Nutritional Interventions and Blood Pressure

Role of specific micronutrients and other food components

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Thesis

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"Es ist nicht genug zu wissen, man muß auch anwenden.
Es ist nicht genug zu wollen, man muß auch tun."

"Knowing is not enough, we must apply.
Willing is not enough, we must do."

(Johann Wolfgang von Goethe)

Abstract

Background

Elevated blood pressure is an important risk factor for cardiovascular diseases (CVD). Modest reductions in blood pressure at the population level, as can be achieved by dietary and lifestyle changes, have a large impact on the burden of CVD. Blood pressure is regulated by several physiological mechanisms, including vascular endothelial function.

This thesis

The studies described in this thesis examined the potential effects of various micronutrients and other food components on blood pressure and endothelial function. The first aim was to assess the importance of selected minerals on population blood pressure levels and the second was to investigate the vascular effects of food components other than minerals that have recently attracted attention in the field of nutrition and blood pressure.

A review of 21 national surveys showed that current dietary potassium intakes are suboptimal (1.7-3.7 gram per day). We estimated that increasing intakes to the recommended level of 4.7 gram per day can reduce population systolic blood pressure by 2-3 mmHg in Western countries, this effect being similar to that which can be achieved by reducing current sodium intakes to recommended levels. Our meta-analysis of 40 randomized controlled intervention studies showed that increasing calcium intake by ~1200 mg per day significantly lowers systolic blood pressure by 2 mmHg and diastolic blood pressure by 1 mmHg. This effect tended to be stronger in populations with lower intakes (<800 mg per day). In an 8-week placebo controlled parallel study in 124 subjects with elevated blood pressure, we found no significant blood pressure lowering effects of skimmed milk enriched in potassium (1500 or 750 mg per daily serving) combined with calcium, magnesium, selenium, vitamin C and vitamin E. However, this study was not designed to detect reductions in systolic blood pressure of 2-3 mmHg, which are still relevant at the population level.

In two 4-week placebo controlled cross-over studies, in 162 subjects with untreated elevated blood pressure, we could not demonstrate an antihypertensive effect of a yogurt drink with lactotripeptides obtained by enzymatic hydrolysis (study 1: 10.2 mg per day; study 2: 4.6 mg per day plus 350 mg added potassium). In another 2-week placebo controlled cross-over study in 35 healthy males we found no consistent effect on

endothelial function and blood pressure of ~800 mg polyphenols per day from either a wine-grape mix or grape seeds. Finally, a meta-analysis of 14 randomized controlled intervention studies showed that folic acid at a high dose (≥5000 μg per day), which can not be attained with a regular diet, significantly improved flow-mediated dilation by 8%.

Conclusion

Adequate potassium and calcium intakes can play an important role in the prevention of hypertension at the population level. Lactotripeptides have no relevant effect on blood pressure in Caucasian populations. The potential of grape polyphenols and folic acid to improve endothelial function is limited.

Multiple actions are needed to lower blood pressure at the population level and reduce the burden of CVD. To improve intakes of potassium and calcium, public health measures should re-emphasize the intake of fruits, vegetables, and low-fat dairy products. Further optimization of mineral intakes, in particular reduction of sodium intake, requires collaborative actions of government and food industry.

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Chapter 1

Introduction

Blood pressure and cardiovascular diseases

Cardiovascular diseases (CVD), including heart disease and stroke, form the world's leading cause of morbidity and mortality. The major risk factors for CVD are age, gender, hypertension, smoking, dyslipidemia and diabetes.[1] Of the single modifiable risk factors, elevated blood pressure poses a greater risk for CVD than elevated serum cholesterol or smoking.[2] Nowadays, systolic blood pressure is considered a more powerful predictor of CVD than diastolic blood pressure, especially after the age of 50 years.[3] Hypertension is also a major risk factor for renal disease.[4]

CVD risk increases linearly with increasing blood pressure, starting from levels as low as 115/75 mmHg.[5] Modest reductions in average population blood pressure level can result in substantial reductions in CVD risk, i.e. a 2 mmHg lower systolic blood pressure on population level is estimated to lower mortality from stroke by ~10% and mortality from ischemic heart disease and other vascular causes by ~7%.[5] It has been estimated that up to 80% of CVD may be preventable by lifestyle changes, including a healthy diet, physical exercise and abstinence from smoking.[6, 7]

This thesis has two general aims, namely 1) to assess the importance of a selected number of minerals on population blood pressure levels, and 2) to study the effect of "emerging" food components on blood pressure and endothelial function.

Elevated blood pressure is an important risk factor for CVD. There is a graded increase in risk of CVD across the entire blood pressure range. At the population level small changes in blood pressure on the population level have a major impact on CVD incidence.

Prevention Strategies

The majority of people with moderately increased risk levels contribute to more cases of disease and mortality than the relatively small number of persons with extremely high risk levels. In 1981 Geoffrey Rose explained that interventions targeting the general population, aimed at shifting the risk curve for major determinants of disease, such as blood pressure, to the left (the population-based approach), may be more effective to reduce the population burden of disease than interventions targeting high-risk individuals (high-risk approach) (*Figure 1*).[8] However, Rose also noted that a population-based strategy only, can much improve overall public health, but has less benefit for the individual with very high risk. Conversely, focus on individuals with high-risk only brings

benefit to an individual, but may have a small impact in the population. This phenomenon was called the "Prevention Paradox"[8] and explains why it is generally recommended that the high-risk approach and the population-based approach should be complementary, which is also supported by recent analyses.[9, 10]

In addition, it has been observed that subgroups that were formerly at lower exposure to risk benefit more from population-based interventions than those who were formerly at greater exposure to risk. Thus, for more substantial reductions of CVD burden, population-based interventions should ideally also alter the underlying risk behaviors and socioeconomic causes of elevated blood pressure levels.[10, 11]

Worldwide just over half the burden of disease attributable to high blood pressure occurs in people with mean systolic blood pressure levels less than 145 mmHg.[12] Current guidelines for the prevention of hypertension recommend a combination of a population-based strategy and an intensive targeted strategy focused on individuals with high risk for hypertension.[13, 14] Lifestyle changes at individual level can help prevent the onset of hypertension, contribute to control other CVD risk factors, and reduce the number and doses of medication in those treated.[4] Population-wide improvements of lifestyle factors could play a crucial role in preventing a rise in population blood pressure levels.

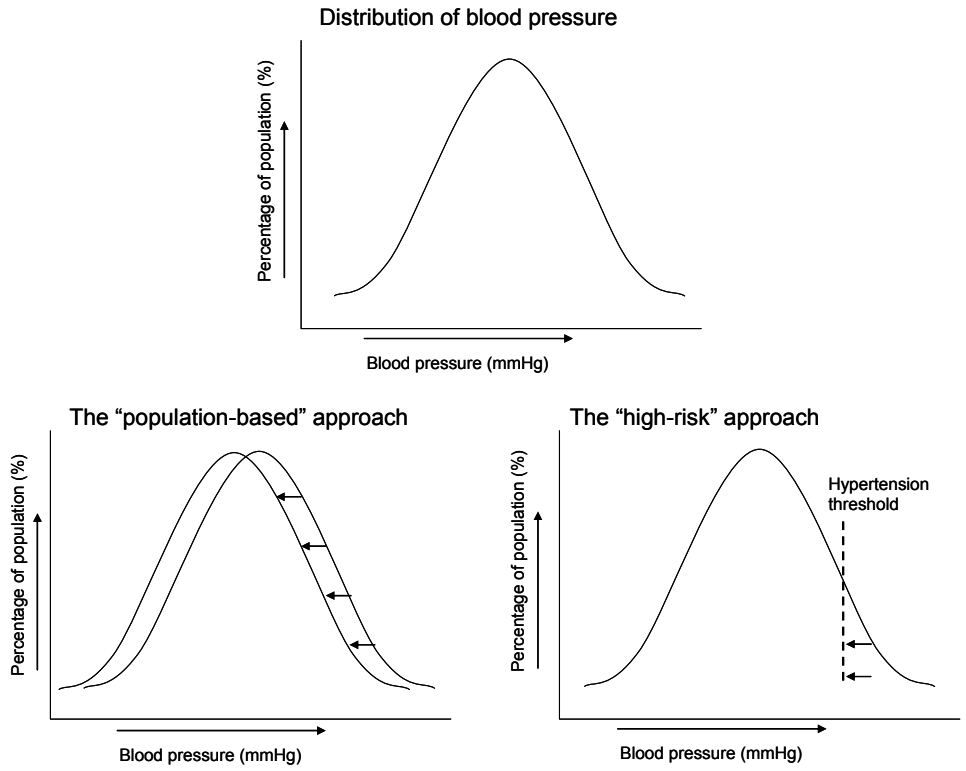

Figure 1 *The "population-based" and "high-risk" strategy for the prevention of CVD illustrated for blood pressure as risk factor for CVD.*[15]

The risk of CVD can be reduced by two complementary strategies; the population-based approach (interventions aimed at reducing the risk factor across the whole population) and the high-risk approach (interventions aimed at reducing risk factors in high-risk individuals). Lifestyle interventions are important both at the individual level and the population level.

Population blood pressure levels

Blood pressure data from the Netherlands show that only 28% of the population (≥12 years) has blood pressure levels classified as optimal (<120/80 mmHg), 35% has normal or high-normal blood pressure levels (120-139/80-89 mmHg) and 37% has blood pressure levels classified as hypertensive (≥140/90 mmHg)[16], as defined in *Table 1*. In Europe, 20-25% of the population has optimal blood pressure levels[17-21], and an average of 44% is classified having hypertension, as defined in *Table 1*.[22] It should be noted that these figures are probably an overestimation of the prevalence of hypertension, as in most population-based surveys blood pressure levels are based on single measurements.[23] In general, blood pressure levels are somewhat higher in men compared to women and tend to increase with age (*Table 2).*[22]

Table 1 *Definitions and classification of blood pressure levels (mmHg).*[1]

Category[2]	Systolic		Diastolic
Optimal	<120	and	<80
Normal	120-129	and/or	80-84
High-normal	130-139	and/or	85-89
Hypertension	≥140	and/or	≥90

[1] According to European Society of Hypertension/European Society of Cardiology 2007 guidelines
[2] In case of use of anti-hypertensive medication classified as hypertension.

Table 2 *Blood pressure distribution (%) in the Netherlands (by gender and 10-year age category) based on the REGENBOOG[1] project (1998-2001).*[16]

		10 years age category (years)						
	Category	<19	20-29	30-39	40-49	50-59	60-69	≥70
Men								
	Optimal	63	30	24	17	11	8	3
	Normal	20	31	30	25	17	12	5
	High-normal	11	23	27	24	20	14	10
	Hypertension	4	15	18	34	52	66	82
Women								
	Optimal	80	58	57	39	22	11	2
	Normal	13	23	19	20	19	16	9
	High-normal	3	11	12	14	13	14	11
	Hypertension	1	8	11	27	45	60	78

[1] REGENBOOG is the acronym for "Risicofactoren En GezondheidsEvaluatie Nederlandse Bevolking een Onderzoek Op GGD-en".

The majority of people (70-80%) in Western countries have above optimal blood pressure levels.

Diet and blood pressure

A variety of diet and lifestyle modifications have been established to lower blood pressure and reduce the incidence of hypertension.[4] These include increased physical activity, weight loss, moderation of alcohol intake, increased dietary potassium intake and reduced dietary sodium intake.[4] Other dietary factors for which effects on blood pressure have been suggested by intervention studies are calcium[24], magnesium[25], fibre[26], vitamin C[27], and fish oil.[28] Regarding macronutrients, the Optimal Macronutrient Intake Trial to Prevent Heart Disease (Omni-Heart) study, showed that replacing part of the carbohydrates with either protein or monounsaturated fat lowers blood pressure.[29] For most interventions, data show that the absolute blood pressure lowering effect is more pronounced in people with higher pre-treatment blood pressure levels.[26, 28, 30, 31]

The Dietary Approaches to Stop Hypertension (DASH) study was a landmark intervention study investigating the effects of dietary patterns, rather than individual nutrients, on blood pressure. After 8 weeks of intervention, substantial reductions in blood pressure were observed among participants assigned to the DASH (combination)

diet, a diet rich in fruit, vegetables, and low-fat dairy products and reduced in saturated fat, total fat and cholesterol, compared to those assigned to the control diet, representing a typical American diet.[32] In addition, as part of the same study a diet rich in fruit and vegetables, and reduced in snacks and sweet, also significantly reduced blood pressure compared to the control diet. This effect was about half the size of that found for the DASH diet.[32] Compared to the typical American diet, the DASH diet also contained substantially higher amounts of the minerals potassium (4415 versus 1752 mg), calcium (1265 versus 443 mg) and magnesium (480 versus 176 mg), as well as some other nutrients, such as vitamins and fiber.[32] The DASH study was not designed to identify the contribution of single nutrients to the observed blood pressure effect. Nevertheless, it is plausible that the minerals potassium, calcium and magnesium were at least partly responsible for blood pressure reduction in this study. Reducing sodium intake in combination with the DASH diet was found to be even more effective.[33] Blood pressure lowering effects of the DASH diet were found in various subgroups of participants, based on race, gender, age, family income, physical activity, and hypertension status.[30] The DASH study showed that blood pressure reductions similar to drug treatment can be achieved with dietary intervention.[30, 34] Interventions aimed at adoption of the DASH diet in free-living populations showed that dietary instructions are effective in increasing adherence to this diet[35-37], especially when the dietary advice is adjusted to fit national food preferences and portion sizes.[35] However, compliance to the original DASH diet in free-living populations is difficult to attain.[36] Greater emphasis on foods that contain high levels of the desired nutrients per calorie, would likely help to reach the DASH intake goals.[36] In this context, it is relevant to investigate which particular nutrients or combination of nutrients are primarily responsible for the blood pressure lowering effects observed with the DASH diet.

Several lifestyle factors, such as weight loss, increased physical activity, moderation of alcohol intake, increased dietary potassium intake and reduced dietary sodium intake lower blood pressure levels. The DASH (combination) diet, a diet rich in fruits, vegetables, and low-fat dairy products and reduced in saturated fat, total fat and cholesterol, is an integrated dietary approach with established blood pressure lowering effects.

Blood pressure and endothelial function

Blood pressure is regulated by various physiological mechanisms[38], which involve endothelial function, water-mineral balance regulated by the kidneys, and autonomic regulation by the heart and brain.[38] Endothelial cells are involved in vasoconstriction and vasodilation, blood clotting, atherosclerosis and inflammation.[39, 40] A large body of evidence supports the fundamental role of nitric oxide as the main endothelium-derived relaxing factor.[41-43]

Endothelial function can be measured by flow-mediated dilation, and reduced values are considered an early marker for endothelial dysfunction. Flow-mediated dilation represents the ability of the brachial artery to dilate in response to ischemia-induced hyperemia in the forearm and as such it reflects the bioavailability of the vasodilator nitric oxide.[44] The application of flow-mediated dilation measurement has grown rapidly over the past decade.

Endothelial function may predict prognosis of disease progression and cardiovascular events in patients with CVD[45], but evidence supporting that endothelial function predicts CVD in the general population, independently of other CVD risk factors, is still limited.[46-53] Endothelium-dependent vasodilatation is sometimes portrayed as a causal mechanism in the development of hypertension, but endothelial dysfunction is also commonly seen together with other CVD risk factors, and thus not specific for elevated blood pressure levels.[54, 55] The relevance of endothelial dysfunction in hypertension needs to be further supported by prospective studies documenting that interventions improving endothelial dysfunction also translate into lower blood pressure levels and a lower risk of developing hypertension.

Blood pressure regulation is dependent on several underlying physiological functions, including endothelial function. Flow-mediated dilation is a technique that measures endothelial function. There is growing evidence that endothelial (dys)function is a marker for cardiovascular risk.

Minerals

Sodium

A large number of studies provide strong evidence for a causal link between high sodium intake, hypertension[56-58] and CVD.[59, 60] A reduction in salt intake of 4-6 gram per day is

estimated to reduce systolic blood pressure by 2-4 mmHg in the general population[56, 57], with a more pronounced effect in hypertensives compared to normotensives.[57] Sodium intakes are high in most populations[61] and the need to reduce sodium intake for decreasing CVD risk is currently being emphasized[58] and translated into public health measures.[62, 63]

Potassium

Potassium has received less attention than sodium. Nevertheless, there is a substantial body of data from both epidemiological and clinical studies indicating that an increase in potassium intake of 2-3 grams per day reduces systolic blood pressure by 3-4 mmHg[31, 64-66] and is also associated with a decreased risk of CVD.[67] Analyses of different subgroups indicate a more pronounced blood pressure effect of potassium in hypertensives compared to normotensives, in non-Caucasians compared to Caucasians and at higher urinary sodium excretion compared to lower sodium excretion levels.[66] Several authoritative bodies acknowledge that increased potassium intake is beneficial to health. The adequate intake of potassium for adolescents and adults in the United States and Canada was set at 4.7 gram per day, based on a review by the Institute of Medicine.[68] An adequate potassium intake could blunt the age-related rise in blood pressure, reduce the adverse effects of high sodium chloride intake on blood pressure, reduce the risk of recurrent kidney stones, and possibly decrease bone loss.[68]

Potassium is abundantly available in diets based on natural foods. However, current diets based on processed foods are generally high in sodium and low in potassium.[69] In the United States and United Kingdom reports of national survey data have clearly indicated that dietary intakes of potassium are low and of concern for public health[70, 71], especially in combination with current high sodium intakes. Several reports have published potassium intake data[72-75], but no recent report has addressed worldwide potassium intakes in populations and/or estimated the impact of potassium intakes on population blood pressure levels.

Sodium to potassium ratio

Several studies indicate that potassium and sodium jointly play an important role in regulating blood pressure.[69, 76] In epidemiological studies, the sodium to potassium intake ratio is more strongly linked to blood pressure and CVD risk than sodium alone.[56, 77, 78] Data from the International Study of Salt and Blood Pressure (INTERSALT) showed that the relation of the urinary sodium to potassium ratio to blood pressure followed a pattern similar to that for sodium, but more strongly and consistently.[78] Recent data from

the Trials of Hypertension Prevention (TOHP) 10-15-year follow up showed that a greater sodium to potassium excretion ratio (mean of multiple 24-hour urinary excretions) was associated with increased risk of subsequent CVD, and this effect was stronger than that of sodium or potassium alone.[67] Several intervention studies found that replacing regular sodium salt with potassium-enriched salt reduces blood pressure and lowers CVD event rates.[79-82] In addition, in societies in which sodium intake is low and potassium intake is high, blood pressure does not rise with age, and the incidence of CVD is generally low.[69]

Calcium

Several meta-analyses of randomized controlled studies on calcium supplementation and blood pressure have been published. Allender *et al.* found a significant reduction in systolic blood pressure of 0.9 mmHg and a non-significant reduction in diastolic blood pressure of 0.2 mmHg in a meta-analysis of 26 randomized controlled intervention studies.[83] Bucher *et al.* found a significant BP reduction of 1.3/0.2 mmHg when pooling 33 calcium intervention studies.[84] Griffith *et al.,* in 1999, included a total of 42 studies, with calcium doses in the range of 500-2000 mg per day.[24] The primary aim of this meta-analysis was to increase precision of the effect estimate and compare the effect of dietary calcium with supplemental calcium. Pooled estimates were significant and larger than in previous meta-analyses, i.e. -1.4 mmHg for systolic blood pressure and –0.8 mmHg for diastolic blood pressure.[24] Food-based calcium supplementation had a larger effect than use of calcium supplements, but this difference was not significant. A Cochrane meta-analysis only included 13 studies with a minimum duration of 8 weeks, whereas the other meta-analyses also included short-term studies (minimum duration of 2 weeks).[85] This meta-analysis found a significant reduction in systolic blood pressure of 2.5 mmHg, while diastolic blood pressure was not significantly affected.[85]

Based on findings from these meta-analyses, the overall effect of calcium on blood pressure in the population as a whole is probably small, but effects in population subgroups may be larger. Substantial heterogeneity was found in all meta-analyses, which could reflect differences in effect among specific patient populations, type of interventions, or methodological aspects of the studies. Most meta-analyses planned subgroup analyses, but due to varying amounts of data available only a few sources of heterogeneity could be addressed.[24, 84, 85] For example, the hypothesis that a blood pressure effect of calcium is stronger with low habitual intakes[24, 84, 86] was not addressed in these meta-analyses.[24, 84]

Combination of minerals

Only a few intervention studies addressed the effect of combinations of the minerals potassium, calcium and magnesium on blood pressure, without influencing sodium intake. Sacks *et al.* performed 2 randomized controlled studies in a large number of participants testing the effect of combinations of these minerals on blood pressure. In the first study 125 subjects with untreated mild or borderline hypertension received either potassium (2346 mg per day) and calcium (1000 mg per day), potassium and magnesium (360 mg per day), calcium and magnesium, or placebo for 6 months.[87] In this study the changes in blood pressure between the treatment and placebo groups were not significant. In the second study, the effect of potassium (1564 mg per day), calcium (1200 mg per day), magnesium (336 mg per day) or a combination of these minerals on blood pressure during 16 weeks was examined in 321 normotensive women with low habitual intakes.[88] Compared to placebo the mean differences in the intervention group were only significant for potassium, with reductions of 2.0 mmHg in systolic blood pressure and 1.7 mmHg in diastolic blood pressure. The administration of calcium and magnesium with potassium did not enhance the effect of potassium alone.[88]

The effect of calcium, magnesium, and potassium in a dairy matrix was investigated in two smaller placebo controlled intervention studies. In the first 6-week intervention study, normal milk treatment (potassium 1650 mg per day, calcium 1180 mg per day, magnesium 110 mg per day) compared to mineral-poor milk treatment (potassium 580 mg per day, calcium 95 mg per day, magnesium 10 mg per day) reduced systolic blood pressure by 2.7 mmHg, but not diastolic blood pressure in 53 normotensive female students on a low calcium diet (<500 mg per day).[89] In the second intervention study, the effect of 4 weeks consumption of a potassium-enriched high-calcium skim milk (1585 mg potassium, 1040 mg calcium, and 71 mg magnesium per day) and a high-calcium skim milk (855 mg potassium, 1075 mg calcium, and 74 mg magnesium per day) on blood pressure was compared to a non-enriched skim milk (885 mg potassium, 720 mg calcium, 64 mg magnesium per day). In this study in 38 participants, systolic blood pressure, but not diastolic blood pressure, decreased significantly in the potassium-enriched high-calcium milk group compared to placebo (8 mmHg). Blood pressure on the high-calcium skim milk diet was non-significantly lower than on placebo.[90]

To summarize, a limited number of studies have examined the effect of combined mineral intake on blood pressure.[91] Most of these studies hypothesized that simultaneous intake of potassium, calcium and magnesium could lower blood pressure more than intervention with individual minerals. This was not confirmed by two large studies

investigating the effect of such a combination of minerals as supplements. The results of two smaller studies addressing the effect of combinations of these minerals in a dairy matrix suggest a possible blood pressure lowering effect of these minerals. However, additional data from larger intervention studies are needed to confirm this. The effects of potassium, calcium, magnesium on blood pressure have not been investigated in combination with other nutrients, such as vitamins.

Meta-analyses of intervention studies showed that increased potassium intake lowers blood pressure. The impact of current potassium intakes on population blood pressure levels has not yet been addressed. A small blood pressure lowering effect of calcium has been found in meta-analyses of intervention studies, but effects in specific population subgroups are largely unknown. Few studies examined effects on blood pressure of combined intake of potassium, calcium and magnesium.

Other food components

Many other food components have been investigated in relation to blood pressure and endothelial function. In recent years an increasing number of studies have addressed the possible beneficial effects of various types of milk-derived bioactive peptides (mainly lactotripeptides), polyphenols (mainly flavonoids), and B-vitamins, in particular folic acid.

