acids for 98% of foods included in the Dutch food composition database. Intake of amino acids per participant was calculated by summing amino acid content of all consumed food items.

blood pressure measurements

blood pressure measurements were taken at the research centre by a trained research assistant at baseline and during follow-up examinations after 2 years and after 6 years.[17,24] blood pressure was measured in duplicate at the right upper arm using a random-zero sphygmomanometer with a 32 x 17 cm cuff, after the participant had been sitting quietly for at least 5 minutes. Systolic blood pressure was recorded at the appearance of sounds (First-phase Korotkoff) and diastolic blood pressure at the disappearance of sounds (Fifth-phase Korotkoff). Systolic and diastolic blood pressure were calculated as the average of the two measurements. Hypertension was defined as systolic blood pressure≥140 mmHg or diastolic blood pressure≥90 mmHg or the use of antihypertensive medication. At the research centre a physician ascertained the indication for which the medication had been prescribed.

Collection of risk factor data

Information on current health status, medical history, medication use, alcohol use, smoking behaviour, and education was obtained by trained research assistants. Height and body

weight were measured while the participant wore indoor clothing without shoes. BMI was calculated as weight in kg divided by the square of height in meters. Information on prevalent cardiovascular disease, defined as a history of myocardial infarction or stroke, was assessed during a home interview and verified in medical records at the office of the general practitioners.

Data analysis

Data analysis was performed using SAS version 9.1 (SAS Institute). Because absolute amino acid intakes are strongly correlated to total protein intake and, as a consequence, strongly positively correlated to each other (correlations in the current study ranging from 0.81 to 0.99), we expressed amino acid intake as percentage of total protein (protein%). We refer to this relative intake of amino acids when 'intake' is mentioned in text and tables. To investigate whether the intakes of amino acids of interest and other characteristics were associated with the proportion of plant protein in the diet, we calculated these baseline characteristics in quartiles of this ratio between plant and animal protein intake. Baseline characteristics are presented in text and tables as mean and standard deviation unless stated otherwise.

Mean blood pressure levels with 95% confidence intervals (95%-CI) were obtained in quartiles of amino acid intake (protein%). The first model included adjustments for age (continuous) and gender. Model 2 additionally included BMI (continuous), education (low, intermediate, or high), smoking status (current, former, or never) and alcohol intake (tertiles). Model 3 (i.e. full model) was additionally adjusted for intake of energy, carbohydrates saturated fatty acids, polyunsaturated fatty acids, fibre, calcium, magnesium, potassium, and sodium (all continuous).

The sample size for our prospective analysis on amino acid intake and hypertension incidence was smaller (n = 1,810) and amino acid intake was therefore divided into tertiles. For participants who did not develop hypertension during follow-up we computed survival time as years from baseline to the end of study period (i.e. 6-year examination visit). For participants who developed hypertension, we allocated 1 year of follow-up if hypertension was identified during the 2-year examination visit, and 4 years of follow-up if hypertension was identified during the 6-year examination visit. Cox proportional hazard models were used to obtain hazard ratios (HR) with 95%-CI for incident hypertension in tertiles of amino acid intake, using the same models as in our cross-sectional analysis.

Because lysine competes with arginine in the transport system we hypothesized that a high intake of lysine compared to arginine might unfavourably influence blood pressure. For this reason we additionally examined whether the ratio of these two amino acids was associated with blood pressure level and hypertension incidence. Furthermore, because we also hy-

pothesized that diet can especially modify blood levels of essential amino acids, we performed a secondary analysis in which we calculated HRs with 95%-CI for incident hypertension in tertiles of essential amino acid intake.

To obtain a p-value for trend, median values of the tertiles or quartiles of amino acid intake were assigned to individuals and entered continuously into the multivariate models. Two-sided p-values <0.05 were considered statistically significant.

RESULTS

Descriptive data

The mean age of 3,086 Dutch adults included in our cross-sectional analysis was 66 ± 7 y and ~40% were men. They had a mean BMI of 26 ± 3 kg/m^2, with 56% of the participants being overweight. Mean blood pressure at baseline was 135/73 mmHg with 38% of participants having a blood pressure >140/90 mmHg. Baseline characteristics across quartiles of relative plant protein intake are shown in **Table 6.1**. With an increasing proportion of plant protein in the diet the proportion of men was higher whereas the percentage of current smokers, alcohol consumers and overweight individuals was lower. Total energy as well as carbohydrate, poly-unsaturated fat, magnesium, and fibre intake increased across quartiles of relative plant protein intake, whereas total protein, saturated fat and calcium intake decreased. Baseline characteristics of 1,810 individuals included in our prospective analysis were very similar, except that mean blood pressure was lower (i.e. 122 ± 12 mmHg systolic and 69 ± 9 mmHg diastolic) because hypertensive participants were excluded at baseline.

Amino acid intake

The contribution of the amino acids of interest to total protein intake is summarized in **Figure 6.1**. Glutamic acid contributed most to protein intake (21 protein%, 17 ± 4 g/d), whereas lysine provided 7 % (6 ± 1 g/d), arginine 5% (4 ± 1 g/d), tyrosine 4% (3 ± 1 g/d) and cysteine 1.5% (1 ± 0.3 g/d). Among those who consumed predominantly plant protein compared with animal protein, intake of glutamic acid, arginine, and cysteine made up a higher percentage of protein, whereas intake of lysine was lower and tyrosine intake was constant over quartiles. (**Table 6.1**). Variations in amino acid intakes were quite small, with differences between medians of the lowest and the highest quartiles ranging from 0.3 protein% for tyrosine to 2.5 protein% for glutamic acid (**Table 6.2**).

Table 6.1. *Baseline characteristics of 3,086 Dutch adults (≥55y) within the ROTTERDAM-cohort*

	Quartiles of the ratio of plant to animal protein			
	<0.43 (n=771)	**0.43-0.53 (n=772)**	**0.53-0.67 (n=772)**	**>0.67 (n=771)**
age, y	66 ± 8	66 ± 7	66 ± 7	67 ± 7
Gender, % men	33	37	42	45
BMI, kg/m2	26 ± 4	26 ± 3	26 ± 3	25 ± 3
Overweight, %	65	57	56	48
systolic BP, mmHg[1]	137 ± 22	135 ± 21	134 ± 20	135 ± 22
Diastolic BP, mmHg[1]	74 ± 11	73 ± 11	73 ± 11	73 ± 11
Current smoker, %	30	28	21	21
Alcohol consumers, %	84	83	85	78
Alcohol intake among consumers, g/d	7.9 (1.4- 20.4)	7.8 (1.5- 17.8)	6.8 (1.5- 18.4)	6.2 (1.4- 15.7)
Dietary intake				
Energy	7925 ± 2100	8251 ± 1946	8543 ± 2031	8853 ± 2266
Total protein, en% (g/d)	19.2 ± 3.2 (88 ± 22)	17.2 ± 2.4 (82 ± 18)	16.2 ± 2.2 (80 ± 17)	14.9 ± 2.5 (77 ± 19)
Plant protein, en% (g/d)	5.0 ± 0.9 (23 ± 6)	5.6 ± 0.8 (27 ± 6)	6.0 ± 0.8 (30 ± 6)	6.9 ± 1.5 (35 ± 10)
animal protein, en% (g/d)	14.2 ± 2.6 (65 ± 18)	11.7 ± 1.7 (56 ± 12)	10.2 ± 1.4 (50 ± 11)	8.1 ± 1.7 (42 ± 12)
glutamic acid, protein% (g/d)	20.2 ± 1.0 (17.8 ± 4.6)	20.6 ± 1.0 (17.0 ± 3.9)	20.9 ± 1.0 (16.7 ± 3.7)	21.4 ± 1.2 (16.4 ± 4.2)
arginine, protein% (g/d)	5.2 ± 0.3 (4.5 ± 1.2)	5.2 ± 0.3 (4.3 ± 1.0)	5.3 ± 0.4 (4.2 ± 0.9)	5.5 ± 0.5 (4.3 ± 1.3)
cysteine, protein% (g/d)	1.3 ± 0.1 (1.2 ± 0.3)	1.4 ± 0.1 (1.2 ± 0.3)	1.5 ± 0.1 (1.2 ± 0.2)	1.5 ± 0.1 (1.2 ± 0.3)
lysine, protein% (g/d)	7.2 ± 0.3 (6.4 ± 1.6)	6.9 ± 0.2 (5.7 ± 1.2)	6.7 ± 0.2 (5.4 ± 1.1)	6.3 ± 0.3 (4.9 ± 1.3)
tyrosine, protein% (g/d)	3.7 ± 0.1 (3.3 ± 0.9)	3.7 ± 0.1 (3.0 ± 0.7)	3.6 ± 0.1 (2.9 ± 0.6)	3.6 ± 0.1 (2.8 ± 0.7)
total fat, en% (g/d)	36.1 ± 6.3 (78 ± 27)	36.2 ± 5.7 (81 ± 26)	35.6 ± 5.7 (83 ± 26)	35.3 ± 6.1 (85 ± 29)
Saturated fat, en% (g/d)	15.0 ± 3.5 (33 ± 13)	14.5 ± 2.8 (33 ± 11)	14.1 ± 2.9 (33 ± 11)	13.3 ± 3.0 (32 ± 12)
mono unsaturated fat, en% (g/d)	12.5 ± 2.7 (27 ± 10)	12.3 ± 2.5 (28 ± 10)	12.1 ± 2.4 (28 ± 10)	11.9 ± 2.9 (29 ± 12)
poly unsaturated fat en% (g/d)	5.9 ± 2.6 (13 ± 6)	6.6 ± 2.7 (15 ± 7)	6.7 ± 2.7 (16 ± 8)	7.3 ± 2.7 (18 ± 9)
Carbohydrates, en% (g/d)	40.9 ± 6.6 (190 ± 60)	43.2 ± 6.2 (209 ± 53)	45.1 ± 6.2 (225 ± 58)	47.3 ± 7.0 (244 ± 65)
Sodium, mg/d[2]	2125 ± 657	2206 ± 659	2270 ± 655	2270 ± 654
Potassium mg/d	3716 ± 873	3657 ± 766	3701 ± 754	3717 ± 880
Magnesium, mg/d	299 ± 75	303 ± 70	315 ± 70	326 ± 80
Calcium, mg/d	1309 ± 487	1153 ± 361	1092 ± 333	968 ± 319
fibre, g/d	15 ± 4	16 ± 4	18 ± 5	20 ± 6

En%= percentage of total energy intake; Protein%=percentage of total protein intake
[1]Antihypertensive medication users have been excluded; [2]Sodium intake only from foods

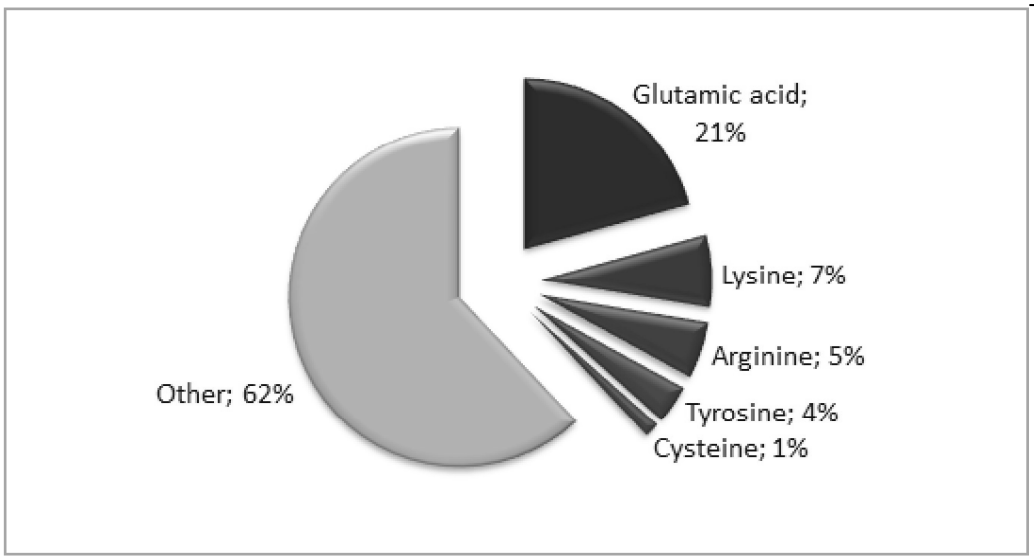

Figure 6.1. *Contribution of each amino acid of interest to total protein intake.*

Amino acid intake and blood pressure levels

Results for the associations between amino acid intake and blood pressure are summarized in **Table 6.2**. After adjustment for age, gender, lifestyle and dietary factors, we did not observe an association between intake of glutamic acid, arginine, or cysteine and blood pressure levels; i.e. systolic blood pressure difference between highest and lowest quartile of intake ranging from -0.6 mmHg to +0.1 mmHg (all p>0.56). Participants with a median intake of 7.3 protein% lysine compared to those with a median intake of 6.3 protein% lysine showed a non-significant higher blood pressure of +1.7 mmHg systolic and +1.0 mmHg diastolic (p_{trend}=0.19 and 0.10 respectively). Participants in the highest quartile of the arginine to lysine ratio (0.86) had a non-significant lower blood pressure compared to participants in the lowest quartile (ratio 0.71); i.e. -1.6 mmHg systolic (p_{trend}=0.35) and -0.3 mmHg diastolic (p_{trend}=0.59). Participants with a median intake of 3.8 protein% of tyrosine, had a 2.4 mmHg lower systolic blood pressure compared to participants with a median intake of 3.5 protein% of tyrosine (p_{trend}=0.05), but without a difference in diastolic blood pressure (-0.4 mmHg, p_{trend}=0.35).

Amino acid intake and hypertension incidence

During 6 years of follow-up, a total of 873 cases were identified (7,292 person-years). None of the amino acids was significantly related to hypertension incidence (**Table 6.3**). Participants with 7.2 protein% of lysine intake showed a non-significant increased risk for incident hypertension, compared with participants with 6.4 protein% of intake (HR 0.15, 95%-CI 0.93-

Table 6.2. *Systolic and diastolic blood pressure levels (95%-CI) in 3,086 untreated Dutch adults aged >55 y in quartiles of amino acid intake.*

	Median intake (protein%)	Model 1		Model 2		Model 3	
		SBP	SBP	SBP	SBP	SBP	SBP
Glutamic acid							
Q1	19.5	135.8 (134.3-137.2)	73.9 (73.1-74.7)	135.3 (133.8-136.7)	73.7 (73.0-74.5)	135.5 (133.9-137.1)	73.8 (72.9-74.7)
Q2	20.4	135.1 (133.7-136.6)	72.8 (72.1-73.6)	134.7 (133.3-136.2)	72.6 (71.9-73.4)	134.9 (133.5-136.4)	72.7 (71.9-73.5)
Q3	21.1	135.4 (134.0-136.8)	73.2 (72.5-74.0)	135.8 (134.4-137.2)	73.4 (72.6-74.2)	135.7 (134.3-137.2)	73.4 (72.6-74.2)
Q4	22.0	134.7 (133.3-136.1)	72.7 (72.0-73.5)	135.2 (133.8-136.7)	73.0 (72.2-73.8)	134.9 (133.2-136.5)	72.8 (72.0-73.7)
p_{trend}		0.34	0.07	0.98	0.29	0.74	0.30
Arginine							
Q1	4.9	135.6 (134.1-137.0)	73.1 (72.4-73.9)	135.7 (134.3-137.2)	73.2 (72.4-74.0)	135.3 (133.6-136.9)	73.0 (72.1-73.9)
Q2	5.1	136.0 (134.6-137.4)	73.5 (72.7-74.2)	136.1 (134.7-137.5)	73.5 (72.7-74.2)	136.1 (134.6-137.5)	73.4 (72.7-74.2)
Q3	5.4	134.7 (133.3-136.2)	72.9 (72.2-73.7)	134.7 (133.3-136.1)	72.9 (72.2-73.7)	134.9 (133.4-136.3)	73.0 (72.2-73.8)
Q4	5.7	134.8 (133.3-136.2)	73.2 (72.4-74.0)	134.5 (133.1-136.0)	73.2 (72.4-73.9)	134.8 (133.2-136.5)	73.4 (72.5-74.2)
p_{trend}		0.29	0.94	0.17	0.76	0.56	0.74
Lysine							
Q1	6.3	134.1 (132.6-135.5)	72.3 (71.6-73.1)	134.7 (133.3-136.2)	72.6 (71.8-73.4)	134.8 (133.1-136.5)	72.8 (71.9-73.7)
Q2	6.7	134.4 (133.0-135.9)	72.6 (71.8-73.3)	134.5 (133.0-135.9)	72.6 (71.8-73.3)	134.5 (133.0-135.9)	72.7 (71.9-73.4)
Q3	7.0	135.4 (134.0-136.8)	73.6 (72.8-74.3)	135.3 (133.9-136.7)	73.5 (72.8-74.3)	135.3 (133.8-136.7)	73.5 (72.7-74.2)
Q4	7.3	137.1 (135.7-138.6)	74.3 (73.5-75.0)	136.6 (135.1-138.0)	74.0 (73.3-74.8)	136.5 (134.8-138.2)	73.8 (72.9-74.7)
p_{trend}		<0.01	<0.01	0.05	<0.01	0.19	0.10

Table 6.2. *Systolic and diastolic blood pressure levels (95%-CI) in 3,086 untreated Dutch adults aged >55 y in quartiles of amino acid intake (continued).*

	Median intake (protein%)	Model 1		Model 2		Model 3	
		SBP	SBP	SBP	SBP	SBP	SBP
Arginine: Lysine							
Q1	0.71	137.1 (135.7-138.6)	73.8 (73.0-74.6)	136.8 (135.3-138.2)	73.6 (72.8-74.4)	136.5 (134.8-138.3)	73.3 (72.3-74.2)
Q2	0.75	135.5 (134.0-136.9)	73.5 (72.8-74.3)	135.5 (134.1-136.9)	73.5 (72.8-74.3)	135.4 (134.0-136.9)	73.4 (72.7-74.2)
Q3	0.79	134.1 (132.6-135.5)	72.9 (72.1-73.7)	134.1 (132.7-135.5)	73.0 (72.2-73.7)	134.2 (132.7-135.7)	73.0 (72.2-73.8)
Q4	0.86	134.4 (132.9-135.8)	72.5 (71.8-73.3)	134.7 (133.2-136.1)	72.7 (71.9-73.4)	134.9 (133.2-136.6)	73.0 (72.1-73.9)
p_{trend}		0.01	0.01	0.04	0.05	0.35	0.59
Cysteine							
Q1	1.3	136.3 (134.8-137.7)	73.5 (72.8-74.3)	135.8 (134.4-137.2)	73.3 (72.6-74.1)	135.4 (133.6-137.2)	73.3 (72.3-74.2)
Q2	1.4	136.6 (135.1-138.0)	74.3 (73.5-75.0)	136.3 (134.8-137.7)	74.1 (73.4-74.9)	136.1 (134.7-137.6)	74.1 (73.3-74.8)
Q3	1.5	133.8 (132.4-135.2)	72.3 (71.5-73.0)	133.9 (132.5-135.3)	72.3 (71.5-73.1)	134.0 (132.5-135.4)	72.3 (71.5-73.1)
Q4	1.6	134.4 (133.0-135.9)	72.7 (71.9-73.5)	135.1 (133.7-136.6)	73.0 (72.2-73.8)	135.5 (133.7-137.3)	73.1 (72.2-74.1)
p_{trend}		0.02	0.02	0.21	0.13	0.79	0.46
Tyrosine							
Q1	3.5	134.8 (133.4-136.3)	73.3 (72.6-74.1)	134.8 (133.4-136.3)	73.3 (72.6-74.1)	135.7 (134.1-137.4)	73.5 (72.6-74.4)
Q2	3.6	136.6 (135.2-138.0)	73.4 (72.7-74.2)	136.7 (135.3-138.2)	73.5 (72.7-74.3)	137.1 (135.6-138.5)	73.6 (72.8-74.3)
Q3	3.7	135.1 (133.7-136.5)	72.6 (71.9-73.4)	135.0 (133.6-136.4)	72.6 (71.9-73.4)	134.9 (133.5-136.4)	72.6 (71.9-73.4)
Q4	3.8	134.5 (133.1-136.0)	73.3 (72.6-74.1)	134.4 (133.0-135.8)	73.3 (72.5-74.1)	133.3 (131.6-135.0)	73.1 (72.1-74.0)
p_{trend}		0.53	0.69	0.42	0.63	0.05	0.35

Values are average blood pressure and 95% confidence interval.
Model 1: Adjusted for age (continuous) and gender; Model 2: Additionally adjusted for BMI (continuous), educational level (low, intermediate, high), smoking (current, former, never), and alcohol consumption (tertiles); Model 3: Additionally adjusted for total energy, carbohydrates, saturated fatty acids poly-unsaturated fatty acids, fibre, calcium, magnesium, sodium (only from food) and potassium (all continuous)..

Table 6.3. *Hazard ratio of hypertension according to tertiles of amino acid intake after 6 years of follow-up.*

	N	Median intake (protein%)	Cases (N)	Person-years	Hazard ratio of hypertension (95%-CI)		
					Model 1	Model 2	Model 3
Glutamic acid							
T1	603	19.7	286	2481			
T2	604	20.7	312	2346	1.09 (0.93- 1.28)	1.13 (0.96- 1.33)	1.18 (0.99- 1.41)
T3	603	21.8	275	2465	0.91 (0.77- 1.07)	0.95 (0.80- 1.13)	1.02 (0.83- 1.26)
p_{trend}					0.23	0.52	0.76
Arginine							
T1	603	5.0	289	2390	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	5.3	289	2401	1.02 (0.87- 1.21)	1.02 (0.87- 1.20)	1.00 (0.83- 1.19)
T3	603	5.6	295	2500	1.08 (0.92- 1.27)	1.07 (0.91- 1.27)	1.06 (0.85- 1.31)
p_{trend}					0.36	0.40	0.81
Lysine							
T1	603	6.4	277	2467	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	6.8	286	2438	1.04 (0.89- 1.23)	1.01 (0.85- 1.19)	1.01 (0.84- 1.21)
T3	603	7.2	310	2387	1.20 (1.02- 1.41)	1.15 (0.98- 1.36)	1.15 (0.93- 1.43)
p_{trend}					0.03	0.10	0.20
Arginine: Lysine							
T1	603	0.72	314	2287	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	0.77	276	2498	0.85 (0.72- 1.00)	0.86 (0.73- 1.01)	0.81 (0.67- 0.97)
T3	603	0.84	283	2506	0.89 (0.76- 1.05)	0.92 (0.78- 1.08)	0.86 (0.69- 1.07)
p_{trend}					0.22	0.39	0.20
Cysteine							
T1	603	1.4	291	2438	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	1.4	291	2462	0.96 (0.82- 1.13)	0.98 (0.83- 1.15)	0.95 (0.79- 1.14)
T3	603	1.5	291	2392	0.97 (0.82- 1.14)	1.02 (0.86- 1.21)	0.98 (0.77- 1.24)
p_{trend}					0.73	0.81	0.83
Tyrosine							
T1	603	3.5	310	2299	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	3.7	275	2507	0.83 (0.71- 0.98)	0.83 (0.71- 0.98)	0.85 (0.71- 1.02)
T3	603	3.8	288	2485	0.87 (0.74- 1.02)	0.86 (0.73- 1.01)	0.92 (0.73- 1.15)
p_{trend}					0.08	0.06	0.17

Model 1: Adjusted for age (continuous) and gender; Model 2: Additionally adjusted for BMI (continuous), educational level (low, intermediate, high), smoking (current, former, never), and alcohol consumption (tertiles); Model 3: Additionally adjusted for total energy, carbohydrates, saturated fatty acids poly-unsaturated fatty acids, fibre, calcium, magnesium, sodium (only from food) and potassium (all continuous).

1.43, p_{trend}=0.20). We observed a non-linear inverse association between the ratio of arginine to lysine and risk of hypertension; 1.00 (ref) for a median ratio of 0.72; a 19% decreased risk for participants in with a median ratio of 0.77 (HR=0.81, 95%-CI=0.67-0.97), and a borderline significant 14% lower risk for participants with a median ratio of 0.84 (0.86, 0.69-1.07; p_{trend}=0.20).

With regard to essential amino acids, none of these amino acids was significantly associated with incident hypertension with HRs ranging from 0.91 to 1.10 (all p_{trend}>0.20; **Supplemental Table I**)

Discussion

In a general older Dutch population we found no association between the habitual intake of glutamic acid, arginine, lysine, and cysteine (expressed as protein%) with blood pressure. For tyrosine intake we found a borderline significant inverse association with systolic blood pressure, but not with diastolic blood pressure. None of the examined amino acids was related to 6-year risk of hypertension.

The Rotterdam Study is a single centre population based cohort in which a wide range of data has been collected. In a validation study using fifteen 24h food records, the FFQ showed a good performance with respect to protein, with 83% of participants being categorised into the same or adjacent quintile of energy adjusted total protein intake, whereas none of the participants was classified into the extreme quintile. For energy adjusted plant protein intake these numbers were 73% and 1.3% respectively.[20]

Our estimate of amino acid intake was based on data from chemical analysis of 150 main foods from the following food groups: grains, milk, eggs, meat, fish, vegetables, fruits, nuts and miscellaneous.[22] This may have introduced measurement error because of potential changes in amino acid composition due to production processes (e.g. production of cheese from milk). Although we expect this measurement error to be small, we cannot exclude that this has led to misclassification of participants, and dilution of the associations between amino acid intake and blood pressure. Another possible explanation for the absence of associations between amino acids and blood pressure or incident hypertension may be the small variation in amino acid intakes in our cohort of older Dutch adults. Furthermore, we studied amino acids as a proportion of total protein intake because absolute intakes were strongly intercorrelated in our study (*r* between 0.81 and 0.99). However, we cannot exclude the possibility that absolute rather than relative amino acid intakes are relevant with regard to blood pressure. This question can only be addressed in randomised controlled trials.