Lactotripeptides
Blood pressure lowering effects of lactotripeptides, specifically Isoleucine-Proline-Proline (IPP) and Valine-Proline-Proline (VPP) have been reported in a considerable number of human intervention studies.[92-100] Two meta-analyses have summarized the studies published in this field between 1996 and 2005.[101, 102] One included intervention studies on lactotripeptides, but also other bioactive peptides derived from food proteins.[101] The other meta-analysis included lactotripeptides intervention studies only.[102] Both meta-analyses reported significant reductions in systolic blood pressure (5 mmHg) and diastolic blood pressure (2 mmHg). Most trials were relatively small studies in Japanese populations.[92-96] Some Finnish studies also showed blood pressure reductions during treatment with lactotripeptides, but the effects were smaller than in Japanese trials.[97-100] Daily doses of lactotripeptides ranged between 3-5 mg in most of these intervention studies.[103] As reviewed by Geleijnse and Engberink, a number of methodological aspects in these intervention studies warrant further consideration.[103] In some studies placebo

effects were not properly accounted for, and in other studies pretreatment blood pressure tended to be higher in the intervention group than in the control group.[103]

Since the publication of these meta-analyses[101, 102] additional randomized controlled studies in other European populations have been reported. These studies found no significant effects of lactotripeptides on blood pressure.[103-108] Recent publications reviewing data in this field conclude that current evidence for an effect of these lactotripeptides on blood pressure is not compelling, and that confirmation of blood pressure lowering effects in additional large scale well-designed intervention studies is needed.[103, 109]

Grape polyphenols

Polyphenols, and especially the subclass of flavonoids, have been examined in many studies for their relation with CVD and vascular outcomes, including blood pressure and endothelial function.[110] Flavonoids occur naturally in plant-based foods and are abundantly present in fruits, vegetables, grains, herbs, and beverages. The strength of the evidence from randomized controlled studies for effects of flavonoids on blood pressure and the CVD risk factors varies among the different flavonoids subclasses.[111]

Several studies indicated that red wine and grape extract consumption might lower CVD risk markers, and in particular endothelial function markers[111], but this evidence is largely based on in vitro findings showing effect on nitric oxide metabolism.[112] In addition, a number of intervention studies investigated the effect of wine on endothelial function. Most of these studies focused on acute rather than the chronic effects of red wine consumption.[112] The studies had conflicting results and it is not clear whether the grape polyphenols, ethanol, or other compounds in red wine are responsible for the possible benefits.[112]

A limited number of small intervention studies addressed the chronic effects (2-8 weeks) of polyphenolic compounds from grape on endothelial function and/or blood pressure.[113-116] Two studies investigated the effect of purple grape juice (~832-1664 mg polyphenols per day[117]) on flow-mediated dilation in patients with coronary artery disease.[113, 114] In these studies improvements in flow-mediated dilation were found, but no control groups were included.[113, 114] Two other small studies were performed in healthy volunteers. Zilkens *et al.*[115] showed that daily consumption of 375 ml of red wine, but not daily consumption of 375 ml of de-alcoholized red wine, increased blood pressure levels compared to abstention from alcohol. No effects were on flow-mediated dilation in this study. Park *et al*.[116] showed that consumption of purple grape juice (885 mg

polyphenols per day) reduced both systolic (3.7 mmHg) and diastolic (3.0 mmHg) blood pressure compared to placebo intervention.

Since evidence from well-controlled intervention studies is still very limited, future placebo-controlled studies should address the effect of the polyphenolic part of grape polyphenols on endothelial function and blood pressure.

Folic acid

Several randomized controlled interventions studies have addressed the possible beneficial effects of folic acid (vitamin B_{11}) on endothelial function and blood pressure.[118] A recent meta-analysis included 12 randomized controlled studies published between 1999 and 2007 in which folic acid was provided at a minimum dose of 5000 µg/d for at least 2 weeks (duration ranged from 2-12 weeks). In 4 of these studies effects on blood pressure as well as endothelial function were addressed, 4 other studies addressed effects on blood pressure only and the remaining 4 studies addressed the effects on endothelial function only. The results of this analysis showed a borderline significant reduction in systolic blood pressure of 2 mmHg, no effect on diastolic blood pressure, and a 1.61% increase in flow-mediated dilation change after supplementation with folic acid.[118] The effect of folic acid on endothelial function over a wider dose range, including lower doses, has not been systematically reviewed.

A number of intervention studies in Japanese and Finnish populations show blood pressure lowering effects of lactotripeptides. Data suggest that grape polyphenols could improve endothelial function, but evidence from randomized controlled intervention studies is still limited. Folic acid at high doses may have beneficial effects on endothelial function and blood pressure; possible effects at lower dose levels have not been systematically reviewed.

Rationale and aim of this thesis

Blood pressure levels show a graded independent relationship with risk of cardiovascular diseases (CVD). The majority of people in Western populations have above optimal blood pressure levels. Therefore, small changes in population blood pressure levels can have a substantial impact on CVD incidence. For related vascular function markers, including endothelial function, it is less clear whether they can independently predict CVD.

Minerals have been investigated in many studies for their effects on blood pressure. Evidence for the impact of current sodium intakes in populations on blood pressure and CVD has clearly been established, which has resulted in several public health measures to reduce salt intake. A vast amount of data from meta-analyses of intervention studies showed that potassium supplementation significantly lowers blood pressure. The potential impact of current potassium intake on population blood pressure, however, has not been systematically assessed. For calcium, meta-analyses of intervention studies showed a small, but consistent blood pressure lowering effect. Whether calcium supplementation could be more effective in specific population subgroups has not yet been established. Many blood pressure trials focused on the effect of individual minerals (i.e., potassium, calcium and magnesium), but only a few studies addressed the effect of combined mineral intake on blood pressure. Two studies suggest that a combination of these minerals in their natural dairy matrix could reduce blood pressure. The effect of combinations of minerals and vitamins in a dairy matrix has not yet been investigated.

In more recent years, several other food components have been studied for their possible effects on blood pressure, including lactotripeptides, polyphenols and folic acid. Intervention studies, mainly in Japanese and Finnish populations showed blood pressure lowering effects of lactotripeptides. However, most of these studies were relatively small and several had methodological shortcomings. Additional large scale well-designed intervention studies are needed to conclude whether lactotripeptides could lower blood pressure. Promising effects of grape polyphenols on blood pressure and endothelial function have been suggested, but evidence is largely based on in-vitro experiments and studies investigating acute effects of red wine on endothelial function. Placebo-controlled intervention studies addressing the chronic effect of grape polyphenols on endothelial function and blood pressure are lacking. The effect of folic acid on endothelial function and blood pressure has been addressed in several intervention studies. A recent meta-analysis found indications for reductions in endothelial function and blood pressure after high-dose supplementation with folic acid. The effects of folic acid over a wider dose

range, including levels that can be attained by diet, on endothelial function, have not been systematically reviewed.

This thesis had 2 general aims:

- To assess the importance of selected mineral intakes for population blood pressure levels
- To assess the effects of "emerging" food components on blood pressure and endothelial function

More specifically, the following research questions were addressed:

- What is the impact of increased potassium intake on population blood pressure? (**Chapter 2**)
- What is the effect of increased calcium intake on blood pressure, overall and in population subgroups? (**Chapter 3**)
- Does potassium combined with other micronutrients at low doses have beneficial effects on blood pressure? (**Chapter 4**)
- Do lactotripeptides improve blood pressure in a European population? (**Chapter 5**)
- Does the polyphenolic fraction of grape extracts improve endothelial function and blood pressure? (**Chapter 6**)
- Does folic acid improve endothelial function? (**Chapter 7**)

The outline of this thesis is depicted in *Figure 2*. In **Chapter 2** the potential impact of increased potassium intake on population blood pressure is described. The meta-analysis in **Chapter 3** focused on calcium supplementation and blood pressure, overall and in population subgroups. **Chapter 4** describes a randomized controlled blood pressure trial of low-dose supplementation of potassium combined with other minerals and vitamins.

Chapter 5 presents the results of two randomized controlled studies of lactotripeptides (IPP and VPP) and blood pressure in European populations. In **Chapter 6** the results of a randomized controlled intervention study of grape polyphenols and changes in endothelial function and blood pressure are presented. **Chapter 7** describes a meta-analysis of randomized controlled trials of folic acid supplementation and endothelial function. In **Chapter 8** the main findings are summarized and interpreted, followed by a discussion of methodological aspects of the studies, future research directions, and the relevance for public health.

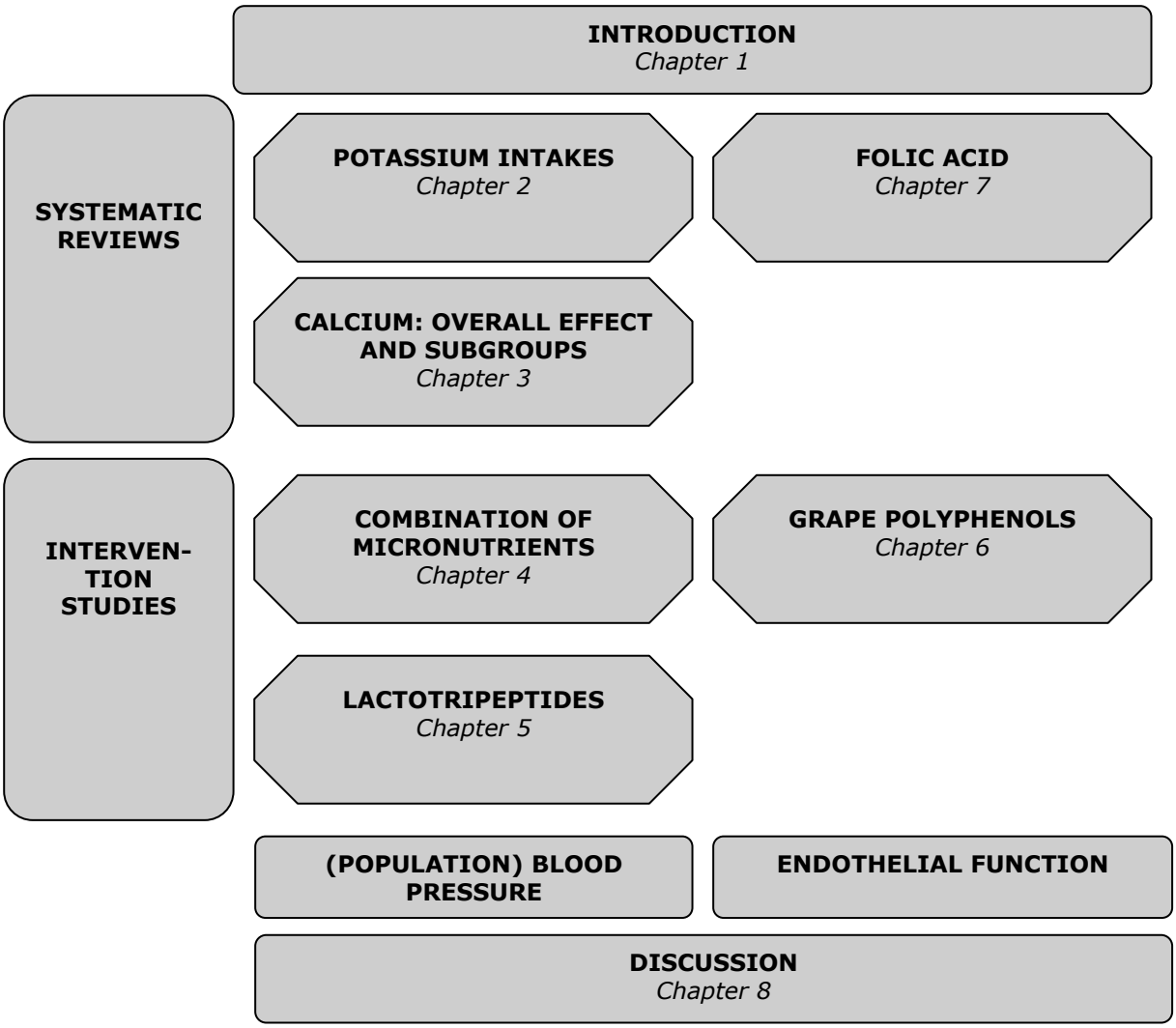

Figure 2 *Schematic overview of the studies described in this thesis.*

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Chapter 2

Suboptimal potassium intakes and potential impact on population blood pressure

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Archives of Internal Medicine; in press

Small reductions in blood pressure (BP) on a population level could have a substantial impact on cardiovascular disease risk.[1] This is especially relevant considering that the majority of the population has suboptimal BP levels. Dietary sodium reduction is a clearly established lifestyle change that has great potential to improve public health. Potassium, on the contrary, received much less attention. Nevertheless, a substantial body of data shows that increasing potassium intake lowers BP.[2] We reviewed population data on potassium intake and estimated the potential impact of increased potassium intake on population BP levels.

Methods

We searched PubMed and contacted health authorities worldwide for national population-based dietary surveys conducted from 1990 to 2009 that included data on potassium intake in more than 1,000 adults. We defined the recommended level of potassium intake at 4.7 gram per day (g/d), based on the Dietary Reference Intakes from the Institute of Medicine.[2] The effect of dietary potassium on systolic BP was set at 1.0 mmHg reduction per 0.6 g/d increase in intake, based on estimates from the INTERSALT study[3] and we assumed this relation to be linear. Population BP data were obtained for Finland, the United Kingdom and the United States, representing populations with relatively high, medium, and low potassium intakes.[4-7] For these countries we estimated the potential impact of increasing potassium intakes on population systolic BP levels and classification in different systolic BP categories, assuming a uniform shift in the population BP distribution, independent of initial BP level.

Results

In 21 countries spread across North America, Europe, Asia and Oceania, the mean potassium intakes ranged from 1.7 (China) to 3.7 g/d (Finland, the Netherlands, and Poland) (*Figure 1, references and data available at http://www.wageningenuniversity.nl/UK/newsagenda/news/*). Mean intakes in women were generally lower than in men. Based on our assumptions and intake data from Finland, United Kingdom and United States, a hypothetical increase in potassium intake to 4.7 g/d would shift the population systolic BP distributions to 1.7 to 3.2 mmHg lower levels in Western countries. This is in the same order of what can be predicted for a

reduction in sodium intake from 9 to 5 g/d. This theoretical increase in potassium intake in these countries would increase the percentage (%) of men and women in the optimal systolic BP category (<120 mmHg) by approximately 2 to 5% and 4 to 8%, respectively, and decrease the percentage of men and women with systolic BP levels in the higher range (≥140 mmHg) by approximately 2 to 5% and 4%, respectively.

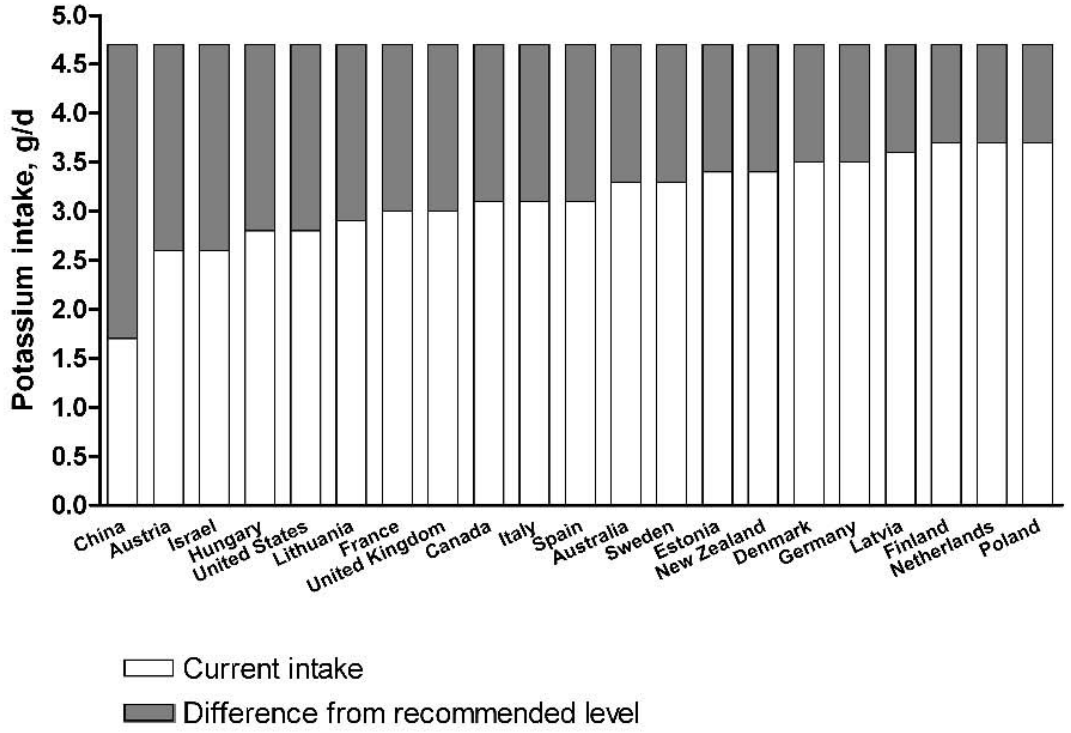

Figure 1 *Current potassium intakes (g/d) and differences from recommended level for the 21 countries included in our review.*

Comment

Increasing current potassium intakes in populations to recommended levels may lower population systolic BP in Western countries by 1.7 to 3.2 mmHg, which can be predicted to reduce the risk of stroke mortality by 8 to 15%, and the risk of heart disease mortality by 6 to 11%.[1] This is of similar magnitude as what can be achieved by lowering sodium intake, and highlights the importance of dietary strategies focussing on both reducing sodium intake and increasing potassium intake. There are various ways to improve intakes of minerals in the population. Adherence to dietary guidelines, with ample fruit

and vegetables, whole grains, and low-fat dairy products, should be promoted. Food companies can help by promoting the availability of healthier foods and also by improving the type and content of minerals in their products.

Acknowledgements

Additional Information: Detailed information on methods and additional results are available at: http://www.wageningenuniversity.nl/UK/newsagenda/news/
Author contributions: Dr Geleijnse had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: van Mierlo, Greyling, Zock, and Geleijnse. Acquisition of data: van Mierlo and Greyling. Analysis and interpretation of data: van Mierlo, Greyling, Zock, Kok, and Geleijnse. Drafting of the manuscript: van Mierlo and Greyling. Critical revision of the manuscript for important intellectual content: van Mierlo, Greyling, Zock, Kok, and Geleijnse. Statistical analysis: van Mierlo. Administrative, technical, and material support: van Mierlo and Greyling. Study supervision: van Mierlo, Zock, and Geleijnse.
Financial Disclosure: Ms van Mierlo and Mr Greyling and Dr Zock are employees of Unilever R&D, Vlaardingen, the Netherlands. Unilever markets foods, some of which are enriched with potassium.
Additional Contributions: Arne Jol, MSc (Unilever R&D, Vlaardingen, the Netherlands) provided expert statistical advice, and Petra Verhoef, PhD (Unilever R&D, Vlaardingen, the Netherlands) critically evaluated the manuscript.

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Supplemental Material

In this document we present additional information on methodology, secondary results and additional comments related to our publication on potassium intakes and population blood pressure levels.

Methods

Search strategy

To estimate dietary potassium intakes we searched for published national survey data on websites of national health authorities and performed a literature search in PubMed. We also contacted researchers to ask for relevant national data through their respective networks and used citation lists from retrieved publications to identify other eligible studies.

The initial search delivered 971 publications and reports. Abstracts were screened to determine eligibility based on the following criteria 1) Representative national survey or observational survey, 2) Sample size > 1,000 subjects, 3) conducted in the time period 1990 – September 2009, 4) Adults (>19 years), and 5) potassium intake estimated from 24 hour urine collections or from dietary questionnaires. This provided us with 83 potential surveys. The full papers were screened by AG and LAJM and 63 surveys were excluded for the following reasons, 1) No mean/median potassium intake reported (for total population or per gender), 2) Studied sample not representative for general population, 3) Surveys estimating potassium intake from food frequency questionnaire (FFQ) or from random ("spot") or overnight urine samples alone, 4) Data included in prior reports (in case of duplicate publication most recent data were used). This left us with 20 studies (representing 21 countries) to be included in this review.

Recommended potassium intake

In human intervention trials on potassium and blood pressure (BP), the average supplementary dose given in addition to intake from the diet was 2-3 gram per day (g/d).[1-4] The average pre-treatment intake from the diet based on urinary potassium excretion in meta-analyses of these randomized controlled trials was approximately 2.4 g/d.[1, 2] Thus the total potassium intake in the treatment groups was approximately 5 g/d.[1, 2] In the Dietary Approaches to Stop Hypertension (DASH) study the target for potassium intake in the intervention group was similar, i.e. 4.7 g/d.[5] In the United States

and Canada the adequate intake for potassium for adolescents and adults was set at 4.7 g/d by the Institute of Medicine.[6] This daily amount was based on scientific evidence showing that this level blunts the age-related rise in BP, reduces the adverse effects of high sodium chloride intake on BP, reduces the risk of recurrent kidney stones, and possibly decreases bone loss.[6] The American Society of Hypertension recently published a position paper in which it also recommends a desirable level of potassium intake of 4.7 g/d.[7]

Estimating the effect of increasing potassium intake on population blood pressure

The design and statistical approach employed in the INTERSALT study allowed for an accurate estimation of the relationship between potassium and BP.[8] In this study of 32 countries, including over 10,000 people, a mean increase in potassium intake of 0.6 g (15 mmol) per day was associated with an estimated mean decrease in systolic BP of 1 mmHg. Assuming that the relation between potassium intake and BP is linear in the normal population ranges of intake, we used this estimate to predict the potential impact on population BP of optimizing potassium intake up to a level of 4.7 g/d.

We selected 3 Western countries (Finland - relatively high intake, United Kingdom - average intake, United States - relatively low intake) for which both potassium intake and BP data (mean ± SD) were available from the same surveys.[9-12] For these countries we estimated the potential impact of optimizing potassium intakes on population systolic BP levels and classification in systolic BP categories (optimal, normal/high-normal, hypertensive). We assumed a normal distribution to estimate the effect on BP distribution. Effects for men and women are reported separately, because BP distribution is different for men and women, and also because combined data were not reported for the United Kingdom and Finland. For the United States, mean population BP data and its distribution were calculated from NHANES 2005-2006 data.[9] We incorporated sample weights and the stratification and clustering of the design into the analysis of the NHANES data.[9]

Results

Overview of data from national surveys

Supplemental Table 1 presents characteristics of the dietary surveys from North America, Europe, Asia and Oceania selected for this review and corresponding data on potassium intakes. The majority of the studies used 24-h dietary recalls to estimate dietary

potassium intake, but multiple day food records and weighing methods were also employed. Sample size ranged from 1,179 (Hungary) to 53,394 (China) and response rates ranged from 27% in Hungary to 86% in Poland.[10-22]

The median value of the average potassium intakes in all countries was 3.2 g/d, and mean potassium intake in the countries ranged from 1.7 g in China to 3.7 g in Finland, the Netherlands and Poland (*Supplemental Table 1, Research Letter Figure 1*). Median potassium intake in women was 2.9 g/d and ranged from 1.6 g in China to 3.4 g in the Netherlands and Finland. Potassium intakes in men were higher. Median intake in men was 3.5 g/d and ranged from 1.8 g in China to 4.4 g in Poland.

Supplemental Table 1 *Characteristics of the national surveys reporting potassium intakes*

Country (survey year)	Population sample	Sample size (N)	Age (y)	Dietary assessment method	Intake in g/d (Mean ± SD)[$]		
					Men	Women	Total
Australia (1995-1996)[19]	Nationally representative sample, 1995 Australia National Nutrition Survey	10,851	≥19	24-h dietary recall (mainly)	3.7 (1.9)	2.8 (1.5)	3.3 (1.6)
Austria (1991-2008)[18]	Nationally representative sample, Austrian Study on Nutritional Status (ASNS).	2,123	19-64	24-h dietary recall	2.7 (1.1)	2.6 (1.0)	2.6 (1.0)
Canada (2004)[14]	Multistage stratified cluster, Canadian Community Health Survey, Cycle 2.2	18,820	≥19	24-h dietary recall	3.5 (3.0)	2.8 (2.3)	3.1 (2.7)
China (2002)[45]	Nationally representative sample, Chinese National Nutrition and Health Status survey 2002	53,394	18-70+	3 consecutive 24-h dietary recalls and weighing methods	1.8	1.6	1.7
Denmark (2000-2002)[17]	Nationally representative sample, Dietary habits in Denmark 2000-2002.	3,151	18-75	7-d food record	3.8 (1.1)	3.2 (0.9)	3.5 (1.0)
Estonia (1997)[21]	Nationally representative samples of each respective population, Baltic Nutrition and Health Surveys 1997	2,108	19-64	24-h dietary recall	3.8 (1.8)	3.1 (1.3)	3.4 (1.6)
Finland (2007)[10]	Random sample drawn from the population register, FINDIET 2007 survey	2,007	25-74	48-h dietary recall; every 2nd respondent 3-d food record.	4.1 (1.3)	3.4 (1.0)	3.7 (1.2)
France (2006-2007)[15]	Nationally representative sample, Afssa, INCA 2 study	2,624	18-79	7-d food record	3.3 (0.9)	2.7 (0.7)	3.0 (0.8)
Germany (2005-2006)[46]	Nationally representative sample, second German National Nutrition Survey (NVS II).	13,959	19-80	2x24-h dietary recalls and 2x4-d weighing protocol in sub-sample	3.8 (1.2)	3.3 (1.0)	3.5 (1.2)
Hungary (2003-2004)[18, 47]	Third Hungarian Nutrition Survey (NHS), national dietary survey of adult population	1,179	≥18	3-d dietary record	3.0 (0.9)	2.7 (1.1)	2.8 (1.0)
Israel (1999-2001)[48]	Random sample drawn from the national population registry (1992 subjects); Further random sample of 4393 subjects (neighbours of the first sample) MABAT First Israeli national health & nutrition survey	3,242	25-64	Questionnaires and 24-h dietary recall	2.9 (1.3)	2.3 (1.0)	2.6 (1.2)
Italy (1994-1996)[18, 64]	INN-CA study (Nationwide Nutritional Survey of Food Behaviour of the Italian population)	1461	19-64	7-d food record	3.4 (0.8)	2.9 (0.8)	3.1 (0.8)
Latvia (1997)[21]	Nationally representative samples of each respective population, Baltic Nutrition and Health Surveys 1997	2,308	19-64	24-h dietary recall	4.2 (2.2)	3.1 (1.6)	3.6 (2.0)
Lithuania (2007)[18]	National Nutrition survey 2007, national, random sample of inhabitants	1,936	19-64	24-h dietary recall	3.3 (1.4)	2.5 (1.0)	2.9 (1.3)

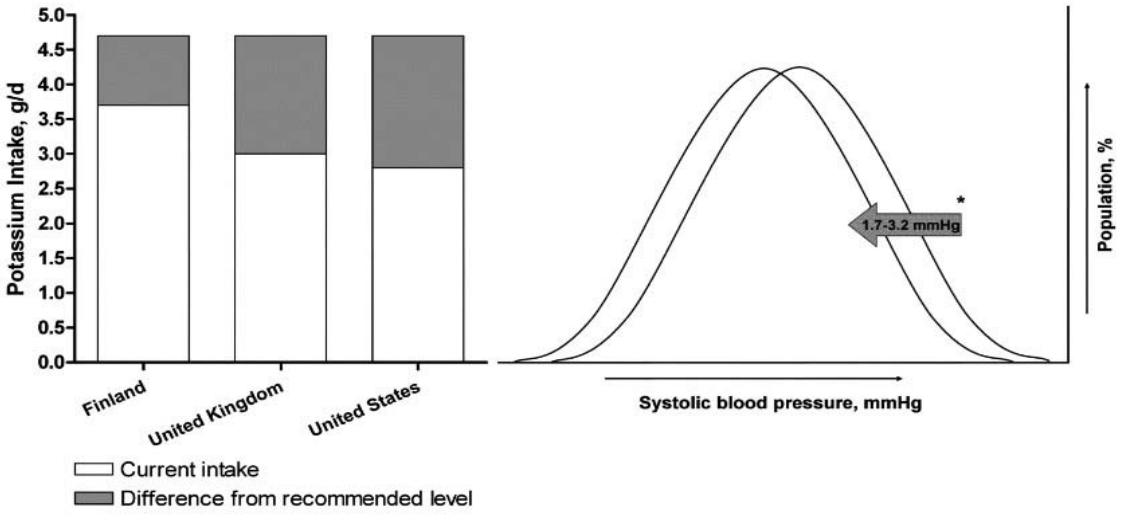
Netherlands (1997-1998)[13]	Nationally representative panel of households (VCP-3)	4,711	19-75+	2-d diary method (2 consecutive days)	4.0 (1.1)	3.4 (0.9)	3.7 (1.0)
New Zealand (1996-1997)[20]	Nationally representative sample, 1997 New Zealand National Nutrition Survey (NNS97)	4,390	19-65+	24 h dietary recall	4.0 (1.8)	3.0 (1.7)	3.4 (1.8)
Poland (2000)[18, 22]	Food consumption and anthropometric survey 2000, randomly selected individuals reflecting the demographic characteristics of the whole population.	2,440	19-64	24-h dietary recall	4.4 (1.5)	3.1 (1.1)	3.7 (1.5)
Spain (1990-1998)[65]	Cross-sectional data from regional population nutrition surveys carried out between 1990 and 1998. Eve study	10,208	25-60	Repeated 24-h dietary recalls (mainly), 3-d food diaries, FFQ	3.3	2.9	3.1
Sweden (1997-1998)[16]	Representative sample of 2000 households, Riksmaten 1997-98. Dietary habits and nutrient intake in Sweden. The second national food consumption survey	1,215	18-74	7-d pre-coded record book	3.5 (1.0)	3.1 (0.8)	3.3 (0.9)
United Kingdom (2000-2001)[11]	Nationally representative sample, The National Diet & Nutrition Survey: Vol. 3.	1,724	19-64	7-d weighed intake dietary record and 24-h urine collection	3.4 (1.0)	2.7 (0.7)	3.0 (0.9)
United States (2005-2006)[12]	Nationally representative sample, NHANES 2005-2006	4,520	≥ 20	24-h dietary recall	3.2 (2.0)	2.4 (1.8)	2.8 (2.0)

[$] For surveys differentiating between potassium intakes including alimentary supplements and excluding supplements we used the data including supplements, unless unavailable. For the survey in the United Kingdom we reported intakes of 7-day weighted intake dietary record to keep data between countries comparable. For Spain and Austria information is based on data published in the respective 2004 and 2009 European Nutrition and Health Report.[18, 65] For countries for which SDs were not reported we calculated the SDs based on data available from the report. For countries for which the SD could not be calculated based on the data available, no SD is reported. For Australia the SD was calculated based on data provided by the Australian Bureau of Statistics.

Estimated impact on population blood pressure distribution

In *Supplemental Figure A* we illustrate the differences between current and recommended potassium intakes for Finland (mean potassium intake of 3.7 g/d), United Kingdom (3.0) and United States (2.8) and the estimated reduction in population systolic BP in these countries if potassium intakes were increased to a level of 4.7 g/d, assuming a 1 mmHg decrease in systolic BP per 0.6 g/d increase in potassium intake as described above. In this way, increasing potassium intake can be estimated to reduce average systolic BP of the population in Finland by 1.7 mmHg, in the United Kingdom by 2.8 mmHg and in the United States by 3.2 mmHg. In women expected reductions are 3.4 mmHg, 2.2 mmHg and 3.9 mmHg, respectively, which is larger than in men (2.2 mmHg, 1.0 mmHg and 2.5 mmHg, respectively), because of lower habitual potassium intake in women.

With the theoretical optimization of potassium intakes, an estimated 2%, 5% and 5% of men in respectively Finland, the United Kingdom and United States would shift to optimal systolic BP levels (<120 mmHg) and 2%, 5% and 3% less men in respectively, Finland, the United Kingdom and United States would have systolic BP in the higher range (≥140 mmHg). For women, 4% in Finland, 8% in United Kingdom, and 6% in United States would shift to optimal systolic BP levels and 4% less women in these three countries would have systolic BP in the higher range (*Supplemental Table 2*). Similar estimations can be performed for other countries and population groups.



Supplemental Figure A *Potassium intakes (current intakes, g/d and differences from recommended level) and illustration of estimated impact on population systolic blood pressure after increasing potassium intake in Finland, United Kingdom and United States.* *[*]Assuming a linear 1 mmHg reduction in population systolic blood pressure per 0.6 g/d increase in intake up to a level of 4.7 g/d.*

Supplemental Table 2 *Predicted percentage (%) of Finnish (25-74 years), United Kingdom (19-64 years) and United States (≥20 years) men and women in systolic BP categories before and after a theoretical increase in potassium intake and the expected change in percentage in the different systolic BP categories.*

		Prevalence in systolic BP categories		
	Systolic BP (mmHg)	Before increase	After increase	Change
Finland				
Men	<120	19%	20%	+2%
	120-139	41%	41%	+1%
	≥140	41%	38%	-2%
Women	<120	30%	34%	+4%
	120-139	38%	38%	0%
	≥140	32%	28%	-4%
United Kingdom				
Men	<120	25%	30%	+5%
	120-139	50%	50%	0%
	≥140	25%	20%	-5%
Women	<120	45%	53%	+8%
	120-139	42%	37%	-4%
	≥140	13%	9%	-4%
United States				
Men	<120	44%	48%	+5%
	120-139	34%	33%	-1%
	≥140	22%	19%	-3%
Women	<120	49%	55%	+6%
	120-139	28%	26%	-2%
	≥140	23%	18%	-4%

Comments

Methodological constraints

Several methodological considerations of our study warrant further consideration. These include the assessment of potassium intakes and assumptions made to predict effects of increasing potassium intakes on BP.

First, it should be mentioned that the quality of the methodology and food composition databases used between the different surveys varies which affects accuracy and makes direct comparison between countries difficult. Furthermore, within most individual surveys potassium intake was only measured via dietary assessment methods. These were found to provide reasonable estimates of intake.[6, 23-27] However measurement of potassium intake by 24-h urinary excretion is the best estimate of intake,[11, 23] but not always practical on large scale though. Due to the nature of dietary questionnaire methods, a systematic error can occur if the entire study population or substantial subgroup - such as overweight persons - consistently over- or underreports dietary intake. In the case of systematic underreporting, our analysis would overestimate the effect on population BP, whereas over-reporting would result in underestimation of the BP effect. Because FFQ are known to underestimate potassium intakes, we excluded such studies from the present review.[24, 25, 27]

For predicting the effect of increasing potassium intake on BP, we set the target recommended potassium intake level for adults at 4.7 g/d based on the recommendations by authoritative bodies in the United States and expert panels, which reviewed all available data on health effects of potassium.[6, 7] For our prediction, we also assumed that increasing dietary potassium intake has a linear effect on BP of -1 mmHg per 0.6 g potassium. This estimate is based on results from the INTERSALT study and has been corroborated by data from several other observational studies.[28-31] Data from meta-analyses of intervention studies in which potassium was supplemented at doses of 2-3 g/d show a 3-4 mm Hg reduction in systolic BP, which is closely in line with -1 mmHg per 0.6 g potassium.[1, 2, 4] Three additional intervention studies were published after these meta-analyses[32-34], including two studies with relatively low doses of potassium (0.9-1.2 g/d)[32, 34], finding similar BP lowering results. Although no formal dose-response intervention studies are available data from intervention studies support that the linear relation we assumed is a reasonable assumption.

Another assumption we made is that the effect of increasing dietary potassium intake is independent of initial BP level. However, dietary interventions to reduce BP are known to be more effective in people with higher initial BP levels.[2, 35-37] Therefore, it is

plausible that improving diet or lifestyle would have more population impact in countries with relatively high BP levels. This also means that with BP lowering interventions, the BP distribution would not simply shift to the left while keeping its shape, but would also become narrower. Taking this phenomenon into account would result in larger estimated effects in countries with higher initial BP levels, such as Finland, and smaller estimated effects in countries with lower initial BP levels, such as the United States. Likewise, the impact in people having high BP levels would be larger, resulting in a larger reduction of the number of people with high blood pressure levels after increasing potassium intake. On the contrary impact on BP effect in the lower ranges would be expected to be smaller.

We found that women have lower potassium intakes (median intake: 2.9 g/d) than men (median intake: 3.5 g/d). It should be noted, however, that the optimal level of potassium intake might be lower in women due to their smaller body size and lower caloric intake. Consequently, we might have overestimated the hypothetical effect of suboptimal potassium intakes in women.[6, 38]

Comparison of the potassium intake data from the national surveys reviewed here with potassium excretion data from the INTERSALT study (1982-1985)[39] and INTERMAP study (1990-1997)[40] suggests that intakes have increased somewhat over the last two decades. However, findings between these and our study should be compared with caution, because of essential differences in methodology of assessing potassium intake and in sampling of participants. A recent paper reported potassium intake data in several European countries based on a random sample of the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Average potassium intakes were similar to data reported here for most countries, except for intakes in Spain and United Kingdom, which were much higher by EPIC compared to the survey data we included in our review for these countries.[41]

Comparison with reductions in sodium intake

The potential of increasing potassium intake levels is considerable. Reducing salt intakes from the concurrent approximately 9 to 5 g/d as advised by the World Health Organisation[42] would reduce systolic BP in the range of 2-4 mmHg, based on observational data from the INTERSALT study[8] and a Cochrane Systematic Review of intervention studies.[43] Our estimate of 1.7-3.2 mmHg for increasing potassium intake is in the same order of magnitude. Thus, improving potassium intake seems to have as much potential for improving BP as reducing sodium intake.

Sodium to potassium intake ratio

Sodium and potassium have opposite effects on BP and other measures of health. Several studies suggest that the sodium to potassium intake ratio is more closely linked to BP and CVD risk than the effect of sodium or potassium alone.[44] In all national surveys for which we could obtain data on both sodium and potassium intake from the same survey, the molar ratio was >1 (range 1.3 – 6.3).[10-18, 45-48] This probably represents an underestimation of the true intake ratio, as intakes were assessed using dietary assessment methods known to systematically underestimate sodium intake.[11, 25, 27, 49] By comparison, the INTERSALT[39] and INTERMAP[40] study found molar ratios >2 (except for the few rural populations included) based on more reliable 24-h urinary excretion data. The recommendations for sodium of <2.0 g/d (equals 87 mmol per day)[50] and for potassium of 4.7 g/d (equals 120 mmol per day)[6] result in a molar ratio of < 0.7. This clearly indicates that current ratios of dietary potassium to sodium intakes are far from optimal.

Feasible options for increasing potassium intake

Potassium is abundantly available in every-day foods that are advised in guidelines for healthy diets[51], for example intake of 5 portions of fruit and vegetables per day already delivers more than 1 g/d.[32] In practice this means that adequate potassium intakes can easily be achieved by following existing dietary guidelines. For instance, adherence to DASH dietary pattern as advised in the Dietary Guidelines for Americans, will result in a potassium intake of 4.7 g/d.[52]

In the general population, dietary potassium intakes above adequate levels pose no potential risk of adverse health effects as excess potassium is readily excreted in the urine.[52, 53] However, some specific subgroups in the population should be careful with increasing potassium intakes. These include specific patients with impaired kidney function; especially those with glomerular filtration rate below to 10–20 ml/min/1.73 m2.[54-56] Another group that should beware of increasing dietary potassium intake are patients on ACE-inhibitors, angiotensin receptor blockers, and potassium-sparing diuretics. However, these patients are typically under medical supervision and follow specific guidelines to control potassium and other nutrients intake[6, 53], and are aware that general population guidelines and campaigns to increase potassium intake do not apply to them.

Estimated effect on cardiovascular disease risk

It is estimated that on a global scale about 62% of stroke and 49% of heart disease are attributable to suboptimal BP.[57] Data from recent national surveys indicate that only 20-28% of people have optimal BP levels (<120/80 mmHg without use of anti-hypertensive medication).[58-62] (personal communication Lucie Viet (RIVM/CBS/ Regenboogproject 1998-2001). This means that a majority of the population can benefit from dietary and lifestyle changes positively influencing BP. BP reductions of 1.7-3.2 mmHg by increasing potassium intakes in the population as estimated here can be predicted to reduce the risk of stroke mortality by 8-15% and heart disease mortality by 6-11%.[63]

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Chapter 3

Blood pressure response to calcium supplementation: a meta-analysis of randomized controlled trials

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Abstract

Background: Calcium plays a role in blood pressure (BP) regulation, but the importance of supplemental calcium intake for the prevention of hypertension is still debated.
Objective: We conducted a meta-analysis of randomized controlled trials to determine the effect of calcium supplementation on BP.
Design: A systematic search for randomized trials of calcium supplementation and BP in non-pregnant subjects was performed in Medline from 1966 to June 2003. Seventy-one trials were identified, 40 of which met the criteria for meta-analysis (total of 2492 subjects). Two persons independently extracted data from original publications on changes in calcium intake and BP. In addition, data were collected on subjects' characteristics, that is, age, gender, initial BP and initial calcium intake. A random effects model was used to obtain the effect of calcium supplementation on BP, overall and in predefined population subgroups.
Results: Calcium supplementation (mean daily dose: 1200 mg) reduced systolic BP by –1.86 mmHg (95% confidence interval: –2.91 to –0.81) and diastolic BP by –0.99 mmHg (–1.61 to –0.37). In people with a relatively low calcium intake (<800 mg per day) somewhat larger BP estimates were obtained, that is, –2.63 (–4.03 to –1.24) for systolic BP and –1.30 (–2.13 to –0.47) for diastolic BP.
Conclusion: Our study suggests that an adequate intake of calcium should be recommended for the prevention of hypertension. More research on BP in people with calcium-deficient diets is warranted.

Introduction

High blood pressure (BP) is a well established risk factor for cardiovascular morbidity and mortality.[1] Calcium is a key factor in the regulation of cardiovascular function and alterations in calcium metabolism have been reported in human and experimental hypertension.[2] Epidemiological studies have shown an association between calcium intake and BP, but findings are inconsistent and could be confounded by healthy lifestyle and diet.[3] Randomized controlled trials (RCTs) that provide stronger evidence for causality suggest that supplemental calcium could indeed exert an effect on BP, but reductions are generally small.[4-6] Because of heterogeneity in BP response among subjects, however, it cannot be excluded that an adequate intake of calcium is important for the prevention of hypertension in specific populations or population subgroups.[7-12] We performed an updated meta-analysis of randomized controlled trials of calcium supplementation and BP, which had sufficient power to detect small treatment effects with high precision. Furthermore, we performed meta-analyses in strata of subject characteristics to examine in more detail heterogeneity of BP response to calcium intake.

Subjects and Methods

Search strategy

The MEDLINE database (1966–June 2003) was searched for RCTs of calcium supplementation and BP using the terms '(calcium NOT calcium antagonis* NOT calcium channel NOT calcium entry NOT calcium blocker) AND (blood pressure OR hypertension)' as Medical Subject Headings (MESH terms) or words in title. Searches were limited to clinical trials or RCTs in humans. Furthermore we performed a manual search using citation lists from retrieved articles, meta-analyses and systematic reviews to identify other eligible studies. No language restriction was used.

Selection of trials

RCTs on the effect of calcium supplementation on BP in non-pregnant normotensive or hypertensive subjects were selected. Studies were excluded if they met any of the following criteria: data included in prior reports (duplicate publication), lack of appropriate BP data, co-intervention (except for co-intervention with vitamin D), study population with renal disease or hyperparathyroidism, lack of control group, or study duration of less than 2 weeks. Seventy-one RCTs were identified (reference list of all

identified studies is available from the authors), of which 40 proved eligible for this meta-analysis (*Figure 1*).[2, 13-51]

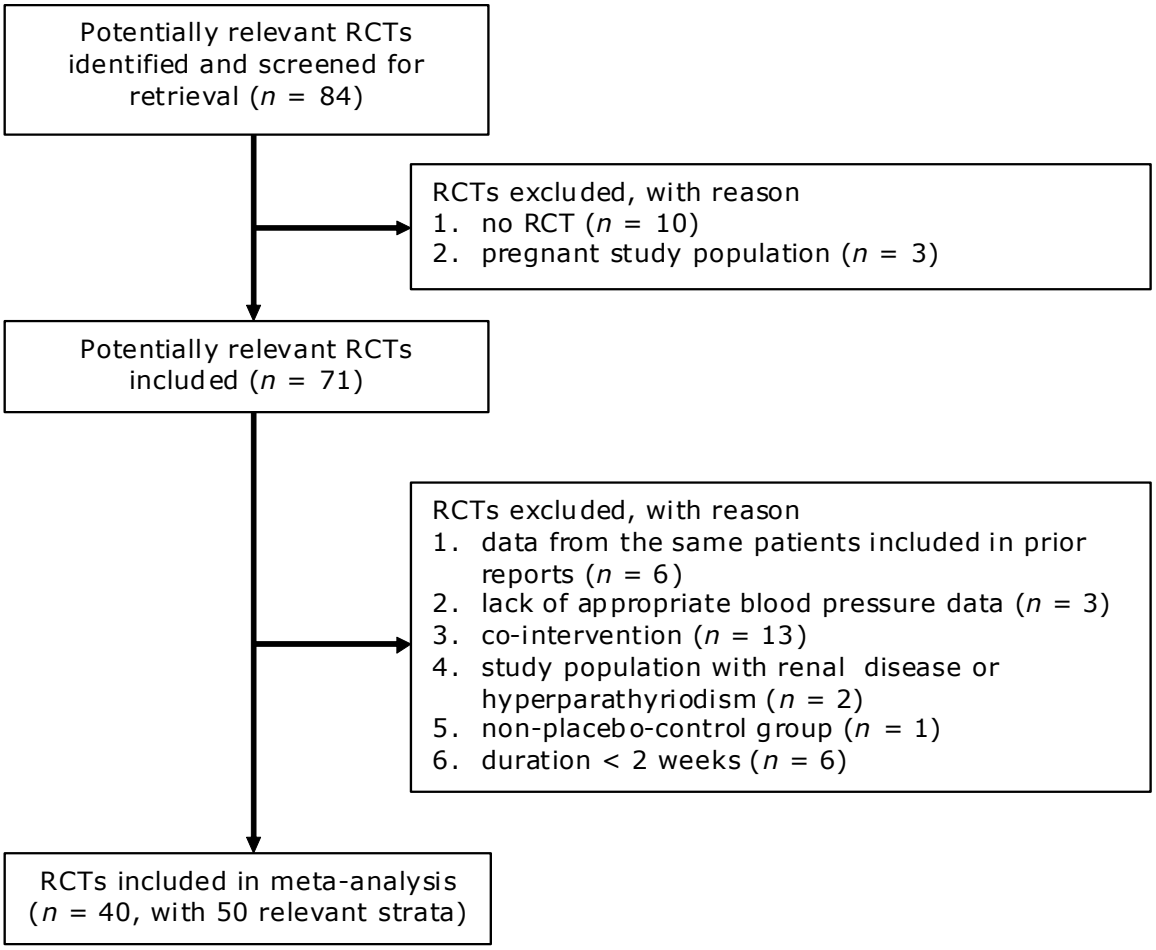

Figure 1 *Selection of studies for meta-analysis of randomized controlled trials of calcium supplementation and blood pressure.*

Validity assessment and data abstraction

We retrieved original articles for data abstraction, except for two trials for which only an abstract was available.[33, 47] Two authors (LAJM and JMG) independently extracted the data. In case of discrepancies consensus was reached, if necessary with the help of a third author (MTS). Studies were scored with regard to blinding towards the type of treatment (i.e. open, single-blind, double-blind).

Extraction of study characteristics

Changes in systolic and diastolic BP and accessory variance measures were abstracted from articles, as were data on calcium dose and the following trial population characteristics: mean age, gender distribution, initial systolic and diastolic BP and initial (i.e. habitual) calcium intake. One trial tested different doses of calcium with a single control group[34] and three trials tested multiple formulations of calcium with a single control group.[32, 33, 46] For these trials, BP changes in the intervention groups were averaged and extracted from BP change in the control group. In trials in which BP was measured at several points in time we used the longest follow-up period during which the patients were still on randomized treatment. With regard to BP, measurements in sitting position were used. If not available, supine BP, standing BP or mean daytime ambulatory BP was taken in that order. For one trial we used BP data that were adjusted for confounders, because raw data were not given in the original article.[28] If different publications on identical studies were found, we used all available information for data abstraction and cited the most recent publication.[34, 52] One trial in which all subjects participated in the parallel phase and part of the subjects in a cross-over phase was treated as if it had a parallel design.[50] Imputations were made for mean age, based on the midpoint of the age range, in one trial.[24] gender (i.e. 50% males) in three trials.[18, 24, 47] and initial calcium intake in 16 trials. The latter imputation was based on the mean calcium intake in the population from which trial participants had been recruited, for which data were derived from population-based dietary surveys (overview of surveys and mean population calcium intakes is available from the authors). If participants had been on a run-in diet for more than 4 weeks before the start of the trial, calcium intake during that period was taken as the habitual calcium intake.[38, 42] For one trial we contacted the authors to obtain additional data.[26] For two other trials[24, 47], we derived data from a meta-analysis[6] and a review[9], respectively.

Meta-analysis

For parallel trials, the change in BP from baseline in the intervention group was subtracted from the change in BP from baseline in the control group to yield the net change in BP due to calcium supplementation. For crossover trials with similar baseline BP for intervention and control periods, the net change in BP was calculated as the final BP during intervention *minus* the final BP during control treatment. Otherwise the net change in BP was calculated similarly as for parallel trials. Variance measures for net changes in systolic and diastolic BP were obtained. If not given, variance measures were derived from confidence intervals, *P* values, or individual variance measures for change

in BP in intervention and control groups or intervention and control periods (crossover design). For parallel trials in which no variance measures for paired differences were given and for crossover trials, the pooled variance was estimated according to the method of Follmann *et al.*[53] assuming a correlation of 0.50 between baseline and final BP values. If variance measures were only reported at baseline, we assumed similar variance at the end of follow-up. The SAS statistical package was used for data-analysis [SAS Version eight, SAS Institute, Inc., Cary, NC, USA]. Homogeneity of effect size was tested by Q statistics[54] and significant heterogeneity was found among trials (P < 0.0001) both for systolic BP (χ^2 = 308.16) and diastolic BP (χ^2 = 334.12). We chose a random-effects model for meta-analysis of continuous outcomes that takes into account both *within* and *between* study variations, using the SAS PROC MIXED statement.[55] For each trial the BP effect was weighted by the reciprocal of its variance, that is, $1/SE^2$. BP estimates are reported with 95% confidence intervals. Two-sided P-values <0.05 were considered statistically significant.