Studies on the association between dietary amino acids and human blood pressure or hypertension incidence are scarce. The relation between glutamic acid intake and blood pressure

was investigated in the INTERMAP study among 4,680 adults.[10] In that study, mean glutamic acid intake was 20.1 protein% (15.7 g/d) ranging from 17.8 protein% in Japan to 24.1 protein% in China. After adjustment for dietary and lifestyle factors, blood pressure was 1.5 mmHg lower with a 4.7 protein% (2 SD) higher glutamic acid intake ($p<0.05$). We could not confirm this association, possibly because INTERMAP included participants from four different countries which resulted in a larger variation in glutamic acid intake (1 SD=2.4 protein%) than in our cohort (1 SD= 1.1 protein%).

We did not find an association between arginine intake and blood pressure. Arginine is a precursor for the vasodilator nitric oxide.[25] In a meta-analysis of 11 trials, the systolic blood pressure effect of arginine supplementation was −5.39 mm Hg (95% CI −8.54 to -2.25, P <0.01).[26] However, arginine doses in these studies ranged between 4 and 24 g/d, which exceeds average dietary intake levels (e.g. 4 ± 1 g/d in the Rotterdam Study). In an observational study among 806 Dutch elderly men (mean age ~71 y) a non-significant systolic blood pressure difference of ~-2 mmHg ($p=0.25$) was found with a 2.2 g/d higher arginine intake.[21] In 1,981 Finnish men with a mean age of 53 ± 5 y, a 2.5 g/d higher arginine intake was related to a 2.6 mmHg lower systolic blood pressure ($p=0.07$).[27] In these studies, however, data were not adjusted for potential confounders.

In those participants included in our study consuming predominantly plant protein compared with animal protein the percentage of lysine intake was lower, whereas the percentage of arginine was somewhat higher. Our results suggested an unfavourable relation between lysine intake and blood pressure or hypertension incidence. Moreover, we observed a tendency towards a beneficial association with hypertension incidence for the ratio of arginine to lysine. This is in line with observational studies in which inverse associations between plant protein and blood pressure were found, whereas no associations were observed for animal protein.[6-9] However, our data were not statistically significant and we therefore cannot draw firm conclusions.

It has been proposed that tyrosine could act as a precursor of norepinephrine in the brain which reduces sympathetic tone, thereby lowering blood pressure.[15] However, in a trial in 13 mildly hypertensive adults 2 weeks supplementation of 7.5 g/d tyrosine did not affect blood pressure.[28] In the present study we observed an inverse association of tyrosine with systolic blood pressure levels, but not with diastolic blood pressure nor with incidence of hypertension. Therefore, we cannot exclude the possibility that our findings for tyrosine are due to chance.

In conclusion, our data do not support a role for relative intakes of the individual amino acids glutamic acid, arginine, lysine, tyrosine, and cysteine in hypertension prevention. Whether absolute intake of these or other amino acids could influence blood pressure still needs to be established. Further evaluations, preferably in cohorts with more hetero-

geneous eating habits and randomised controlled trials, could clarify the role that protein intake and specific amino acids might play in the prevention and treatment of hypertension.

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***Supplemental table I**: Hazard ratio of hypertension according to tertiles of essential amino acid intake in 1,810 Dutch adults (≥55y) within the ROTTERDAM-cohort after 6 years of follow-up*

	N	Median intake (protein%)	Cases (n)	Person-years	Hazard ratio of hypertension (95% CI)		
					Model 1	Model 2	Model 3
Histidine							
T1	603	2.8	284	2459	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	2.9	274	2476	1.00 (0.84-1.18)	0.96 (0.81-1.14)	0.94 (0.79-1.12)
T3	603	3.0	315	2356	1.19 (1.01-1.40)	1.14 (0.97-1.35)	1.10 (0.91-1.33)
p_{trend}					0.03	0.10	0.27
Isoleucine							
T1	603	4.53	291	2370	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	4.68	279	2546	0.87 (0.74-1.03)	0.86 (0.73-1.01)	0.88 (0.73-1.06)
T3	603	4.82	303	2375	0.97 (0.82-1.14)	0.93 (0.79-1.10)	1.00 (0.80-1.27)
p_{trend}					0.69	0.42	0.64
Leucine							
T1	603	7.75	307	2336	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	8.06	276	2500	0.84 (0.71-0.99)	0.81 (0.69-0.96)	0.83 (0.69-1.00)
T3	603	8.37	290	2456	0.86 (0.73-1.02)	0.86 (0.73-1.01)	0.91 (0.71-1.17)
p_{trend}					0.08	0.07	0.22
Methionine							
T1	603	2.20	285	2502	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	2.30	291	2440	1.01 (0.86-1.19)	0.98 (0.84-1.16)	0.96 (0.81-1.15)
T3	603	2.39	297	2350	1.08 (0.91-1.26)	1.04 (0.88-1.23)	1.04 (0.85-1.28)
p_{trend}					0.39	0.66	0.61
Phenylalanine							
T1	603	4.60	307	2347	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	4.74	280	2444	0.87 (0.74-1.02)	0.86 (0.73-1.02)	0.92 (0.77-1.10)
T3	603	4.87	286	2500	0.85 (0.72-1.00)	0.87 (0.74-1.03)	1.00 (0.80-1.24)
p_{trend}					0.05	0.11	0.54
Threonine							
T1	603	3.99	302	2379	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	4.12	274	2521	0.84 (0.71-0.99)	0.81 (0.69-0.96)	0.80 (0.67-0.96)
T3	603	4.24	297	2392	0.96 (0.82-1.13)	0.94 (0.80-1.10)	0.92 (0.75-1.15)
p_{trend}					0.59	0.39	0.30

***Supplemental table I**: Hazard ratio of hypertension according to tertiles of essential amino acid intake in 1,810 Dutch adults (≥55y) within the ROTTERDAM-cohort after 6 years of follow-up (continued).*

	N	Median intake (protein%)	Cases (n)	Person-years	Hazard ratio of hypertension (95% CI)		
					Model 1	Model 2	Model 3
Valine							
T1	603	5.38	292	2377	1.00 (ref)	1.00 (ref)	1.00 (ref)
T2	604	5.61	291	2487	0.94 (0.80-1.10)	0.91 (0.77-1.07)	0.97 (0.81-1.18)
T3	603	5.83	290	2427	0.92 (0.78-1.08)	0.92 (0.78-1.08)	1.07 (0.83-1.39)
p_{trend}					0.32	0.32	0.96

Model 1: Adjusted for age (continuous) and gender; Model 2: Additionally adjusted for BMI (continuous), educational level (low, intermediate, high), smoking (current, former, never), and alcohol consumption (tertiles); Model 3: Additionally adjusted for total energy, carbohydrates, saturated fatty acids poly-unsaturated fatty acids, fibre, calcium, magnesium, sodium (only from food) and potassium (all continuous).

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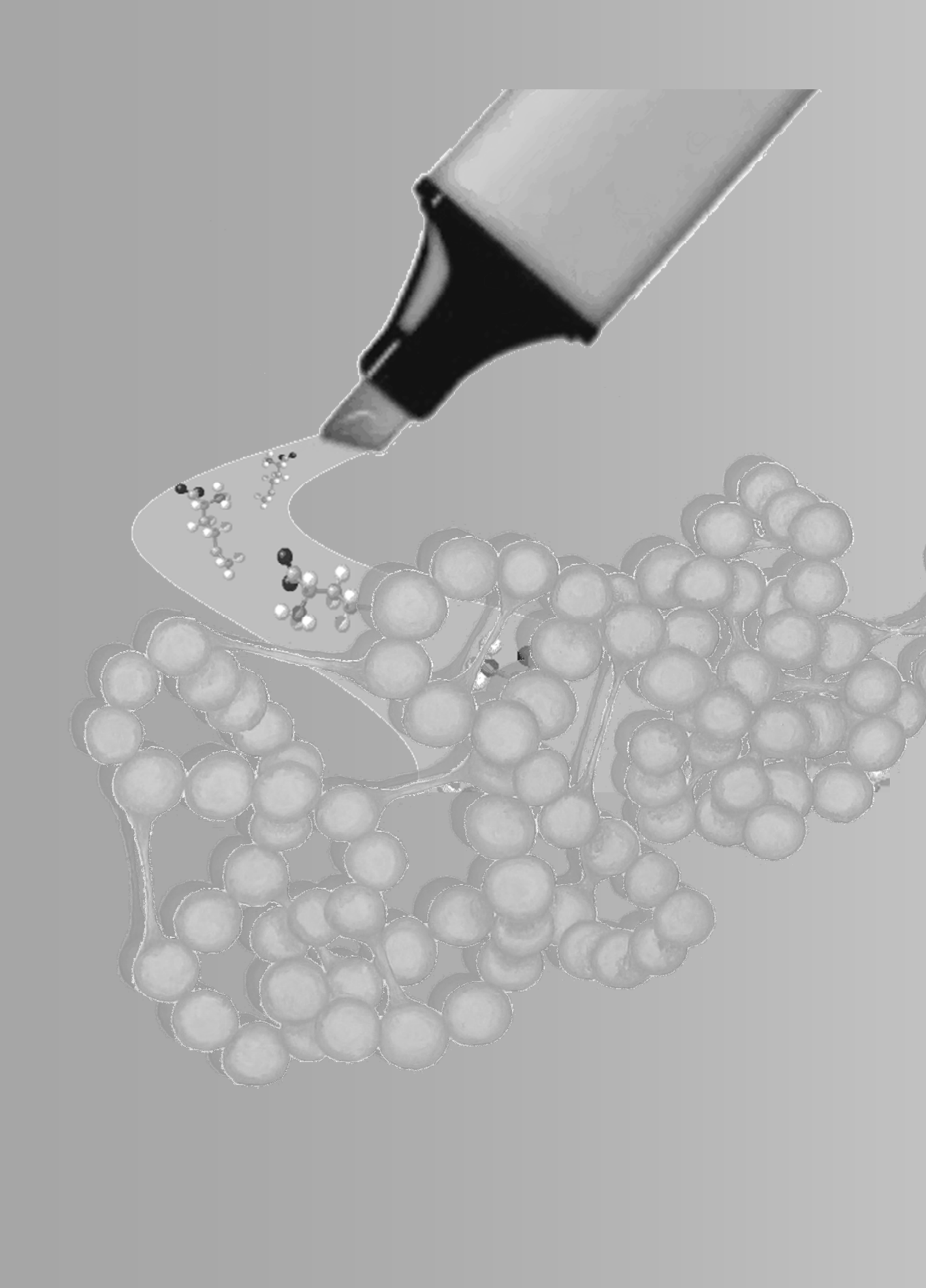

Chapter 7

Identification of biomarkers for intake of protein from meat, dairy and grains: a fully controlled dietary intervention study

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Submitted

Abstract

Objective

In this fully controlled randomized multiple cross-over dietary intervention study we aimed to identify potential biomarkers for dietary protein from dairy, meat, and grain, which could be useful to estimate intake of these protein types in epidemiological studies.

Methods

After 9 days run-in, 13 men and 17 women (22±4y) received three high protein diets (aimed at ~18 en%) in random order for 1 week each, with ~14 en% originating from either meat, dairy, or grain. We used a two-step approach to identify biomarkers in urine and plasma. With principal component discriminant analysis (PCDA) we identified amino acids (AA) from the plasma or urinary amino acid profile that were distinctive between diets. Subsequently, after pooling total study data we applied mixed models to estimate the predictive value of those AAs for intake of protein types.

Results

A very good prediction could be made for the intake of meat protein by a regression model that included urinary carnosine, 1-methylhistidine, and 3-methylhistidine (98% of variation in intake explained). Furthermore, for dietary grain protein a model that included 7 amino acids (plasma lysine, valine, threonine, α-amino-butyric acid, proline, ornithine and arginine) made a good prediction (75% of variation explained). We could not identify biomarkers for dairy protein intake.

Conclusion

Specific combinations of urinary and plasma AAs may be potentially useful biomarkers for meat and grain protein intake, respectively. These findings need to be cross-validated in other dietary intervention studies.

Introduction

There is increasing interest in the role of dietary protein and specific types of protein (e.g. from animal or plant sources) in health and disease[1-4]. Observational epidemiological studies in this field often rely on food frequency questionnaires (FFQ) or dietary recalls to estimate habitual intake of (types of) protein. Such memory-based methods, however, are prone to errors which can lead to misclassification of participants which could weaken the associations between intake of protein types and health outcomes. [5,6] Therefore, markers of intake for these protein types in biological tissues or fluids, could provide more objective indices of true intake. Several metabolic compounds, i.e. urinary carnosine[7], 1-methylhistidine[8], 3-methylhistidine[7,8], taurine[9,10], sulphate[7], creatinine[7], and serum creatine[7,11], have been proposed as biomarkers for meat protein intake (**Table 7.1**). Furthermore, the ratio between natural stable isotopes of nitrogen ($^{14}N/^{15}N$) may be an indicator for the ratio between plant and animal protein intake.[12,13] However, none of these potential biomarkers have sufficiently been validated. Biomarkers for other major protein types, i.e. meat, dairy and grain protein, are lacking.

***Table 7.1**; Overview of postulated biomarkers.*

Urine	
Carnosine	The dipeptide, beta-alanyl-histidine (carnosine), is present in muscle and nerve tissues in most vertebrates.[7] Because dietary intake of nerve tissues usually are limited, urinary carnosine might be a potential marker of muscle intake from animals.[7]
1-Methylhistidine	1-Methylhistidine (1MH) forms a dipeptide with β-alanine, anserine.[8] Anserine occurs in the skeletal muscle of several species but not in man. Therefore, urinary 1-methylhistidine is a potential biomarker for meat protein intake.
3-Methylhistidine	Urinary excretion of 3-methylhistidine (3-MH) has been suggested as marker of meat consumption because it is synthesized in the muscle of mammals and released and excreted in urine after intake of muscle protein.[23]
Taurine	Taurine is present in animal tissues in high levels.[9] About 40% of taurine, fed as such, is recovered in the urine.[10]
Sulphate	A high content of cysteine and methionine in proteins leads to an increased degradation to sulphate and sulphite by the intestinal microbiota. Since animal proteins are rich in sulphur -containing amino acids, urinary excretion of inorganic sulphate might reflect meat protein intake.[7]
Creatinine	Meat contains creatine and creatine phosphate, which partially decomposes to creatinine during cooking.[7] Urinary creatinine excretion may increase after (cooked) meat intake.[7]
Ratio of natural stable isotopes of Nitrogen ($^{14}N/^{15}N$)	Cattle urine has shown that there is a depletion of ^{15}N relative to their diet.[12] It has therefore been hypothesised that animals incorporate dietary ^{15}N preferentially over dietary ^{14}N. Indeed it has been found that the level of the ^{15}N stable natural isotope increases by 3‰ up every step in the food chain.[13] Possibly the proportion of ^{15}N in urine reflects the ratio of animal and plant protein in the diet. However, data on this subject are scarce.
Blood	
Creatine	Meat contains creatine and creatine phosphate.[7] In a study of 60 male and female vegetarians and 99 age-matched omnivores, omnivorous individuals had a higher serum creatine compared to vegetarians.[7,11] Therefore plasma creatine might be a biomarker for meat protein intake.

We conducted a fully controlled dietary intervention study to identify potential biomarkers for intake of dairy protein, meat protein, and grain protein, which could be useful for further epidemiological studies. We focused on these types of protein because these are the main sources of protein in the Dutch population, with approximately 26% of total protein intake originating from dairy, 25% from meat, and 18% from grain[14]. The proteins were provided to the participants in a food-based setting in order to mimic a real life situation.

Materials and methods

Study population

Participants were recruited within a 10-km radius from the university campus. Men and women between 18-40 years old with a BMI between 18.5 and 30 kg/m2 were invited to participate. We used questionnaires to collect information about general characteristics and medical status. Individuals suffering from chronic disease(s) or using prescribed medication were excluded. We also excluded women who were pregnant, lactating or not using oral contraceptives. Liver- and kidney function markers were checked for abnormalities in a fasted blood sample before the start of the study. All participants gave written informed consent before the screening was performed.

Study design

The Biomarker Study was a fully controlled randomized multiple cross-over dietary intervention study, which was conducted between 21 March and 20 April 2011 at Wageningen University, The Netherlands. An overview of the study design is given in **Figure 7.1**. The study lasted 30 days and consisted of four dietary periods: a run-in period of 9 days and three subsequent intervention periods of 7 days each that were applied in random order. The participants were allocated to one of the six diet orders by block randomisation with a block size of 5 and with stratification for gender. On the last day of each treatment period urine was collected for 24 hours and a fasting blood sample was taken. The medical ethics committee of Wageningen University, The Netherlands approved the design and the aim of the study, which was registered in the NIH clinical trial database (ClinicalTrials.gov number. NCT01314040).

Dietary intervention

Menus were designed for ten levels of energy intake ranging from 7 to 16 MJ/d. The participants were allocated to an energy intake level close to their habitual energy intake, which

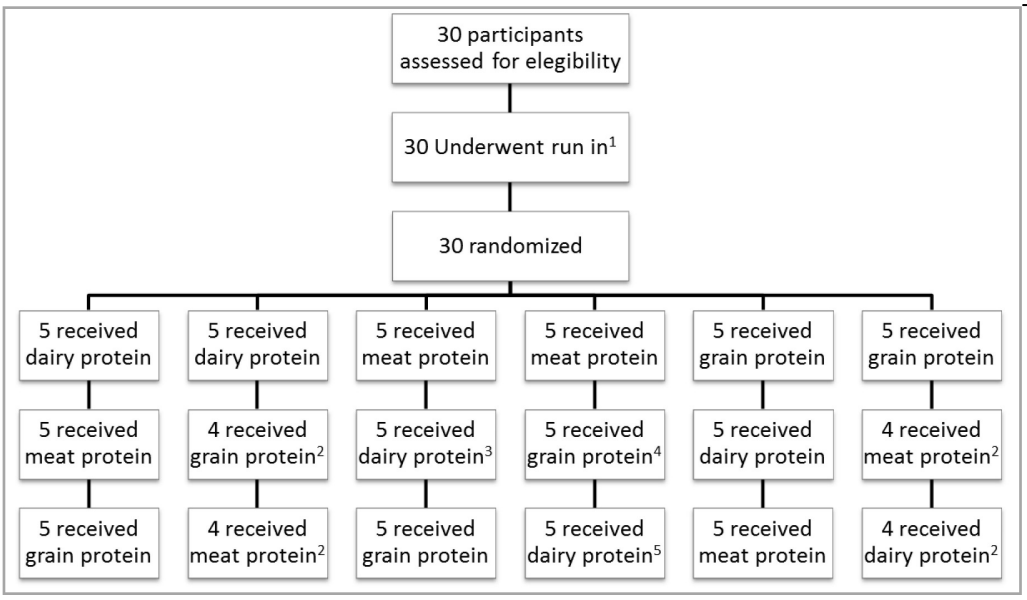

Figure 7.1. *Flow diagram of participants in the Biomarker Study.*

After 9 days run-in participants were randomised in one of six diet orders. Each intervention diet was consumed for 7 days. The run in diet was aimed at ~15 en% protein, whereas the intervention diets were aimed at ~18 en% protein of which ~14 en% originated from the source of interest. After each dietary period 24h urine and blood were collected.

[1]Urine data of the run in period of one participant was excluded because he reported incomplete urine collection; [2]Two participants (a man and a woman) discontinued the intervention because of difficulties with the fact that they were not allowed to choose their own food; [3]The data of the dairy protein period of one participant was excluded from analysis because of a 130% higher nitrogen excretion than expected based on chemical analysis of the diet; [4]The data of the grain protein period of one participant was excluded because of a knee surgery on the day before collection; [5]The urine data of the dairy protein period of one participant was excluded because of a mistake in urine handling.

was estimated before start of the study using an FFQ[15]. From Monday till Friday participants consumed their hot meal at lunchtime at Wageningen University supervised by dieticians who ensured that the complete meal was consumed. Breakfast, bread meals, snacks, beverages, and all meals for Saturdays and Sundays were provided in take-home packages. Participants were carefully instructed how to prepare the hot meals during the weekends. When participants had incidentally increased energy requirements, e.g. because of sports, a bread bun (500 kJ/bun) was provided with the same relative macronutrient composition as the intervention diet of the participant. During the whole study we supplied 90% of daily energy intake to the participants. To cover the remaining 10% of daily energy needs participants were obliged to choose foods that were low in protein content (< 0.6 g protein per portion) from a restricted list. They recorded these foods in a diary in which they also noted any deviations from the study protocol. Body weight was measured twice every week with indoor clothing, without shoes and with empty pockets on a digital balance accurate to 0.1 kg (Seck Bascule MT, USA). If necessary, energy intake was adjusted to limit changes in weight to less than 0.2 kg.

Diet composition and chemical analyses of duplicate portions

The total protein content of the run-in diet was aimed at 15 en%. The intervention diets had a protein content aimed at ~18 en% with ~14 en% coming from either dairy, meat, or grain. During the dairy protein based diet the main sources of protein were milk and milk products, yoghurt, and cheese. In addition, a whey protein isolate was added to the dessert (~4 en%, Nectar, Syntrax, Scott City, MO, USA). In the meat protein based diet the main protein sources were pork, beef, and chicken. The main protein sources in the grain protein based diet were wheat, bran, rice, and corn. Additionally, the diet contained legumes (chickpeas, lentils), contributing 3.6 en% of protein. A wheat protein isolate was added to the dessert, the dressing and a drink (~7 en%, Ultimate Nutrition Inc., USA).

Duplicate portions of each intervention diet with an energy level of 11 MJ were collected daily and analysed for energy, fat, dry matter, ash, and dietary fiber, according to official methods of analysis (AOAC).[16] Furthermore, nitrogen was determined by the Kjeldahl method (Kjeltec 2300, Foss, Denmark), and the amount of protein was calculated using a conversion factor of 6.25. Carbohydrate content of the diets was calculated by difference.

Amino acid composition was measured using ion-exchange chromatography and derivatised post-column (TRIS/AZA, JEOL AminoTac JLC/500-V, Jeol, Japan), after hydrolysis of the samples with hydrochloric acid (6 mol/L) using norvaline as internal standard. Detection was performed at 570 nm (proline at 440 nm). For the determination of cystine and methionine, hydrolysis was preceded by oxidation with performic acid. For analysis of tryptophan samples were hydrolysed by heating at 119 °C in a nitrogen atmosphere with a barium hydroxide solution using 5-methyl tryptophan as internal standard. Samples were analysed by HPLC (HPLC-pump: Waters 616, auto sampler Waters 717, Waters Corporation, Milford, USA) with fluorescence detection excitation 300 nm, emission 330 nm (fluorescence detector: Jasco FP -1520, Jasco Benelux b.v., De Meern, The Netherlands; column: Nucleosil C18, PN 89161, Grace Davison Discovery Science, Deerfield, Ireland). The nutrients in the free-choice items were calculated (NEVO, 2006[17]) and added to the analysed values **(Table 7.2)**.

Because actual intake of total protein did not exactly meet the target intake, leading to differences across the diet periods, we adjusted all our analyses for nitrogen excretion, so that biomarkers for protein types could be identified independent of protein quantity of the diets.

Urine sampling and analysis

Urine was collected during 24h at the final day of each intervention period. Before collection 3 ml of chlorhexidine digluconate (19-21% m/V) was added to each urine container of 2 litres as a preservative. Participants were instructed to discard the first voiding in the morning

Table 7.2. *Mean daily intakes of energy, macronutrients, and amino acids during the Biomarker study.*

	Run in	Dairy protein based diet	Meat protein based diet	Grain protein based diet
Energy, MJ/d	10.9	11.1	11.0	11.1
Macronutrients				
Total protein (analysed), en% (g/kg/d)	15.1 (1.4)	19.1 (1.8)	22.5 (2.1)	16.7 (1.6)
Animal protein[1], en% (g/kg/d)	9.2 (0.9)	15.9 (1.5)	17.2 (1.6)	3.1 (0.3)
Dairy protein[1], en% (g/kg/d)	4.4 (0.4)	15.2 (1.4)	1.5 (0.1)	1.5 (0.1)
Meat protein[1], en% (g/kg/d)	4.5 (0.4)	1.2 (0.1)	15.7 (1.5)	1.5 (0.1)
Plant protein[1], en% (g/kg/d)	4.9 (0.5)	3.3 (0.3)	3.7 (0.3)	15.6 (1.5)
Grain protein[1], en% (g/kg/d)	4.3 (0.4)	1.3 (0.1)	1.5 (0.1)	14.2 (1.3)[2]
Fat, en% (g/kg/d)	30.4 (1.3)	30.9 (1.3)	29.9 (1.3)	27.8 (1.2)
Carbohydrate, en% (g/kg/d)	53.0 (4.9)	48.5 (4.6)	43.0 (4.1)	54.1 (5.1)
Amino acids				
Isoleucine, protein% (mg/kg/d)	4.3 (60)	5.3 (96)	4.3 (92)	3.7 (59)
Leucine, protein% (mg/kg/d)	7.8 (110)	9.8 (177)	7.5 (160)	7.1 (112)
Lysine, protein% (mg/kg/d)	6.2 (87)	8.1 (146)	7.4 (158)	3.4 (53)
Methionine, protein% (mg/kg/d)	2.2 (31)	2.5 (45)	2.4 (51)	1.8 (28)
Cysteine, protein% (mg/kg/d)	1.3 (18)	1.4 (25)	1.0 (22)	1.9 (30)
Phenylalanine, protein% (mg/kg/d)	4.4 (61)	4.6 (84)	3.9 (82)	4.7 (74)
Tyrosine, protein% (mg/kg/d)	3.5 (49)	4.3 (78)	3.0 (63)	3.2 (51)
Threonine, protein% (mg/kg/d)	3.8 (53)	4.9 (88)	4.0 (86)	3.1 (49)
Tryptophan, protein% (mg/kg/d)	1.2 (17)	1.5 (27)	1.2 (25)	1.1 (18)
Valine, protein% (mg/kg/d)	5.0 (70)	6.1 (110)	4.8 (101)	4.4 (69)
Arginine, protein% (mg/kg/d)	4.9 (69)	3.9 (71)	5.7 (121)	4.6 (73)
Histidine, protein% (mg/kg/d)	2.7 (38)	2.5 (46)	3.0 (64)	2.2 (35)
Alanine, protein% (mg/kg/d)	4.3 (60)	4.2 (76)	5.2 (111)	3.6 (56)
Aspartic acid, protein% (mg/kg/d)	7.7 (108)	9.4 (170)	8.9 (189)	5.7 (90)
Glutamic acid, protein% (mg/kg/d)	20.9 (293)	21.7 (391)	16.3 (346)	27.9 (440)
Glycine, protein% (mg/kg/d)	3.7 (52)	2.7 (49)	4.7 (99)	4.0 (63)
Proline, protein% (mg/kg/d)	7.2 (102)	9.0 (163)	5.0 (106)	9.6 (151)
Serine, protein% (mg/kg/d)	4.4 (62)	5.3 (95)	3.9 (82)	4.6 (73)

Mean nutrient intakes are based on chemical analysis of duplicate portions and calculations of free choice foods. Mean intakes are given as energy percentage with mean intake per kilogram bodyweight between brackets.
[1]Mean nutrient intake only based on calculated nutrient content of foods, because types of protein cannot be distinguished in chemical analysis; [2]3.6 en% of protein came from chickpeas and lentils (food based)
Protein%=percentage of total protein intake.

after waking up, and to note the time. Subsequently they collected all urine up to and including the voiding on the same time the next day. Urine was kept cool in a cooling bag with a cooling element during the 24 hours of collection. Subsequently, urine samples were stored at -80°C until analyses. Total nitrogen was analysed by the Kjeldahl method (Kjeltec 2300, Foss, Denmark) and used as a marker of dietary compliance. Participants with 50% higher or lower nitrogen excretion than expected based on chemical analysis of the diets were considered non-compliant and excluded from analysis.