We performed predefined subgroup analyses to study modification of BP response to calcium supplementation. Subgroups were based on calcium dose (≤1000 mg *vs* >1000 mg/day) and trial population characteristics, that is, median age (<45 *vs* ≥45 years), initial BP (<140/90 mmHg *vs* ≥140/90 mmHg), gender (≤50% *vs* >50% males) and initial calcium intake (<800 mg *vs* ≥800 mg/day). Analysis by calcium dose, age and initial calcium intake were based on the median of the frequency distribution of these variables.

Analyses in strata of trial population characteristics and calcium dose were repeated using a multivariate random effects model adjusting for potential confounders (except when used as a stratification variable), that is, mean age (years), gender distribution (%males), initial BP (dichotomous variable, i.e. <140/90 mmHg *vs* ≥140/90 mmHg), initial calcium intake (mg/day) and calcium dose (mg/day).

Publication bias was visually examined after construction of a funnel plot in which weight factors ($1/SE^2$) were plotted against net changes in systolic BP. In addition a non-parametric 'trim and fill' method was used to adjust for publication bias.[56]

Results

Study characteristics

Table 1 displays characteristics of 40 trials selected for meta-analysis, comprising 50 different strata and a total of 2492 subjects. Eighteen strata had a cross-over design (572 subjects in total) and 32 were double-blind. Trial sample size ranged from 7 to 452 subjects. Duration of intervention ranged from 3 to 208 weeks (median: 9.5 weeks) and daily calcium dose from 355 mg to 2000 mg (mean: 1200 mg, median: 1055 mg). In four trials, calcium intake was increased by dietary intervention[14,21,22,26] and in one trial both by dietary intervention and calcium supplements.[33] Trials were mainly conducted in white populations (39 strata), and mostly included both men and women (31 strata). Mean age of trial populations ranged from 11 to 77 years (43.7 ± 14.3 years (mean ± s.d.)). Based on mean BP at baseline (cut-off: 140/90 mmHg), 27 strata were derived from normotensive populations and 23 strata from hypertensive populations. From visual examination of the funnel plot it was concluded that small trials with large systolic BP reductions are possibly overrepresented (*Figure 2*). A non-parametric 'trim and fill' method revealed that two trials might have been missing. After adjustment for putative missing data, the overall effect on systolic BP was attenuated to –1.33 mmHg (95% confidence interval (CI); -2.66 to 0.00).

Table 1 *Overview of randomized trials of calcium supplementation and blood pressure included in the meta-analysis.*

	First author, stratum	Year	Design	n[1]	Duration (wks)	Age (years)	Men (%)	Race[2]	Systolic BP (mmHg)			Diastolic BP (mmHg)			Calcium dose (mg/day)	Intervention
									Initial	Change (SD)[3]		Initial	Change (SD)[3]			
13[a]	Belizan, men	1983	p,db	23	22	26	100	White	113	−0.80	(5.26)	71	−6.02	(5.96)	1000	Calcium gluconate
13[b]	Belizan, women	1983	p,db	20	22	24	0	White	102	−2.40	(5.06)	68	−4.50	(5.30)	1000	Calcium gluconate
14	Bierenbaum	1988	c,o	50	8	43	50	White	119	−4.00	(11.16)	79	−1.00	(7.38)	1150	Dairy products
15	Bloomfield	1986	p,db	32	4	53	19	White	139	5.30	(15.01)	92	0.00	(5.44)	1500	Calcium carbonate
16	Bostick	2000	p,db	185	26	59	63	White	130	−2.20	(17.65)	76	−1.30	(10.30)	1500	Calcium carbonate
17	Cappuccio	1987	c,db	18	4	49	61	White	154	3.50	(11.73)	103	1.10	(8.29)	1600	Calcium gluconate
18	Davis	1996	p,o	34	4	17	50	Black	125	−1.70	(7.85)	91	−0.50	(5.77)	1500	Calcium gluconate
19	Dwyer	1998	c,db	99	8	16	44	Black	116	0.74	(13.75)	66	−1.68	(18.39)	1500	Calcium carbonate
20	Galloe	1993	c,db	20	12	63	66	White	169	2.20	(20.1)	97	3.30	(12.3)	2000	Calcium gluconate
21	Gillman	1995	p,db	101	12	11	50	Black	102	−1.30	(7.21)	58	−0.20	(5.93)	600	Calcium citrate malate enriched juice
22	Green	2000	c,db	38	4	53	50	White	123	−4.00	(15.72)	77	−1.00	(9.27)	355	Dairy products
23	Grobbee	1986	p,db	90	12	24	86	White	143	−0.40	(11.34)	83	−2.40	(9.95)	1000	Calcium citrate
24	Jespersen	1993	c,db	7	8	46	50	White	148	−0.57	(7.2)	93	−0.86	(3.88)	1000	Calcium carbonate
25[a]	Johnson, ht	1985	p,db	34	208	53	0	White	141	−14.0	(22.31)	86	0.00	(10.21)	1000	Calcium carbonate
25[b]	Johnson, nt	1985	p,db	81	208	53	0	White	120	1.00	(13.34)	74	2.00	(8.37)	1000	Calcium carbonate
2	Kawano	1998	c,o	60	8	58	58	Asian	149	−2.00	(13.15)	90	−1.10	(7.67)	1000	Calcium carbonate
26	Kynast-Gales	1992	c,o	13	4	61	100	White	136	3.62	(10.37)	83	0.15	(7.01)	1109	Dairy products
27	Lijnen	1996	p,db	32	16	24	100	White	114	−2.00	(6.93)	73	−1.00	(7.09)	2000	Calcium gluconate
28[a]	Lyle, blacks	1987	p,db	21	12	28	100	Black	114	−6.90	(10.03)	71	1.20	(7.24)	1500	Calcium carbonate
28[b]	Lyle, whites	1987	p,db	54	12	35	100	White	115	−3.70	(8.65)	75	−1.10	(7.39)	1500	Calcium carbonate
29	Lyle	1992	p,db	42	8	34	76	White	133	−6.00	(8.53)	87	−7.20	(5.89)	1500	Calcium carbonate
30	Martinez	1989	p,o	51	8	44	39	White	157	−4.00	(10.75)	99	−2.00	(6.60)	1000	Not given
31[a]	McCarron, ht	1985	c,db	47	8	52	52	White	152	−3.80	(15.07)	94	0.00	(7.94)	1000	Calcium carbonate
31[b]	McCarron, nt	1985	c,db	31	8	48	38	White	121	−2.00	(15.57)	75	−3.30	(8.19)	1000	Calcium carbonate
32	Meese	1987	c,db	26	8	49	38	Black	143	−1.00	(7.94)	96	−3.00	(4.89)	800	Calcium carbonate, calcium citrate
33[a]	Morris, ht men	1988	p,o	46	12	45	100	White	138	−4.35	(6.10)	94	−2.25	(4.07)	1500	Dietary calcium, calcium carbonate
33[b]	Morris, ht women	1988	p,o	36	12	45	0	White	138	−2.90	(5.76)	94	0.05	(4.94)	1500	Dietary calcium,

														calcium carbonate
33[c]	Morris, nt men	1988	p,o	26	12	45	100	White	118	−4.70	(4.74)	74	0.50	(4.39)	1500	Dietary calcium, calcium carbonate
33[d]	Morris, nt women	1988	p,o	19	12	45	0	White	118	−1.75	(5.29)	74	−0.10	(2.73)	1500	Dietary calcium, calcium carbonate
34[a]	Nowson, ht	1989	p,db	47	8	60	87	White	155	−1.50	(11.10)	91	0.50	(6.25)	800/1600	Calcium carbonate
34[b]	Nowson, nt	1989	p,db	48	8	42	58	White	123	0.00	(19.4)	74	0.30	(9.01)	800/1600	Calcium carbonate
35	Orwoll	1990	p,db	65	156	56	100	White	131	1.88	(14.73)	84	3.75	(10.61)	1000	Calcium carbonate[4]
36	Pan	1993	c,db	29	11	74	72	Asian	136	−7.09	(13.05)	72	−0.87	(11.05)	800	Calcium citrate
37	Sacks	1998	p,db	153	16	39	0	White	116	−0.70	(5.84)	74	−0.90	(4.49)	1200	Calcium carbonate
38	Sanchez	1997	p,db	20	8	45	60	White	166	1.10	(23.84)	99	−2.10	(13.97)	1500	Calcium gluconate
39	Siani	1987	c,db	8	3	40	75	White	154	5.10	(13.86)	96	1.30	(2.4)	1000	Calcium gluconate
40	Siani	1988	c,db	14	4	41	80	White	139	2.20	(12.19)	91	0.70	(9.08)	1000	Calcium gluconate
41	Strazzullo	1986	c,db	17	15	43	61	White	145	−1.00	(11.78)	91	−1.00	(6.08)	1000	Calcium gluconate
42	Takagi	1991	c,o	9	8	77	33	Asian	146	−12.1	(13.06)	78	−6.00	(8.89)	1000	AA calcium
43	Tanji	1991	c,db	19	13	48	33	White	146	−1.00	(16.66)	95	0.00	(7.11)	1200	Calcium carbonate
44	Thomsen	1987	p,db	28	52	50	0	White	124	−4.10	(16.55)	76	0.20	(10.38)	2000	Calcium gluconate
45	Van Beresteyn	1986	p,db	58	6	21	0	White	115	−1.70	(9.22)	65	0.40	(6.68)	1500	Calcium carbonate
46	Vinson	1987	p,o	14	7	21	100	White	116	4.70	(5.22)	75	−2.70	(3.07)	500	Calcium gluconate, calcium yeast
47	Waal-Manning	1987	p,db	52	39	61	50	White	143	−2.00	(28.64)	84	−2.00	(15.91)	1000	Calcium supplement
48[a]	Weinberger, ht	1993	c,db	17	8	45	41	White	131	−2.00	(15.48)	87	−1.00	(8.28)	1500	Calcium carbonate
48[b]	Weinberger, nt	1993	c,db	29	8	45	41	White	116	1.00	(12.41)	72	−1.00	(9.59)	1500	Calcium carbonate
49[a]	Yamamoto, men	1995	p,db	306	26	42	100	White	126	0.50	(3.37)	84	1.00	(2.83)	1000	Calcium carbonate
49[b]	Yamamoto, women	1995	p,db	146	26	45	0	White	125	−2.10	(3.83)	84	−1.60	(2.77)	1000	Calcium carbonate
50	Zhou	1994	p,db	56	14	46	51	Asian	157	−18.9	(15.90)	103	−8.56	(9.33)	1000	Calcium carbonate
51	Zoccali	1988	c,db	21	8	43	70	white	142	2.00	(14.39)	88	2.00	(9.59)	1000	Calcium gluconate

Abbreviations: c, crossover; db, double-blind; ht, hypertensive; nt, normotensive; o, open; p, parallel
[1] Number of subjects that completed the trial. [2] Predominant race in study population. [3] Values represent net BP effects of calcium supplementation with standard deviations (SD) in parentheses. [4] Combined with cholecalciferol treatment (1000 IU/day).

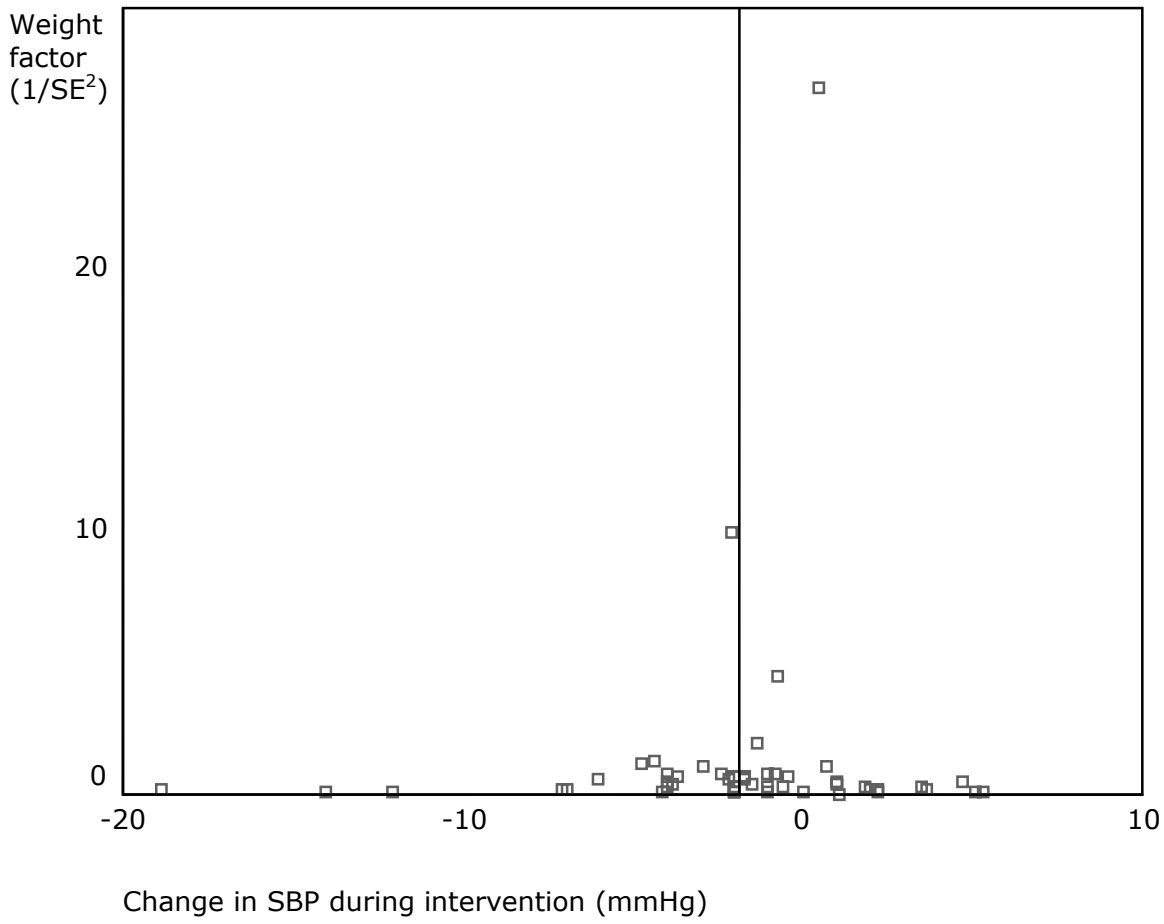

Figure 2 *Funnel plot of change in systolic blood pressure (BP) (mmHg) against weight factor (1/SE^2) in randomized controlled trials of calcium supplementation and BP.*

Quantitative data analysis

Forest plots of BP effects in individual trials with 95% CIs are shown in *Figure 3*. Meta-analysis of randomized controlled trials yielded a weighted estimate for the overall effect of calcium supplementation of –1.86 mmHg (–2.91 to –0.81) on systolic BP and of –0.99 mmHg (–1.61 to –0.37) on diastolic BP. If only double-blind trials were included, reductions were –1.67 mmHg (–2.87 to –0.47) and –0.93 mmHg (–1.64 to –0.22), respectively. BP estimates were not significantly different among subgroups (*Table 2*), but there was a tendency towards an increased BP sensitivity to calcium in populations with a low initial calcium intake (<800 mg/day) compared to populations with higher intakes (≥800 mg/day), both for systolic BP (–2.68 mmHg (–4.07 to –1.28) *vs* –0.90 mmHg (–2.41 to 0.61), respectively) and diastolic BP (–1.30 mmHg (–2.15 to –0.46) *vs* –0.63 mmHg (–1.53 to 0.28), respectively). BP responses to calcium supplementation

did not differ significantly in strata of mean age (<45 *vs* ≥45 years), calcium dose (≤1000 mg *vs* >1000 mg/d), initial BP (<140/90 mmHg *vs* ≥140/90 mmHg) and gender (≤50% *vs* >50% males). Dietary increases in calcium intake (five trials) tended to have a larger effect on systolic BP than calcium supplements (–2.56 mmHg (–4.98 to –0.13) *vs* –1.70 mmHg (–2.85 to –0.55)), but this was not the case for diastolic BP (–0.51 mmHg (–1.96 to 0.95) *vs* –1.10 mmHg (–1.78 to –0.41)). Blood pressure effects of calcium supplementation were particularly pronounced in four trials among Asian populations, comprising a total of 154 subjects with a habitual calcium intake of 400-550 mg/day. This discrepancy persisted after adjustment for age, gender, initial BP, calcium dose and habitual calcium intake, that is, BP estimates of -9.66/-4.62 mmHg in Asian subjects compared to -1.54/0.32 mmHg in blacks and -1.21/-0.78 mmHg in whites.

Multivariate, stratified meta-analysis (*Table 2*) with adjustment for subgroup differences in mean age, gender distribution, initial BP, initial calcium intake and calcium dose, yielded essentially similar results. BP response did not differ among subgroups, but again a tendency towards larger BP reductions was observed in populations with a low initial calcium intake (<800 mg/day) which could not be explained by differences in mean age, gender distribution, initial BP or calcium dose.

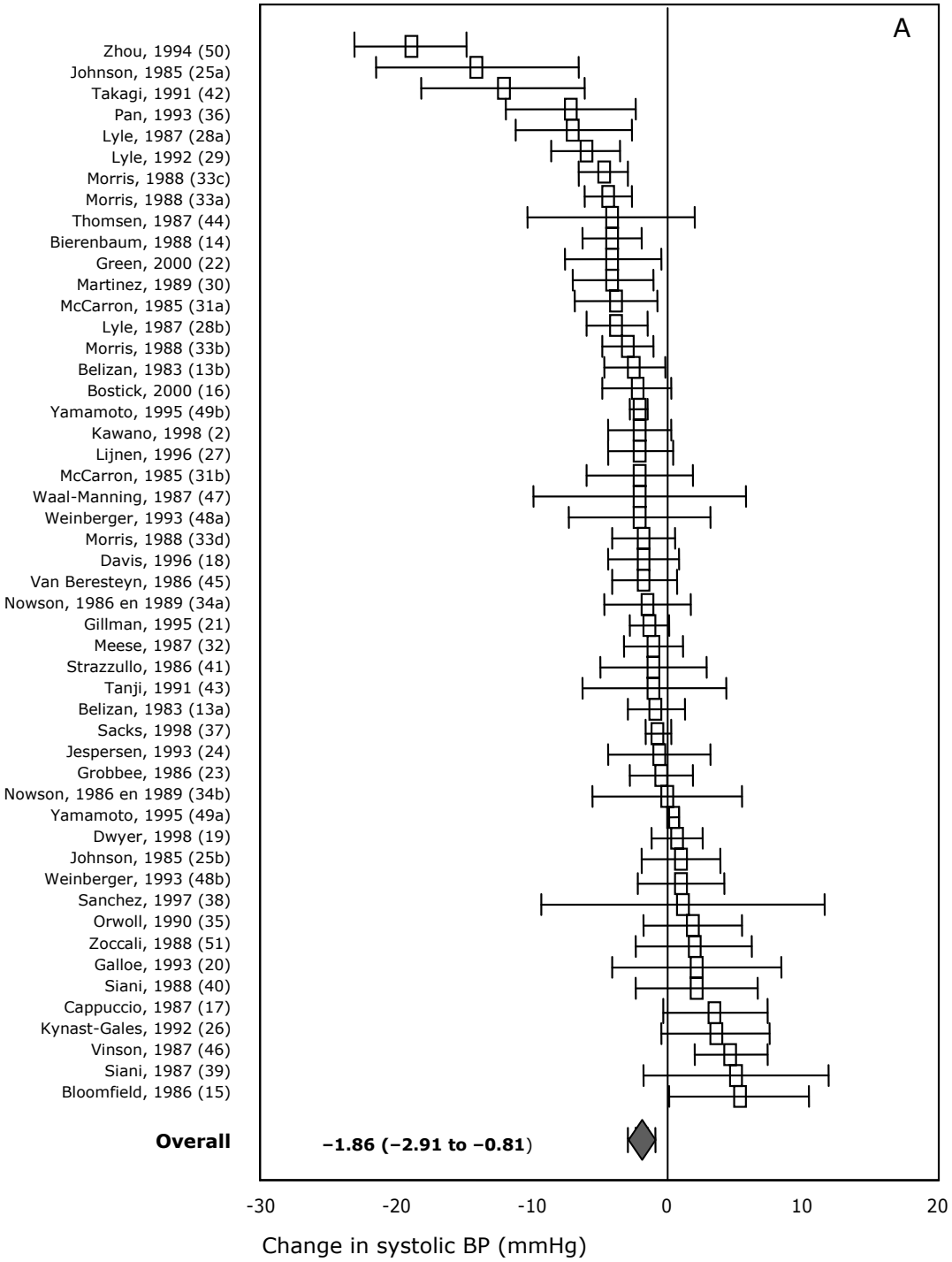

Figure 3 *Blood pressure (BP) response to calcium supplementation in randomized controlled trials. BP effects in individual trials are depicted as open squares with 95% confidence intervals (CIs), for systolic BP (Forest plot A) and diastolic BP (Forest plot B), respectively. Pooled estimates with 95% CIs are depicted as diamonds.*

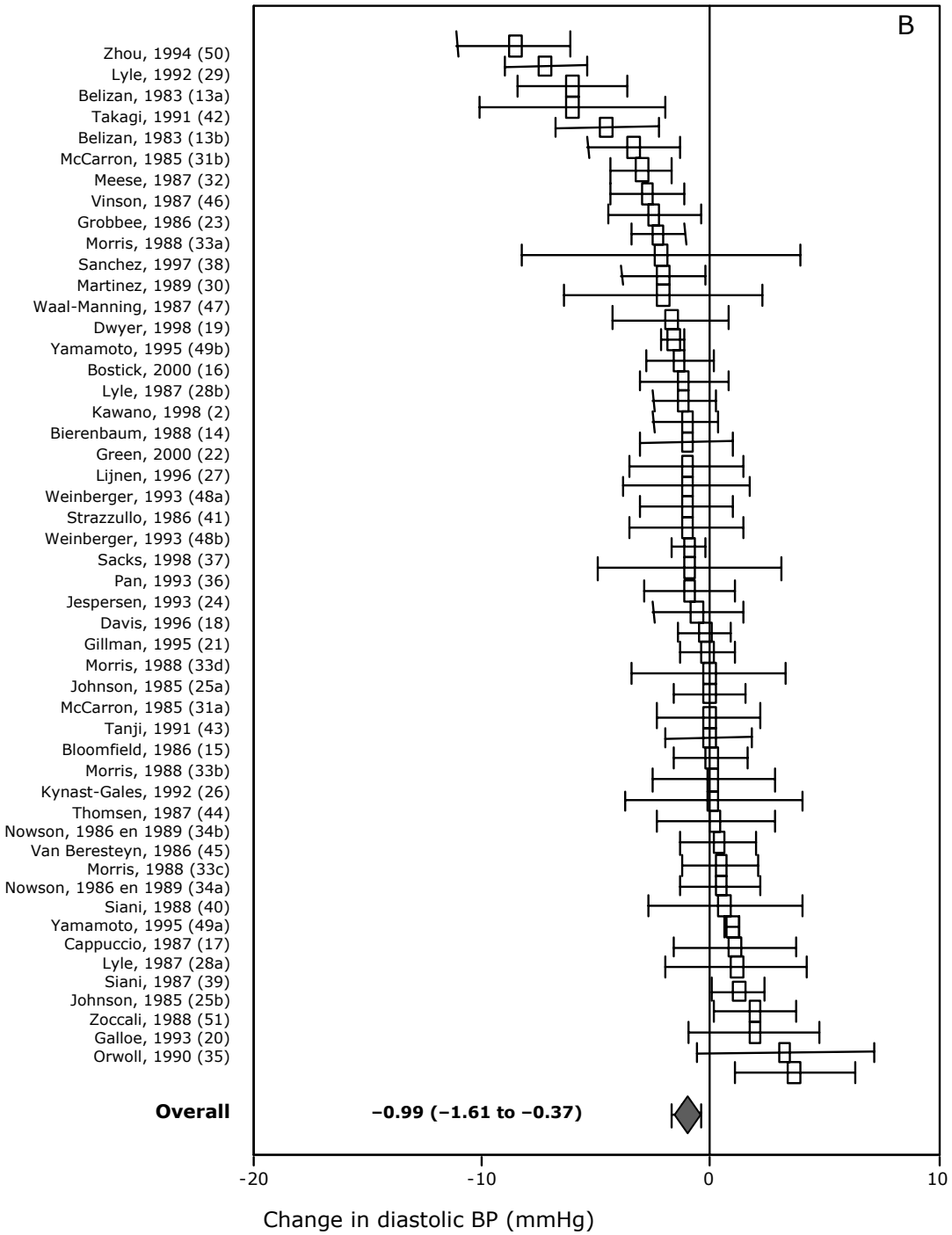
B
Zhou, 1994 (50)
Lyle, 1992 (29)
Belizan, 1983 (13a)
Takagi, 1991 (42)
Belizan, 1983 (13b)
McCarron, 1985 (31b)
Meese, 1987 (32)
Vinson, 1987 (46)
Grobbee, 1986 (23)
Morris, 1988 (33a)
Sanchez, 1997 (38)
Martinez, 1989 (30)
Waal-Manning, 1987 (47)
Dwyer, 1998 (19)
Yamamoto, 1995 (49b)
Bostick, 2000 (16)
Lyle, 1987 (28b)
Kawano, 1998 (2)
Bierenbaum, 1988 (14)
Green, 2000 (22)
Lijnen, 1996 (27)
Weinberger, 1993 (48a)
Strazzullo, 1986 (41)
Weinberger, 1993 (48b)
Sacks, 1998 (37)
Pan, 1993 (36)
Jespersen, 1993 (24)
Davis, 1996 (18)
Gillman, 1995 (21)
Morris, 1988 (33d)
Johnson, 1985 (25a)
McCarron, 1985 (31a)
Tanji, 1991 (43)
Bloomfield, 1986 (15)
Morris, 1988 (33b)
Kynast-Gales, 1992 (26)
Thomsen, 1987 (44)
Nowson, 1986 en 1989 (34b)
Van Beresteyn, 1986 (45)
Morris, 1988 (33c)
Nowson, 1986 en 1989 (34a)
Siani, 1988 (40)
Yamamoto, 1995 (49a)
Cappuccio, 1987 (17)
Lyle, 1987 (28a)
Siani, 1987 (39)
Johnson, 1985 (25b)
Zoccali, 1988 (51)
Galloe, 1993 (20)
Orwoll, 1990 (35)
Overall
–0.99 (–1.61 to –0.37)
-20
-10
0
10
Change in diastolic BP (mmHg)