Urinary creatinine was analysed by the Jaffé reaction using reagents from Roche Diagnostics (Mannheim, Germany) on a Roche-Hitachi Modular P device (Roche, Mannheim, Germany) from the same manufacturer. Furthermore, the levels of urinary amino acids were analysed by a triple-quadrupole mass spectrometer (type API 4000 AB SCIEX, Foster City, California 94404-1121, USA) after separation of amino acids by isocratic HPLC (Agilent 1100LC, Agilent Technologies Deutschland GmbH, Böblingen, Germany). Isotope analysis ($^{14}N/^{15}N$) was conducted by Europe 20/20 Stable Isotope Analyser coupled with a ^{15}N sample combustion unit (Europa Scientific Ltd, Crewe, Cheshire, UK).

Blood sampling and analysis

At the final morning of each study period a fasting blood sample was obtained from the antecubital vein of the forearm. From 22.00 hours the evening before, participants were not allowed to consume foods or drinks except for water. Blood was sampled in vacutainer tubes (BD Vacutainer, Plymouth, UK) containing clot activator for serum and in tubes containing Potassium Ethylene Diamine Tetra Acid (K_2EDTA) for plasma. K_2EDTA plasma tubes were stored ice-chilled and centrifuged for 10 minutes at 1190xg at 4°C, within 60 minutes after venepuncture. Serum tubes were stored in a dark condition for approximately 1.5 hours and then centrifuged for 10 minutes at 1550xg at 20°C. Plasma and urine samples were stored at -80°C until analysis.

Creatine in serum was analysed measuring the Barrit reaction after addition of 1-naphthol and photometrically quantified at 546 nm (Hitachi U-1800 spectrophotometer, Hitachi High-Technologies Europe GmbH, Mannheim, Germany). Finally, amino acid profile in plasma was analysed by a triple-quadrupole mass spectrometer (type API 4000 AB SCIEX, Foster City, California 94404-1121, USA) after separation of amino acids by isocratic HPLC (Agilent 1100LC, Agilent Technologies Deutschland GmbH, Böblingen, Germany).

Statistical analysis

To identify biomarkers that may be useful to estimate intake of protein types we used a two -step approach. With principal component discriminant analysis (PCDA) we identified amino

acids from the urinary and plasma amino acid profiles that were distinctive between diets. For individual biomarkers that did not belong to the amino acid profile (i.e. urinary creatinine, sulphate, ‰^{15}N and serum creatine) we investigated whether there were differences between intervention diets using ANCOVA. As a second step we applied mixed models after pooling total study data to estimate the predictive value of selected amino acids and individual biomarkers for intake of protein types.

PCDA analyses were performed in the Matlab environment (R2008b, 1984-2008, The Mathworks Inc, Natick, MA, USA) using the PLS toolbox for Matlab version 5.0.3 (r 6466, 1995-2008, Eigenvector Research Inc, Wenatchee, WA, USA). We performed ANCOVA and mixed models using the SAS statistical software package (SAS version 9.2, SAS Institute, Cary, NC).

Preparation of data

Urinary excretion data of amino acids and sulphate were adjusted for creatinine excretion to account for potential incompleteness of 24h urine collections. Furthermore these data were adjusted for total nitrogen excretion to take into account the unintended differences in protein content of intervention diets that were revealed by chemical analysis of the duplicate portions. Plasma amino acid levels and serum creatine were not correlated to total protein intake and were therefore not adjusted for differences in protein content of the diets. Missing data due to levels below detection limit were replaced by detection limit divided by 2. For PCDA analysis of amino acid profiles, levels of amino acids were calculated relative to the run in period [(diet-run-in)/ run in*100] and data were mean-centered per person to remove between subject variation. Furthermore, auto scaling of all amino acids was performed by dividing the values by their own standard deviation.

Identification of amino acids that are distinctive between diets

Principal component analysis (PCA)[18] was used to screen all data sets in order to detect outliers or patterns present in the data. Principal component discriminant analysis (PCDA) classification was applied to investigate diet differences. The validity of the PCDA model was tested using a ten-fold venetian blind cross validation (CV). This resulted in a percentage of samples that could be classified in the right diet based on the urinary or plasma amino acid profiles.

In PCDA analysis, loadings of the discriminant (a linear combination of all amino acids from the profile) reflect the influence of the original variables on differences between diets, which allowed us to identify specific amino acids that might be distinctive for intake of one of the protein types of interest.[19,20] We considered loadings >4 for further analysis.

Identification of individual biomarkers that are different between diets

For individual biomarkers that did not belong to the amino acid profile (i.e. urinary creatinine, sulphate, ‰^{15}N and serum creatine) we investigated whether there were differences between intervention diets using ANCOVA. Because of non-normality data were log-transformed. In case a significant diet effect was found, partial tests, corrected for multiple comparisons using Tukey-Kramer, were used to identify the differences. We considered a two sided p-value<0.05 statistically significant.

Predictive value of selected amino acids and individual markers

To explore whether amino acids with a loading>4 or individual compounds that were significantly different between diets would be interesting as biomarkers, we evaluated their predictive value for the intake of one of the protein types. We modelled the compounds of interest against intake of protein types using mixed model analysis with participant number as random factor and data of all diets in one model. Subsequently we calculated the amount of explained variation in intake using the method of Snijders and Bosker.[21]

Results

The study involved 13 men and 17 women with a mean age of 22±4 y and a BMI of 21.6±2.2 kg/m^2 (**Figure 7.1**). After the first intervention period one male and one female participant withdrew, because they could no longer adhere to the prescribed diet. Furthermore, for one participant urine data of the run in period were excluded from analysis because he reported incomplete urine collection. For another participant data of the grain protein period were excluded because of a knee surgery on the day before collection, and data of the dairy protein period of a third participant were excluded because of a 130% higher nitrogen excretion than expected based on chemical analysis of the diet. Finally for one participant urine data of the dairy protein period were excluded because of a mistake in urine handling.

In **Table 7.3** amino acid intake and amino acid levels in plasma and in urine are shown after adjustment for total protein intake (amino acid intake) or total nitrogen and creatinine excretion (levels in urine). Baseline values without adjustment are given in **Supplemental table 1**. PCA analysis revealed no gender differences or other patterns that were not due to diet differences.

Table 7.3. *Overview of mean amino acid intake (adjusted for total protein intake), plasma levels, and urinary excretion (adjusted for total nitrogen excretion).*

		Dairy protein based diet	Meat protein based diet	Grain protein based diet
	Total energy (MJ/d)	11.1	11.0	11.1
	Total protein (g/kg/d)	1.8	2.1	1.6
Dietary amino acids				
Isoleucine	Intake (mg/kg/day[1])	95.1	86.6	61.0
	Plasma levels (mg/dl)	0.86 ±0.03	0.84 ±0.03	0.84 ±0.03
	Urinary excretion (mg/24h[2])	1.6 ±0.1	1.6 ±0.1	1.8 ±0.1
Leucine	Intake (mg/kg/day[1])	174.7	149.5	115.1
	Plasma levels (mg/dl)	1.46 ±0.06	1.45 ±0.06	1.38 ±0.06
	Urinary excretion (mg/24h[2])	5.7 ±0.3	4.7 ±0.3	4.5 ±0.3
Lysine	Intake (mg/kg/day[1])	144.3	149.7	56.1
	Plasma levels (mg/dl)	2.60 ±0.07	2.61 ±0.07	1.90 ±0.07
	Urinary excretion (mg/24h[2])	15.2 ±2.7	18.3 ±2.6	11.5 ±2.4
Methionine	Intake (mg/kg/day[1])	44.2	48.1	29.3
	Plasma levels (mg/dl)	0.41 ±0.01	0.39 ±0.01	0.40 ±0.01
	Urinary excretion (mg/24h[2])	2.6 ±0.2	2.7 ±0.2	2.6 ±0.1
Cystine	Intake (mg/kg/day[1])	25.1	20.0	30.6
	Plasma levels (mg/dl)	0.49 ±0.02	0.51 ±0.02	0.47 ±0.02
	Urinary excretion (mg/24h[2])	10.2 ±0.5	8.9 ±0.5	10.8 ±0.4
Phenylalanine	Intake (mg/kg/day[1])	82.3	76.7	76.2
	Plasma levels (mg/dl)	0.93 ±0.02	0.87 ±0.02	0.87 ±0.02
	Urinary excretion (mg/24h[2])	8.8 ±0.4	8.1 ±0.4	9.9 ±0.4
Tyrosine	Intake (mg/kg/day[1])	77.2	58.4	52.1
	Plasma levels (mg/dl)	0.81 ±0.04	0.74 ±0.04	0.82 ±0.04
	Urinary excretion (mg/24h[2])	13.3 ±1.1	9.5 ±1.1	15.5 ±1.0
Threonine	Intake (mg/kg/day[1])	86.4	80.8	50.7
	Plasma levels (mg/dl)	1.72 ±0.06	1.75 ±0.06	1.38 ±0.06
	Urinary excretion (mg/24h[2])	28.0 ±3.2	30.6 ±3.1	19.6 ±2.9
Tryptophan	Intake (mg/kg/day[1])	26.4	23.1	18.2
	Plasma levels (mg/dl)	1.17 ±0.03	1.12 ±0.03	1.10 ±0.03
	Urinary excretion (mg/24h[2])	13.2 ±1.1	11.9 ±1.1	13.5 ±1.0
Valine	Intake (mg/kg/day[1])	108.2	95.2	71.3
	Plasma levels (mg/dl)	2.64 ±0.08	2.60 ±0.08	2.23 ±0.08
	Urinary excretion (mg/24h[2])	7.9 ±0.3	6.7 ±0.3	6.3 ±0.3
Arginine	Intake (mg/kg/day[1])	69.4	115.5	75.2
	Plasma levels (mg/dl)	1.34 ±0.05	1.37 ±0.05	1.50 ±0.05
	Urinary excretion (mg/24h[2])	4.4 ±0.2	3.5 ±0.2	2.9 ±0.2
Histidine	Intake (mg/kg/day[1])	45.0	61.2	36.1
	Plasma levels (mg/dl)	1.26 ±0.04	1.33 ±0.04	1.28 ±0.04
	Urinary excretion (mg/24h[2])	109.2 ±11.2	138.1 ±10.8	128.5 ±10.2
Alanine	Intake (mg/kg/day[1])	74.6	105.6	58.2
	Plasma levels (mg/dl)	2.86 ±0.11	2.84 ±0.11	2.94 ±0.11
	Urinary excretion (mg/24h[2])	31.4 ±2.6	29.3 ±2.5	31.2 ±2.3

Table 7.3. *Overview of mean amino acid intake (adjusted for total protein intake), plasma levels, and urinary excretion (adjusted for total nitrogen excretion), (continued).*

		Dairy protein based diet	Meat protein based diet	Grain protein based diet
Dietary amino acids (continued)				
Aspartic acid	Intake (mg/kg/day[1])	167.8	179.4	93.1
	Plasma levels (mg/dl)	0.10 ±0.01	0.11 ±0.01	0.09 ±0.01
	Urinary excretion (mg/24h[2])	1.4 ±0.1	1.3 ±0.1	1.0 ±0.1
Asparagine	Plasma levels (mg/dl)	0.70 ±0.02	0.68 ±0.02	0.65 ±0.02
	Urinary excretion (mg/24h[2])	19.8 ±3.2	20.4 ±3.1	15.0 ±2.9
Glutamic acid	Intake (mg/kg/day[1])	384.8	319.4	449.2
	Plasma levels (mg/dl)	0.87 ±0.04	0.90 ±0.04	0.89 ±0.04
	Urinary excretion (mg/24h[2])	7.0 ±0.4	6.8 ±0.4	6.1 ±0.4
Glutamine	Plasma levels (mg/dl)	7.26 ±0.25	7.18 ±0.25	7.48 ±0.25
	Urinary excretion (mg/24h[2])	121.4 ±7.7	113.6 ±7.4	126.8 ±7.0
Glycine	Intake (mg/kg/day[1])	48.5	95.0	64.1
	Plasma levels (mg/dl)	1.25 ±0.07	1.36 ±0.07	1.43 ±0.07
	Urinary excretion (mg/24h[2])	75.7 ±6.7	81.0 ±6.4	88.3 ±6.1
Proline	Intake (mg/kg/day[1])	160.4	97.3	154.1
	Plasma levels (mg/dl)	2.45 ±0.11	1.95 ±0.11	2.83 ±0.11
	Urinary excretion (mg/24h[2])	1.7 ±0.2	1.2 ±0.2	2.0 ±0.2
Serine	Intake (mg/kg/day[1])	93.5	76.9	74.5
	Plasma levels (mg/dl)	1.06 ±0.05	1.19 ±0.05	1.19 ±0.05
	Urinary excretion (mg/24h[2])	43.4 ±3.4	44.9 ±3.3	48.4 ±3.1
Metabolites				
Orinithine	Plasma levels (mg/dl)	0.41 ±0.03	0.42 ±0.03	0.49 ±0.03
	Urinary excretion (mg/24h[2])	1.5 ±0.1	1.3 ±0.1	1.6 ±0.1
Citrulline	Plasma levels (mg/dl)	0.47 ±0.02	0.47 ±0.02	0.47 ±0.02
	Urinary excretion (mg/24h[2])	1.0 ±0.1	1.2 ±0.1	1.0 ±0.1
Hydroxyproline	Plasma levels (mg/dl)	0.11 ±0.01	0.22 ±0.01	0.15 ±0.01
	Urinary excretion (mg/24h[2])	1.0 ±0.1	1.0 ±0.1	1.0 ±0.1
Phosphoethanolamine	Plasma levels (mg/dl)	0.10 ±0.01	0.11 ±0.01	0.11 ±0.01
	Urinary excretion (mg/24h[2])	3.2 ±0.3	3.6 ±0.3	3.0 ±0.3
α-Aminobutyric acid	Plasma levels (mg/dl)	0.21 ±0.01	0.23 ±0.01	0.16 ±0.01
	Urinary excretion (mg/24h[2])	1.7 ±0.2	1.6 ±0.2	1.7 ±0.1
Taurine	Plasma levels (mg/dl)	1.4 ±0.1	1.4 ±0.1	1.3 ±0.1
	Urinary excretion (mg/24h[2])	63.7 ±29.5	125.6 ±28.4	88.2 ±26.7
Sarcosine	Urinary excretion (mg/24h[2])	1.0 ±0.1	1.0 ±0.1	1.0 ±0.1
Carnosine	Urinary excretion (mg/24h[2])	3.9 ±3.1	41.1 ±3.0	8.6 ±2.8
1-Methylhistidine	Urinary excretion (mg/24h[2])	28.3 ±8.1	178.9 ±7.8	44.4 ±7.3
3-Methylhistidine	Urinary excretion (mg/24h[2])	36.2 ±2.4	81.0 ±2.3	44.7 ±2.2

[1]Adjusted for total protein intake by means of ANCOVA; [2]Adjusted for total nitrogen and creatinine excretion by means of ANCOVA

Identification of urinary amino acids that are distinctive between diets

The results from PCDA analysis of urinary amino acid profiles are depicted in F**igure 7.2**. In cross validation of the PCDA model 70% of participants were correctly classified in the dairy protein based diet, 93% in the meat protein based diet, and 80% in the grain protein based diet. The differences between the meat protein based diet and the other two diets were mainly observed in the values of discriminant 1. Several of the amino acids that have been suggested as biomarkers for meat protein had an absolute loading >4 in the direction of the meat protein based diet (i.e. 1-methylhistidine, 3-methylhistidine, and carnosine, **Table 7.4**). Amino acids that had an absolute loading >4 in the direction of the other two diets were proline and cysteine. Because in the values of discriminant 2 diets could not be separated it was not possible to identify potential biomarkers for the other two diets.

Identification of plasma amino acids that largely influence diet differences

For the plasma amino acid profiles results are depicted in **Figure 7.3**. The percentage of participants that was correctly classified was 86% for the dairy protein based diet, 88% for the meat protein based diet, and 96% for the grain protein based diet. The differences between the grain protein based diet and the other two diets were mainly observed in the values of discriminant 1, and amino acids that had an absolute loading >4 in the direction of the grain protein diet were proline, ornithine, and arginine (**Table 7.5**). Amino acids with an absolute loading in the direction of the other two diets were lysine, valine, threonine, and α-aminobutyric acid. Because in the values of discriminant 2 diets could not be separated it was not possible to identify potential biomarkers for the other two diets.

Identification of individual biomarkers that are different between diets

In **Table 7.6** the 24h urinary excretion of nitrogen, sulphate, and creatinine are shown. After adjustment for total nitrogen and creatinine excretion, 24h urinary sulphate was 3.4 to 4.0 mmol lower during the meat protein based diet compared to the other two diets ($p<0.01$). Furthermore, urinary creatinine levels were 0.2 to 0.3 g lower in the dairy protein based diet ($p<0.01$), and the proportion of ^{15}N was slightly lower in the grain protein based diet compared to the other two diets (0.002 to 0.003 ‰) with a borderline significant diet-effect ($p=0.06$). The partial tests, however, did not show a significant difference ($p=0.08$ for grain vs. dairy, $p=0.11$ for grain vs. meat).

The serum creatine levels during the different diets are shown in **Table 7.6**. During the meat protein diet creatine levels were 0.16 to 0.19mg/dl higher than during the other two diets ($p<0.01$).

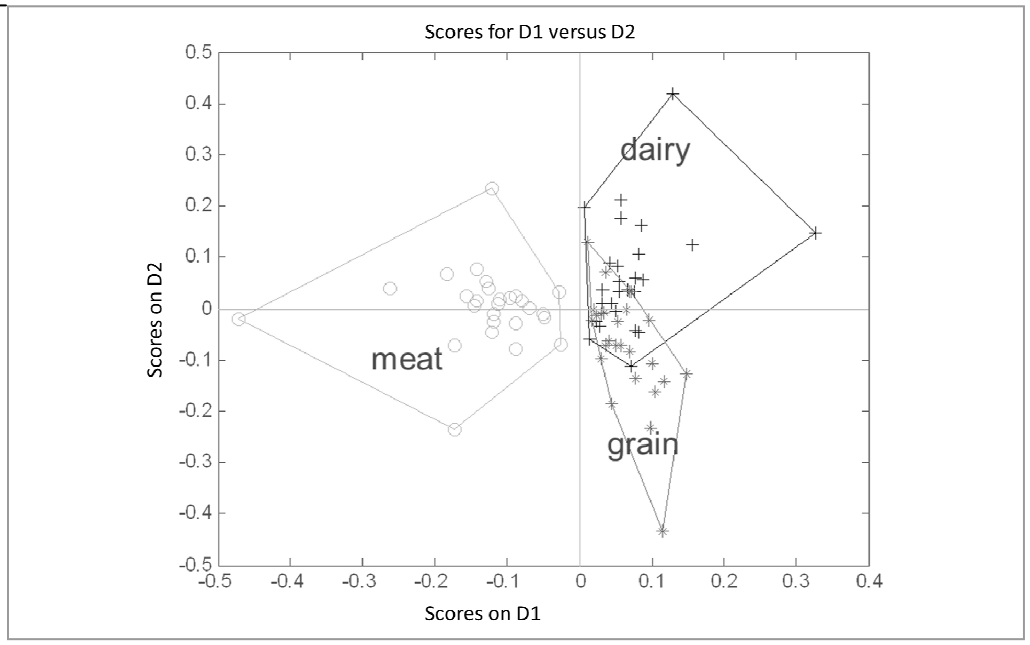

Figure 7.2. *PCDA score plot for urinary amino acid profiles.*

Values of the two discriminant components from PCDA analysis that explained most variation in urinary amino acid profiles. Each dot represents a linear combination of all urinary amino acid levels in one participant during one dietary period. Based on their urinary amino acid profiles 93% of participants was correctly classified in the meat protein based diet, 70% in the dairy protein based diet and 80% in the grain protein based diet.
D1=discriminant 1 and D2=discriminant 2.

Table 7.4. *Urinary amino acid excretion relative to run in: PCDA loadings of D1 in Figure 7.2*

Variable	Loadings D1[1]	Variable (continued)	Loadings D1[1] (continued)
Carnosine	-7.99	Glutamine	2.83
1-Methylhistidine	-7.26	Serine	2.84
3-Methylhistidine	-4.24	Citrulline	2.90
Taurine	-3.92	Leucine	2.91
Lysine	0.18	Isoleucine	3.13
Threonine	1.56	Tryptophan	3.14
Histidine	1.67	Aspartic acid	3.23
Phosphoethanolamine	1.85	Phenylalanine	3.25
Methionine	2.14	Sarcosine	3.35
Aspargarine	2.21	Hydroxyproline	3.35
Glycine	2.39	Ornithine	3.78
Arginine	2.46	Glutamic acid	3.82
Alanine	2.48	Tyrosine	3.98
α-Aminobutyric acid	2.69	Proline	4.02
Valine	2.78	Cystine	4.03

In PCDA analysis, loadings (or weights) of the discriminant (a linear combination of all amino acids from the profile) reflect the influence of the original variables on differences between diets.
[1]High negative values indicate a high influence of the amino acid on classification in the meat protein based diet, whereas high positive values indicate a high influence on classification in one of the two other diets.

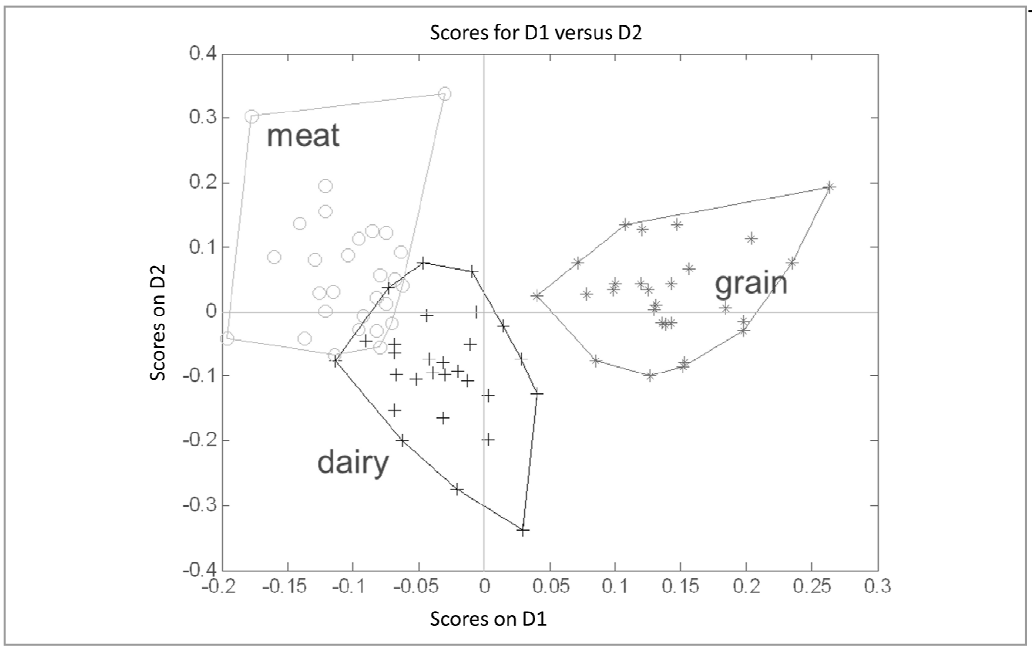

Figure 7.3. *PCDA score plot for plasma amino acid profiles.*
Values of the two discriminant components from PCDA analysis that explained most variation in urinary amino acid profiles. Each dot represents a linear combination of all plasma amino acid levels in one participant during one dietary period. Based on their plasma amino acid profiles 96% of participants was correctly classified in the grain protein based diet, 88% in the meat protein based diet and 86% in the dairy protein based diet.
D1=discriminant 1 and D2=discriminant 2.

Table 7.5. *Plasma amino acid levels relative to run in: PCDA loadings of D1 in Figure 7.3*

Variable	Loadings D1[1]	Variable (continued)	Loadings D1[1] (continued)
Lysine	-6.84	Glutamic acid	0.05
Valine	-6.60	Phosphoethanolamine	0.83
Threonine	-6.32	Citrulline	0.87
α-Aminobutyric acid	-6.26	Methionine	1.09
Cystine	-3.97	Serine	1.24
Leucine	-3.21	Isoleucine	1.45
Hydroxyproline	-3.04	Glycine	1.46
Aspargarine	-2.39	Alanine	1.48
Taurine	-2.05	Glutamine	2.55
Aspartic acid	-1.67	Tyrosine	2.67
Phenylalanine	-1.49	Arginine	4.17
Histidine	-1.08	Ornithine	4.37
Tryptophan	-0.46	Proline	7.20

In PCDA analysis, loadings (or weights) of the discriminant (a linear combination of all amino acids from the profile) reflect the influence of the original variables on differences between diets.
[1]High positive values indicate a high influence of the concerned amino acid on the classification in the grain protein based diet, whereas high negative values indicate a high influence on classification in one of the two other diets.

Table 7.6. *Levels of Postulated biomarkers during each diet.*

	Dairy protein based diet	Meat protein based diet	Grain protein based diet	p-value
Urine				
Sulphate, mmol/24h[1]	35.9 ±1.4	31.9 ±1.3[a]	35.3 ±1.2	<0.01
Creatinine g/24h[2]	1.3 ±0.1[a]	1.6 ±0.1	1.5 ±0.1	<0.01
‰15N	3.685 ±0.002	3.684 ±0.002	3.683 ±0.002	0.06
Serum				
Creatine, mg/dl	0.56 ±0.04	0.72 ±0.04[a]	0.53 ±0.04	<0.01

Data are presented as mean ± SE.
[1]Adjusted for creatinine excretion and nitrogen excretion; [2]Adjusted for nitrogen excretion.
[a]Diet different from both other diets p<0.01.

Predictive value of selected amino acids and individual markers

A combination of the three urinary AAs with absolute loadings >4 in the direction of the meat protein diet, i.e. 1-methylhistidine, 3-methylhistidine, and carnosine, explained 98% of variation in meat protein intake during the study (**Table 7.7**), which was more than was explained by each of these amino acids separately (69%, 72%, and 34% respectively). Adding proline and cystine to the model did not explain extra variation. For dietary grain protein the combination of proline, arginine, and ornithine, explained 24% of variation in grain protein intake, whereas a combination of all 7 amino acids with the highest loadings in PCDA analysis (plasma proline, lysine, valine, threonine and α-aminobuteric acid, ornithine and arginine) explained 75% of variation in intake. With regard to variation in dairy protein intake, urinary creatinine did not explain any variation in intake.

Discussion

In this fully diet-controlled intervention study among 30 young healthy adults a very good prediction could be made for the intake of meat protein by a regression model that included urinary carnosine, 1-methylhistidine, and 3-methylhistidine (98% of variation in intake explained). Furthermore, for dietary grain protein a model that included 7 amino acids (plasma lysine, valine, threonine, α-amino-butyric acid, proline, ornithine and arginine) made a good prediction (75% of variation explained). We could not identify biomarkers for dairy protein intake.

Strengths of this study were the strictly controlled diets, the low dropout rate (n=2) and good compliance to the diets as indicated by nitrogen excretion. In addition, the multivariate analysis of amino acid profiles made it possible to study a wide range of biomarkers at the

Table 7.7. *Regression models of potentially interesting biomarkers from ANOVA and PCDA analysis with protein types, and explained variation in intake.*

Intake variable (protein%)	Specimen	Regression model	% explained variation in intake (R^2)
Regression models for amino acids with loadings >4 in PCDA analysis			
Meat protein	Urine	-16.5+1.0 * Carnosine (mg/24h)+ 0.2 * 1-methylhistidine (mg/24h)+ 0.5 * 3-methylhistidine (mg/24h)[1]	98
Meat protein	Urine	-10.9+0.9 * Carnosine (mg/24h)+0.2 * 1-methylhistidine (mg/24h) +0.5 * 3-methylhistidine (mg/24h)-2.8 * proline (mg/24h)-0.3 * cystine (mg/24h)[2]	98
Grain protein	Plasma	-42.4+23.3 * Proline (mg/dl)+13.5 * Arginine (mg/dl)+6.8 * Ornithine (mg/dl)[1]	24
Grain protein	Plasma	99.0+ 19.9 * Proline (mg/dl)+ 43.1 * Arginine (mg/dl)+ 39.9 * Ornithine (mg/dl)- 32.9 * Lysine (mg/dl)- 42.1 * α-aminobutyric acid (mg/dl)- 20.9 * threonine (mg/dl)- 27.9 * valine (mg/dl)[2]	75
Regression models for individual biomarkers that were significantly different between diets			
Meat protein	Urine	-2.4+1.2 * Sulphate (mg/24h)	11
Meat protein	Serum	11.4+ 42.5 * Creatine (mg/dl)	4
Dairy protein	Urine	43.7-8.2 * Creatinine (mg/24h)	0

[1]Regression model containing AAs with PCDA loadings >4 in the direction of the diet of interest; [2]Regression model containing all AAs with absolute PCDA loadings >4.

same time, taking correlations between these biomarkers into account. Because each protein type contains all amino acids in different proportions it is not possible to identify a single amino acid or amino acid derivate that indicates whether or not a certain protein type is consumed. However, in this study we could identify combinations of amino acids that may be used to rank individuals according to intake of a protein type.

A limitation of the study, however, was the difference in total protein intake across the intervention periods. We accounted for this difference by adjusting urinary data for total nitrogen excretion so that biomarkers for protein intake could be identified independent of protein quantity of the diets. Furthermore, we observed a significantly lower creatinine excretion during the dairy protein based diet. Nevertheless, in regression analysis urinary creatinine did not explain any variation in dairy protein intake. We therefore considered these differences between diets to be chance findings and adjusted all urinary excretion data for creatinine excretion to account for incompleteness of urine collection.

Meat protein intake was best predicted by a regression model that included urinary carnosine, 1-methylhistidine, and 3-methylhistidine. In literature, urinary carnosine, 1-methylthistidine and 3-methylhistidine have been proposed as biomarkers for meat protein intake. In an exploratory study in one healthy man urinary carnosine was increased after ingestion of muscle protein, although the increase was only a small proportion of carnosine in-

gested.[22] In 33 non-diabetic obese participants a linear relationship was found between meat protein intake and 3-methylhistidine excretion with an increment of 1.34 μmoles/g of ingested protein[23], and in a Swedish study among 5 healthy adults, a strong linear relationship was found between meat intake (beef, pork, chicken and plaice) and 3-methylhistidine and 1-methylhistidine excretion.[8] In the current study, a combination of these three amino acids explained 98% of variation in meat protein intake, which was more than the variation explained by each of these amino acids per se. The combination of the three amino acids may be a useful biomarker for intake of meat protein that warrants validation in controlled studies with different levels of meat protein intake.

It has been shown that after intake of 1-methylhistidine, and 3-methylhistidine from meat, these amino acids are rapidly excreted in urine and fasting plasma levels are therefore very low [7,22-24], which is why these plasma levels were not measured in the current study. This may partly explain why in the plasma amino acid profile the grain protein diet showed the best separation from the other diets, in contrast to the urinary profile where the meat protein diet showed the best separation. A regression model with a combination of plasma concentrations of 7 amino acids (lysine, valine, threonine, α-aminobutyric acid, proline, ornithine and arginine) explained 75% of variation in grain protein intake. Compared with the other two diets our grain protein diet had a lower content of the essential amino acids lysine, threonine, and valine, methionine which was reflected in lower plasma levels of the first three amino acids and in the level of plasma α-aminobutyric acid, which is derived primarily from methionine and serine.[25] Furthermore, glutamic acid was relatively high in the grain protein diet which was reflected in a higher excretion of proline, arginine and ornithine for which glutamic acid is a precursor.[26] Nevertheless, we should be careful in interpreting these results. Because grains added much bulk to the diet we replenished the grain protein based diet with legumes (chickpeas, lentils; 3.6 en% legume protein) to reach 14 en% of plant protein. Additionally, this was the only diet that focused on protein of plant origin, and markers that we identified as potential biomarkers for grain protein may in reality reflect plant protein in general. These results need confirmation in other studies with a range in grain protein intake closer to the habitual intake, in which it is not necessary to add protein from other plant sources. Furthermore, plasma amino acid levels need to be compared between a grain protein based diet and a diet that contains protein from other plant sources.

A potential marker for which data in humans up to date are scarce is the ratio of $^{14}N/^{15}N$ stable isotopes in urine as a biomarker for the proportion of plant and animal protein in the diet. There is evidence that human hair and bones reflect the proportion of animal protein in the diet[27] and in cattle urine differences in ^{15}N isotope levels have been found in response to a maize or a grass diet.[28] In line with the hypothesis that the proportion of ^{15}N increases with higher animal protein intake, we observed in the current study a tendency toward a lower percentage of urinary ^{15}N during the grain protein based diet compared to the other two diets. However, this difference was too small to be significant. Possibly a dietary period of

one week was too short to reach the maximum effect of diet on urinary stable isotope ratio. In cattle the urinary ^{15}N required 12 days to reach the new equilibrium after dietary changes.[28] This potential biomarker needs to be investigated in a study with longer dietary periods.

In the current study among 30 young healthy adults we identified a combination of three amino acids in urine as potentially useful biomarkers for the intake of meat protein and a combination of seven amino acids in plasma as potentially useful biomarkers for the intake of grain protein. We did not find biomarkers for dairy protein intake. Further studies are needed to validate these findings and to investigate whether these biomarkers are also useful within lower ranges of intake as observed in population based studies.

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Supplemental table 1. *Overview of amino acid intake, plasma levels, and urinary excretion, unadjusted for total protein intake and total nitrogen and creatinine excretion.*

		Dairy protein based diet	Meat protein based diet	Grain protein based diet
	Total energy (MJ/d)	11.1	11.0	11.1
	Total protein (g/kg/d)	1.8	2.1	1.6
Dietary amino acids				
Isoleucine	Intake (mg/kg/day)	96.4	92.2	59.1
	Plasma levels (mg/dl)	0.86 ±0.04	0.84 ±0.04	0.84 ±0.04
	Urinary levels (mg/24h)	1.6 ±0.2	2.1 ±0.2	1.5 ±0.2
Leucine	Intake (mg/kg/day)	177.0	159.6	111.6
	Plasma levels (mg/dl)	1.46 ±0.06	1.45 ±0.06	1.38 ±0.05
	Urinary levels (mg/24h)	5.7 ±0.5	6.0 ±0.5	3.9 ±0.3
Lysine	Intake (mg/kg/day)	146.2	157.7	53.2
	Plasma levels (mg/dl)	2.60 ±0.07	2.61 ±0.06	1.90 ±0.07
	Urinary levels (mg/24h)	17.4 ±3.9	24.1 ±3.0	9.0 ±1.3
Methionine	Intake (mg/kg/day)	44.8	50.9	28.3
	Plasma levels (mg/dl)	0.41 ±0.01	0.39 ±0.01	0.40 ±0.01
	Urinary levels (mg/24h)	2.6 ±0.2	3.3 ±0.3	2.3 ±0.2
Cystine	Intake (mg/kg/day)	25.5	21.7	30.0
	Plasma levels (mg/dl)	0.49 ±0.02	0.51 ±0.01	0.47 ±0.01
	Urinary levels (mg/24h)	9.8 ±0.7	11.0 ±0.7	9.7 ±0.7
Phenylalanine	Intake (mg/kg/day)	83.5	82.0	74.3
	Plasma levels (mg/dl)	0.93 ±0.02	0.87 ±0.02	0.87 ±0.02
	Urinary levels (mg/24h)	8.8 ±0.5	10.1 ±0.6	8.9 ±0.6
Tyrosine	Intake (mg/kg/day)	78.2	62.8	50.5
	Plasma levels (mg/dl)	0.81 ±0.04	0.74 ±0.04	0.82 ±0.05
	Urinary levels (mg/24h)	13.5 ±1.6	14.4 ±1.5	13.1 ±1.5
Threonine	Intake (mg/kg/day)	87.5	85.8	48.9
	Plasma levels (mg/dl)	1.72 ±0.07	1.75 ±0.07	1.38 ±0.05
	Urinary levels (mg/24h)	27.7 ±3.4	34.4 ±4.5	17.7 ±1.4
Tryptophan	Intake (mg/kg/day)	26.8	24.8	17.6
	Plasma levels (mg/dl)	1.17 ±0.04	1.12 ±0.02	1.10 ±0.04
	Urinary levels (mg/24h)	12.6 ±1.2	15.1 ±1.4	11.8 ±1.2
Valine	Intake (mg/kg/day)	109.6	101.5	69.1
	Plasma levels (mg/dl)	2.64 ±0.08	2.60 ±0.09	2.23 ±0.08
	Urinary levels (mg/24h)	7.7 ±0.4	8.2 ±0.5	5.5 ±0.4
Arginine	Intake (mg/kg/day)	70.7	121.2	73.2
	Plasma levels (mg/dl)	1.34 ±0.05	1.37 ±0.05	1.50 ±0.06
	Urinary levels (mg/24h)	4.3 ±0.3	4.0 ±0.3	2.7 ±0.2
Histidine	Intake (mg/kg/day)	45.7	64.5	35.0
	Plasma levels (mg/dl)	1.26 ±0.06	1.33 ±0.04	1.28 ±0.04
	Urinary levels (mg/24h)	113.7 ±11.7	164.9 ±13.5	116.0 ±9.8
Alanine	Intake (mg/kg/day)	75.8	110.7	56.4
	Plasma levels (mg/dl)	2.86 ±0.11	2.84 ±0.09	2.94 ±0.11
	Urinary levels (mg/24h)	31.3 ±3.1	36.5 ±3.2	27.6 ±2.6

Supplemental table 1. *Overview of amino acid intake, plasma levels, and urinary excretion, unadjusted for total protein intake and total nitrogen and creatinine excretion (continued).*

		Dairy protein based diet	Meat protein based diet	Grain protein based diet
Dietary amino acids (continued)				
Aspartic acid	Intake (mg/kg/day)	170.0	189.0	89.6
	Plasma levels (mg/dl)	0.10 ±0.01	0.11 ±0.02	0.09 ±0.01
	Urinary levels (mg/24h)	1.4 ±0.1	1.4 ±0.2	1.0 ±0.1
Asparagine	Plasma levels (mg/dl)	0.70 ±0.02	0.68 ±0.02	0.65 ±0.02
	Urinary levels (mg/24h)	19.4 ±3.2	23.4 ±4.1	13.5 ±1.6
Glutamic acid	Intake (mg/kg/day)	390.8	345.5	440.0
	Plasma levels (mg/dl)	0.87 ±0.04	0.90 ±0.04	0.89 ±0.04
	Urinary levels (mg/24h)	6.7 ±0.4	7.3 ±0.4	5.9 ±0.3
Glutamine	Plasma levels (mg/dl)	7.26 ±0.25	7.18 ±0.24	7.48 ±0.26
	Urinary levels (mg/24h)	119.9 ±9.9	145.6 ±11.2	110.6 ±9.7
Glycine	Intake (mg/kg/day)	49.5	99.1	62.7
	Plasma levels (mg/dl)	1.25 ±0.06	1.36 ±0.07	1.43 ±0.07
	Urinary levels (mg/24h)	76.6 ±6.6	103.9 ±9.4	77.1 ±7.9
Proline	Intake (mg/kg/day)	162.5	106.4	150.9
	Plasma levels (mg/dl)	2.45 ±0.11	1.95 ±0.07	2.83 ±0.15
	Urinary levels (mg/24h)	1.6 ±0.2	1.4 ±0.1	1.9 ±0.4
Serine	Intake (mg/kg/day)	94.8	82.4	72.6
	Plasma levels (mg/dl)	1.06 ±0.03	1.19 ±0.06	1.19 ±0.05
	Urinary levels (mg/24h)	42.3 ±3.6	55.2 ±5.2	43.1 ±3.3
metabolites				
Orinithine	Plasma levels (mg/dl)	0.41 ±0.02	0.42 ±0.02	0.49 ±0.03
	Urinary levels (mg/24h)	1.5 ±0.2	1.6 ±0.2	1.4 ±0.1
Citrulline	Plasma levels (mg/dl)	0.47 ±0.02	0.47 ±0.03	0.47 ±0.02
	Urinary levels (mg/24h)	1.1 ±0.1	1.3 ±0.1	0.9 ±0.1
Hydroxyproline	Plasma levels (mg/dl)	0.11 ±0.01	0.22 ±0.01	0.15 ±0.01
	Urinary levels (mg/24h)	1.1 ±0.1	1.1 ±0.1	0.9 ±0.1
Phsophoethanolamine	Plasma levels (mg/dl)	0.10 ±0.01	0.11 ±0.01	0.11 ±0.01
	Urinary levels (mg/24h)	3.4 ±0.3	4.4 ±0.4	2.7 ±0.3
Sarcosine	Plasma levels (mg/dl)			
	Urinary levels (mg/24h)	1.1 ±0.1	1.1 ±0.1	0.9 ±0.1
α-aminobutyric acid	Plasma levels (mg/dl)	0.21 ±0.01	0.23 ±0.01	0.16 ±0.01
	Urinary levels (mg/24h)	1.8 ±0.2	2.2 ±0.2	1.4 ±0.1
Taurine	Plasma levels (mg/dl)	1.37 ±0.09	1.37 ±0.08	1.35 ±0.07
	Urinary excretion (mg/24h)	71.8 ±20.5	138.9 ±25.8	82.9 ±26.3
Sarcosine	Urinary excretion (mg/24h)	1.1 ±0.1	1.1 ±0.1	0.9 ±0.1
Carnosine	Urinary excretion (mg/24h)	4.5 ±0.4	47.5 ±5.8	5.5 ±0.8
1-Methylhistidine	Urinary excretion (mg/24h)	33.9 ±2.9	1.95.3 ±14.2	37.2 ±3.4
3-Methylhistidine	Urinary excretion (mg/24h)	35.7 ±2.5	95.2 ±5.5	37.6 ±2.9

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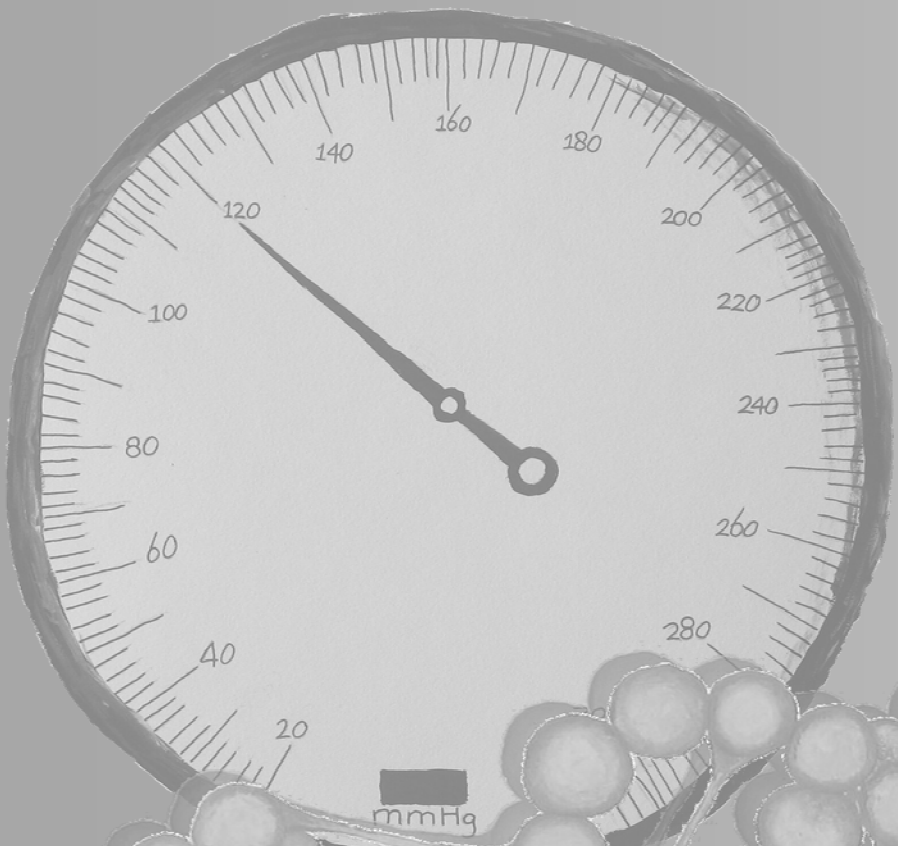
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Chapter 8

General discussion

Elevated blood pressure is a major risk factor for cardiovascular diseases. In 2002 the World Health Organisation estimated that worldwide about 62% of cerebrovascular disease and 49% of ischaemic heart disease were attributable to suboptimal blood pressure (i.e. systolic blood pressure levels >115 mmHg).[1,2] Prevention of high blood pressure by healthy lifestyle and diet, therefore, can have a substantial public health impact; it has been estimated that a population-wide reduction in systolic blood pressure of only 2 mmHg is expected to result in a 6% reduction in fatal stroke, and a 4% reduction in fatal coronary heart disease.[3]

The present thesis focused on the potential role of dietary protein in reducing population blood pressure. As discussed in **Chapter 1** the objectives were 1) to examine whether habitual intake of dietary protein is related to blood pressure level or incidence of hypertension, 2) to examine whether plant and animal protein, protein from specific sources (dairy, meat, and grain), or specific amino acids are differentially related to blood pressure levels or hypertension incidence, and 3) to examine whether subject characteristics like age, gender, BMI, and hypertensive status, could modify the association between dietary protein and blood pressure. In this last chapter we first give a brief overview of the main findings. Subsequently, we present several meta-analyses that we conducted for total protein and protein types in relation to blood pressure or incident hypertension, based on our own findings and data presented in the literature until January 2012. Finally, the implications of our findings for public health are discussed.

Main findings

In a systematic review of existing literature on protein intake and blood pressure (**Chapter 2**) we concluded that dietary protein could have a small beneficial effect on blood pressure. This conclusion was mainly based on observational studies that used urinary biomarkers for protein intake and randomized controlled trials that used carbohydrates as the control treatment. Observational data suggested a more beneficial role of plant protein compared to animal protein, although residual confounding (e.g. from other macronutrients, fiber, or flavonoids) could not be excluded. Little was known about protein from specific sources (e.g. dairy, meat, and grain) in relation to blood pressure. There was some evidence that blood pressure in hypertensives and at an older age could be more sensitive to dietary protein, but more data was needed on subject characteristics that can modify the blood pressure effect of dietary protein.

We conducted a cross-sectional study in ~20,000 Dutch adults of the MORGEN cohort (**Chapter 3**) and two longitudinal studies in ~3,500 Dutch adults (Doetinchem cohort, **Chapter 4**) and in ~2,000 older Dutch adults of the Rotterdam Study (**Chapter 5 and 6**) to examine the associations of different types of protein and amino acids with blood pressure or incident hypertension and to identify potential effect modifiers. The main results of these studies are summarized in **Table 8.1.** Intake of total protein and animal protein were not related to blood pressure or incident hypertension. A higher energy adjusted plant protein intake of 14 grams (2.5 en%) was associated with 1.8/1.0 mmHg lower blood pressure in our cross-sectional analysis (**Chapter 3**), and this association was more pronounced in hypertensives ($p_{interaction}$<0.01). In longitudinal analysis, however, there was no association between plant protein and incident hypertension (**Chapter 4 and 5**). When focusing more in detail on protein from specific sources (**Chapter 3-6, Table 8.1**), meat protein was not associated with blood pressure or hypertension. Results for dairy protein were inconsistent across the different studies, and the significant associations that we observed were probably due to chance. Cross-sectionally, grain protein was inversely associated with diastolic blood pressure only (**Chapter 3**) and in longitudinal analysis a higher grain protein intake was associated with a lower risk of hypertension in a general population (HR: 0.85, 95% CI: 0.73 to 1.00, **Chapter 4**), but not in older adults (**Chapter 5**). Although findings for grain protein were inconclusive, we cannot exclude an inverse association. Finally, none of the investigated amino acids (i.e. glutamic acid, arginine, lysine, tyrosine, cysteine, and essential amino acids) were associated with blood pressure levels or incidence of hypertension (**Chapter 6**).