Table 2 *Blood pressure response to calcium supplementation in strata of subjects' characteristics and calcium dose.*

	No. of strata (subjects)	Systolic blood pressure[1]		Diastolic blood pressure[1]	
		Univariate model	Multivariate model[2]	Univariate model	Multivariate model[2]
Age (years)					
<45	21 (1256)	−1.17 (−2.71;0.36)	−1.45 (−2.99;0.09)	−1.21 (−2.15;−0.28)	−1.26 (−2.20;−0.33)
≥45	29 (1236)	−2.43 (−3.83;−1.03)	−2.33 (−3.69;−0.96)	−0.81(−1.64;0.02)	−0.80 (−1.62;0.02)
		P = 0.24	*P = 0.42*	*P = 0.53*	*P = 0.48*
Gender (males)					
≤50%	23 (1170)	−2.08 (−3.63;−0.53)	−2.20 (−3.68;−0.72)	−1.12 (−2.04;−0.21)	−1.12 (−1.98;−0.26)
>50%	27 (1322)	−1.67 (−3.10;−0.24)	−1.77 (−3.13;−0.42)	−0.88 (−1.73;−0.02)	−0.84 (−1.65;−0.04)
		P = 0.70	*P = 0.67*	*P = 0.70*	*P = 0.65*
Initial blood pressure (mmHg)					
<140/90	27 (1728)	−1.64 (−3.01;−0.27)	−2.04 (−3.40;−0.68)	−1.02 (−1.85;−0.19)	−1.04 (−1.86;−0.22)
≥140/90	23 (764)	−2.17 (−3.78;−0.55)	−1.85 (−3.45;−0.32)	−0.95 (−1.89;−0.01)	−0.89 (−1.79;0.01)
		P = 0.62	*P = 0.89*	*P = 0.91*	*P = 0.81*
Initial calcium intake (mg/day)					
<800	27 (1388)	−2.68 (−4.07;−1.28)	−2.63 (−4.03;−1.24)	−1.30 (−2.15;−0.46)	−1.30 (−2.13;−0.47)
≥800	23 (1104)	−0.90 (−2.41;0.61)	−1.07 (−2.62;0.48)	−0.63 (−1.53; 0.28)	−0.53 (−1.44;0.38)
		P = 0.089	*P = 0.15*	*P = 0.28*	*P = 0.23*
Calcium dose (mg/day)					
≤1000	25 (1346)	−2.13 (−3.62;−0.64)	−2.17 (−3.59;−0.75)	−1.36 (−2.23;−0.49)	−1.41 (−2.24;−0.59)
>1000	25 (1146)	−1.59 (−3.08;−0.11)	−1.75 (−3.20;−0.31)	−0.61 (−1.49;0.26)	−0.56 (−1.40;0.29)
		P = 0.62	*P = 0.69*	*P = 0.23*	*P = 0.16*

[1] Mean BP effects with 95% confidence interval obtained from random-effects model. *P*-values are given for difference in BP response between strata. [2] Adjusted for age, gender distribution, initial calcium intake, calcium dose and initial BP.

Discussion

This meta-analysis of 40 RCTs shows that calcium supplementation (~1 g/day) may significantly reduce systolic BP by 1.9 mmHg and diastolic BP by 1.0 mmHg. The BP effect tended to be more pronounced in populations with a habitually low calcium intake (2.6/1.3 mmHg). Blood pressure showed no further decrease when calcium doses exceeded 1 g/day.

Compared to previous meta-analyses of calcium and BP, this study included six RCTs[2, 16, 19, 22, 30, 37] that had not been analyzed previously. A random effects model was used to account for heterogeneity; using an advanced statistical approach.[55] Power of this meta-analysis was sufficient to allow subgroup analyses to study modification of BP response to calcium supplementation. Our pooled BP estimate of –1.9/–1.0 mmHg was somewhat larger than in previous meta-analyses of calcium supplementation trials (ranging from –1.4 to –0.8 mmHg systolic and –0.8 to –0.2 mmHg diastolic).[4-6] If only double-blind trials with high internal validity were included in meta-analysis, our pooled BP estimate was slightly attenuated to –1.7/–0.9 mmHg.

It has been suggested supplemental calcium exerts a stronger effect on BP in calcium-deficient subjects.[5, 11, 57] In our meta-analysis, BP response to calcium tended to be stronger in people with a low habitual calcium intake (–2.7/–1.3 mmHg) compared to people with a higher intake (–0.9/–0.6 mmHg). However, it should be noted that initial calcium intake was not reported in 16 trials, for which we imputed the mean intake of the population from which subjects were recruited. These findings should therefore be interpreted with caution. Previous meta-analyses showed stronger associations of calcium with BP in hypertensive subjects[4], at high doses of calcium[58] and for food-based increases in calcium intake.[6] This could not be confirmed in our study, except for a potentially larger effect of calcium-enriched foods on systolic BP. Stratified analysis of dietary compared to supplemental calcium, however, included only five dietary intervention studies and may have lacked power.

High intake of calcium is often accompanied by an increased intake of other nutrients, including saturated fat and sodium, which may elevate BP. In the Dietary Approaches to Stop Hypertension (DASH) trial the effect of dietary patterns on BP was assessed.[59] The DASH combination diet, rich in fruits, vegetables and low-fat dairy products, reduced systolic and diastolic BP to a greater extent (–5.5/–3.0 mmHg) than the "fruits and vegetables diet" (–2.8/–1.1 mmHg). This may partly be attributable to extra dietary calcium from low-fat dairy products (1240 mg/day) in this US population with a daily calcium intake of only 400–500 mg.

Prospective cohort studies provide better insight in the long-term BP effect of calcium intake than trials, that are mostly of short duration. However, observational data must be interpreted with caution because of inaccuracies in the assessment of habitual calcium intake, potential confounding by other dietary and lifestyle factors, and the fact that the range of calcium intake within a single population may be too limited to demonstrate an effect on BP. In the Third National Health and Nutrition Examination Survey (NHANES III) a higher intake of calcium was associated with a reduced rate of rise in systolic BP with age, suggesting an effect of dietary calcium also on long-term BP control.[60]

Minor reductions in population BP could have a substantial impact on cardiovascular disease in westernized societies. MacMahon *et al* showed that a prolonged difference in usual diastolic BP of only 5 mmHg is associated with at least 34% less stroke and 21% less coronary heart disease.[61] This meta-analysis showed a small favorable effect of calcium supplementation on BP. This beneficial effect may be larger in populations with a habitually low calcium intake, for example in Asian subjects. We recommend that more research is carried out on the potential health benefits of increased calcium intake, especially in vulnerable subgroups.

What is known about this topic

- Epidemiological studies suggest that an adequate intake of calcium may prevent hypertension, but findings are inconsistent.[3]
- Randomized controlled trials have shown a small effect of calcium supplementation on BP.[4]
- There may be heterogeneity in BP response to calcium intake among individuals.[11]

What this study adds

- Meta-analysis of 40 randomized controlled trials showed that calcium supplementation (~1 g/day) significantly reduces systolic BP by 2 mmHg and diastolic BP by 1 mmHg.
- BP response to calcium supplementation tended to be stronger in populations with a low habitual calcium intake (<800 mg/day) than in populations with an adequate intake.
- BP response to calcium supplementation was not modified by age, gender and presence of hypertension.

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Chapter 4

The effect of vitamins and minerals enriched milk on blood pressure in mildly hypertensive subjects

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In a randomized controlled study, we found no effect of a dairy drink containing additional amounts of potassium, calcium, magnesium, selenium and vitamins C and E on blood pressure (BP). The results of this study confirm the discrepancy between dietary intervention studies showing reasonable BP lowering effects and intervention studies with a combination of minerals or vitamins showing no effect. Future studies should focus on assessing the effect of subsets of combinations of minerals and vitamins in natural matrices to get a better understanding of the possible antagonistic action between some of these ingredients.

The Dietary Approaches to Stop Hypertension (DASH) study showed an impressive reduction on BP.[1] The effective components of this diet probably include minerals and vitamins. Several meta-analyses and intervention studies investigated the relationship between individual minerals and vitamins and their effect on BP. A meta-analysis showed a significant effect of potassium supplementation on systolic blood pressure (SBP) of -3.11mm Hg and of diastolic blood pressure (DBP) of -1.97mm Hg.[2] Studies on the effect of a combination of minerals[3–6] and vitamins[7] showed mixed results. The present study is, to the best of our knowledge, the first study investigating the effect of a combination of minerals and vitamins on BP.

We tested the BP lowering effect of skimmed milk with added vitamins and minerals (calcium 446 mg, magnesium 100 mg, selenium 40 mg, vitamin C 180 mg, vitamin E 30 mg and tocopherol equivalents and either of two levels of potassium (high-K combination 1500 mg/serving and low-K combination 750 mg/serving)) in an 8 week trial. Untreated subjects with elevated BP were enrolled. The placebo and the run-in product consisted of 250 g of water. By adding titaniumoxide, xanthan and pectin, the appearance of the placebo product was comparable to the other test products. No BP effects have been described for these additives. Products were provided in nontransparent cups and lids. Office BP was measured three times with an oscillometric automatic device (OMRON HEM-907) following an overnight fast and after 15 min of rest. The mean of the second and third measurement was used for statistics. Office BP was measured at 2 days at baseline (week 0) and at the end of the intervention (week 8), and once at week 2, week 4, and after 2 weeks of wash-out. Twenty-four hours ambulatory blood pressure (24 h ABP) was measured once at baseline and at week 8 using an oscillometric automatic device (Spacelab monitor type 90217). Body weight and 24 h urinary sodium and potassium excretion were measured at baseline and week 8. The primary outcome of this study was the reduction in SBP. Secondary outcomes included DBP and 24 h ABP. The target sample size of 40 subjects per treatment group

was estimated to provide 80% power and α = 0.05 (one-sided) to detect a reduction in SBP of 5 mmHg. Data are expressed as mean ± SEM. Analyses were performed using analysis of variance. To compare differences between groups, the Tukey multiple comparison method was used. A total of 124 subjects completed the study. Data of nine subjects were excluded (use of medication interfering with BP (n = 5), the uptake of the product (n = 1), arrhythmia (n = 1) and other (n = 2)). Baseline characteristics (BP levels, body weight and urinary excretion levels) were comparable in each of the groups. After 8 weeks, office SBP decreased by 4.6 ± 1.4 (high-K combination), 4.5 ± 1.4 (low-K combination) and 5.1 ± 1.4mm Hg (placebo). These changes were not significantly different between the groups. The decrease in office DBP, pulse pressure (PP), 24 h SBP, 24 h DBP and HR were not significantly different either (for more information about office DBP and PP data see *Table 1*). Office BP levels after 2 weeks of wash-out did not change significantly when compared to values at week 8. Body weight showed an increase in the intervention groups compared to baseline, while it showed a decrease in the placebo group. There was, however, no interaction between weight change and intervention group on BP changes. At the end of treatment, sodium excretion increased somewhat in the high-K combination group and urinary potassium excretion increased during treatment, depending on the potassium content in the product.

Table 1 *Office SBP, DBP and PP (mean ± SEM).*

	Placebo	High-K combination	Low-K combination
Office SBP			
Week 0	143.1 ± 1.4	145.4 ± 1.8	144.2 ± 1.8
Week 8	139.4 ± 1.8	141.5 ± 1.7	140.4 ± 1.7
Office DBP			
Week 0	84.9 ± 1.2	85.4 ± 1.6	87.8 ± 1.2
Week 8	83.0 ± 1.4	84.0 ± 1.3	86.9 ± 1.2
PP			
Week 0	58.3 ± 1.4	60.0 ± 1.7	56.5 ± 1.5
Week 8	56.4 ± 1.3	57.5 ± 1.6	53.5 ± 1.2

Abbreviations: DBP, diastolic blood pressure; PP, pulse pressure; SBP, systolic blood pressure.

The results found in the present study are in contrast with many studies addressing interventions with the individual components. One explanation for this may be that the amount of minerals and vitamins in the intervention products used in this study is smaller than the amounts usually tested.[2,8–10] Studies exploring the effect of combinations of minerals or vitamins on BP, hypothesized that the reductions would be

greater than the effect reported in studies of each mineral/vitamin alone.[3–7] However, although most of these studies have shown a decrease in BP, they did not find a clear additive or synergistic effect of the nutrients. Notably, in two studies, in which supplements of combinations of minerals were given to the subjects, the effect found was even lower than expected from one of the minerals (that is potassium).[4,5] As an explanation for these unexpected findings, the possibility of a counteracting effect of the combination of minerals on the effect of potassium on BP was mentioned.[5] This phenomenon might have influenced the results of our study as well.

The results of the present study are also in contrast with the substantial effects found in dietary intervention studies. Several aspects might explain this difference. First, studies replacing complete diets not only add potentially beneficial components, but reduce potential negative components in the diet as well. Second, there may be other components in foods 'not known' yet to be beneficial for BP, which are unchanged in supplementation trials, but are higher in studies replacing (part of) a diet. Finally, nutrients in dietary supplements/products may not reduce BP to the same extent as nutrients in food because of altered bioavailability. The dairy matrix used in this study may be not the optimal matrix for the nutrients to show their effect.

The question now is whether or not to continue exploring the efficacy of mineral and vitamin supplements/products on BP. In the PREMIER study, the effect of the DASH diet was explored when subjects purchased their own food. The effect found was much smaller than expected. One of the explanations mentioned was that subjects in real life do not fully comply to the guidelines.[11] This illustrates that it is hard to change someone's diet and difficult to extrapolate findings from well-controlled studies and motivated volunteers to the population at large. Therefore, supplements or products containing a combination of nutrients resulting in a reasonable BP reduction in combination with guidelines for a healthy diet could help many people with slightly elevated BP levels, and may have a substantial impact on the total number of cardiovascular disease in Westernized societies.[12]

In conclusion, we were not able to demonstrate a BP lowering effect of a vitamin and mineral combination drink. Future studies should focus on assessing the effect of subsets of combinations of minerals and vitamins in natural matrices to get a better understanding of the possible antagonistic action between some of these ingredients.

What is known about this topic

- Several meta-analyses and intervention studies have shown a beneficial effect of individual minerals and vitamins on BP.
- Studies exploring the effect of combinations of minerals on BP could not prove clear additive or synergistic effects. The same counts for studies exploring the effect of combinations of vitamins on BP whereas dietary intervention studies did find substantial BP lowering results, for example, the DASH diet.
- The effect of a dairy product enriched in vitamins and minerals on BP has not been tested before.

What this study adds

- In this randomized double-blind placebo-controlled parallel study, we did not find a BP lowering effect of a dairy drink containing an additional amount of potassium, calcium, magnesium, selenium and the vitamins C and E.
- The results of this study confirm the discrepancy in results between dietary intervention studies and intervention studies with a combination of minerals or vitamins and do not suggest an additive BP effect of combinations of vitamins and/or minerals.

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Conflicts of interest: LAJ van Mierlo, HCM van der Knaap, MMG Koning and J Kloek are working at the Unilever Food & Health Research Institute, Vlaardingen, The Netherlands.

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Chapter 5

Lactotripeptides do not lower ambulatory blood pressure in untreated Caucasians: results from two multi-centre controlled cross-over studies

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Abstract

Background: Dietary factors directly influence blood pressure (BP). The lactotripeptides (LTPs) IPP (isoleucine-proline-proline) and VPP (valine-proline-proline), formed by hydrolyzing dairy proteins, and potassium, a mineral mainly found in fruit, vegetables, and dairy products, are extensively studied for their BP-lowering effect. The efficacy of LTPs seems modest in whites compared with that in Asians.

Objective: The objective was to study the effects of enzymatically produced LTPs alone or in combination with potassium on ambulatory BP in whites.

Design: Two multicenter, placebo-controlled, randomized, crossover studies were conducted; each consisted of two 4-wk intervention periods separated by a 4-wk washout period. In study 1, 69 subjects received 200 g/d of a dairy drink with 5.8 mg IPP and 4.4 mg VPP or placebo. In study 2, 93 subjects received 100 g/d of a dairy drink with 2.7 mg IPP, 1.9 mg VPP, and 350 mg added potassium or placebo. The subjects were randomly assigned according to their daytime ambulatory BP.

Results: Mean 24-h systolic and diastolic BP (baseline values - study 1: 137.1/81.6 mmHg; study 2: 139.2/80.9 mmHg) remained similar with no significant differences between treatments in either study ($P > 0.10$). Office BP decreased over the course of both studies (systolic BP > 5 mmHg), but differences between interventions were not significant ($P > 0.10$). In both studies, nighttime BP dipped during all treatments (≥15%) but was statistically more significant with placebo ($P < 0.05$). Sodium excretion increased significantly after consumption of LTPs and potassium compared with after placebo intervention ($P = 0.01$), but not after consumption of LTPs alone.

Conclusion: The data do not support a BP-lowering effect of LTPs in whites.

Introduction

Elevated blood pressure (BP) is an important public health challenge because of its high prevalence worldwide[1], poor treatment and control rate[2], and consistent relation with the risk of cardiovascular disease.[3] Health authorities recommend adoption of a healthy diet and lifestyle for persons with a high normal BP and for patients who require drug treatment.[3,4] Dietary and lifestyle factors can directly influence BP, particularly a diet rich in fruit and vegetables and in low-fat dairy products, as was shown in the Dietary Approaches to Stop Hypertension (DASH) Study.[5] Furthermore a considerable number of human studies have addressed the BP-lowering effect of individual nutrients. Peptides, particularly the milk-derived lactotripeptides (LTPs) IPP (isoleucine-proline-proline) and VPP (valine-proline-proline), and potassium, a mineral mainly found in fruit, vegetables, and dairy products, have been extensively studied for their BP-lowering effect.

Several meta-analyses of >30 intervention studies have shown significant BP-lowering effects of potassium supplementation at relatively high doses.[6-9] Most studies that examined the effect of IPP and VPP have also reported significant decreases in BP, although the reported magnitude of the effect varies considerably between data derived from Asian[10-19] and white[20-26] populations.

Two recent meta-analyses provided evidence of a significant effect of peptides on BP.[27,28] The meta-analysis by Pripp[28] included intervention studies on both IPP and VPP and other peptides derived from food proteins, and found a significant pooled effect on systolic BP (SBP) of -5.1 mmHg and on diastolic BP (DBP) of -2.4 mmHg. A second meta-analysis by Xu *et al.*[27], which only included IPP and VPP intervention studies, also found a significant effect on SBP of -4.8 mmHg and on DBP of -2.2 mmHg. Recently, several new IPP and VPP intervention studies were published, most of which found no significant BP-lowering effect.[20,22,25,26] These studies were not included in the meta-analyses; thus, it is likely that findings from these meta-analyses overestimated the true underlying effect.

LTPs can be produced by 2 processes: fermentation and enzymatic hydrolysis. In fermented milk, IPP, VPP, and many other dairy peptides are produced from milk casein by the complex proteolytic activity of lactic acid bacteria. Enzymatic LTPs are formed by enzymatic hydrolysis of dairy protein (milk casein) by a single protease. Enzymatic LTPs have a less complex peptide breakdown pattern than does fermented LTP.

To further investigate the BP-lowering effect of IPP and VPP, we studied the effect of enzymatic LTPs on BP in a white population and performed 2 multicenter, randomized, double-blind, placebo controlled, crossover studies with two 4-wk intervention periods separated by a 4-wk washout period. In total, 162 Scottish subjects not receiving

antihypertensive treatment were included. We used ambulatory BP as the primary outcome measure to improve the precision of the BP values and to be able to detect any possible transient effects. In study 1, the effect of 5.8 mg IPP and 4.4 mg VPP was tested. In study 2, the effect of 2.7 mg IPP, 1.9 mg VPP, and 350 mg added potassium - similar to the level in one serving of fruit, vegetables, or a dairy product - was tested.

Materials and methods

Subjects

The studies were performed subsequently at 9 general practitioner sites located in Scotland. The Contract Research Organization (CRO; Quintiles Ltd, Bracknell, United Kingdom) was responsible for the management of both studies. The subjects were screened between November 2005 and April 2006. Subjects with untreated elevated BP were recruited in collaboration with general practitioners and qualified for inclusion in the studies if they were white and between 35 and 70 y of age. To minimize the risk of misclassification of a person's BP, office BP (inclusion criteria: SBP > 135 mmHg) and mean daytime (0900–2100) ambulatory BP (inclusion criteria: SBP 130–160 mmHg, DBP <100 mmHg) were measured at prescreening and at screening. In both studies, subjects were excluded if they had a body mass index (in kg/m2) <18 or >32, used protein supplements during the study or <4 wk before the study, had a recorded history of cardiovascular disease or other medical conditions, or had lifestyle habits that could influence the primary outcome of the studies.

All participants received both written and oral information and gave their written consent. Written approval for the studies was obtained from the Multicentre Research Ethics Committee (study 1: Central Manchester Local Research Ethics Committee, South Manchester, United Kingdom; study 2: West Glasgow Research Ethics Committee, Glasgow, United Kingdom). The studies were executed in accordance with Good Clinical Practice policy, according to the principles of the Declaration of Helsinki, revision 2000.

Design

The 2 studies had a double-blind, multicenter, placebo controlled, randomized full-crossover design and involved 2 treatments and two 4-wk intervention periods separated by a 4-wk washout period. Thus, each subject participated for 12 wk in the study. The studies were designed to compare the BP-lowering effect of a dairy product with LTPs and a dairy product with LTPs and enriched in potassium with that of a placebo product in

subjects with elevated BP. The recruited subjects were randomly assigned according to their daytime (0900–2100) ambulatory BP values, which were obtained at baseline. The subjects then consumed the active or placebo product for 4 wk. The treatments were crossed over after the washout and continued in the study for another 4 wk (*Figure 1*).

Test products

The test products were ready-to-drink yogurt drinks. The active products and placebo products used in the studies were similar in appearance, color, taste, and smell and were provided in nontransparent white cups that differed only in terms of the coding on the bottles. All products were provided by Unilever (Vlaardingen, Netherlands). The products were made from pasteurized semiskim milk that was acidified with a standard yogurt strain to produce a yogurt drink. The active substance was LTPs obtained by enzymatic hydrolysis of sodium caseinate. After hydrolysis, the powder was produced by spray-drying. This powder was added to the yogurt. The extra protein content achieved by adding hydrolyzed casein in the active product was corrected for in the placebo product by adding whey-protein isolate. Pectin for stability and sugar, fruit puree, and flavoring for taste were added. The dairy products contained naturally present potassium (150 mg/100 g). The active product used in study 2 was enriched with food-grade potassium gluconate, which provided 350 mg K. The nutritional composition of the products is shown in *Table 1*. In study 1, the subjects randomly received a daily dose of 200 g of dairy drink with 5.8 mg IPP and 4.4 mg VPP or placebo. In study 2, the subjects randomly received a daily dose of 100 g of dairy drink with 2.7 mg IPP and 1.9 mg VPP enriched with potassium (350 mg) or placebo. To assess compliance, the subjects were asked to return used and unused cups of the test products. The research staff and subjects remained blinded to the type of treatment during the study.

Table 1 *Nutrient Composition of the test products per 100 g of Dairy Drink.*[1]

Product	Study 1[2]		Study 2[3]	
	LTP	Placebo	LTP + potassium	Placebo
Energy (kcal)	81 (162)	82 (164)	85 (85)	85 (85)
Protein (g)	3.7 (7.4)	3.4 (6.8)	3.5 (3.5)	3.6 (3.6)
Carbohydrates (g)	13.0 (26.0)	12.2 (24.4)	13.5 (13.5)	13.1 (13.1)
Fat (g)	0.6 (1.2)	0.6 (1.2)	0.6 (0.6)	0.7 (0.7)
Calcium (mg)	97 (194)	91 (182)	93 (93)	97 (97)
Sodium (mg)	57 (114)	38 (76)	58 (58)	39 (39)
Potassium (mg)	142 (284)	140 (280)	471 (471)	138 (138)
Magnesium (mg)	9 (18)	9 (18)	9 (9)	10 (10)
LTP				
IPP (mg)	2.9 (5.8)	---	2.7 (2.7)	---
VPP (mg)	2.2 (4.4)	---	1.9 (1.9)	---

[1] Daily dose of the product is in parentheses. LTP, lactotripeptides; IPP, Isoleucine-Proline-Proline; VPP, Valine-Proline-Proline. [2] Treatment: 200 g of dairy drink/d. [3] Treatment: 100 g of dairy drink/d.

Diet

For the duration of the studies, the subjects were asked to maintain their normal diet and lifestyle, except that the subjects were instructed to not consume protein supplements. On the measurement days, the subjects were asked to refrain from strenuous exercise and to refrain from consuming specific fermented foods (eg, meat, fish and soy products, and foods containing licorice). The subjects were asked to consume fermented milk products and alcoholic beverages as accustomed to, but not to vary more than one volume unit between the visit days. Furthermore, the subjects were asked to fast and consume only water from 2200 onward on the evening before a visit day and were instructed to eat breakfast and consume the test products daily in the morning with breakfast and standardized between 2.5 and 3 h before the office BP measurement. On a visit day, no food or drink (except for water) was allowed after breakfast until after the office BP was measured. The subjects were asked to eat, on all subsequent visit days, exactly the same breakfast that they had consumed on the day of visit 1. Because of a possible BP effect of caffeine, each subject was asked to consume no more than one caffeinated beverage as part of his or her breakfast.

Blood pressure measurements

At baseline and after both intervention periods, the subject's 24-h ambulatory BP was measured (study 1: monitor A/A type Spacelabs 90217; Spacelabs Medical, Issaquah, WA; study 2: monitor A/A type TM-2430; A&D Company Ltd, Tokyo, Japan). The monitor was programmed to take readings on the nondominant arm every 20 min during the day and every hour during the night for a length of ≥25 h. The values obtained from the first hour were discarded. The subjects were instructed on how to operate the monitor and were asked to refrain from any strenuous activity. The subjects were asked to sit down if possible or stand still and relax their arms during the readings. Other than refraining from strenuous activity and avoiding the consumption of particular standardized food products, the subjects were instructed to maintain their usual lifestyle habits. The ambulatory BP measurement was automatically repeated if the measurement was not successful (eg, if the monitor malfunctioned or if the subject moved his or her arm during the assessment). It was preferable that all 24-h ambulatory BP measurements per subject were made on the same day of the week during the study. Daytime (0900–2100), nighttime (0000–0600), and the first 2 h after consumption of the product ambulatory BP were derived from the 24-h ambulatory BP measurements.

At baseline, after both intervention periods and after the washout period, office BP was measured on the subject's dominant arm on each visit. The subjects were asked to rest for 15 min; an automatic arm cuff (monitor A/A type Omron HEM 907; Omron Healthcare Europe BV, Hoofddorp, Netherlands) was fitted to obtain 6 successive measurements of office BP. When possible, the same monitor was used for each subject during the study. If an error occurred during any of the first 6 measurements of office BP, additional measurements were made. The mean of the last 4 successful measurements was calculated and used in the studies. For all subjects, measurements were made at a fixed time point during the day, preferably 2.5–3 h after consumption of the product. Measurements were performed between 0900 and 1300 on weekdays to reduce variability.

Additional measurements

Body weight was measured at baseline, after both intervention periods and after the washout period. The subjects were weighed after voiding and while not wearing shoes. Well-being was measured on a 5-point scale (1 = excellent and 5 = very poor) at baseline, after both the intervention periods and after the washout period. Safety indicators (measures of liver function, kidney function, and hematology) were analyzed in blood collected at baseline and after the last intervention period into a 4-mL EDTA

containing tube, an 8-mL clotting tube, and a 2-mL NaF containing tube. The subjects were asked to collect 24-h urine samples at baseline, after both intervention periods, and after the washout period. The urine was weighed, and the volume was measured. Urinary sodium, potassium, creatinine and, albumin concentrations were measured on a Roche Modular instrument (Hoffman La-Roche, Basel, Switzerland). The Roche ISE indirect method was used to measure sodium and potassium, the Roche Jaffe Kinetic Colormetric assay for creatinine, and the Roche BCG method for albumin.

All adverse events (AEs) experienced during the study were reported on a clinical report form. The intensity of the AE was graded on a 3-point scale (mild, moderate, or severe) and was reported in detail on the clinical report form. The relation of the adverse event to the treatment was also assessed by the investigator.

Statistical analysis

Raw data were collected by electronic data capture entry, and the resulting spreadsheets were archived at the Contract Research Organization; 100% of the inclusion and exclusion criteria and main outcome variables were verified, and 10% of all other data were verified. The treatment codes were broken after the blind data analysis.

Two statistical analyses were performed based on the intention-to-treat (ITT) population and on the per protocol population. The ITT population (primary population) consisted of all subjects who had been randomly assigned for treatment and had received at least one dose of the study product. Decisions about which subjects were to be excluded from the per protocol population were made during 2 data review meetings before deblinding of the data.

The values reported in text and tables are least-squares means ± SEMs based on the ITT population, except for baseline data, which are reported as means ± SD. Mixed linear models (analysis of covariance) were used to compare the differences between treatments. This procedure corrected for the imbalance between groups and missing values and was used to test the period effect and the carryover effect. The following variables were included in the models: treatment, period, and interaction. The following covariates were included as continuous variables and random effects and were left in the model if the variables were shown to have a significant effect: baseline BP value, age, sex, and weight change during the study. The absolute values for 24-h ambulatory BP and office SBP and DBP were statistically evaluated for differences between the active period and the placebo period within each subject.

Results of the power calculation showed that, for each crossover study, 48 subjects should have been sufficient to detect a change in SBP of 3 mmHg with a power

of 0.9 and one-sided α = 0.05. The analyses were performed by using SAS software (version 9.1.3; SAS Institute, Cary, NC).

Results

Subjects and compliance

A total of 263 subjects underwent screening for ambulatory BP, and 162 subjects were enrolled in the 2 studies: 69 subjects were randomly assigned to treatment in study 1 and 93 to treatment in study 2. Study 1 was completed by 64 subjects (1 person was excluded because of an abnormal laboratory results, 4 persons withdrew consent, and 2 persons were excluded because they could not comply with the visit schedule). Study 2 was completed by 91 subjects (1 person was excluded because of lymphedema, and 1 person was excluded because they did not tolerate the intervention product). The number of subjects screened and randomized overall and per study is shown in *Figure 1*. Treatment compliance was excellent. In both studies >95% of the drinks were consumed as indicated by the number of (empty) bottles returned. Scores for well-being were high at baseline and remained high throughout the studies (mean: < 2). Both Scottish study populations consisted of slightly overweight and older men and women with untreated elevated BP (*Table 2*).

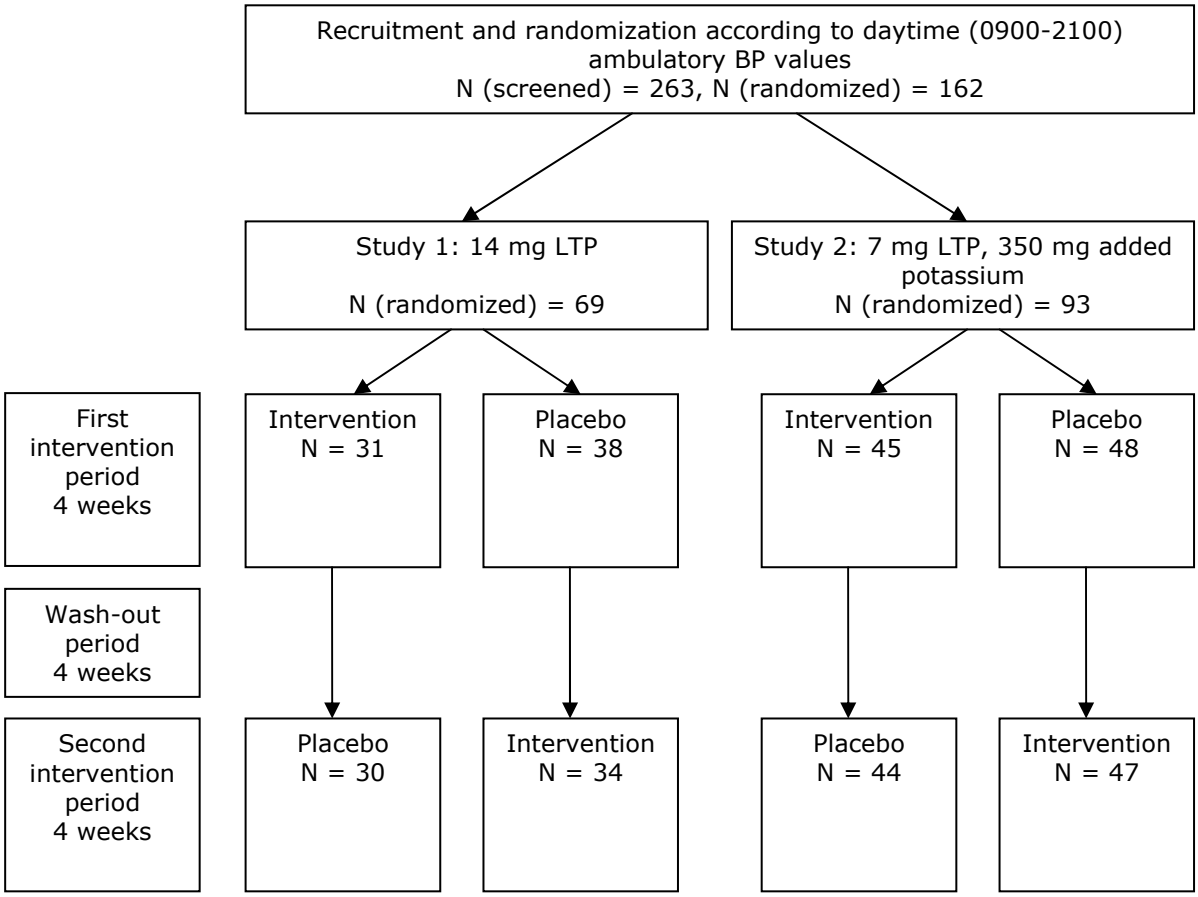

Figure 1 *Flow chart and design of the two double-blind, randomized, placebo-controlled cross-over studies in 162 Scottish subjects with untreated elevated blood pressure. LTP, lactotripeptides; BP, blood pressure.*

Table 2 *Baseline characteristics of the participants in study 1 and study 2.*[1]

	Study 1	Study 2
No. of subjects (male/female)	69 (45/24)	93 (47/46)
Age (y)	61.7 ± 6.7[2]	61.1 ± 7.0
Weight (kg)	78.8 ± 14.1	78.7 ± 14.4
Height (cm)	170.7 ± 8.8	170.4 ± 10.3
BMI (kg/m^2)	26.9 ± 3.2	26.9 ± 3.0
Ambulatory blood pressure (mmHg)		
- 24-h SBP	137.1 ± 8.1	139.2 ± 7.8
- 24-h DBP	81.6 ± 6.6	80.9 ± 6.4
- HR (beats/min)	75.0 ± 9.7	73.1 ± 8.9
Office blood pressure (mmHg)		
- SBP	147.4 ± 9.0	144.9 ± 8.3
- DBP	89.0 ± 5.9	87.0 ± 6.4
Urinary excretion (mmol/24h)		
- Sodium	131.8 ± 52.1	119.1 ± 47.7
- Potassium	75.2 ± 27.9	67.3 ± 20.5

[1] BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.
[2] Mean ± SD (all such values).

Blood pressure results

In study 1, no significant differences in 24-h SBP/DBP were found between treatments (LTPs compared with placebo) after 4 wk of intervention. Also, daytime SBP/DBP, nighttime DBP, and SBP/DBP 2 h after intake did not change significantly, except for nighttime SBP, for which the decrease was less pronounced after the LTP treatment than after placebo (*Table 3*). In study 2 also, 24-h SBP/DBP, daytime SBP/DBP, nighttime DBP, and SBP/DBP 2 h after intake did not change significantly between treatments (LTPs plus potassium compared with placebo) after 4 wk of intervention. Again, nighttime SBP decreased significantly less after the LTPs plus potassium treatment than after placebo (*Table 4*). In both studies, nighttime BP decreased by ≥15% after each treatment. In contrast with 24-h ambulatory BP, office BP decreased during both studies (SBP >5 mmHg), regardless of the intervention. In both studies, heart rate did not change significantly during the intervention, and no significant differences were found between treatments (LTPs compared with placebo) after 4 wk of intervention. Also, no carryover effects were found for the primary outcome models with covariates, except for a significant carryover effect in study 1 for nighttime ambulatory DBP (P = 0.001). Similar results were obtained in both studies in the per protocol analysis and when the covariates were not included (data not shown). Post hoc analysis of daytime BP during the first 4, 8,

and 12 h after consumption of the enzymatic LTP-containing drink showed no immediate and/or transient BP-lowering effects (data not shown).

Table 3 *Study 1: Ambulatory blood pressure (BP), office BP, and heart rate after 4 wk of intervention with the placebo drink or the lactotripeptides (LTP) dairy drink.*[1]

	Placebo (*n = 68*)	LTP[2] (*n = 65*)	Difference (95% CI)	*P* value[3]
24-h Ambulatory BP (mmHg)				
- SBP	135.8 ± 0.9[4]	136.4 ± 0.9	0.6 (-1.4;2.7)	0.55
- DBP	79.9 ± 0.6	80.2 ± 0.6	0.3 (-0.9;1.5)	0.63
- Heart rate (beats/min)	74.6 ± 0.8	75.0 ± 0.8	0.4 (-1.4;2.2)	0.68
Daytime (0900-2100) BP (mmHg)				
- SBP	141.0 ± 1.0	141.2 ± 1.0	0.2 (-2.2;2.6)	0.86
- DBP	83.1 ± 0.6	83.3 ± 0.6	0.2 (-1.2;1.6)	0.75
Night-time (0000-0600) BP (mmHg)				
- SBP	114.5 ± 1.2	117.7 ± 1.2	3.2 (0.3;6.1)	0.03
- DBP	67.0 ± 0.7	68.3 ± 0.7	1.2 (-0.8;3.3)	0.23
2-hour after intake (mmHg)				
- SBP	137.6 ± 1.6	138.1 ± 1.6	0.5 (-3.1;4.0)	0.80
- DBP	81.5 ± 1.2	82.2 ± 1.2	0.7 (-1.6;3.0)	0.54
Office BP (mmHg)				
- SBP	142.3 ± 1.0	142.3 ± 1.0	0.0 (-2.4;2.4)	0.98
- DBP	86.2 ± 0.8	85.7 ± 0.9	-0.6 (-1.9;0.7)	0.37

[1] SBP, systolic blood pressure; DBP, diastolic blood pressure. [2] Isoleucine-Proline-Proline and Valine-Proline-Proline. [3] ANCOVA, with baseline BP, age, sex and weight change as covariates. *P* values Are 2-sided. [4] Least-squares mean ± SEM (all such values).

Table 4 *Study 2: Ambulatory blood pressure (BP), office BP, and heart rate after 4 wk of intervention with the placebo drink or the lactotripeptides (LTP) + potassium dairy drink.*[1]

	Placebo (*n = 92*)	LTP[2] + potassium (*n = 92*)	Difference (95% CI)	*P* value[3]
24-h Ambulatory BP (mmHg)				
- SBP	138.0 ± 1.0[4]	137.1 ± 1.0	-0.9 (-3.0;1.3)	0.42
- DBP	80.7 ± 0.6	80.8 ± 0.6	0.1 (-1.4;1.6)	0.89
- Heart rate (beats/min)	73.7 ± 0.7	74.4 ± 0.7	0.8 (-1.2;2.7)	0.44
Daytime (0900-2100) BP (mmHg)				
- SBP	141.3 ± 1.1	140.3 ± 1.1	-1.0 (-3.5;1.5)	0.43
- DBP	82.8 ± 0.6	83.0 ± 0.7	0.3 (-1.4;1.9)	0.74
Night-time (0000-0600) BP mmHg)				
- SBP	115.7 ± 1.5	119.8 ± 1.4	4.1 (0.7;7.6)	0.02
- DBP	67.6 ± 1.1	68.3 ± 1.1	0.7 (-2.0;3.3)	0.60
2-hour after intake (mmHg)				
- SBP	138.5 ± 1.8	140.1 ± 1.7	1.5 (-3.0;6.1)	0.50
- DBP	80.0 ± 1.6	83.3 ± 1.5	3.3 (-1.0;7.6)	0.13
Office BP (mmHg)				
- SBP	135.6 ± 0.8	134.7 ± 0.9	-1.0 (-3.4;1.5)	0.43
- DBP	83.4 ± 0.7	82.3 ± 0.8	-1.1 (-2.6;0.3)	0.12

[1] SBP, systolic blood pressure; DBP, diastolic blood pressure. [2] Isoleucine-Proline-Proline and Valine-Proline-Proline. [3] ANCOVA, with baseline BP, age, sex and weight change as covariates. *P* values are 2-sided. [4] Least-squares mean ± SEM (all such values).

Other outcomes

Weight did not change significantly between the different treatment groups in the 2 studies. Also, no significant changes in urinary albumin, creatinine, and potassium were found between the treatment periods in both studies; urinary sodium was significantly higher after the LTP plus potassium treatment than after placebo in study 2 (*Table 5*).

In study 1, 44 subjects experienced a total of 81 AEs, which were determined not to be related to treatment. Most of the AEs were mild, and all subjects recovered. The 3 most frequent AEs were acute nasopharyngitis (n = 14), joint pain (n = 4), and dizziness and giddiness (n = 4). Two serious AEs (SAEs) were experienced, both of which lasted for 1 d and were determined not to be related to treatment; the subjects recovered from both SAEs. In study 2, 55 subjects experienced 101 AEs, which were determined not to be related to treatment. Most AEs were mild and all subjects recovered. The 4 most frequent AEs were hay fever (n = 8), head cold (n = 8), headaches (n = 7), and lower

back pain (n = 5). One SAE was experienced, which was mild; the subject recovered, and the SAE was determined not to be related to the study treatment.

Table 5 *Studies 1 and 2: urinary excretion of sodium and potassium (mmol/24-h) and the ratio of sodium to potassium at the end of the intervention periods.*

Urinary excretion (mmol/24 h)	Placebo	LTP[1] + Potassium	Difference (95% CI)	*P*-value[2]
Study 1				
- Sodium	122.1 ± 5.7[3]	132.0 ± 5.7	9.9 (-5.4;25.2)	0.20
- Potassium	73.6 ± 2.7	74.2 ± 2.7	0.5 (-6.7;7.8)	0.88
- Sodium:potassium	1.8 ± 0.1	1.9 ± 0.1	0.1 (0.0;0.3)	0.26
Study 2				
- Sodium	115.1 ± 5.8	132.7± 5.6	17.6 (3.6;31.5)	0.01
- Potassium	75.4 ± 3.0	78.5 ± 2.9	3.1 (-5.3;11.4)	0.47
- Sodium:potassium	1.6 ± 0.1	1.8 ± 0.1	0.2 (0.0;0.3)	0.08

[1] LTP, lactotripeptides (Isoleucine-Proline-Proline and Valine-Proline-Proline). [2] ANCOVA, with baseline BP, age, sex, and weight change as covariates. P values are 2-sided. [3] Least-squares mean ± SEM (all such values).

Discussion

The results of the present 2 multicenter crossover studies showed no antihypertensive effect of daily consumption of a dairy drink with enzymatic LTPs (5.8 mg IPP and 4.4 mg VPP) or enzymatic LTPs (2.7 mg IPP and 1.9 mg VPP) plus potassium (350 mg added) in a population of 162 Scottish subjects. The lack of effect on 24-h ambulatory and office BP confirm that whites may not benefit from LTP treatment to the extent previously reported for Asians. Possible explanations for the discrepant findings include differences in diet, genetics, or physical background between Asians and whites, such as intakes of sodium and fermented products, LTP dose per kilogram body weight, and baseline BP values.

We found a significantly greater reduction in nighttime ambulatory SBP in the placebo group than in the active group in both studies ($P < 0.05$), but no effect was found in the studies of nighttime ambulatory DBP. Nighttime BP values might have been less reliable than daytime and 24-h BP values because fewer measurements of nighttime BP were made every hour (from 0000 to 0600); daytime BP was measured every 20 min during the day (from 0900 to 2100). Whether the decrease in nighttime BP has any

physiologic relevance with respect to endorgan damage is unclear: during LTP treatment in both studies, BP still decreased by ≥15%, which is considered to be a healthy pattern.[29] This finding requires additional research focusing on LTPs and nighttime BP.

In study 2, the urinary excretion of sodium increased significantly with the LTPs plus potassium intervention compared with the placebo. Potassium excretion did not change significantly in any of the studies. Therefore, the 350-mg difference in potassium between the intervention and placebo groups was probably too small to reliably measure because other factors that can influence potassium excretion can have a larger effect. The increase in sodium excretion in study 2 might have been due to the potassium content of the intervention product, which triggered greater sodium excretion.[30,31] However, per the literature, a dose of 350 mg potassium might be too small to demonstrate a natriuretic effect.

A strength of the present studies was their randomized, double blind, placebo-controlled design. We chose to measure ambulatory BP during the screening period and to use this value as the primary study outcome. Studies have shown that ambulatory BP more accurately reflects treatment-induced decreases in BP than does office BP, because of a higher reproducibility over time and an absent or negligible ''white coat'' or placebo effect.[4] We observed in both studies a continuous decrease in office BP, regardless of the intervention. In contrast, 24-h ambulatory BP levels remained stable during the intervention periods. This finding supports the importance of using ambulatory BP to detect small changes in BP. Furthermore ambulatory BP has shown to be a better predictor of cardiovascular disease risk.[4] Compliance with the test product was excellent, and dropout rates were low. High dropout rates are considered to be one of the major drawbacks of a crossover design. Also, baseline characteristics, including initial BP levels, were similar between the treatment groups, and body weight did not change during the intervention periods in either study. No carryover effects were observed in any of the models with covariates, except for a significant carryover effect in study 1 for nighttime ambulatory DBP ($P = 0.001$).

A limitation of the present studies was the relatively short intervention period (4 wk). Although previous studies of LTPs observed BP changes after intervention periods as short as 1 or 2 wk, many showed significant effects on BP after 4- or 8-wk intervention periods. Furthermore, the doses of LTPs (2.7 mg IPP and 1.9 mg VPP/d) and potassium (350 mg added/d) in study 2 were chosen based on a hypothesized additive or synergistic effect of the combination of LTPs and potassium. However, on the basis of the results in study 1 (5.8 mg IPP and 4.4 mg VPP/d), it is unlikely that LTPs contributed to

an effect on BP in study 2. Therefore, the current design of study 2 may not have been sufficiently powered to detect BP changes due to a solely small increase in potassium intake (*Table 4*). A higher level of potassium, similar to 3 servings of good food sources of potassium (approximately 1 g/d) and proven to reduce BP in intervention studies[32,33], would have been a more appropriate amount to test.

In conclusion, we were unable to show a BP-lowering effect in whites after daily consumption of enzymatic LTPs (5.8 mg IPP and 4.4 mg VPP) or enzymatic LTPs (2.7 mg IPP and 1.9 mg VPP) with an additional amount of potassium (350 mg) using ambulatory BP as the primary outcome. The data do not support a BP-lowering effect of LTPs in whites.