Table 8.1. *Main findings of the observational studies described in this thesis.*

Chap-ter	Cohort[1]	Design	N	Mean intake[2]	Delta intake[3]		Results[4]	Effect modification
Total protein								
3	MORGEN	CS	20,820	15	29	(Q5 vs. Q1)	-0.8/-0.3	
4	Doetinchem	P	3,588	15	20	(T3 vs. T1)	1.01 (0.85-1.19)	
5	Rotterdam	P	2,241	17	13	(1 SD)	1.03 (0.92-1.15)	1.34* for age >70y
Plant protein								
3	MORGEN	CS	20,820	6	14	(Q5 vs. Q1)	-1.8*/-1*	-3.6* in hypertensives
4	Doetinchem	P	3,588	6	9	(T3 vs. T1)	0.96 (0.80-1.16)	
5	Rotterdam	P	2,241	6	6	(1 SD)	1.03 (0.93-1.13)	
Animal protein								
3	MORGEN	CS	20,820	9	32	(Q5 vs. Q1)	-0.6/-0.1	
4	Doetinchem	P	3,588	9	22	(T3 vs. T1)	0.97 (0.81-1.15)	
5	Rotterdam	P	2,241	11	13	(1 SD)	1.02 (0.91-1.15)	1.37* for age >70y
Dairy protein								
3	MORGEN	CS	20,820	4	27	(Q5 vs. Q1)	+1.6*/+0.4	
4	Doetinchem	P	3,588	4	19	(T3 vs. T1)	1.00 (0.81-1.25)	
5	Rotterdam	P	2,241	5	11	(1 SD)	0.91 (0.82-1.01)	
Meat protein								
3	MORGEN	CS	20,820	4	23	(Q5 vs. Q1)	0.0/-0.2	
4	Doetinchem	P	3,588	4	16	(T3 vs. T1)	0.99 (0.85-1.16)	
5	Rotterdam	P	2,241	4	9	(1 SD)	1.02 (0.93-1.10)	1.29* for age >70y
Grain protein								
3	MORGEN	CS	20,820	3	13	(Q5 vs. Q1)	-0.2/-0.6*	
4	Doetinchem	P	3,588	3	8	(T3 vs. T1)	0.85 (0.73-1.00)*	
5	Rotterdam	P	2,241	3	3	(1 SD)	1.02 (0.95-1.08)	
Glutamic acid								
6	Rotterdam	CS	3,086	21	2.1	(Q4 vs. Q1)	-0.6/-1	
6	Rotterdam	P	1,810	21	2.1	(T3 vs. T1)	1.02 (0.83-1.26)	
Arginine								
6	Rotterdam	CS	3,086	5	0.7	(Q4 vs. Q1)	-0.5/+0.4	
6	Rotterdam	P	1,810	5	0.6	(T3 vs. T1)	1.06 (0.85-1.31)	
Lysine								
6	Rotterdam	CS	3,086	7	0.8	(Q4 vs. Q1)	+1.7/+1	
6	Rotterdam	P	1,810	7	0.8	(T3 vs. T1)	1.15 (0.93-1.43)	
Cysteine								
6	Rotterdam	CS	3,086	1	0.2	(Q4 vs. Q1)	+0.1/-0.2	
6	Rotterdam	P	1,810	1	0.1	(T3 vs. T1)	0.98 (0.77-1.24)	
Tyrosine								
6	Rotterdam	CS	3,086	4	0.2	(Q4 vs. Q1)	-2.4*/-0.4	
6	Rotterdam	P	1,810	4	0.3	(T3 vs. T1)	0.92 (0.73-1.15)	

CS=cross-sectional; P=prospective; Q5=quintiles; Q4 is quartiles; T3=tertiles; SD=standard deviation
*P_{trend} <0.05.
[1]MORGEN and Doetinchem: Population based cohort of Dutch adults aged 25 to 65 y; Rotterdam: Population based cohort of Dutch older adults aged ≥55 y; [2]In percentage of energy for studies on protein and in percentage of protein for studies on amino acids; [3]Difference in intake between the highest and the lowest quantile in grams per day (adjusted for energy according to the residual method) for studies on protein and in percentage of protein for studies on amino acids.; [4]For cross-sectional studies: Δsystolic blood pressure/Δdiastolic blood pressure (mmHg); for prospective studies: HR (95%-CI).

TOTAL BODY OF EVIDENCE AND INTERPRETATION OF FINDINGS

To put our findings in the context of all available literature we summarized the total body of evidence in a series of meta-analyses. We identified 43 studies on the relation between protein intake and blood pressure levels or hypertension incidence that were published until January 2012. Five papers of cross-sectional studies were excluded because data on systolic blood pressure or standard errors were missing.[4-8] Two papers of prospective studies were excluded because a yearly change in blood pressure was reported instead of a relative risk.[9,10] Six trials were excluded because 1) exact data on actual protein intake could not be extracted[11], 2) no isocaloric macronutrient replacement occurred[12-15], or 3) data were not sufficient to calculate the blood pressure response[16]. Finally, one trial could not be included in the meta-analysis because in the control diet protein was replaced by a mix of carbohydrates and fat instead of one macronutrient only.[17]

We aggregated data from 8 cross-sectional studies (**Table 8.2**) and 4 prospective studies (**Table 8.3**) in a meta-analysis on intake of total protein or protein types and blood pressure levels or hypertension incidence. In addition, we pooled the results of 17 randomized controlled trials (**Table 8.4**) for which we also conducted a metaregression analysis on protein dose and study duration. Furthermore, to check whether blood pressure response to protein supplementation was modified by subject characteristics we conducted a meta-regression analysis on age, gender (% males), BMI, and initial systolic blood pressure level. For each type of protein the meta-analysis findings are summarized below, followed by a critical discussion of methodological issues, and discrepancies between studies.

Total dietary protein

Summary of results

The combined results of cross-sectional studies showed a significant inverse association of total dietary protein with blood pressure, although the association was small with a pooled estimate of -0.20 mmHg systolic (95%-CI: -0.39 to -0.01) per 25 grams (~1 SD) of protein intake (**Figure 8.1**). Prospectively, there was no association between total protein intake and incidence of hypertension (pooled HR=0.99, 95%-CI=0.96 to 1.02, **Figure 8.2**). In intervention studies that used carbohydrate as the control treatment, the pooled blood pressure effect was -2.11 mmHg systolic (95%-CI=-2.86 to -1.37, **Figure 8.3**) for a weighed mean contrast in protein intake of 41 g/d. Metaregression analyses showed no associations of dose or study duration with blood pressure response (**Table 8.5**). Trials with a fat control (mainly monounsaturated fatty acids) showed no effect of protein intake on blood pressure (pooled estimate=-0.04 mmHg, 95%-CI=-2.20 to +2.12, **Figure 8.4**).

Table 8.2. *Design and study population characteristics of cross-sectional studies on protein intake and blood pressure that were included in the meta-analysis.*

Source	Country	N	Charac-teristics	Age, y	Men,%	Mean intake (g/d)	Mean blood pressur e	Adjustment
Total protein								
He, 1995[59]	China	827	men	38	100	88	107/66	age, BMI, alcohol, urinary Na, energy intake, region
Stamler, 1996[60]	USA	11342	men	46	100	87	125/84	Age, race, BMI, education, smoking, serum cholesterol, antihypertensive drugs, intake of Na, K, alcohol, caffeine
Masala, 2008[61]	Italy	7601	women	51	0	91	123/79	Age, BMI, waist circumference, smoking, education, physical activity, energy intake
Wang, 2008[24]	USA	810	HBP	50	38	74	135/85	age, race, gender, treatment, education, income, weight, waist, PA, intake of Ca, K
Umesawa, 2009[20]	Japan	7585	general	53	46	74	135/82	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of Na, K, Ca
Altorf, 2012[62]	Netherlan ds	20820	General	42	45	84	120/76	age gender BMI education smoking alcohol, intake of energy, SFA carbohydrates, fiber, Ca, Mg, K
Plant protein								
Joffres, 1987[63]	Hawaii	615	men	55	100	28	135/79	Age, BMI
He, 1995[59]	China	827	general	38	100	72	107/66	age, BMI, alcohol, urinary NA, energy intake, region
Elliott, 2006[23]	China, Japan, USA, UK	4680	general	49	50	36	119/74	Age, sex, weight, height, exercise, alcohol, sample, history CVD or DM, family history of hypertension, special diet, supplement use, urinary Na, K, intake of Ca, SFA, PUFA, cholesterol, fiber
Masala, 2008[61]	Italy	7601	women	51	0	29	123/79	Age, BMI, waist circumference, smoking, education, physical activity, energy intake
Wang, 2008[24]	USA	810	HBP	50	38	23	135/85	age, race, gender, treatment, education, income, weight, waist, PA, intake of Ca, K
Umesawa, 2009[20]	Japan	7585	general	53	46	38	135/82	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of Na, K, Ca
Altorf, 2012[62]	Netherlan ds	20820	general	42	45	32	120/76	age gender BMI education smoking alcohol consumption intake of energy, SFA carbohydrates, fiber, Ca, Mg, K, animal protein

Table 8.2. *Design and study population characteristics of cross-sectional studies on protein intake and blood pressure that were included in the meta-analysis (continued).*

Source	Country	N	Charac-teristics	Age, y	Men,%	Mean intake (g/d)	Mean blood pressure	Adjustment
Animal protein								
Elliott, 2006[23]	China, Japan, USA, UK	4680	general	49	50	45	119/74	Age, sex, weight, height, exercise, alcohol, sample, history CVD or DM, family history of hypertension, special diet, supplement use, 24h urinary Na, K, intake of Ca, SFA, PUFA, cholesterol, fiber
Masala, 2008[61]	Italy	7601	women	51	0	59	123/79	Age, BMI, waist circumference, smoking, education, physical activity, energy intake
Wang, 2008[24]	USA	810	HBP	50	38	50	135/85	age, race, gender, treatment, education, income, weight, waist, PA, intake of Ca, K
Umesawa, 2009[20]	Japan	7585	general	53	46	35	135/82	age, gender, BMI, smoking, alcohol, community, use of antihypertensive medication, intake of sodium, potassium, and calcium
Altorf, 2012[62]	Netherlan ds	20820	general	42	45	52	120/76	age gender BMI education smoking alcohol consumption intake of energy, SFA carbohydrates, fiber, Ca, Mg, K, plant protein

NR=not reported; BP= blood pressure; HBP=untreated pre- or mild hypertensives; CVD=cardiovascular disease; DM=diabetes mellitus.

Table 8.3. *Design and study population characteristics of prospective studies on protein intake and blood pressure that were included in the meta-analysis.*

Source	Country	N	Characteristics	Age, y	men ,%	Habitual intake, en%	BP[1]	Follow-up time, y	adjustment
Total protein									
Alonso, 2006[25]	Spain	5880	grads	36	39	18	NR	2	Age, gender, BMI, exercise, alcohol, smoking, hypercholesterolemia, intake of total energy, Na, fruit, vegetables, fiber, caffeine, Mg, K, low-fat dairy, MUFA, SFA
Altorf, 2010[64]	Netherlands	2241	older	65	43	16	122/68	6	age, gender, BMI, baseline SBP, smoking, alcohol, education, intake of total energy, K, Na, Ca, Mg, fiber, CH, SFA, PUFA
Altorf, 2012[65]	Netherlands	3640	general	44	44	15	118/76	10	age, gender, BMI, education, smoking, alcohol, baseline SBP, intake of total energy, SFA, PUFA, CH, fibre, Ca, Mg, K
Plant protein									
Alonso, 2006[25]	Spain	5880	grads	36	39	NR	NR	2	Age, gender, BMI, exercise, alcohol, smoking, hypercholesterolemia, intake of total energy, Na, fruit, vegetables, fibre, caffeine, Mg, K, low-fat dairy, MUFA, SFA
Wang, 2008[24]	Australia, EUR, USA	810	Healthy	50	38	5	135/85	0.5	Age, gender, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline BP, alcohol, intake of Ca, K, urinary creatinine and Na
Altorf, 2010[64]	Netherlands	2241	older	65	43	6	122/68	6	age, gender, BMI, baseline SBP, smoking, alcohol, education, intake of total energy, K, Na, Ca, Mg, fiber, CH, SFA, PUFA, animal protein
Altorf, 2012[65]	Netherlands	3640	general	44	44	6	118/76	10	age, gender, BMI, education, smoking, alcohol, baseline SBP, intake of total energy, SFA, PUFA, CH, fibre, Ca, Mg, K, animal protein
Animal protein									
Alonso, 2006[25]	Spain	5880	grads	36	39	NR	NR	2	Age, gender, BMI, exercise, alcohol, smoking, hypercholesterolemia, intake of total energy, Na, fruit, vegetables, fiber, caffeine, Mg, K, low-fat dairy, MUFA, SFA
Wang, 2008[24]	Australia, EUR, USA	810	Healthy	50	38	11	135/85	0.5	Age, gender, race, weight, waist, exercise, education, income, antihypertensive drugs, study site, baseline BP, alcohol, intake of Ca, K, urinary creatinine and Na
Altorf, 2010[64]	Netherlands	2241	older	65	43	10	122/68	6	age, gender, BMI, baseline SBP, smoking, alcohol, education, intake of total energy, K, Na, Ca, Mg, fiber, CH, SFA, PUFA, animal protein
Altorf, 2012[65]	Netherlands	3640	general	44	44	10	118/76	10	age, gender, BMI, education, smoking, alcohol, baseline SBP, intake of total energy, SFA, PUFA, CH, fibre, Ca, Mg, K, animal protein

[1]*Mean blood pressure at baseline*

NR=not reported; EUR=Europe, Grads=graduate university students; general=general population

Table 8.4. *Design and study population characteristics of trials on protein intake and blood pressure that were included in the meta-analysis.*

Source	Country	Design	N[3]	Men, %	Charac-teristics[4]	Age, y	Dura-tion, wk	ΔProtein, g/d	Protein source[6] and type	Control[7]	Mean baseline BP (intervention/ control, mmHg)
Sacks, 1984[66]	USA	X-SB	18	39	Vegan	32	6	56	S-soy/wheat	CH-NR	112/74
Appel, 2005[19]	USA	X-DB	161	55	HBP	54	6	53	D-mixed	CH-NR	131/77
He, 2011[30]	USA	X-DB	273	58	HBP	48	8	31	S-soy[8]	CH-mixed[9]	126/81
He, 2011[30]	USA	X-DB	273	58	HBP	48	8	33	S-milk	CH-mixed[9]	126/82
Pal, 2010[67]	Australia	P-SB	20/25	14	OV	48	12	63	S-casein	CH-glucose	118/67 vs. 115/66
Pal, 2010[67]	Australia	P-SB	25/25	14	OV	48	12	74	S-whey	CH-glucose	119/64 vs. 115/66
Hodgson, 2006[68]	Australia	P-0	29/31	63	HBP[5]	59	8	38	D-red meat	CH-NR	134/79 vs. 138/77
Hendler, 1988[69]	USA	P-NR[1]	8/9	NR	OV	31	3	60	D-mixed	CH-fructose	120/79 vs. 121/79
Meckling, 2007[70]	Canada	P-NR[1]	10/8	0	OV	46	12	28	D-mixed	CH-NR	128/79 vs. 127/81
Meckling, 2007[70]	Canada	P-NR[1]	14/11	0	OV	39	12	56	D-mixed[10]	CH-NR	134/82 vs. 129/82
Burke, 2001[71]	Australia	P-O	18/18	50	HBP[5]	57	8	60	S-soy	CH-maltodextrin	134/75 vs. 132/76
Leidy, 2007[72]	USA	P-O[1]	21/25	0	OV	50	12	48	D-mixed	CH-NR	109/68 vs. 114/72
Brinkworth, 2004[73]	Australia	P-NR[2]	19/19	18	OV;DM2[5]	62	12	69	D-mixed	CH-NR	148/83 vs. 140/76
Larsen, 2011[74]	Australia	P-DB[2]	53/46	48	OV;DM2	59	52	30	D-mixed	CH-NR	132/82 vs. 127/82
Delbridge, 2009[75]	Australia	P-NR[2]	42/40	50	OV	44	48	26	D-mixed	CH-NR	135/85 vs. 131/83
Teunissen, 2012[76]	Netherlands	P-DB	43/51	70	OV+HBP	55	4	61	S-mixed	CH-maltodextrin	143/93 vs. 143/92
Hodgson, 2011[77]	Australia	P-DB	101/95	0	Older[5]	74	104	28	S-Whey	CH-maltodextrin	143/70 vs. 143/70
Papakonstantinou, 2010[78]	Greece	X-SB[1]	17	29	OV+DM2	46	4	56	D-mixed	Fat-MUFA	134/86 vs. 134/80
Appel, 2005[19]	USA	X-DB	160	55	HBP	54	6	53	D-mixed	Fat-MUFA	131/77 vs. 131/77
Hochstenbach, 2010[79]	Netherlands	P-SB[2]	19/26	22	OV	43	8	32	D-milk	Fat-NR	118/75 vs. 116/72

[1]Weight loss trial with hypocaloric diets in both groups; [2]Weight maintenance after a period of weight loss; [3]In parallel studies: number of participants in the intervention group versus number of participants in the control group; [4]HBP=pre- or mild hypertensives, OV=overweight or obese, DM2=diabetes mellitus type 2; [5]Antihypertensive drug users included; [6]S=supplement, D=diet; [7]Carbohydrates or fat and type of carbohydrate or fat; CH=carbohydrates; MUFA=mono-unsaturated fatty acids; [8]Isoflavones were not removed from the soy protein [9]Sucrose, fructose, maltodextrine; [10]In both intervention and control group also an exercise intervention was conducted.
NR=Not reported

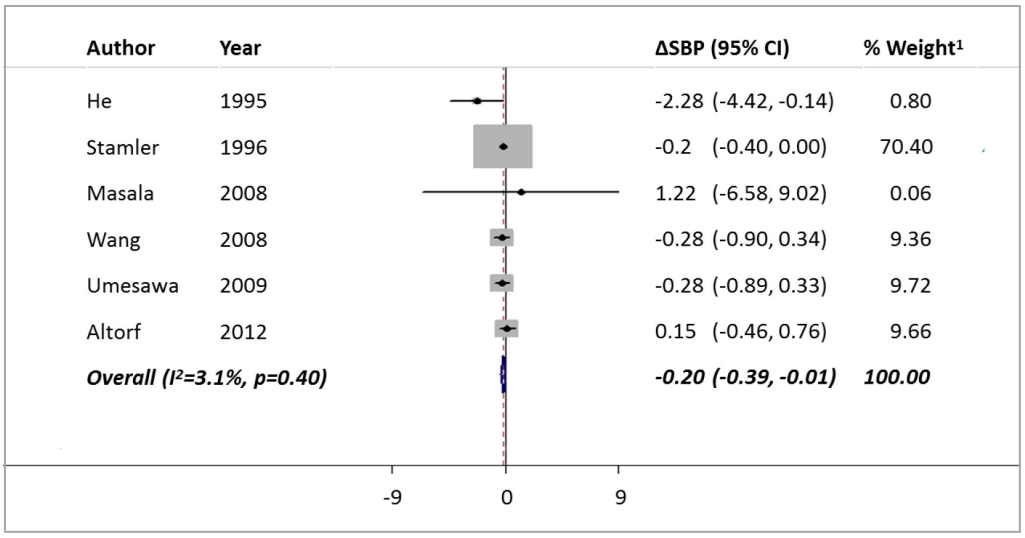

Figure 8.1. *Fully adjusted difference in systolic blood pressure (ΔSBP) with consumption of 25 grams (~1 SD) higher total protein intake in 6 cross-sectional studies.*
For details of the studies see ***Table 8.2.***
[1]Weights are from random effects analysis.

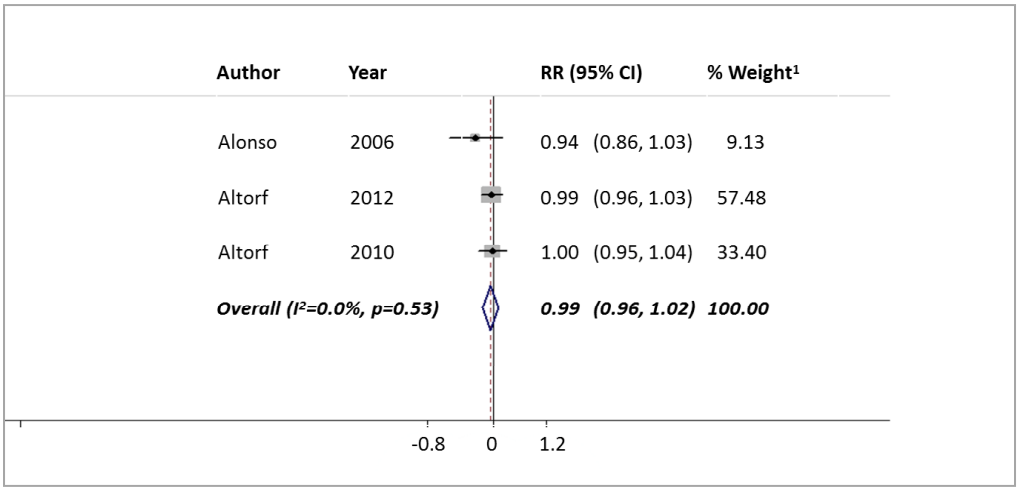

Figure 8.2. *Fully adjusted relative risk of hypertension (RR) with 25 grams (~1 SD) higher total protein intake in 3 longitudinal studies.*
For details of the studies see ***Table 8.3.***
[1]Weights are from random effects analysis.

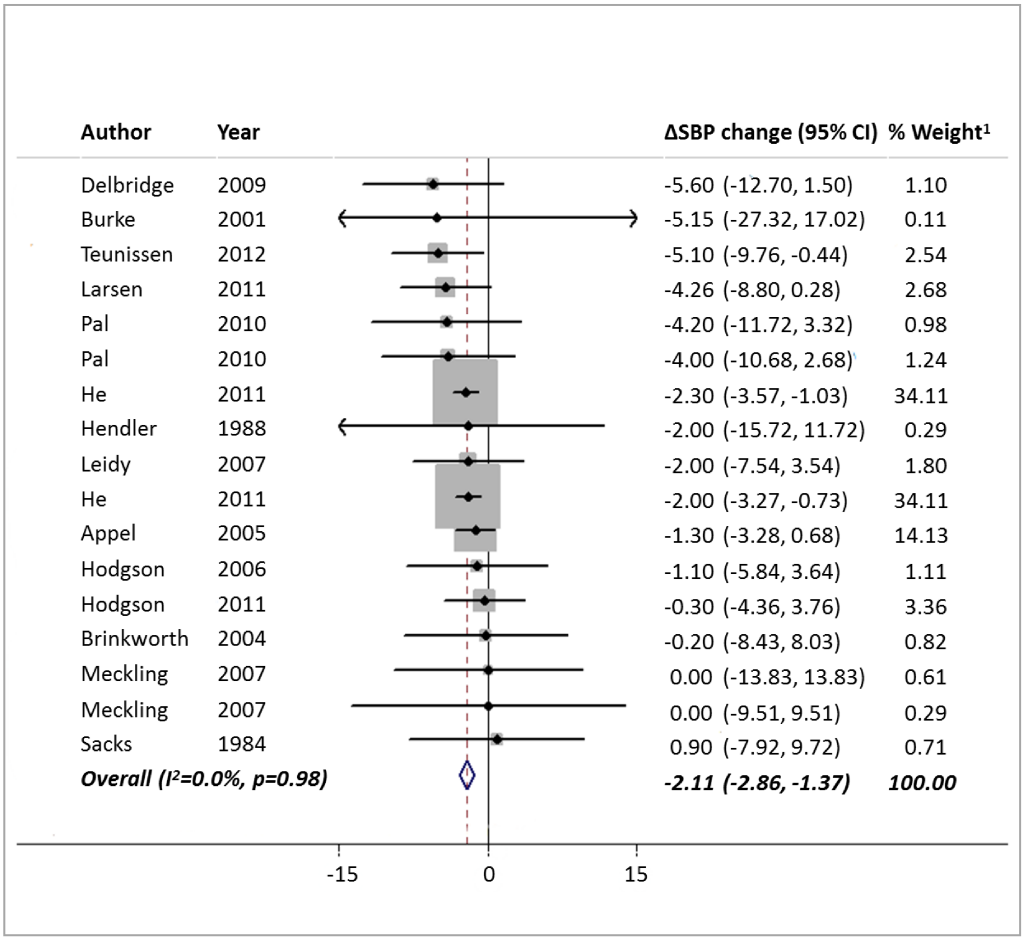

Figure 8.3. *Net change in systolic blood pressure (ΔSBP change) with consumption of protein compared to carbohydrates in 14 randomized controlled trials.*
For details of the studies see ***Table 8.4.***
In the studies of Pal et al.[67], and He et al.[30] two intervention arms were included that were compared to the same control group. In the study of Meckling et al. two intervention arms were included that were each compared to their own control group (intervention with and without exercise in both intervention and control group).
[1]Weights are from random effects analysis.

Table 8.5. *Characteristics associated with net change in blood pressure in trials with carbohydrates as control: univariate meta-regression analysis.*

	Coefficient	95%-CI	p-value
Mean age, y	+0.2	-0.3 to +0.8	0.30
Men, %	-5.7	-20 to +9	0.37
BMI	+0.1	-0.9 to +1.0	0.86
Mean baseline SBP	+0.04	-0.3 to +0.4	0.81
Duration, wk	-0.1	-0.3 to +0.1	0.30
Δprotein, g/d	-0.1	-0.3 to +0.1	0.30

SBP=systolic blood pressure; Δprotein=difference in protein intake between intervention and control group.

Methodological issues of observational studies on total protein intake

In most observational studies dietary protein intake was adjusted for total energy to account for confounding by caloric intake or energy-related determinants of blood pressure, such as physical activity. However, it cannot be excluded that the absolute amount of protein intake (per kilogram of body weight) rather than energy adjusted protein is the determinant of interest in relation to blood pressure (see the discussion on amino acids below). If so, there is a possibility that adjustment for total energy, which we considered necessary, resulted in misclassification for protein intake and attenuation of the associations.

Results from cross-sectional studies suggest a (small) beneficial association of total protein with blood pressure levels, whereas results of prospective studies did not show an association with incidence of hypertension. Possibly, the associations in prospective studies were weaker because of a lower blood pressure in these populations due to the exclusion of hypertensive participants at baseline. Furthermore, small associations may have been missed in prospective studies because of the use of incident hypertension as a dichotomous endpoint (defined as blood pressure ≥140/90 mmHg or use of antihypertensive medication). This approach has the advantage that participants who started antihypertensive medication during follow-up can be included in the analysis without causing bias, but a disadvantage is that blood pressure changes closely around the cut-off point are emphasized, whereas changes further away from the cut-off point are ignored. Consequently, small blood pressure differences may have been more difficult to detect.