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Contribution of the authors: The authors' responsibilities were as follows—LAJvM and RD: designed and monitored the studies, analyzed and interpreted the data, and wrote the manuscript; MMGK: designed the studies, interpreted the data, and critically evaluated the manuscript; and KvdZ: designed the studies and interpreted the data. The sponsor monitored the studies but was not involved in on-site data collection. All authors were working at the Unilever Food & Health Research Institute (Vlaardingen, Netherlands) at the time the studies were conducted. Unilever markets food products, some of which address cardiovascular risk factors.

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Chapter 6

Grape polyphenols do not improve vascular function in healthy men

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Abstract

Data suggest that polyphenol-rich products may improve endothelial function and other cardiovascular health risk factors. Grape and wine contain high amounts of polyphenols, but effects of these polyphenols have hardly been investigated in isolation in randomized controlled studies. The objective of this study was to test the chronic effect of polyphenol rich solids either derived from a wine-grape mix or grape seed on flow-mediated dilation (FMD). Blood pressure (BP) and other vascular function measures, platelet function and blood lipids were secondary outcomes. 35 healthy males were randomized in a double-blind, placebo-controlled crossover study, consisting of three 2-wk intervention periods separated by 1-wk washout periods. The test products, containing 800 mg of polyphenols, were consumed as capsules. At the end of each intervention period, effects were measured after consumption of a low fat breakfast (on average 751 kJ, 25% fat) and a high fat lunch (on average 3136 kJ, 78% fat). After the low fat breakfast the treatments did not significantly affect FMD. The absolute difference after wine-grape solids was -0.4% (95% CI -1.8 to 0.9; P = 0.77) and after grape seed solids 0.2% (-1.2 to 1.5; P = 0.94) compared to after the placebo treatment. FMD effects after the high fat lunch and effects on secondary outcomes showed also no consistent differences between grape solids and placebo treatment. In conclusion, consumption of grape polyphenols has no major impact on FMD in healthy men. Future studies should address whether grape polyphenols can improve FMD and other cardiovascular health risk factors in populations with increased cardiovascular risk.

Introduction

Endothelial function has predictive value for future cardiovascular events.[1-6] Non-invasive flow-mediated dilation (FMD) is a frequently used method to assess endothelial function in humans.[7,8] FMD is the difference in brachial artery diameter measured by ultrasound before and after a period of induced ischemia of the forearm by inflation of a blood pressure (BP) cuff. FMD is expressed as the percentage increase in brachial artery diameter after release of the BP cuff. As the dilation is mediated by nitric oxide, FMD is thought to reflect the bioavailability of nitric oxide.[7]

Evidence for a role of dietary flavonoids in the prevention of cardiovascular diseases (CVD) from epidemiologic and intervention studies is increasing.[9,10] Consumption of red wine has been proposed as explanation of the "French paradox" of relatively low coronary heart disease mortality rates in France, despite high intake of saturated fat.[10-12] This association has been attributed mainly to the high levels of grape polyphenols in the wine, although other wine constituents and ethanol could also have beneficial effects.[13] In vitro data suggest that red wine and its constituents induce vasodilatation by increasing production of nitric oxide. Furthermore, although several studies investigated the acute effects on endothelial function of red wine and other grape products in humans[14], evidence from randomized controlled intervention studies is still limited.[9,10,15] In addition, most studies have been performed in participants with elevated CVD risk[14,16-23], rather than in healthy participants and in the majority of studies the contribution of grape polyphenols to the observed effects has not been addressed.[14]

The objective of our study was to assess the effect of polyphenol rich grape solids on endothelial function and other cardiovascular health markers in healthy participants. To that end, we conducted a placebo-controlled, double-blind crossover study to measure whether daily intake of polyphenol rich solids either derived from a wine-grape mix or grape seed, provided at a dose of 800 mg of polyphenols, would affect responses on endothelial function and other CVD risk markers compared to placebo after 2 wk intervention. The wine-grape mix represents a product with a broad range of polyphenols (monomeric anthocyanins, catechins, flavonols, procyanidins and stillbenes and unidentified oligomers and polymers), while the grape seed primarily consists of monomeric catechins and unidentified oligomers and polymers.[24] Both the red wine (Provinols™) and the grape seed (Leucoselect™) solids have been shown to affect vascular function in animals studies.[25,26] We studied effects after a low fat breakfast and after a high fat lunch as it has been suggested that red wine might counteract endothelial

dysfunction caused by a vascular function stressor, such as cigarette smoking or a high-fat meal.[14,19,27-29]

Methods

Participants

35 Apparently healthy males, aged between 18 and 45 y were recruited. Participants were not included if they reported current or previous metabolic diseases, gastrointestinal disorders, or CVD, had a body mass index (in kg/m^2) <18 or >32, had blood markers (WBC, RBC, haemoglobin, haematocrit and platelet count), plasma liver enzymes (ALAT, ASAT and γ-GT), BP (systolic BP ≥160 mmHg and/or diastolic BP ≥95 mmHg), fasting blood lipids in serum (total cholesterol >8 mmol/L, total cholesterol/HDL cholesterol ratio >8 and triglycerides >4 mmol/L), urinary protein and glucose markers outside normal reference range and/or lifestyle habits that could influence the primary outcome of the studies. All participants received both written and oral information about the study and gave their written consent. The study took place from November-December 2003 at Unilever Research and Development Vlaardingen, the Netherlands. The protocol was approved by the Medical Ethics Committee of Wageningen University, the Netherlands.

Design and randomization procedure

The study had a double-blind, placebo-controlled randomized full crossover design with a three days run-in period, 3 two wk intervention- and 2 one wk washout periods. Participants were randomly divided over the 3 treatment orders according to a complete balanced design.[30] The 3 treatments consisted of two different grape solids and a placebo. Measurements were performed following the run-in period (baseline assessment), and at the end of each of the intervention periods. The effect of the treatment under basal conditions and after a fat-load was investigated by measurements after a low fat breakfast and 3 hours after a high fat lunch, and these responses were compared to the placebo response (*Supplemental Figure 1*). Staff and participants remained blinded toward the type of treatment during the study and data analysis.

Test products

The placebo and grape solids (wine-grape and grape seed) were consumed in 6 capsules per d each of 500 mg. The placebo capsules contained micro-crystalline cellulose. The

total polyphenol content of both grape solids in the 6 daily capsules was 800 mg, according to gallic acid equivalents; 1405 mg of the wine-grape solids contained 550 mg polyphenols of Provinols™ (Seppic, France), and 250 mg polyphenols of MegaNatural™ Rubired grape juice (Polyphenolics, US); 2547 mg of the grape seed solids contained 800 mg polyphenols of Leucoselect™ Phytosome® (Indena, Italy).

Identification by HPLC analysis of monomeric polyphenols revealed that the 550 mg of wine solids contained 18.8 mg anthocyanins, 6.9 mg phenolic acids, 4.0 mg catechins, 0.4 mg flavonols and 0.1 mg stilbenes and that 250 mg of grape juice contained 118.5 mg anthocyanins, 2.7 mg phenolic acids, 0.2 mg catechins, 0.5 mg flavonols and 0.1 mg stilbenes. However, the majority of polyphenols in these solids are oligomers and polymers that could not be identified by this analysis. According to analysis by Gabetta *et al.*[31] the polyphenolic profile of the grape seed consists of approximately 15% (+)-catechin and (-)-epicatechin and 85% (-)-epicatechin 3-O-gallate and unidentified oligomers and polymers.

During the 2-wk treatment periods, volunteers consumed daily three capsules with breakfast and three with dinner. On the test day after each treatment period, participants ingested three capsules with a low fat breakfast and three capsules with a high fat lunch. To assess compliance, participants were asked to register their intake of the capsules on each day in the intervention period.

Background diet and test meals

Volunteers were asked to refrain from vitamin supplementation as from the day of the screening till the end of the study. On the days prior to a test day standardized meals were provided to the participants. On the day prior to the test day participants were asked to avoid eating fruit, drinks high in fruit, apple-sauce and alcoholic drinks, chocolate and to limit their intake of tea to two cups. To assess compliance, participants were asked to record their food intake on the day prior to the first test day, and were requested to follow a similar dietary pattern on the day prior to the 3 remaining test days.

To start a test day in a fasting state, participants had to refrain from foods for 10 hours and drinks for 8 hours, except (mineral) water. During the test days all foods and drinks were provided, including breakfast (water and max 2 sandwiches with low energy filling, 410-1092 kJ, fat 1-9 g, protein 2-15 g, carbohydrates 16-32 g per d), and all products were low in antioxidants, flavonoids, fat, glucose and caffeine. The high fat lunch was similar to the low fat breakfast, but consumed together with 143 mL liquid whipping cream (2385 kJ, fat 61 g, protein 3 g, carbohydrates 4 g).

Measurements

BP was assessed at the left arm by a calibrated Omron IC device. The BP cuff around the left arm was three times inflated and BP values were recorded. FMD was assessed by echo-Doppler (Esaote, Genova, Italy) on each test day at baseline and 3 h after the fat challenge at the subject's right arm in recumbent position by using a 7.5MHz transducer and automatic tracking vessel wall movement software (Wall Track System™). The brachial artery was imaged in the upper arm (about 5 cm above the antecubital crease) in B-mode. The baseline arterial diameter was measured in M-mode (motion-mode). Following this, a cuff placed around the upper arm was inflated to 200 mmHg for 4 minutes. After deflation of the cuff the arterial diameter was measured at 20-second intervals for 5 minutes. FMD was defined as the maximal percentage diameter change of the post-occlusion arterial diameter measurement relative to the mean of the corresponding 3 baseline measurements. The device automatically calculated pulse wave transit time starting with the electrical signal reflecting the ventricular systole to the arterial wall movement of the brachial artery measured by the echo-transducer. The wall movement results from the pulse wave propagation initiated by each ventricular contraction, and is a measure of arterial stiffness.

As an explorative vascular function tool, the HDI/PulseWave™ CR-2000 Research Cardio Vascular Profiling System (Hypertension Diagnostics, Minnesota, USA) was included in the study protocol, and used according to the indications of the supplier. The device generates a radial artery waveform by tonometry, and by using an algorithm, calculates the large (C1) and small (C2) artery elasticity index. C1 reflects capacitive arterial compliance and C2 oscillatory or reflective arterial compliance. Detailed information on measurement of platelet function and blood lipids is available at http://jn.nutrition.org.

Additional measurements

Body weight was measured without shoes and heavy clothing at each visit on an electronic device. Height was measured during screening using a wall-mounted stadiometer. Body mass index was computed based on these two measurements. All adverse events experienced during the study were reported and coded by the study physician.

Statistical analysis

Data collected on paper were 100% verified and all data were archived at Unilever Research and Development Vlaardingen, The Netherlands. Data were analyzed according

to the intention-to-treat principle. The values reported in text and tables are Least-Squares Means (LSMeans) ± SEMs, except for baseline data, which are reported as means ± SD. Results of the power calculation showed that 35 participants would be sufficient to detect a clinically relevant FMD improvement of 2.5% (power of 0.8, α = 0.05). Data were analyzed using an analysis of covariance (Proc Mixed, SAS software V9.1, Cary, NC USA) to compare the absolute differences after the intervention periods between the two treatments and placebo. The model terms were treatment, period and their interaction. Baseline values were used as covariables. For the FMD analyses baseline diameter of the brachial artery was also included in the model. To test the differences of both interventions with the placebo treatment a multiple comparison according to Dunnett was performed.

Results

Participants and compliance

Of the 53 volunteers who responded to advertisements and underwent screening, 11 were excluded based on their blood and urine results, and 7 were excluded by lottery. The remaining 35 participants were randomized and completed the study. Compliance of the test products was 86% as indicated by registration of test product consumption by participants. The participants were young healthy males with BMI, vascular function parameters and lipid levels in the normal range and not receiving medication that might affect outcome variables or bioavailability of the test products (*Table 1*).

Table 1 *Baseline characteristics of the healthy male participants.*[1]

Characteristics	
Age (y)	31.4 ± 9.0
Height (cm)	183.6 ± 7.2
Weight (kg)	78.1 ± 10.5
BMI (kg/m^2)	23.2 ± 2.5
FMD (%)	5.6 ± 3.6
Brachial artery diameter (mm)	4.22 ± 0.54
Systolic BP (mmHg)	123.0 ± 10.5
Diastolic BP (mmHg)	72.6 ± 9.2
Heart Rate (beats/min)	62.7 ± 7.8
Serum Total cholesterol (mmol/L)	4.8 ± 1.1
Serum HDL cholesterol (mmol/L)	1.4 ± 0.3
Serum LDL cholesterol (mmol/L)	3.2 ± 1.0
Serum Triglycerides (mmol/L)	1.0 ± 0.4

[1] Values are means ± SD, n=35.

Vascular function

Neither FMD after low fat breakfast, nor FMD after high fat lunch were significantly affected by the treatments (*Table 2*). The mean difference in maximal response in diameter within 5 minutes of measurement after breakfast between the end of the intervention period and the end of the placebo period treatment was -0.4% (95% Confidence Interval (CI) -1.8; 0.9, P = 0.77) for the wine-grape solids and 0.2% (-1.2; 1.5, P = 0.94) for the grape seed solids. After the high fat challenge, the mean differences in FMD vs. placebo were 0.7% (-0.6; 2.0, P = 0.49) after the wine-grape solids intervention and -0.2% (-1.5; 1.1, P = 0.94) after the grape seed solids intervention.

No significant effects on BP were found. The mean difference between wine-grape and placebo in systolic BP was -1.3 mmHg (-4.7; 2.2, P = 0.70) and for diastolic BP -1.6 mmHg (-4.4; 1.3, P = 0.44). For grape seed differences were -0.6 mmHg (-4.0; 2.9, P = 0.92) for systolic BP and -0.6 mmHg (-3.4; 2.2, P = 0.88) for diastolic BP. In addition no significant differences were found for heart rate, pulse wave transit time and large arterial elasticity and small arterial elasticity (*Table 2*). Results for all these vascular function measures were similar after the fat challenge (data not shown, except for FMD).

Table 2 *Vascular function of men after 2-wk interventions with wine-grape solids, grape seed solids, and placebo.*[1]

	Placebo	Difference compared to placebo	
		Wine-grape	Grape seed
FMDmax (%)			
after low fat breakfast	3.9 ± 0.5	-0.4 (-1.8;0.9)	0.2 (-1.2;1.5)
after high fat lunch	4.5 ± 0.5	0.7 (-0.6;2.0)	-0.2 (-1.5;1.1)
Systolic BP (mmHg)	118.6 ± 1.2	-1.3 (-4.7;2.2)	-0.6 (-4.0;2.9)
Diastolic BP (mmHg)	70.8 ± 1.0	-1.6 (-4.4;1.3)	-0.6 (-3.4;2.2)
Heart Rate (beats/min)	61.4 ± 1.1	1.2 (-1.9;4.3)	0.0 (-3.1;3.1)
Pulse wave transit time (ms)	168.7 ± 2.9	4.2 (-4.0;12.5)	0.8 (-7.4;9.1)
Large arterial elasticity (C1, mL/mmHgx10)	18.3 ± 0.7	-0.1 (-1.5;1.2)	-0.2 (-1.6;1.1)
Small arterial elasticity (C2, mL/mmHgx100)	10.3 ± 0.3	0.5 (-0.3;1.3)	0.3 (-0.5;1.1)

[1] Values are LSmeans ± SEM or LSmeans (95% CI), n=35; Data were analyzed by ANCOVA with the baseline value as the covariable or for FMD, baseline FMD and baseline brachial artery diameter as covariables.

Platelet function and blood lipids

Measures of platelet function did not differ after the treatments compared to placebo although platelet activation in whole blood induced by a combination of collagen and epinephrine after the fat challenge tended to be enhanced due to the wine-grape treatment compared to placebo [14.5 s (1.3 to 27.6; P = 0.06)] (*Supplemental Table 1*). Platelet aggregation in platelet rich plasma induced by ADP (10 μmol/L) after the fat challenge also tended to be reduced [-6% (-12.1 to 0.1; P < 0.10) after the wine-grape treatment compared to placebo (*Supplemental Table 1*).

After the wine-grape intervention, serum total cholesterol was lower after the low-fat breakfast [-0.16 mmol/L (-0.31 to -0.01; P = 0.07)] and after the high-fat lunch [-0.20 mmol/L (-0.33 to -0.07; P < 0.01)] compared to after the placebo treatment. The serum total cholesterol concentration did not differ after the grape seed and placebo treatments. Compared to the placebo treatment, serum triglycerides were reduced by the wine-grape treatment after the high fat lunch [-0.29 mmol/L (-0.54 to -0.04; P < 0.05)] and tended to be reduced by the grape seed treatment [-0.25 mmol/L (-0.51 to 0.00; P ~ 0.1)]. Serum LDL- and HDL-cholesterol concentrations did not differ after the treatments compared to after placebo (*Supplemental Table 2*).

Other outcomes

Body weights did not differ after the treatment periods compared to after placebo. Participants experienced a total of 39 adverse events, which were determined to be not related to any of the test products. Most of the adverse events were mild, and all participants recovered. The 4 most frequent events were acute nasopharingitis (*n* = 7), headache (*n* = 6), diarrhea (*n* = 4) and flatulence (*n* = 4).

Discussion

Possible protective effects of wine (constituents) on CVD risk factors have been suggested by in vitro, and animal studies[15], whereas data from well-controlled human studies are scarce.[9] The present hypothesis-testing study in 35 healthy males investigated the potential of the polyphenolic part of two different grape solids in a randomized, double-blind, placebo-controlled cross-over setting. We found no significant differences in FMD, and other vascular function markers after grape solids treatment compared to after placebo treatment, despite the large range of indicators assessed (FMD, BP, heart rate, pulse wave transit time, large and small arterial elasticity) and the considerable high polyphenol content of the solids.

The strength of this study is its randomized double-blind placebo-controlled design. In addition, all participants completed the study and compliance to the test capsules was high. Furthermore, unlike most other studies in this field, encapsulation of the solids ensured blinding of the test products. To reduce variation we measured FMD on all test days in each participant on the same day of the week at the same time point and by the same observer. We only included men in this study to decrease variation in FMD measurements due to hormonal fluctuations in women.[32] Effects of the grape solids on FMD and other outcomes were measured after a low fat breakfast and after a high fat lunch as it has been suggested that a high fat meal or other vascular function stressors, such as smoking, may acutely impair FMD and that this impairment may be prevented by intake of red wine constituents.[14,19,27-29] For this proof of principle study we selected extracts with a high polyphenols content (approximately 80% of the dry weight) and with a large range of polyphenols subtypes. The polyphenol dose (800 mg polyphenols per d), was similar to the dose tested in other intervention studies addressing the chronic effects (2-8 wks) of the polyphenolic fraction of grape consumption on endothelial function and/or BP.[23,33-37]

The study design also had its limitations. The intervention period was relatively short, but we expected to show benefits of polyphenols within 2 weeks, because previous studies investigated acute and several weeks effects on FMD with positive outcomes.[16,28,29,33-35,37,38] The wash-out period was 1 wk, which was considered more than sufficient to prevent carry-over effects, because polyphenols are rapidly metabolized and their maximum plasma concentrations are usually reached within hours.[39] Furthermore, only participants without elevated CVD risk factors were included, whereas in most other studies investigating the effects of grape polyphenols on vascular function, the focus was on participants with elevated CVD risk.[14] Indeed, our study population appeared to be largely insensitive to the fat challenge, indicating a balanced and healthy vascular function. Consequently, grape solids may still protect vascular function in participants at increased risk for CVD. In addition, our study may have not been optimally designed to answer the secondary objectives. The trends we found on platelet function were not consistent across the different methods we applied. This may reflect differences in sensitivity of the applied methods towards specific platelet activation pathways.[40] More consistent seem the total cholesterol-lowering effects after wine-grape treatment in this normolipidemic population. Some human studies also found beneficial effects of grape products on lipid profile, however evidence is not consistent.[10,13] Moreover, triglycerides were significantly lower after the high fat challenge for the wine-grape treatment compared to placebo treatment and tended to be lower after grape seed consumption. This effect may be explained by inhibition of chylomicron secretion from intestinal cells by the grape polyphenols.[41] It should be noted that the observed benefits could well be chance findings and require confirmation in a separate study, including multiple blood sampling in the postprandial phase. We investigated effects on blood lipids as secondary outcome with the same wine-grape treatment in 30 hypertensive participants and were not able to confirm the lipid lowering effects (unpublished data). To directly test effects on blood lipids, it would require a cross-over study with about 60 subjects to detect differences of a similar magnitude as observed here.

In conclusion, this randomized controlled study does not support that grape polyphenolic compounds have important potential to improve endothelial function and related cardiovascular health markers in a healthy population. Future studies should focus on the potential of grape polyphenols for improving endothelial function and cardiovascular health in participants with an elevated cardiovascular risk profile with interventions of sufficient duration to accurately allow assessment of longer term effects.

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Conflicts of interest: LAJM, PLZ, HCMK and RD are employees at Unilever R&D Vlaardingen. Unilever markets food products, some of which address cardiovascular risk factors.

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Online Supplemental Material

Blood platelet and blood lipids measurements

Blood was collected 1.75 h after the low fat breakfast and 3.75 h after the high fat lunch. Platelet activation was determined in citrated blood using several experimental approaches. First, it was measured in whole blood to ensure a physiological environment as close as possible using the Platelet Function Analyser (PFA 100). PFA-100 represents the different processes that are involved in thrombus formation with increased PFA-100 values indicating a less thrombotic state of the blood. Whole blood is aspirated through a collagen plus epinephrine or collagen plus ADP coated filter that induces platelet activation leading to thrombus formation and occlusion of the filter. The time required to occlude the filter reflects the capacity of blood platelets to contribute to thrombus formation. Additionally, platelet aggregation in an optical aggregometer was measured in platelet-rich plasma derived from citrated blood using a centrifugation step, according to the method of Born (Born, 1967). The platelet count was adjusted to 200,000 platelets/µL. After a resting period of 30 min at room temperature, the suspension was used for experiments. Platelets were activated separately by two concentrations of adenosine diphosphate (ADP; 5 and 10 µM) or collagen. Total cholesterol, LDL, HDL and triglycerides were measured by means of a Hitachi 912 auto analyzer using the enzymatic colorimetric test CHOD-PAP (Roche, cat. no. 1489232), the homogenous enzymatic colorimetric test "LDL cholesterol plus 2nd generation" (Roche cat. no. 04714423190), the "HDL cholesterol plus 3rd generation" (Roche, cat. no. 04713109 190), and the enzymatic colorimetric test GPO-PAP (Roche, cat. no. 1488872), respectively.

All measurements were performed in each participant on the same day of the week at the same time of the day by the same observer.