Discrepancies between observational and trial data on total protein intake

Trials with a carbohydrate control provided stronger evidence for an inverse relation of protein intake with blood pressure than observational studies, which may be partly attributable to the inclusion of more sensitive individuals with (pre)hypertension, overweight, or obesity

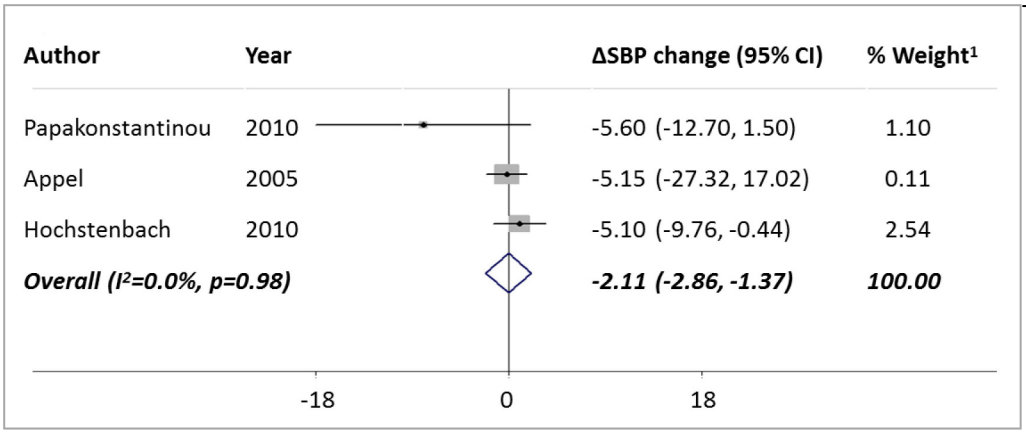

Figure 8.4. *Net change in systolic blood pressure (ΔSBP change) with consumption of protein compared to fat (mainly mono-unsaturated fatty acids) in 3 randomized controlled trials.*
For details of the studies see ***Table 8.4.***
[1]Weights are from random effects analysis.

(**Table 8.4**). Furthermore, in trials mostly supplements or fully controlled diets were used, and attenuation of blood pressure effects because of exposure misclassification does not occur, in contrast to observational studies where protein intake is measured using memory based methods. Finally, the contrast in protein intake was generally larger in trials with a weighed mean contrast in intake of 41 g/d (range: 28 to 74 g/d, **Table 8.4**) versus a contrast of 25 g/d (~1 SD) that was used in the meta-analyses of the observational studies. The high doses that were used in trials may also explain the lack of a dose response effect if blood pressure would mainly respond to protein within the low intake range or below a certain threshold.

Substitution of macronutrients

Given a constant energy intake, a blood pressure effect after intake of protein will be relative to the intake of fat, carbohydrates, or both. The results of the meta-analysis indicate a stronger blood pressure effect of protein when it is exchanged for carbohydrates (**Figure 8.3**) than when it is exchanged for fat (mainly mono-unsaturated fatty acids, **Figure 8.4**). It is therefore well possible that a decreased carbohydrate intake rather than an increased protein intake plays a role in blood pressure reduction. In observational studies in which associations are adjusted for energy, however, a higher protein is likely to be accompanied with a lower intake of both carbohydrates and fatty acids. Because protein may not reduce blood pressure compared to fat this may explain the generally weaker associations in observational studies.

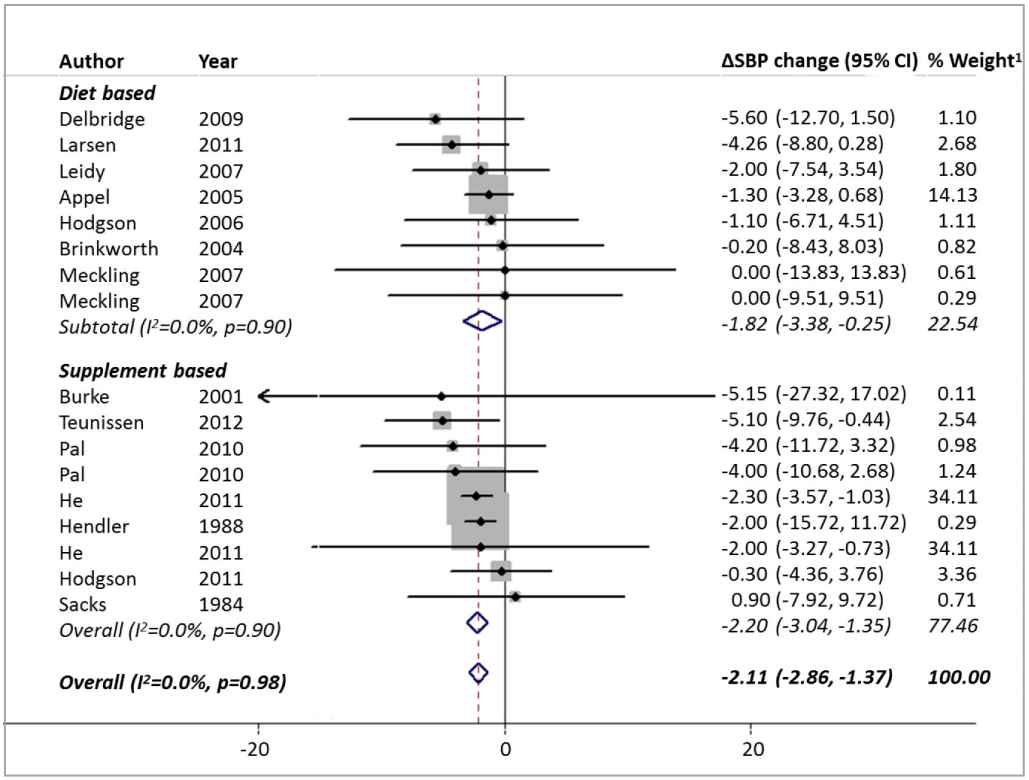

Figure 8.5. *Net change in systolic blood pressure (ΔSBP change) with consumption of protein compared to carbohydrates in 14 randomized controlled trials, stratified by intervention type.*
For details of the studies see ***Table 8.4.***
[1]*Weights are from random effects analysis.*

Possibly, the type of carbohydrate in the control diet also is a determinant of the blood pressure effect of protein. Blood pressure effects were more pronounced in trials in which glucose or maltodextrine were used as a control than in trials that were diet-based and had a mix of carbohydrates in the control diet (**Figure 8.5**). The increase of protein at the expense of carbohydrates (especially 'fast' carbohydrates like sucrose and maltodextrine) reduces the glycemic index of diets, which may result in an attenuated insulin response. Because there is some evidence for an unfavourable effect of insulin on blood pressure this may explain a blood pressure lowering effect of such diets.[18] However, it cannot be excluded that the generally more controlled dose in the supplement-based trials rather than the type of carbohydrates accounted for the stronger blood pressure effects (**Figure 8.5**).

Total protein; conclusions and suggestions for further research

The totality of evidence, especially from trials, indicates that total dietary protein may have a beneficial effect on blood pressure if it is consumed instead of carbohydrates, although no

dose-response relationship could be found. However, it cannot be excluded that a lower carbohydrate intake, rather than a higher protein intake reduces blood pressure. The question whether dietary protein per se influences blood pressure is difficult to answer on basis of observational studies. Trials with multiple control treatments like OmniHeart[19] may shed light on this complex issue. Also unravelling of blood pressure regulating pathways that can be linked to dietary protein could help to solve this question. Furthermore, it is worthwhile to investigate whether replacement of protein by different types of carbohydrate, e.g. 'fast' carbohydrates (e.g. sucrose and maltodextrine), or complex carbohydrates differentially affects blood pressure.

Plant versus animal protein

Summary of results

Results of the meta-analysis did not suggest different effects of plant protein or animal protein on blood pressure or risk of hypertension. In cross-sectional studies a small, but non-significant, inverse association of -0.52 mmHg systolic per 11 grams (~1 SD) was found for plant protein (95%-CI; -1.10 to +0.05, **Figure 8.6**), whereas animal protein was not associated with blood pressure (**Figure 8.7**). The pooled estimates in prospective studies did not show a relation with incident hypertension for plant protein (HR: 0.96, 95%-CI 0.89 to 1.03; **Figure 8.8)** or animal protein (HR: 0.98, 0.95 to 1.02; **Figure 8.9**). When analysing trials in strata of protein type there was no significant difference between the blood pressure effects of plant and animal protein (plant protein: -1.95 mmHg systolic, 95%-CI= -3.21 to -0.69; animal protein: -2.20 mmHg, 95%-CI= -3.36 to -1.03, **Figure 8.10**).

Heterogeneity between cross-sectional studies on plant and animal protein

There was substantial heterogeneity in the meta-analysis of cross-sectional studies on plant protein (I^2=75%, p=0.01, **Figure 8.6**) and animal protein (I^2=55%, p=0.07, **Figure 8.7**). This was mainly due to the study of Umesawa et al.[20] in 7,585 Japanese adults that showed an inverse association with blood pressure for animal protein and a direct association for plant protein. After exclusion of that study heterogeneity was strongly reduced to 17% for plant protein (p=0.31) and 0% for animal protein (p=0.61). In addition, pooled estimates changed toward a larger and significant difference between protein types, i.e. -0.73 mmHg systolic per SD (95%-CI: -1.08 to -0.38) for plant protein and +0.24 mmHg (-0.09 to +0.57) for animal protein. The deviant estimates in the study of Umesawa et al.[20] may be attributable to the eating habits in Japan, where ~ 24% of animal protein intake comes from fish[21], whereas in China and in Western countries ~6% of animal protein intake is derived from fish.[21] Fish may be more beneficial to blood pressure than meat[22], which may explain the inverse association

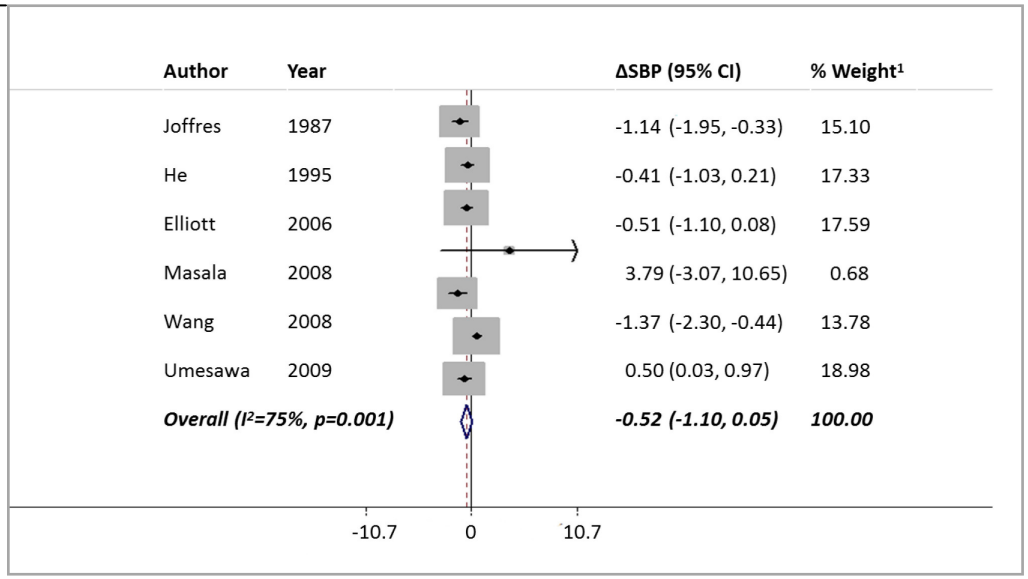

Figure 8.6. *Fully adjusted difference in systolic blood pressure (ΔSBP) with consumption of 11 grams (~1 SD) higher plant protein intake in 7 cross-sectional studies.*
For details of the studies see ***Table 8.2****).*
[1]Weights are from random effects analysis.

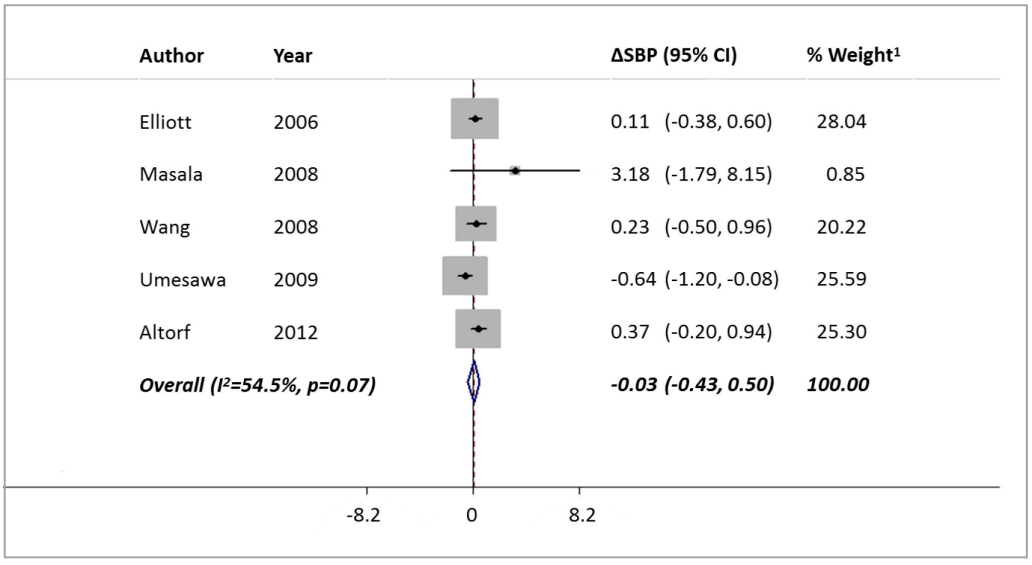

Figure 8.7. *Fully adjusted difference in systolic blood pressure (ΔSBP) with consumption of 23 grams (~1 SD) higher animal protein intake in 5 cross-sectional studies.*
For details of the studies see ***Table 8.2****).*
[1]Weights are from random effects analysis.

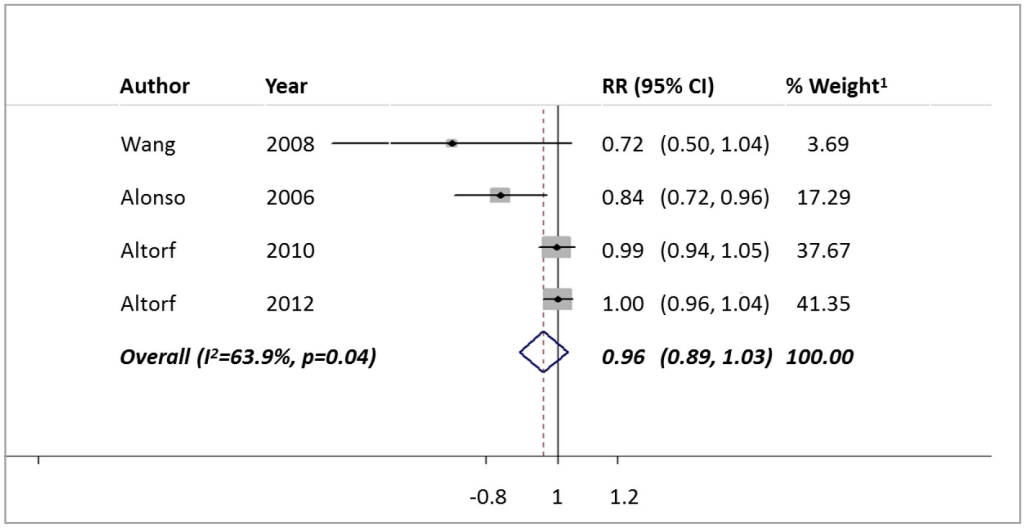

Figure 8.8. *Fully adjusted relative risk of hypertension (RR) with 11 grams (~1 SD) higher plant protein intake in 4 longitudinal studies.*
For details of the studies see ***Table 8.3****).*
[1]Weights are from random effects analysis.

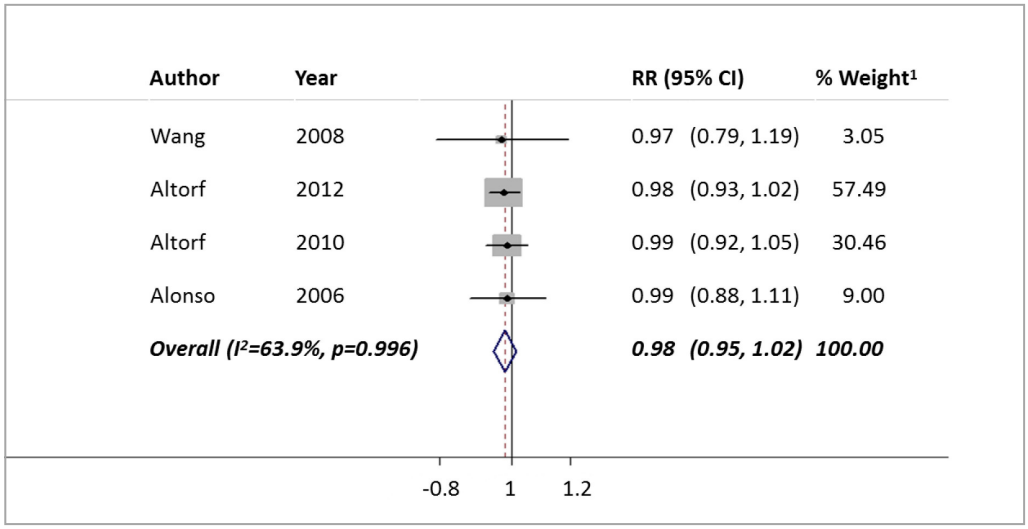

Figure 8.9. *Fully adjusted relative risk of hypertension (RR) with 23 grams (~1 SD) higher animal protein intake in 4 longitudinal studies.*
For details of the studies see ***Table 8.3****).*
[1]Weights are from random effects analysis.

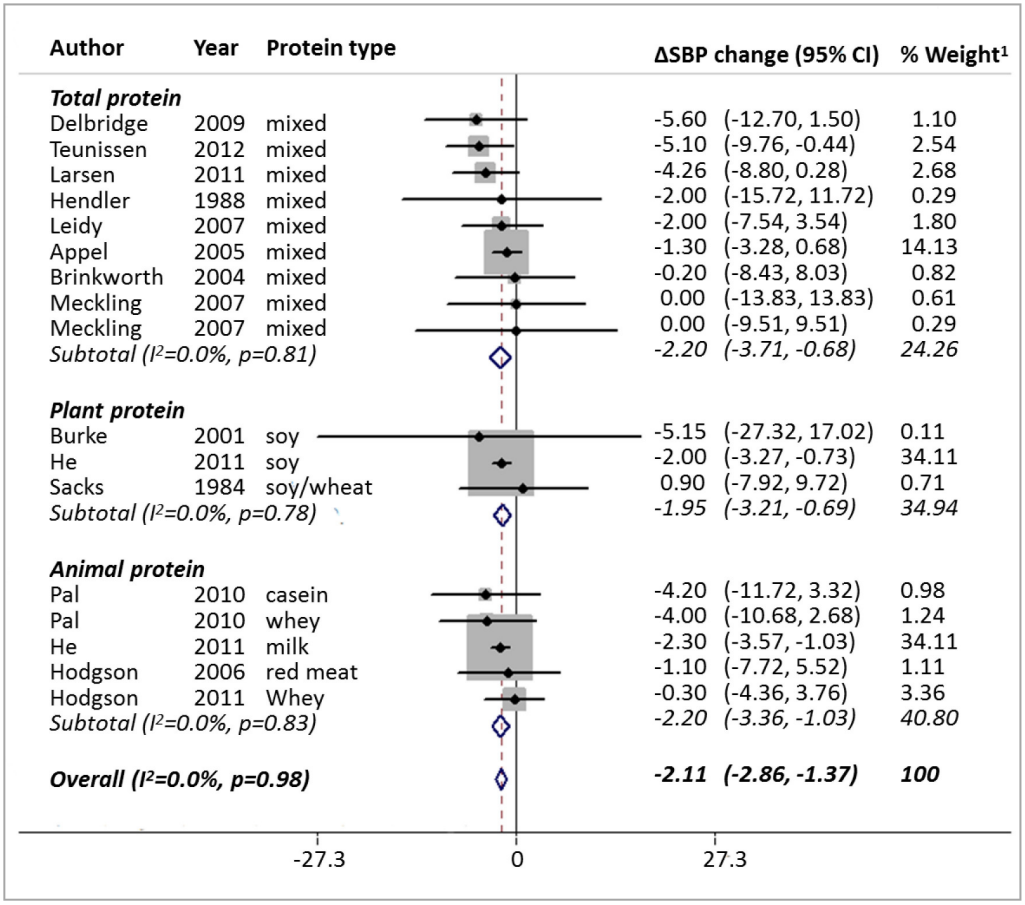

Figure 8.10. *Net change in systolic blood pressure (ΔSBP change) with consumption of protein compared to carbohydrates in 14 randomized controlled trials, stratified by protein type.*
For details of the studies see ***Table 8.4****).*
[1]Weights are from random effects analysis.

between animal protein and blood pressure in the study of Umesawa et al. A more beneficial influence of plant protein compared to animal protein on blood pressure may, therefore, only be present in countries with a more westernized diet.

Plant protein: a healthy diet indicator?

In cross-sectional studies an inverse association was found for plant protein, whereas there was no association for animal protein.[23,24] A major drawback of a cross-sectional design is that dietary intake and blood pressure are measured at the same moment in time. It is possible that participants at increased cardiovascular risk intentionally changed their diets toward a more plant based diet that is known to be healthy. This could have biased the associations for plant protein toward no association. However, individuals on antihypertensive

treatment were excluded from the analysis and elevated blood pressure is often asymptomatic, which makes intentional dietary changes unlikely. Therefore, we think that the observed differences between plant and animal protein in cross-sectional studies cannot be explained on basis of reverse causation.

Another methodological aspect of observational studies is residual confounding from factors that are strongly correlated to intake of protein types. Individuals in Western countries who consume a diet rich in plant protein probably have a healthier lifestyle than those who consume much animal protein. In most observational studies, including the ones described in this thesis, adjustments were made for nutrients that are indicators of a healthy lifestyle, such as dietary fibre and potassium. However, incomplete adjustment for lifestyle factors or dietary factors, such as polyphenols that are abundant in plant food, may have resulted in residual confounding. This could have influenced the findings for plant protein toward a more beneficial association with blood pressure.

Does plant protein decrease the risk of hypertension?

Two prospective studies on protein types and hypertension incidence from the literature showed inverse associations for plant protein intake, whereas for animal protein no such association was observed. In 810 untreated pre- or mild hypertensives of the PREMIER study a ~28% reduction in hypertension risk after 18 months of follow-up was observed per 11 grams of plant protein intake (~1 SD, p=0.08, **Figure 8.8**) after adjustment for major confounders like sodium and potassium.[24] In 5,880 university graduates of the SUN cohort 2-year risk of hypertension was 16% lower per 11 grams of plant protein intake (p=0.06).[25] The prospective studies presented in this thesis that involved over 3,500 general Dutch adults and over 2,000 older adults (**Chapter 4 and 5** respectively), however, did not show differences between plant and animal protein. When pooling all data, the relative risk for hypertension per SD was 0.96 (95%-CI: 0.89 to 1.03) for plant protein and 0.98 (0.95 to 1.02) for animal protein. There are several possible explanations why our findings differ from other prospective studies. The lower risk with higher plant protein intake in the study of Wang et al.[24] may be related to the fact that only individuals with elevated blood pressure were included who could be more sensitive to blood pressure lowering effects of plant protein. In the study by Alonso et al.[25] among Spanish university graduates, the type of plant protein sources may play a role. In Spain, on average, more legumes are consumed[26], and residual confounding from correlated healthy nutrients cannot be excluded. Whether high intake of plant protein indeed reduces the risk of hypertension thus still needs to be established.

Randomized controlled trials of plant versus animal protein

In a well-designed randomized trial the influence of residual confounding is minimized. Four trials have been published in which the blood pressure effects of protein from plant and animal sources were directly compared.[27-30] Three trials included only a small number of participants (n≤25), which may explain why no significant effect on blood pressure was found in those trials.[27-29] In a large cross-over trial among 352 adults with elevated blood pressure (~127/82 mmHg), 40 grams of soy protein per day for 8 weeks did not change blood pressure compared to 40 grams of milk protein (+0.4, 95%-CI: -1.0 to +1.7) However, in a Western diet soy protein makes only a small contribution to total intake of plant protein (~2.5% in the MORGEN cohort, unpublished data), and it is therefore not justified to draw the conclusion that plant and animal protein have similar blood pressure effects. It has been estimated that grain protein contributes ~53% to plant protein intake in the Netherlands (**Chapter 3**), with other important sources being potatoes (10%), vegetables (8%), and fruits (10%). Up to date no trial has been conducted that examined the blood pressure effect of dietary plant protein originating from these sources compared with a balanced mix of animal protein.

To gain more insight in the effect of plant and animal protein we conducted a meta-analysis of trials with a carbohydrate control, stratified by type of protein in the intervention diet (**Figure 8.10**). We did not find a significant different effect between trials with protein interventions from plant or animal sources. However, the protein source in all plant protein trials was again soy, and results cannot be generalised to total plant protein.

Plant versus animal protein; conclusions and suggestions for further research

In the past, several observational studies that investigated dietary protein types in relation to blood pressure levels or incidence of hypertension have found an inverse association for plant protein, but not for animal protein.[9,23-25] On the other hand, results from the meta-analyses did not provide evidence for a differential effect. Definitive conclusions cannot be drawn because of methodological issues that have been described above, and because trials investigating plant protein used soy as the sole source, whereas soy intake is low in the Netherlands and in many other Western countries. Future trials should therefore include a mix of plant and animal protein sources that better reflect habitual intakes in Western populations.

Protein from specific sources

Summary of results

The number of available blood pressure studies on protein from specific food sources was insufficient to conduct a meta-analysis. A parallel trial involving 64 hospital staff members[31] and a cross-over trial in 35 men[32] have been published on meat protein compared to other protein sources, showing no significant results (**Chapter 2**). This is in line with the lack of association for meat protein in our own observational studies (**Chapter 3-5, and Table 8.1**). Also for dairy protein we did not find an association with blood pressure or incident hypertension (**Chapter 3-5, Table 8.1**). One cross-over trial was published on dairy protein that showed a blood pressure lowering effect of -2.30 (95% CI: -3.36 to -1.03) after 33 gram milk protein supplementation compared to a carbohydrate supplement in 352 US adults.[30] For grain protein we found a small inverse association with diastolic (but not systolic) blood pressure levels in over 20,000 Dutch adults (**Chapter 3**). In addition, in our prospective analysis in 3,588 Dutch adults 8 grams higher energy adjusted grain protein intake was associated with 15% lower hypertension risk (95%-CI: 0.73 to 1.00, **Chapter 4**). However, we could not confirm this association in a prospective analysis among 2,241 Dutch older adults (**Chapter 5**). In summary, we were the first to study protein from several specific sources in relation to blood pressure showing a possible beneficial association with grain protein but not with protein from other major sources (**Table 8.1**).

Exposure assessment and biomarkers for protein from specific sources

The FFQs that were used in the studies described in this thesis were not designed to estimate intake of protein from specific sources. Moreover, the intake of plant foods, and consequently plant protein, may have been over-reported because of social desirability. Such errors could partly explain attenuated associations and inconclusive findings in our epidemiological studies. Objective biomarkers of intake could provide a better estimation of dietary protein from specific sources. However, such biomarkers are currently not available. This thesis includes a fully controlled dietary intervention study (**Chapter 7**) in which we aimed to identify new biomarkers for meat protein, dairy protein and grain protein. This resulted in a combination of 3 urinary amino acids as a potential biomarker for meat protein intake and a combination of 7 plasma amino acids as a potential biomarker for grain protein intake. We could not identify a reliable biomarker for dairy protein intake. These biomarkers need to be confirmed in a trial in which different levels of these protein types are given under strictly controlled conditions. After such a validation study these biomarkers may be used to calibrate or validate FFQs in future epidemiological studies, to assess intake of these protein types more accurately.

Protein from specific sources; conclusions and suggestions for further research

The studies described in **Chapters 3 to 5** of this thesis were the first that examined protein from several specific sources (meat, dairy, grain) in relation to blood pressure levels or incidence of hypertension. The results for these protein types were inconclusive, which may be due to errors in exposure assessment because FFQs were not designed to estimate intakes for these specific protein types. We conducted a study in which we identified combinations of urinary or plasma amino acids as potential biomarkers for meat and grain protein intake. If these biomarkers prove to be valid within normal ranges of dietary intake, they may be used to calibrate or validate FFQs in future epidemiological studies to assess intake of these protein types more accurately.

Amino acids

Summary of results

The number of available observational blood pressure studies on specific amino acids was insufficient to conduct a meta-analysis. One observational study has been published showing an inverse association with blood pressure for glutamic acid[33], whereas in other studies no association was observed for arginine (two studies)[34,35] and methionine (one study)[36]. Also trials on the blood pressure effect of specific amino acids in humans are scarce, except for trials on arginine which is a precursor for the vasodilator nitric oxide. In a recently published meta-analysis of 11 arginine supplementation trials, a pooled blood pressure effect of -5.4/-2.7 mmHg was found.[37] Furthermore, in a trial involving 13 untreated hypertensives two weeks with 7.5 g/d tyrosine supplementation did not affect blood pressure.[38] In **Chapter 6** of this thesis we examined whether dietary intakes of the individual amino acids glutamic acid, arginine, lysine, cysteine, and tyrosine were associated with blood pressure and incidence of hypertension in a population of ~3,000 older Dutch adults of the Rotterdam Study. We found no association of the habitual intake of glutamic acid, arginine, lysine, cysteine, and essential amino acids (expressed as protein%) with blood pressure level (**Table 8.1**). For tyrosine intake we observed a borderline significant inverse association with systolic blood pressure, but not with diastolic blood pressure. None of the examined amino acids was related to 6-year risk of hypertension (**Table 8.1**).

Intake of amino acids relative to total dietary protein

All amino acids are available from almost all types of food that contain protein, although in different proportions. Absolute amino acid intakes (i.e. expressed in g/d) are, therefore, strongly correlated to total protein intake and consequently to each other. Because of multicollinearity it is not possible to estimate the association with blood pressure for absolute in-

takes of single amino acids in an observational study. We therefore expressed amino acid intake as a percentage of total protein intake and assessed the relation between relative amino acid intake and blood pressure. However, for those amino acids that are precursors for blood pressure regulating compounds (e.g. arginine which is a precursor for nitric oxide) absolute intakes may be more important than relative intakes for blood pressure. If this is the case, participants in our study have not been correctly classified for absolute amino acid intake, which could explain the null findings.

The relation between absolute amino acid intake and blood pressure can be investigated in randomized controlled trials in which individual amino acids are supplemented. Until now, trials have mainly focussed on arginine. However, in the meta-analysis on these trials there was substantial heterogeneity (I^2=73%, p<0.001) due to two studies with large systolic blood pressure reductions of -18 mmHg after 9 g/d arginine supplementation and -23 mmHg after 6 g/d arginine supplementation, respectively.[39,40] In a sensitivity analysis excluding these two studies, the beneficial blood pressure effect was still significant (pooled blood pressure effect of −3.3 mmHg; 95% CI −4.9 to −1.9).[37] It should be noted, however, that arginine doses in these studies ranged between 4 and 24 g/d, which exceeds contrasts that can be reached by diet (e.g. 1 SD in the Rotterdam Study was 1 g/d). Whether arginine from the usual diet influences blood pressure levels is not yet clear. In another trial, tyrosine supplementation was investigated in relation to blood pressure. After 2 weeks of 7.5 g/d supplementation in 13 mildly hypertensive adults, no significant effect on blood pressure was found compared to placebo (lactose).[38] However, the lack of a significant effect (-3 mmHg as estimated from graph) may be due to the small sample size of this study.

Amino acids; conclusions and suggestions for further research

In an observational analysis in a population-based cohort of ~3,000 Dutch older adults, we did not observe significant associations for glutamic acid, arginine, lysine, tyrosine, cysteine, or essential amino acids with blood pressure. Because of multicollinearity we expressed intake of these amino acids as a percentage of total protein intake, which may explain the null findings if absolute intakes (i.e. expressed in g/d) are more important for blood pressure. Arginine supplementation was significantly related to blood pressure in a recently published meta-analysis of randomized controlled trials.[37] However, in these trials high doses of arginine were given. Blood pressure trials with dietary doses of arginine are needed to judge the relevance of this amino acid for population blood pressure. The same holds for other amino acids, for which little is known in relation to blood pressure.

Subject characteristics that modify the blood pressure response to dietary protein

We conducted a metaregression analysis of protein trials with a carbohydrate control to identify subject characteristics that may modify the blood pressure response to dietary protein. However, mean age, gender (% males), BMI, and initial blood pressure were not significantly related to treatment effect. For age, gender, and BMI this is in agreement with our findings in this thesis. However with regard to initial blood pressure there is a contrast with the cross-sectional analysis in the MORGEN cohort in which the inverse associations of plant protein with blood pressure were more pronounced in hypertensives than in normotensives. Similarly, in the OmniHeart trial the blood pressure effect of a high protein diet (about half from plant sources) compared to a high carbohydrate diet was stronger in hypertensives than in prehypertensives. The fact that our metaregression analysis was based on aggregate trial data with possibly large blood pressure ranges within individual studies may have blurred the associations of baseline blood pressure with treatment effect. Presenting results of observational studies and trials in strata of baseline blood pressure is warranted to find out whether those with higher blood pressure show a stronger response to increased protein intake.

PUBLIC HEALTH IMPLICATIONS

The prevalence of hypertension is high and increasing. In 2008, the World Health Organisation estimated that ~33% of Dutch adult men and 23% of women had a high blood pressure (≥140/90).[41] In 2002 approximately 7.1 million deaths, about 13% of the total, were estima-

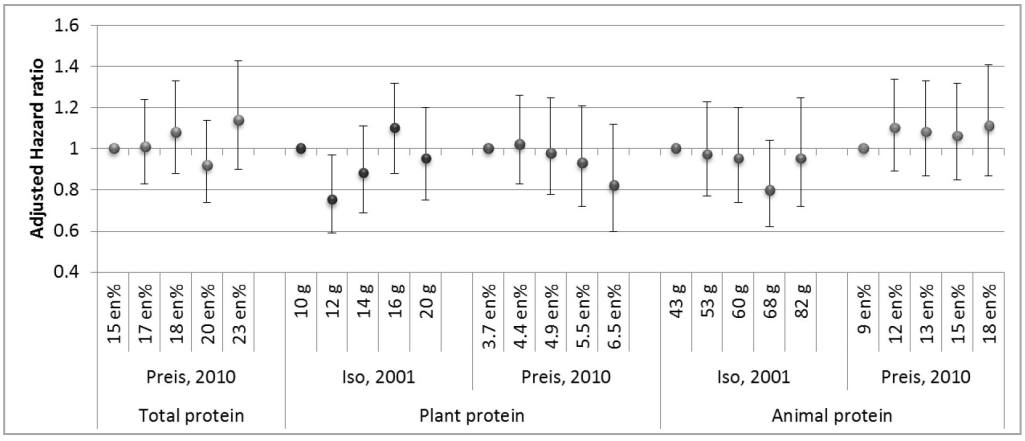

Figure 8.11. *Risk for stroke in the Nurses' Health Study (Iso, 2001[80]) and the Health Professionals Follow-up Study (Preis, 2010[42]) in quintiles of total, plant, and animal protein intake.*
For each quintile the median protein intake is given.

ted to be attributable to high blood pressure.[2] Hypertension is usually without symptoms and remains often undetected, whereas cardiovascular risk already increases from a systolic blood pressure of 115 mmHg.[1] Population-wide lifestyle and dietary changes that effectively prevent a rise in blood pressure, starting already in youth, will have a substantial public health impact. In the present thesis we investigated the influence of dietary protein on blood pressure. In this paragraph we discuss our findings in the context of recommended protein intake, taking into account types of dietary protein.

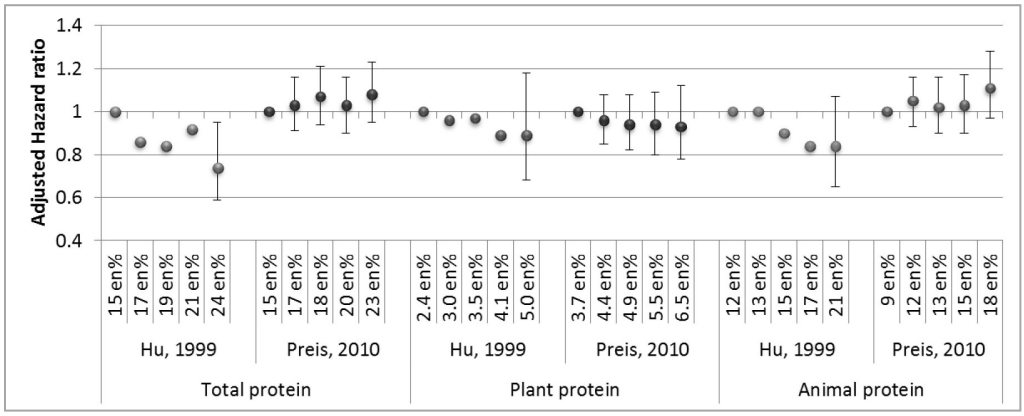

Figure 8.12. *Hazard ratio for Coronary heart disease in the Nurses' Health Study (Hu, 1999[45]) and the Health Professionals Follow-up Study (Preis, 2010[43]) in quintiles of total, plant, and animal protein intake.*
For each quintile the median protein intake is given.

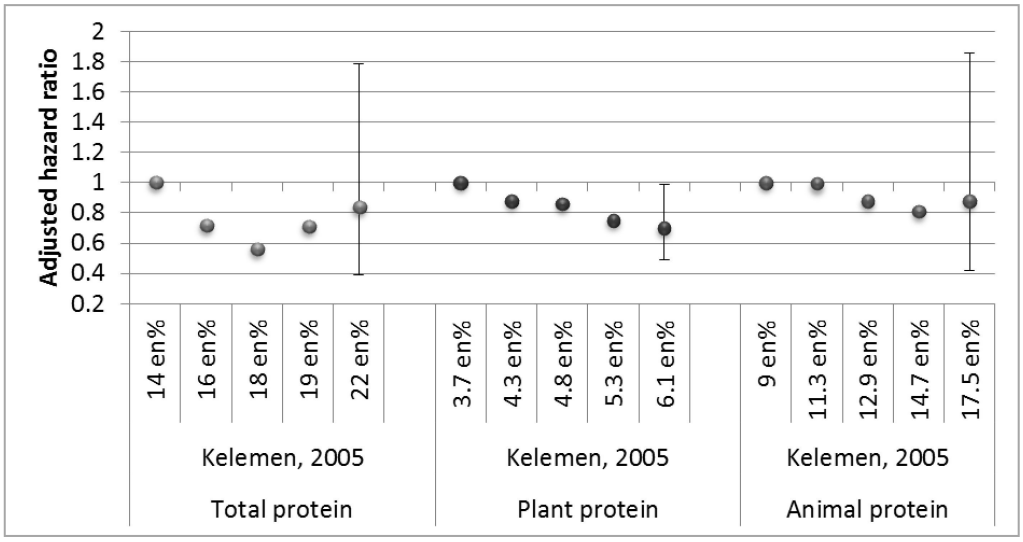

Figure 8.13. *Hazard ratio for total mortality from coronary heart disease in the Iowa Women's Health Study (Kelemen, 2005)[46] in quintiles of total, plant, and animal protein intake.*
For each quintile the median protein intake is given.

Should we increase total protein intake?

Results from this thesis suggests a small beneficial effect of protein on blood pressure if it is consumed instead of carbohydrates, and this beneficial effect may be most pronounced in hypertensives. Whether total dietary protein also influences cardiovascular disease risk has been examined by several large observational studies, but no consistent associations were found.[42-46] In the Health Professionals Follow-Up study among 43,960 US men no association was observed between total protein intake and risk of stroke (**Figure 8.11**).[42] For coronary heart disease, a total protein intake of 24 en% compared to an intake of 15 en% resulted in a hazard ratio of 0.74 (95%-CI: 0.59 to 0.95) in the Nurses' Health Study among 85,764 US women (**Figure 8.12**).[45] However, in the Health Professionals Follow-Up study and in the Iowa Women's Health study in 29,017 postmenopausal women there was no association between total protein intake and coronary heart disease (**Figures 8.12 and 8.13**).[43,46] It may be concluded, therefore, that a high intake of protein does not increase the risk of cardiovascular diseases. Protein intake, especially intake from animal sources, was relatively high in the US studies, i.e. ~19 en% (~13 en% from animal sources) in the Health Professionals Follow-Up study[42,43], and ~18 en% (~13 en% from animal sources) in the Iowa Women's Health Study[46]. For the Nurses' Health Study total protein intake was not reported, but animal protein intake was ~61 g/d (intake in en% not reported).[44,45] Protein intake in the Netherlands is considerably lower, being ~15 en% (~73 g/d for women and ~96 g/d for men), with ~10 en% (~46 g/d for women and ~60 g/d for men) originating from animal sources.[47] Possibly, the high intake of protein from animal sources, and concomitant intake of saturated fat, could explain why no association with coronary heart disease was found in several large US studies.

Although protein may be beneficial for blood pressure, there is also concern that high protein intake may promote renal damage by chronically increased glomerular pressure and hyperfiltration.[48] In the Nurses' Health Study, total protein intake was associated with accelerated renal function decline during 11 years of follow-up in 489 women with mild renal insufficiency (defined as glomerular filtration rate (GFR) between 55 and 80 mL/ min per 1.73 m^2) with a change in estimated GFR of -1.69 mL/min with 10 g/d higher protein intake.[49] Trials with high protein diets in patients with mild kidney impairment are lacking, possibly because this type of intervention is considered unethical. In those with healthy kidneys, on the other hand, protein intake is unlikely to be harmful. This was confirmed in 1,135 women from the Nurses' Health Study with normal renal function in whom protein intake was not related to renal function decline.[49] It is possible that protein-induced changes in renal filtration are a normal adaptive mechanism within the function limits of a healthy kidney.[50]

In 2006 the Health Council of the Netherlands recommended a dietary protein intake between 8 and 11 en% (~0.8 g/kg body weight) dependent on age and gender, with an upper level of 25 en%.[51,52] These recommendations are primarily intended to guarantee an adequate intake of essential amino acids and nitrogen to build up necessary proteins in the

body.[51,52] In Europe total protein intake is ample sufficient, ranging between 12 and 23 en% with intake in the Netherlands being ~15 en%.[26,47] Whether an increased protein intake at the expense of carbohydrates should be considered for the purpose of hypertension prevention is unclear and premature because there are no data available on the optimal protein dose. Trials in which several doses of protein are consumed instead of carbohydrates are warranted. In addition, in these trials attention should be paid to what is the best type of carbohydrate to be replaced.

Should protein mainly be derived from plant sources?

This thesis provides evidence for a more beneficial effect of plant protein (e.g. from grain) on blood pressure compared to animal protein, although data were not conclusive. In the Iowa Women's Health study an inverse association with fatal coronary heart disease was found for plant protein with a 30% lower risk in the highest quintile (6.1 en% of intake) compared to the lowest quintile (3.7 en% of intake, p_{trend}=0.02, **Figure 8.13**).[46] On the other hand, in the Nurses' Health study and the Health Professional Follow-up Study no significant associations were found between plant protein and stroke or total coronary heart disease (**Figure 8.11 and 8.12**).[42, 43, 45, 80] However, as discussed previously, plant protein intake in the US cohorts was low compared to animal protein, which may have influenced the associations. Prospective data on plant protein intake and risk of cardiovascular diseases in the Netherlands are lacking.

Randomized controlled trials are warranted that directly compare plant protein with animal protein in relation to blood pressure, using protein sources that reflect habitual intakes in Western populations. Furthermore, the use of biomarkers in future observational studies may result in more robust estimates for protein intake from specific sources. Finally, whether a differential effect of plant protein and animal protein is due to specific amino acids remains to be established in trials.

The Dutch dietary guidelines do not include recommendations for the intake of specific types of protein. For the purpose of hypertension prevention, recommendations to increase plant protein intake would be premature based on data presented in this thesis. Nevertheless, increased intake of plant foods is desirable because it is a major source of vitamins, polyphenols, fiber, potassium and magnesium, all being nutrients that have been associated with lower blood pressure and a better cardiovascular health profile.[53,54] Also from an ecological perspective a more plant based diet is preferable. The ecologic load of animal protein, especially from meat, is large compared to that of plant protein with a need of 6 kg plant protein for the production of 1 kg meat protein.[55,56] Therefore, the Health Council of the Netherlands recommended in 2011 a diet in which plant foods are emphasized, although they considered it not necessary to remove dairy and meat completely from the

diet.[55] A point of concern with regard to a more plant based diet, however, might be the low amount of lysine, an essential amino acid. Where recommendations for protein intake in omnivores are between 8 and 11 en%, lacto-ovo vegetarians may have a 1.2 times higher need of dietary proteins. Nevertheless, it has been estimated that vegetarians in the US and the UK have an average protein intake of ~13 en%, which is sufficient according to the guidelines.[57,58] Therefore, lysine deficiency may not be a point of concern in a diet that is rich in plant foods. On the other hand, most plant foods are not only low in lysine, but also in other nutrients like iron, calcium, vitamin B12 and riboflavin. Legumes, that are relatively high in lysine, and meat replacers that are usually enriched with these micronutrients may be used to ensure sufficient intakes.[55]

Concluding remarks

Results from this thesis suggest a small beneficial effect of protein on blood pressure if consumed instead of carbohydrates. Plant protein, e.g. from grain, may be more beneficial to blood pressure than animal protein but data are too limited to draw firm conclusions. After validation, future epidemiological studies could make use of biomarkers as more robust estimates for protein from specific sources and amino acid intakes. Furthermore, randomized controlled trials are warranted to examine the blood pressure effect of specific types of protein, reflecting habitual intakes in western societies. Furthermore, trials should include different types of carbohydrate as control. At present, a prudent diet for the prevention of hypertension with adequate amounts of dietary protein, preferable from plant sources, is recommended.

What is already known

- A healthy diet can substantially lower blood pressure.
- Findings on dietary protein and blood pressure are inconsistent, although data from studies using biomarkers for intake and trials suggest a small inverse association.
- In the large OmniHeart trial blood pressure decreased more after a high protein diet than after a high carbohydrate diet, but no difference in blood pressure effect was found compared to a diet high in mono-unsaturated fatty acids. (Appel et al, N Engl J Med 1997)
- Observational data suggest that plant protein may be beneficial to blood pressure.
- The effect of protein intake from specific sources like dairy, meat or grains on blood pressure is largely unknown.
- The blood pressure effect of amino acids, within the normal range of dietary intake, is unknown.
- Data on subject characteristics that may modulate the blood pressure effect of dietary protein are scarce.

What this thesis adds

- Data from a comprehensive meta-analysis show a beneficial blood pressure effect of protein compared to carbohydrates, but not compared to (mono-unsaturated) fat.
- Plant protein, e.g. from grain, may beneficially influence blood pressure.
- Specific combinations of urinary and plasma amino acids may be potentially useful biomarkers for meat and grain protein intake.
- Individuals who already have an elevated blood pressure are likely to benefit more from a beneficial effect of (plant) protein on blood pressure.

What still needs to be done

- More data are needed to conclude whether protein from different sources are important for population blood pressure.
- Trials are warranted that compare plant and animal protein from a mix of protein sources that reflect habitual intakes in Western populations.
- Trials are warranted in which the effect of protein is compared to different types of carbohydrates.
- Urinary and plasma biomarkers of specific types of protein need to be validated in in a trial in which different levels of meat and grain protein are given under strictly controlled conditions.

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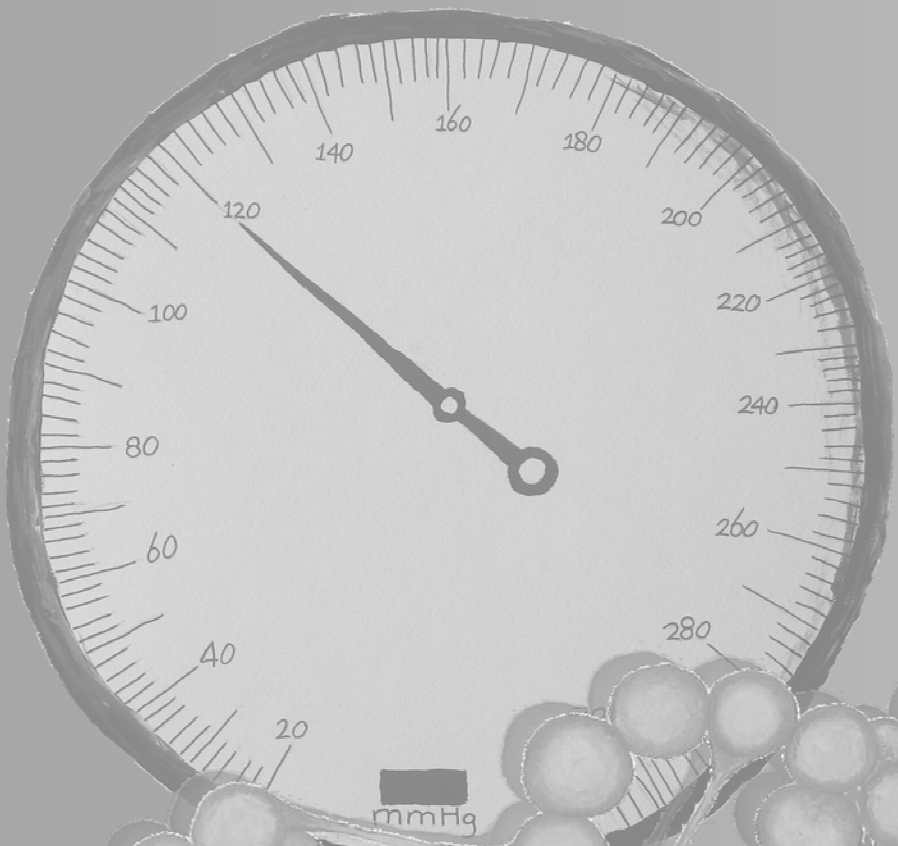
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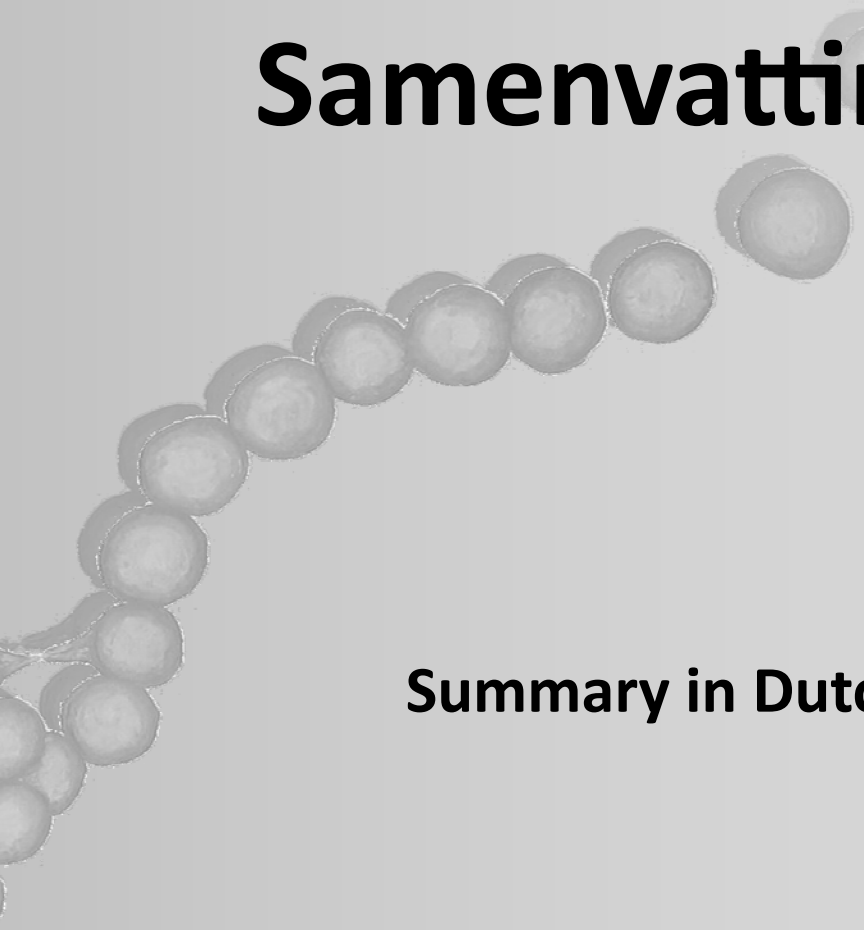


Summary in Dutch

Een verhoogde bloeddruk is een belangrijke risicofactor voor het krijgen van hart- en vaatziekten en nierschade. Een bloeddruk lager dan 120 mmHg systolisch en 80 mmHg diastolisch wordt gezien als optimaal. Er is sprake van hypertensie bij bloeddrukwaarden van 140/90 mmHg of hoger, of wanneer antihypertensieve medicatie gebruikt wordt. Naar schatting is 33% van de mannen en 23% van de vrouwen in Nederland hypertensief. De toename in cardiovasculair risico beperkt zich echter niet tot deze groep, maar is al meetbaar vanaf 'normale' systolische bloeddrukwaarden van 115 mmHg. Een geringe daling van 2 mmHg in de gemiddelde systolische bloeddruk in de algehele bevolking kan het aantal fatale hersenbloedingen met 6% verlagen en het aantal fatale coronaire hartziekten met 4%.

Voeding en leefstijl zijn van groot belang voor een gezonde bloeddruk. Bekende maatregelen zijn voldoende beweging, een gezond gewicht, het eten van voldoende groente en fruit, en matig zout– en alcoholgebruik. Ook zijn er aanwijzingen dat voedingseiwit een rol speelt bij het handhaven van een gezonde bloeddruk. In Nederland wordt ongeveer 85 gram per dag aan eiwit gegeten, hetgeen overeenkomt met 15% van de totale dagelijkse energie-inname. Twee derde van dit eiwit is van dierlijke oorsprong en een derde is van plantaardige oorsprong. De belangrijkste bronnen van dierlijk eiwit zijn zuivel (42%, ~24 gram/dag) en vlees (40%, ~22 gram/dag) terwijl het plantaardige eiwit vooral uit granen komt (48%, ~13 gram/dag).

Dit proefschrift richt zich op de mogelijke rol van voedingseiwit in relatie tot de bloeddruk in de Nederlandse bevolking. Deze relatie is onderzocht voor de totale eiwitinname, als ook voor de inname van plantaardig en dierlijk eiwit, eiwit uit specifieke bronnen (in het bijzonder zuivel, vlees en granen) en specifieke aminozuren. Daarnaast is onderzocht of leeftijd, geslacht, overgewicht en de hoogte van de bloeddruk deze verbanden kunnen beïnvloeden.

Hoofdstuk 2 van dit proefschrift bestaat uit een systematisch literatuuroverzicht over de mogelijke invloed van voedingseiwit op de bloeddruk. Uit interventiestudies bleek dat extra eiwit de bloeddruk kan verlagen. Ook waren er aanwijzingen dat plantaardig eiwit gunstiger is voor de bloeddruk dan dierlijk eiwit. Er was weinig bekend over de bloeddrukeffecten van eiwit uit specifieke bronnen zoals zuivel, vlees en granen.

In de **hoofdstukken 3, 4 en 5** van dit proefschrift worden drie epidemiologische studies beschreven waarin de inname van totaal en typen eiwit in relatie tot de bloeddruk en/of het risico op hypertensie is onderzocht. In **hoofdstuk 3** zijn dwarsdoorsnede-gegevens gebruikt van 20.820 Nederlandse volwassenen in de leeftijd van 25 tot en met 65 jaar uit het MORGEN onderzoek (Monitoring van Risicofactoren en Gezondheid in Nederland) van het RIVM. Vervolgens is in **hoofdstuk 4** het verband tussen totaal eiwit en typen eiwit en het risico op hypertensie onderzocht in 3.588 van deze deelnemers die 15 jaar waren gevolgd (Doetinchem Cohort Studie). Hetzelfde verband is bestudeerd bij 2.241 deelnemers van 55 jaar en ouder van de Rotterdam Study die 6 jaar waren gevolgd (**hoofdstuk 5**). Voor totaal of dierlijk eiwit vonden we in geen van deze studies een verband met de bloeddruk of het risico op hypertensie. In personen met een relatief hoge inname van plantaardig eiwit (>36 gram / dag) was de bloeddruk circa 2 mmHg lager dan in personen met een relatief lage inname (<27 gram/dag). Plantaardig eiwit was echter niet gerelateerd aan het risico op hypertensie (**hoofdstuk 4 en 5**). Er was geen duidelijk verband van zuiveleiwit of vleeseiwit met de bloeddruk of het risico op hypertensie (**hoofdstuk 3, 4, 5**). Wel was voor graaneiwit het risico op hypertensie ongeveer 15% lager bij een relatief hoge inname (>18 gram/dag) vergele-

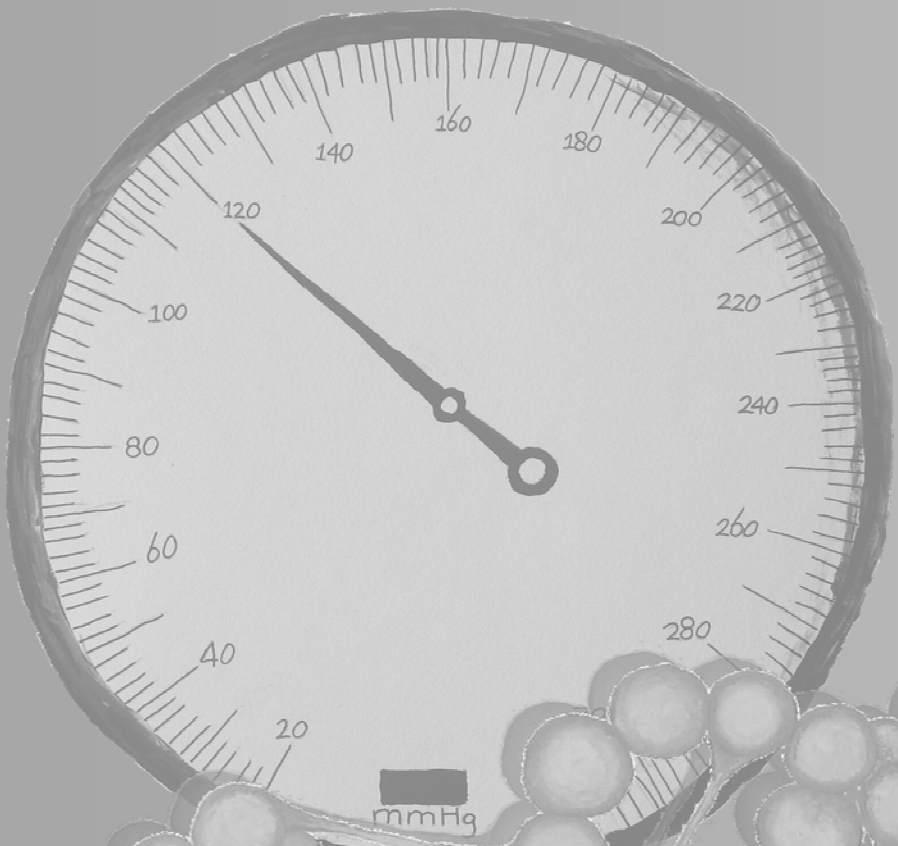
ken met een lage inname (<14 gram/dag)

In **hoofdstuk 6** onderzochten we bij 3.086 deelnemers van 55 jaar en ouder van de Rotterdam Study of specifieke aminozuren uit de voeding samenhingen met het risico op hypertensie, waarbij de aminozuurinname werd uitgedrukt als percentage van de totale eiwitinname. Van de bestudeerde aminozuren droeg glutaminezuur het meest bij aan de totale eiwitinname (21%), gevolgd door lysine (7%), arginine (5%), tyrosine (4%) en cysteine (1,5%). Geen van deze aminozuren liet een significant verband zien met hypertensie (relatieve risico's variërend tussen 0,81 – 1,18 voor het hoogste versus het laagste kwartiel van inname).

Door middel van biomerkers in lichaamsweefsels kan de inname van voedingsstoffen op een objectieve manier worden geschat, wat de validiteit van epidemiologisch onderzoek kan vergroten. Voor de inname van typen eiwit waren geen gevalideerde biomerkers bekend. In een gecontroleerde voedingsinterventie **(hoofdstuk 7)** is bij 30 personen in de leeftijd van 18 tot en met 40 jaar gedurende 4 weken de inname van verschillende typen voedingseiwit sterk verhoogd met als doel het vaststellen van biomerkers voor eiwit uit zuivel, vlees en granen. Hieruit bleek dat een combinatie van aminozuren in 24-uursurine (carnosine, 1-methylhistidine en 3-methylhistidine) een betrouwbare schatting levert van de inname van vleeseiwit. Een combinatie van 7 aminozuren in het bloedplasma (lysine, valine, threonine, α -aminoboterzuur, proline, ornithine en arginine) is mogelijk geschikt voor het schatten van de inname van graaneiwit. Voor eiwit uit zuivel konden we geen biomerkers vaststellen.

In **hoofdstuk 8** is een kwantitatieve samenvatting gegeven van de stand van zaken rondom eiwit en bloeddruk, na toevoeging van de studies uit dit proefschrift. In meta-analyses van epidemiologische studies werd geen verband gevonden tussen de totale inname van eiwit en de bloeddruk of het risico op hypertensie. In een meta-analyse van 14 gecontroleerde interventiestudies was een verhoogde eiwitinname (~41 gram/dag) ten koste van koolhydraten echter gerelateerd aan een gemiddeld 2,1 mmHg (95%-betrouwbaarheidsinterval: -2,9 tot -1,4 mmHg) lagere systolische bloeddruk. Wat betreft typen eiwit zagen we voor plantaardig eiwit in epidemiologische studies een klein gunstig verband met de bloeddruk, maar niet voor dierlijk eiwit. Hoewel oudere studies een gunstig verband lieten zien tussen plantaardig eiwit en het risico op hypertensie, was die samenhang verdwenen na toevoeging van de resultaten uit dit proefschrift. Er waren nog te weinig studies uitgevoerd om een meta-analyse te kunnen doen voor eiwit uit specifieke bronnen zoals granen, vlees en zuivel.

Samengevat suggereren de diverse onderzoeksresultaten dat eiwit gunstig is voor de bloeddruk als het wordt geconsumeerd in plaats van koolhydraten. Toekomstig onderzoek moet uitwijzen welke typen koolhydraten het beste vervangen kunnen worden. Het vervangen van dierlijk door plantaardig eiwit, bijvoorbeeld uit granen, kan mogelijk ook bijdragen aan een gezondere bloeddruk, maar op basis van de huidige resultaten is het prematuur om plantaardig eiwit aan te bevelen voor de preventie van hypertensie. Wel is bekend dat een voedingspatroon met meer plantaardige producten het risico op hypertensie en hart- en vaatziekten verlaagt. Het is daarom aan te bevelen om voldoende eiwit uit voornamelijk plantaardige bronnen te consumeren.


20
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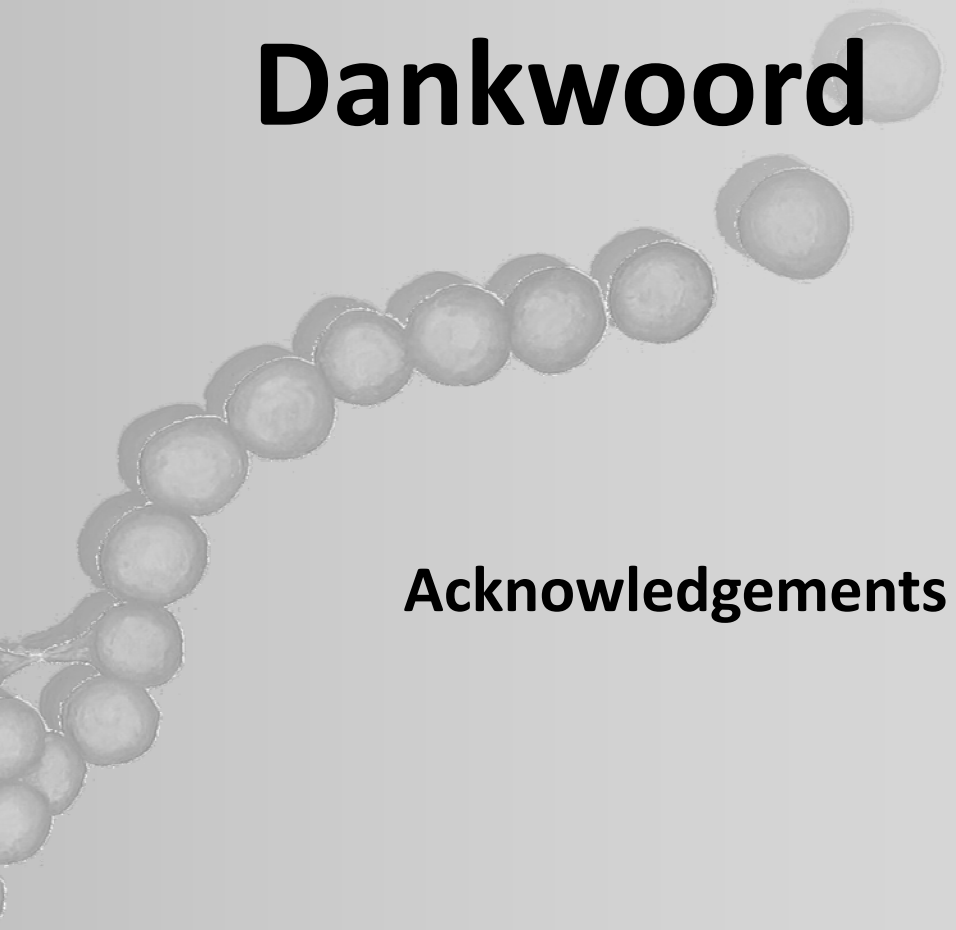
mmHg

Dankwoord



Acknowledgements

Al weer een paar jaar geleden kwam ik de volgende uitspraak van Einstein tegen:

> *"Het opstellen van een nieuwe theorie is niet hetzelfde als het slopen van een oude schuur om op die plaats een wolkenkrabber te bouwen. Het lijkt meer op het beklimmen van een berg, waarbij we nieuwe en weidse gezichten aanschouwen en waarbij we onverwachte verbindingen ontdekken tussen ons uitgangspunt en zijn rijke omgeving".*

Ik zelf heb in de afgelopen vier jaar weliswaar geen nieuwe theorie opgesteld, maar ik heb wel een stukje van de berg mogen beklimmen. Ik heb prachtige uitzichten gezien en soms ook wel eens over paden gelopen waar ik nog geen meter voor mij uit kon kijken. Gelukkig was ik hierbij in goed gezelschap, en ik wil dan ook de gelegenheid aangrijpen om al die verschillende mensen te bedanken.

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ste moeten ontgelden, maar reageerde steeds weer even opgewekt. Bedankt!

In het kader van de Biomerker Studie zijn er nog veel meer mensen om te bedanken. Zo'n volledig gecontroleerde voedingsproef is niet niks om in een korte tijdsperiode op te zetten en draaiend te houden. Maar hierbij had ik een enorme steun aan mijn onderzoeksassistent *Martine*. Martine, je bent een enorm harde werker, en je bleef altijd opgewekt, ook in de periode dat het werk ons behoorlijk boven het hoofd groeide. Het was fijn om de grote berg werk samen aan te kunnen pakken. *Els, Ingrid, Yvonne, Karin, Corine, Jelle en Laura,* ondanks de onmogelijke eisen wat betreft eiwitgehalten hebben jullie toch weer lekkere menu's samen weten te stellen en enorm hard gewerkt om iedere dag weer op tijd een warme maaltijd en een meeneempakket voor iedere deelnemer klaar te zetten. Dank jullie wel voor de gezellige middagmaaltijden en alle behulpzaamheid. *Anita en Lucie*, jullie hebben flink moeten puzzelen om mijn bloedprikmomenten nog tussen de andere studies door te plannen. Dank jullie wel voor al die moeite. Ook wil ik de studentassistenten (*Gerdien, Ilse, Anneke, Ipa, Josje, Anne, en Marijke*) en de laboratoriummedewerkers (in het bijzonder *Robert, Betty en Marlies*), enorm bedanken voor de hulp bij het bestellen van de benodigdheden en het verwerken en analyseren van de urines en het bloed. *Sabina Bijlsma en Carina Rubingh (TNO),* dank jullie wel voor de multivariate data-analyse en het onvermoeibaar steeds weer opnieuw uitleggen wat jullie nou precies hadden gedaan en hoe we de figuren moesten lezen. *Professor Daniel Tomé*, thank you for the meetings we could have with you on the Biomarker Study and for your advices.

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Verschillende studenten hebben in het kader van hun afstudeervak literatuuronderzoek, data-analyse of praktisch werk gedaan. *Moniek, Lisette, Elles, Evren en Melissa,* dank jullie wel voor jullie bijdrage aan mijn project.

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De *eerste verdieping van het agrotechnion* moet even apart genoemd worden. Ik vond dat

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About the author

Curriculum vitae

Publications

Educational program

CURRICULUM VITAE

Wieke Altorf – van der Kuil was born on December 25th 1980 in Utrecht, The Netherlands. After finishing the secondary school at the 'Christelijk Gymnasium' in Utrecht, she started the study 'Nutrition and health' at Wageningen University. She conducted her MSc thesis in the specialization epidemiology and public health at Wageningen University on Meat, meat products, NAT2 genotype and K-ras mutations in colorectal adenoma's and was second author on a publication on this subject. Her internship consisted of literature research and practical work in the VISIE study at the Dutch cancer institution in Amsterdam. In November 2005 she obtained her MSc degree.

After her studies Wieke first did some practical voluntary work in Taizé, a Christian community in France with over time more responsibilities: from cleaning to leading a team in the non-profit shop. After that she worked as datamanager at the integral cancer center in Nijmegen. In March 2008 Wieke started as PhD fellow at Wageningen University with detachment to Top Institute Food and Nutrition on a project investigating the effects of dietary protein on blood pressure. This project is conducted in collaboration with Maastricht University and University Medical Center Groningen. Within this project Wieke conducted a systematic review and performed epidemiologic analyses on the intake of protein types and blood pressure level or hypertension risk in three different population-based cohorts. For this, she collaborated with research groups at the Dutch National Institute for Public Health and the Environment (RIVM) and Erasmus MC Rotterdam.

A paper entitled 'Dietary protein and risk of hypertension in a Dutch older population: the Rotterdam study' (J Hypertens 28:2394–2400) was nominated for the publication prize of Top Institute Food and Nutrition in 2010, and a poster entitled 'Dietary protein and risk of hypertension in a general Dutch population' was nominated for the poster prize of Top Institute Food and Nutrition in 2012. In 2011 Wieke designed and performed a trial that aimed to validate postulated biomarkers of protein from meat, and to identify new potential biomarkers for protein from meat, dairy and grain. The abstract of this study was nominated for the 'Foppe ten Hoor' young investigator's award in 2011.

As part of the educational program of the graduate school VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences) Wieke joined several (international) conferences and courses in the field of nutrition, epidemiology and cardiovascular diseases and she was involved in teaching. In 2008 she organized a retreat for the division of Human Nutrition (Human Nutrition Research Update 2008).

PUBLICATIONS

Full papers

- Wark PA, van der Kuil W, Ploemacher J et al. (2006) Diet, lifestyle and risk of K-ras mutation-positive and -negative colorectal adenomas. International Journal of Cancer 119, 398-405.
- Altorf - van der Kuil W, Engberink MF, Brink EJ et al. (2010) Dietary Protein and Blood Pressure: A Systematic Review. PLoS ONE 5, e12102.
- Altorf-van der Kuil W, Engberink MF, van Rooij FJ et al. (2010) Dietary protein and risk of hypertension in a Dutch older population: the Rotterdam study. Journal of Hypertension 28, 2394-2400.
- Altorf – van der Kuil W, Engberink MF, Vedder MM, Boer JMA, Verschuren WMM, Geleijnse JM. Sources of dietary protein in relation to blood pressure in a general Dutch population. PLoS One 7 (2012)2.
- Altorf-van der Kuil W, Engberink MF, Geleijnse JM, Boer JMA, Verschuren WMM. Sources of dietary protein and risk of hypertension in a general Dutch population. Br J Nutr. 2012 Jan 25:1-7. [Epub ahead of print].

Full papers submitted for publication

- Altorf-van der Kuil W, Engberink MF, van Rooij FJ, Hofman A, van't Veer P, Witteman JCM, Geleijnse JM, Dietary amino acids and risk of hypertension in a Dutch older population: the Rotterdam Study. Submitted.
- Altorf–van der Kuil W, Brink EJ, Boetje M, Siebelink E, Bijlsma S, Engberink MF, van 't Veer P, Tomé D, Bakker SJL, van Baak MA, Geleijnse JM, Identification of biomarkers for intake of protein from meat, dairy and grains: a fully controlled dietary intervention study. Submitted.
- Altorf-van der Kuil W, Engberink MF, IJpma I, Verberne L, Toeller M, Chaturvedi N, Fuller J, Soedamah-Muthu S. Protein intake in relation to risk of hypertension and microalbuminuria in patients with type 1 diabetes: the EURODIAB Prospective Complications Study. Submitted.

Abstracts

- Altorf-van der Kuil W, Engberink MF, Witteman JC, et al. Dietary Protein and Risk of Hypertension in a General Older Population: The Rotterdam Study. HYPERTENSION 54(4) E59-E59; 2009.
- Altorf–van der Kuil W, Engberink MF, Vedder MM, Boer JMA, van 't Veer P, Verschuren WMM, Geleijnse JM. Dietary Protein and Blood Pressure in a General Dutch Population. Joint Conference - 50th Cardiovascular Disease Epidemiology and Prevention– and – Nutrition, Physical Activity and Metabolism San Francisco, USA 2010.

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- Altorf–van der Kuil W, Engberink MF, Vedder MM, Boer JMA, van 't Veer P, Verschuren WMM, Geleijnse JM. Dietary Protein and Blood Pressure in a General Dutch Population. WEON Nijmegen, Netherlands 2010.
- De Neve M, Altorf-van der Kuil W, Engberink MF, et al. Amino Acids and Incidence of Hypertension in a Dutch older population: The Rotterdam Study. Journal of Epidemiology and Community Health65(S1) A222-A222 2011.
- Altorf-van der Kuil W, De Neve M, Engberink MF, van Rooij FJA, Hofman A, Witteman JCM, Geleijnse JM. Amino Acids and Incidence of Hypertension: the Rotterdam Study. 11th European Nutrition Conference (FENS); Madrid, Spain 2011.

Educational Program

Discipline specific activities

Courses

- Erasmus Summer School: 'Regression analysis', Netherlands Institute for Health Sciences (NIHES), Rotterdam (NL) 2008.
- Erasmus Summer School: 'Survival analysis', Netherlands Institute for Health Sciences (NIHES), Rotterdam (NL) 2008.
- Erasmus Winter School: 'Basic principles of epidemiologic data analysis', Netherlands Institute for Health Sciences (NIHES), Rotterdam (NL) 2010.
- Masterclass 'Multilevel analysis', Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences), Wageningen (NL) 2011.

Meetings

- Annual meetings of the Netherlands Epidemiology Society (WEON), Groningen (NL) 2008, Nijmegen (NL) 2010.
- Annual meetings of NWO Nutrition, Deurne (NL) 2008, 2009, 2011.
- Symposium 'Dairy and blood pressure', NZO, Ede (NL) 2008.
- 49th Annual Conference on Cardiovascular Disease Epidemiology and Prevention, American Heart Association, Palm-Harbour, Florida (USA) 2009.
- 63rd Annual High Blood Pressure Research Conference, American Heart Association, Chicago, Illinois (USA) 2009.
- 20th Scientific Meeting of the European Society of Hypertension, Oslo (NO) 2010.
- 19th Annual World Congress of Epidemiology, International Epidemiology Association, Edinburgh (UK) 2011.

General courses

- VLAG PhD-week, Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences), Bergeijk (NL) 2008.
- 'Organising and supervising an MSc thesis', Education Institute (OWI), Wageningen (NL) 2009.
- 'Presentations', TIFN, Wageningen (NL) 2008.
- 'Writing and presenting a scientific paper', Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences), Wageningen (NL) 2009.
- NWO talent day, NWO, Den Haag (NL) 2010.
- Clinic 'coping with conflicts of interest', VU, Amsterdam (NL) 2011.

Optional courses and activities

- Preparing a PhD research proposal.
- Symposium 'sustainable nutrition', young NAV, Utrecht (NL) 2008.
- Participation in research meetings, TIFN.
- Participation in literature group: 'oldsmobiles', Wageningen (NL), 2009-2012.
- Participation in discussion group, 'Concepts and Methods of Epidemiology', Wageningen (NL), 2010-2011.
- Organisation of and participation in 'Human Nutrition Research Update' (research meeting of the division of Human Nutrition), Wageningen (NL), 2009.

COLOPHON

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