References

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Supplemental Table 1 *Platelet function of men after 2-wk interventions with wine-grape solids, grape seed solids, and placebo.*[1]

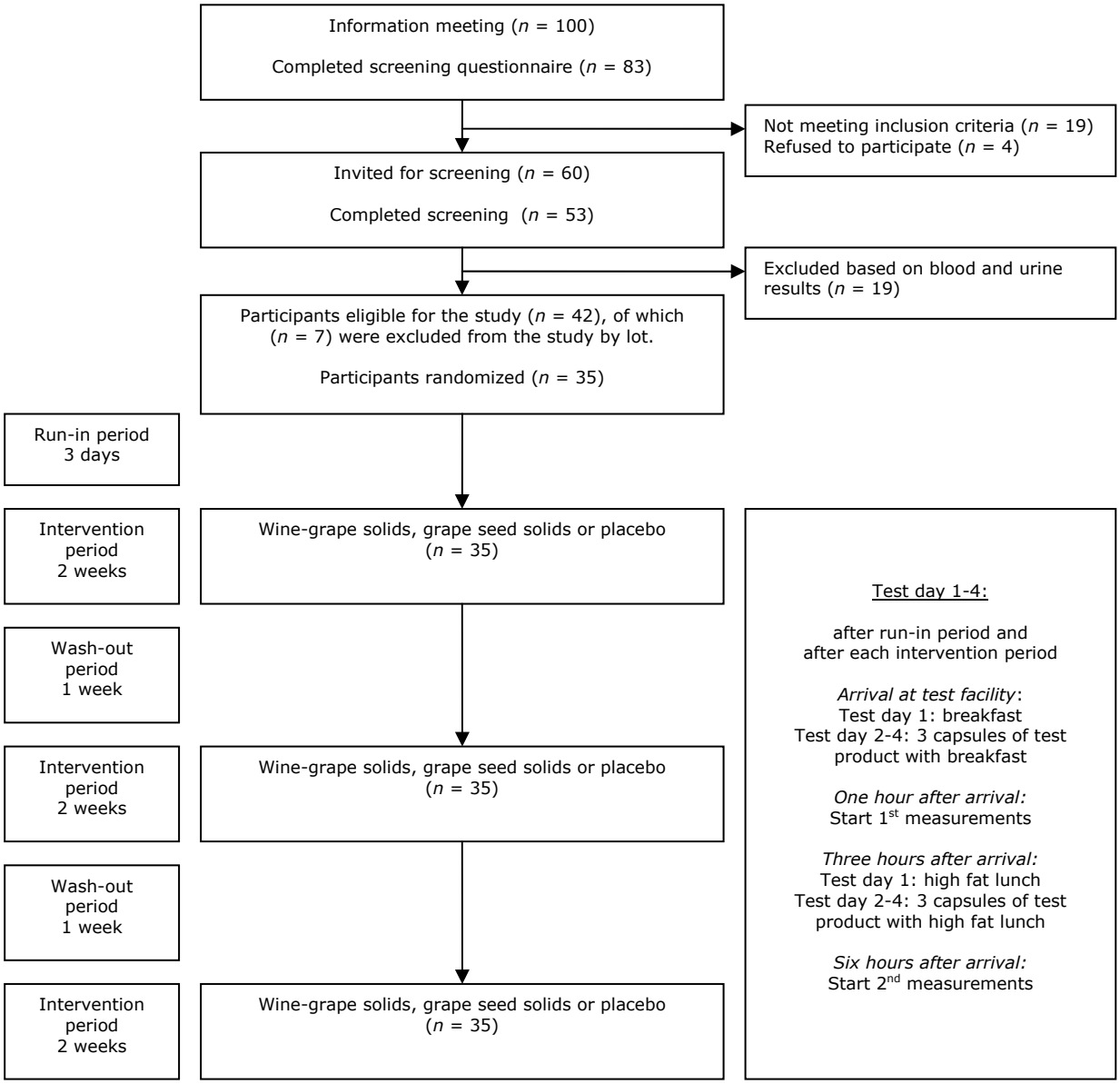
	Placebo	Difference compared to placebo	
		Wine-grape	Grape seed
PFA[2] 100 epinephrine coated cartridge (s)			
after low fat breakfast	145.4 ± 7.3	7.8 (-5.0;20.6)	6.5 (-6.4;19.3)
after high fat lunch	138.9 ± 6.3	14.5 (1.3;27.6)	7.5 (-5.8;20.7)
PFA 100 ADP[3] coated cartridge (s)			
after low fat breakfast	80.4 ± 1.6	0.4 (-3.5;4.3)	0.9 (-3.0;4.7)
after high fat lunch	77.9 ± 1.9	2.1 (-2.5;6.8)	0.5 (-4.2;5.1)
Aggregometry ADP 10 μmol/L-induced (%)[4]			
after low fat breakfast	71.3 ± 2.4	1.0 (-4.5;6.5)	1.2 (-4.5;6.8)
after high fat lunch	72.9 ± 2.5	-6.0 (-12.1;0.1)	-4.2 (-10.2;1.7)
Aggregometry Collagen-induced (%)			
after low fat breakfast	73.6 ± 3.1	-2.6 (-9.3;4.0)	-2.0 (-8.8;4.7)
after high fat lunch	70.2 ± 4.2	-5.9 (-15.5;3.7)	-4.5 (-13.9;4.9)

[1] Values are LSmeans ± SEM or LSmeans (95% CI), n=35; Data were analyzed by ANCOVA with the baseline value as the covariable. [2] PFA, Blood platelet function. [3] ADP, Adenosine diphosphate. [4] Aggregometry ADP 5 μmol/L-induced (%), was also measured, but is not reported.

Supplemental Table 2 *Lipid levels of men after 2-wk interventions with wine-grape solids, grape seed solids, and placebo.*[1]

	Placebo	Difference compared to placebo	
		Wine-grape	Grape seed
Total cholesterol (mmol/L)			
after low fat breakfast	4.66 ± 0.07	-0.16 (-0.31 ; -0.01)	-0.10 (-0.25 ; 0.06)
after high fat lunch	4.78 ± 0.07	-0.20 (-0.33 ; -0.07)[§]	-0.08 (-0.21 ; 0.06)
LDL-C (mmol/L)			
after low fat breakfast	3.07 ± 0.06	-0.12 (-0.27 ; 0.04)	-0.07 (-0.23; 0.08)
after high fat lunch	3.02 ± 0.06	-0.11 (-0.25 ; 0.02)	-0.03 (-0.17 ; 0.10)
HDL-C (mmol/L)			
after low fat breakfast	1.35 ± 0.03	-0.00 (-0.05 ; 0.05)	0.03 (-0.02 ; 0.08)
after high fat lunch	1.31 ± 0.03	-0.01 (-0.06 ; 0.05)	0.02 (-0.04 ; 0.08)
Triglycerides (mmol/L)			
after low fat breakfast	1.06 ± 0.08	-0.13 (-0.33 ; 0.06)	-0.13 (-0.33 ; 0.06)
after high fat lunch	1.91 ± 0.15	-0.29 (-0.54 ; -0.04)[#]	-0.25 (-0.51 ; 0.01)

[1] Values are LSmeans ± SEM or LSmeans (95% CI), n=35; Data were analyzed by ANCOVA with the baseline value as the covariable; [#] $P < 0.05$; [§] $P < 0.01$; 2-sided P values for the difference from placebo (Dunnett test).



Supplemental Figure 1 *Flowchart and design of the randomized controlled cross-over study in 35 healthy men.*

Chapter 7

Folic acid improves vascular reactivity in humans: a meta-analysis of randomized controlled trials

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Abstract

Background: The effect of folic acid on endothelial function, a prognostic factor for cardiovascular diseases, is not well established. We calculated this effect in a meta-analysis of randomized, double-blind, placebo-controlled trials in humans.

Objective: The objective of the study was to quantify the effect of folic acid on endothelial function, as measured with the use of flow-mediated dilatation (FMD).

Design: We conducted a meta-analysis of randomized, double-blind, placebo-controlled folic acid trials evaluating endothelial function. Trials were identified through MEDLINE (1966–15 Sept 2005), by hand-searching of references, and by contact with investigators for unpublished results. Two of us (AdB and RD) independently extracted trial data. A pooled estimate was calculated by using random-effects meta-analysis. Previously defined stratified analyses were conducted to explore the influence of study characteristics.

Results: Of 163 identified studies, 14 met inclusion criteria and provided data on 732 persons. Evidence for publication bias was not obvious. In the overall pooled estimate, folic acid improved FMD by 1.08 (95% CI: 0.57, 1.59; P = 0.0005) percentage points over placebo. Of the study characteristics, only folic acid dose significantly influenced the outcome. Post hoc analysis, which should be interpreted with caution, seemed to indicate a dose-response effect: the change in FMD was -0.07 (95% CI: -0.37, 0.22) percentage points at doses between 400 and 800 μg/d, 1.37 (95% CI: 1.12, 1.54) percentage points at doses of 5000 μg/d, and 2.04 (95% CI: 1.43, 2.65) percentage points at doses of 10 000 μg/d.

Conclusion: This study indicates that high doses of folic acid improve endothelial function, which could potentially reduce the risk of cardiovascular disease.

Introduction

A large amount of epidemiologic evidence links elevated homocysteine concentrations to an increased risk of cardiovascular disease (CVD).[1,2] This linkage has initiated the execution of secondary prevention trials testing whether homocysteine lowering therapy reduces the risk of recurrent CVD events.[3] Because the B vitamin folic acid and, to a lesser extent, vitamin B-6 and vitamin B-12 lower homocysteine concentrations[4], they are used in such trials. Many of these trials are ongoing, and data from 4 of them have been published, showing disappointing results.[5-8] A combined analysis of these 4 trials lacked the power to detect significant differences. However, the CIs around the summary risk estimates of these 4 trials were compatible with a 10% lower risk of ischemic heart disease and a 20% lower risk of stroke associated with a 25% lower homocysteine concentration.[9] Furthermore, we should also bear in mind that these secondary prevention trials typically look at risk reduction after short-term treatment in high-risk subjects, and thus these results should not be generalized to the overall population. Indeed, a beneficial effect of folic acid fortification on stroke mortality in the United States and Canada was recently reported.[10]

Although folic acid may not be able to reverse advanced atherosclerosis in CVD patients, it may affect the early stages of the CVD process, such as endothelial dysfunction.[11] This possibility has not been investigated in a systematic way. Endothelial function can be measured by the degree of flow-mediated dilatation (FMD).[12] FMD represents the ability of the brachial artery to dilate in response to ischemia-induced hyperemia in the forearm, and as such it reflects the bioavailability of the endogenous vasodilator nitric oxide (NO). In the present study, we systematically evaluated the effect of folic acid (with or without vitamin B-6, vitamin B-12, or both) on FMD in humans by performing a meta-analysis of randomized, placebo controlled clinical trials.

Study methods

Strategy to search randomized trials

The Quality of Reporting of Meta-analyses standards[13] were followed during all phases of the design and implementation of the present analysis. Included studies were randomized clinical trials that measured vascular reactivity by using the percentage of FMD (%FMD) after folic acid supplementation without a vascular challenge (such as a methionine or fat load). Trials were identified by searching the MEDLINE database from

1966 to 15 September 2005 with the use of the search terms homocysteine OR folate OR folic acid OR vitamin B-12 OR cobalamin OR vitamin B-6 OR B-6 and flow mediated OR flow-mediated OR endothelium-dependent OR vasomotor OR vasoacti* OR "blood flow" OR brachial* OR intima OR vasodilat* OR dilat* OR circulation OR endothel* OR distensibility OR microcirculat* OR micro-circulation OR vascular resistance OR wave OR plethysmography OR "blood supply" OR claudication OR cold hands.

We limited the search to clinical trials conducted in humans aged >19 y and published in the English language. Furthermore, we hand-searched the reference lists of the articles obtained through MEDLINE and of conference abstract books for additional studies.

Trial review

A flow chart of the selection of the included trials is given in *Figure 1*. The MEDLINE search identified 158 studies. The abstracts of these studies were screened independently by 2 of us (AdB and RD). After the exclusion of 127 studies, the remaining 31 studies were assessed more specifically, including a review of their reference lists. This resulted in the identification of 4 additional studies for inclusion.[14-17] Finally, 1 other trial was identified in a conference abstract book, for a total of 36 studies. Two of us (AdB and RD) independently evaluated these studies in detail - reviewing the complete publication, if available - which left 13 published trials[18-30] and 1 then-unpublished trial (M Olthof, personal communication, 22 November 2005; now:[31]) that met the inclusion criteria. The inclusion and exclusion decisions were unanimous.

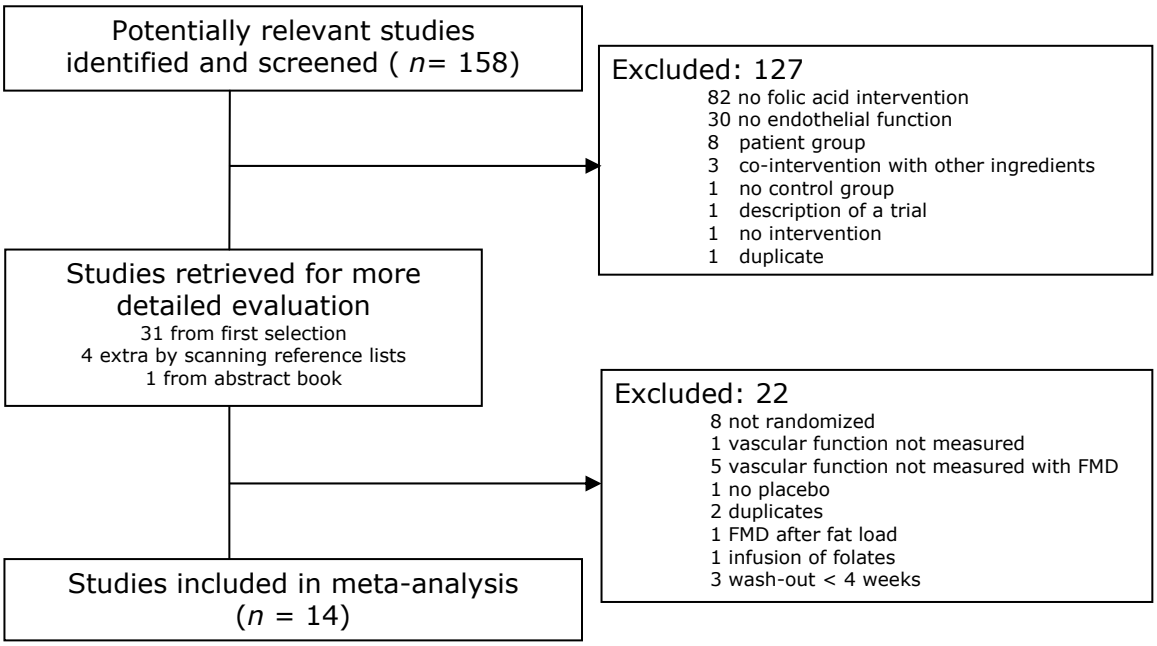

Figure 1 *Flow diagram of showing the process (and the reasons) of selecting and excluding studies for this meta-analysis.*

Data extraction

For each of the 14 studies meeting the inclusion criteria, data on study design, population, sample size and number of dropouts, intervention type, dose, and duration were independently extracted (by AdB and RD). Standard forms in EXCEL were used to calculate the net change in %FMD after folic acid supplementation compared with that after administration of placebo and also to calculate the SE of this change (*see* Statistical analysis). Study quality was also independently assessed (by AdB and RD) according to the criteria for quality assessment of randomized clinical trials developed by Delphi consensus.[32] The 9 criteria were treatment allocation (randomized = 1 point), similarity of groups at baseline with respect to the most important prognostic indicators (1 point), eligibility criteria (specified = 1 point), blinding (treatment allocation blinded = 1 point, outcome assessment blinded = 1 point, care provided blinded = 1 point, and patients blinded = 1 point), measures of variability presented for FMD measurement (yes = 1 point), and intention-to-treat analysis (yes = 1 point; studies with no drop-outs received 1 point for this criterion). Thus, the highest score a trial could get was 9 points.

We assessed the validity of data extraction by comparing the independently abstracted results for concordance. Discussion and review of the original manuscript resolved any discrepancies between the results abstracted by the 2 independent investigators.

Exposure and outcome variables

Supplementation with folic acid, vitamin B-6, and vitamin B-12

As exposure variable, we used the dose of folic acid and vitamin B-6 or vitamin B-12 (or both) rather than the plasma concentration of these B vitamins or of homocysteine. We did this because the absence of a gold standard method makes it very difficult to compare plasma concentrations between studies, and thus there are great variations between different laboratories.[33,34] In addition, circulating concentrations of B vitamins are known to vary and therefore are not very stable markers for actual intake.[35-37]

Flow-mediated dilatation

As outcome variable, we used the net change in FMD. In most of the articles, FMD was presented as the %FMD, which is calculated by the following equation:

$$\%FMD = [(\text{maximum diameter} - \text{baseline diameter}) / \text{baseline diameter}] \times 100\% \quad (1)$$

For consistency, we have used that formulation throughout as the unit of FMD measurement.

Statistical analysis

Net change in flow-mediated dilatation

Our primary outcome was the net change in FMD due to folic acid (with or without vitamin B-6 and vitamin B-12) treatment - ie, %FMD after folic acid - %FMD after placebo. For the 6 included crossover trials, %FMD at the end of the control period was subtracted from vascular reactivity at the end of the treatment period. For parallel design trials, the %FMD change from baseline to study end in the control group was subtracted from the %FMD change from baseline to study end in the treatment group.

Standard error of the net change in flow-mediated dilatation

For the crossover trials, the SE of the net change in %FMD was derived from the *P* value[18,19], from the SD[20], or directly from the author of one of the studies.[31] Two crossover trials did not report %FMD but reported the baseline diameter (in mm) and the increase (in μm) after occlusion.[24,26] For these studies we estimated the %FMD for the

end of the control period and the end of the treatment period. Then we calculated the variance in these %FMD values by using the following equation:

$$\text{Variance}_{FMD} = [\text{variance}_{increase} + FMD^2 \times \text{variance}_{baseline\ diameter} - 2 \times FMD \times \text{covariance}_{baseline\ diameter\ increase}]/(\text{baseline diameter}^2) \quad (2)$$

An estimate of the covariance between the baseline diameter and the increase after occlusion for these calculations was obtained from unpublished FMD data (ie -16, based on 316 data points; R Draijer, personal communication, 16 November 2005). The SE at the end of the control period (SE_C) and the SE at the end of the treatment period (SE_T) were calculated by taking the square root of the estimated variance in %FMD at the end of the control period and at the end of the treatment period. The pooled SE of the net difference was then calculated according to the equation of Follmann *et al.*[38]:

$$\text{SE of the net difference} = \sqrt{[(SE_T^2 + SE_C^2) - 2(r)(SE_T)(SE_C)]} \quad (3)$$

where *r* is the within-subject correlation in %FMD between the treatment and control period, which is estimated to be 0.5.

For the 8 included parallel designs, the SE of the net change in %FMD was estimated with the *P* value for 2 studies.[23,30] Six of the parallel trials provided either the SE[27] or the SD[17, 21, 22, 29, 30] at baseline and at the end of the study for the control and treatment groups. Respective SEs were calculated by using the following equation[17, 21, 22, 29, 30]:

$$SE = SD/\sqrt{(n)} \quad (4)$$

The SEs were used to calculate the SE for the change within the treatment group (SE_{TG}) and within the control group (SE_{CG}), again with the method of Follmann *et al.*[38] For example, for the treatment group, we used the following equation:

$$SE_{TG} = \sqrt{[(SE_{T\text{baseline}}^2 + SE_{T\text{end of study}}^2) - 2(r)(SE_{T\text{baseline}})(SE_{T\text{end of study}})]} \quad (5)$$

where *r* was estimated to be 0.5. Finally, the SE of the net change was calculated by using the following equation:

$$\text{SE of the net change} = \sqrt{[(SE_{TG}^2 + SE_{CG}^2)} \quad (6)$$

One parallel trial[28] did not report %FMD values, but reported the baseline diameter (in mm) and the increase (in μm) after occlusion. The %FMD values and respective SEs were calculated as described above for the crossover trials. We used these values and equations *5* and *6* to calculate the SE of the net change. Data for the calculation of the change in %FMD and the SE of this change were not missing from any trial.

Because this meta-analysis brings together studies that are diverse both clinically (eg, dose and type of subjects) and methodologically (eg, design and quality), heterogeneity in their results is expected. We calculated that the proportion of total variation between studies due to heterogeneity rather than to chance was 26%.[39] Although a value >50% is considered to represent substantial heterogeneity, we used a random-effects model (SAS PROC MIXED) with inverse variance weighting for each trial.[40] In this way, we addressed the question "What is the average effect of folic acid supplementation on %FMD?" rather than using a fixed-effects model that addresses the question "What is the best estimate of the effect of folic acid on %FMD?" In addition, a random effects model results in a more conservative estimate of statistical significance than does a fixed-effects model.

We performed defined stratified meta-analyses to roughly explore the potential effect of study design (crossover or parallel), mean population age (≤55 or >55 y old), general health (healthy or at greater risk of CVD), folic acid dose (400–800, 5000, or 10 000 μg/d), duration of treatment (≤8 or >8 wk), additional vitamin B-6 or vitamin B-12 or both (no or yes), and study quality (≤7 or >7 Delphi criteria).

To assess publication bias, a funnel plot of the treatment effect versus $1/SE^2$ was visually inspected as described earlier.[41] In addition, the symmetry of the funnel plot was judged by regressing the standard normal deviate (ie, effect of folic acid supplementation on %FMD/SE of this effect) against the estimate precision (1/SE) (standard normal deviate = $\alpha + \beta$ x precision). A symmetrical funnel plot should give a regression equation in which α is close to 0 and β indicates the size and direction of effect.[41]

We used SAS software (version 8.2; SAS Institute, Cary, NC) for the statistical analyses. The effect of folic acid on %FMD was reported with the use of 95% CIs. Two-sided *P* values < 0.05 were considered significant.

Results

Trial characteristics

Of the 14 trials meeting inclusion criteria, 1 trial[29] had 2 separate intervention groups, and thus 15 intervention groups are presented in *Table 1*. Six of these trials had a crossover design, and 8 had a parallel design. In total, this meta-analysis was based on 732 persons treated with folic acid (with or without vitamin B-6 or vitamin B-12 or both) or placebo for a median of 8 wk, with a median study size of 34 participants. Most trials included middle-aged male subjects: the mean age in the individual studies ranged from 29.3 to 69.1 y (overall median: 55.8 y), and the median percentage of males was 86%. Seven intervention groups were composed of populations with CVD or high cholesterol concentrations, 5 groups were generally healthy, and 3 were healthy but with slightly elevated plasma homocysteine concentrations. The median folic acid dose was 5000μg/d (range: 400–10 000 μg/d). In 5 studies, additional vitamin B-6 and vitamin B-12 were supplied. All studies that supplied information on the plasma homocysteine concentration showed a drop in this concentration after intervention. The lowest Delphi score was 7 points; 5 trials had that score. Three trials fulfilled all 9 Delphi criteria.

Table 1 *Characteristics of the 15 intervention groups (14 trials) included in the meta-analysis.*[1]

Reference	Design	Subjects[2]		Age	Male	General health	Folic acid dose	Vitamin B-6 dose	Vitamin B-12 dose	tHcy		Duration	Drop-out	Delphi criteria met[3]
		Folic acid group	Control group							Placebo group	Folic acid group			
		n	*n*	*y*	*%*		*µg/d*	*mg/d*	*µg/d*	*µmol/L*	*µmol/L*	*wk*	*%*	*n*
Bellamy, 1999[19]	CO	18	19	NA	NA	Healthy, tHcy>13 µmol/L	5000	-	-	~11	~7	6	10	7
Woo, 1999[20]	CO	17	17	54	88	Healthy	10.000	-	-	9.5 ± 2.5[4]	8.1 ± 3.0[5]	8	0	9
Title, 2000[21]	P	25	25	58.9	80	CAD	5000	-	-	11.8	10.9	16	0	9
Chambers, 2000[22]	P	59	30	56	100	CAD	5000	-	1000	14.9 ± 6.5	9.3 ± 1.9[5]	8	0	7
Thambyrajah, 2001[23]	P	43	43	63	87	CAD	5000	-	-	12.3	9.3[5]	12	4.4	7
Pullin, 2001[24]	CO	106	106	39	42	Healthy	400	-	-	10.9 ± 6.9	8.5 ± 3.1[6]	16	15.9	8
Van Dijk, 2001[25]	P	63	67	45.3	51	Healthy	5000	250	-	12.3 ± 5.5	7.5 ± 1.9[6]	52	6.5	8
Doshi, 2001[26]	CO	48	48	57	88	CAD	5000	-	-	10.8 ± 2.4	9.3 ± 2.4[5]	6	3.8	7
Sydow, 2003[27]	P	8	8	69.1	78	PAOD	10.000	20	200	NA	NA	8	11	8
Doshi, 2002[28]	P	16	17	55.5	91	CAD	5000	-	-	10.8 ± 2.1	8.3 ± 1.3[5]	6	0	7
Hirsch, 2002[29] [7]	P	9	11	29.3	100	Healthy	600	2	800	~9	~8	8	0	8
Hirsch, 2002[29]	P	9	11	29.3	100	Healthy, tHcy ≥ 15 µmol/L	600	2	800	~22	~10	8	0	8
Woodman, 2004[18]	CO	26	26	49	69	Healthy, mean tHcy 15.6 µmol/L	5000	-	-	~12.8	~8.4	8	0	9
Lekakis, 2004 [30]	P	17	17	56.5	85	HCHOL, 50% with CAD, all taking statins	5000	-	-	NA	NA	4	0	8
Olthof, 2006 [31]	CO	39	39	59	58	Healthy	800	-	-	9.9 ±1.6	8.0 ±1.3[5]	6	2.5	8

[1] tHcy, total homocysteine; CO, crossover; NA, not available; P, parallel; CAD, coronary artery disease; PAOD, peripheral arterial occlusive disease; HCHOL, hypercholesterolemia. [2] Subjects who completed the trial. [3] Total Delphi criteria = 9. [4] *x* ± SD (all such values). [5] Significantly different from tHcy value in placebo group, $P < 0.05$. The tHcy concentration was estimated from data given in the referenced article. [6] Significance level of difference not available. [7] Half of the study population had high concentrations of homocysteine.

Effect of folic acid on flow-mediated dilatation

The individual trial results and the pooled estimate are shown in *Figure 2*. In the overall pooled estimate, compared with placebo, folic acid improved FMD with 1.08%FMD (95% CI: 0.57, 1.59; *P* = 0.0005). There was no effect of design, mean age of the study population, study duration, or the addition of vitamin B-6 or vitamin B-12 on the estimated change in %FMD due to folic acid (*Table 2*). However, there was a tendency that subjects at greater risk of CVD had a larger improvement in %FMD, and studies that met a higher number of Delphi criteria had a smaller improvement in %FMD. The dose of folic acid was clearly important. The trials using a lower dose - ie, <5000 μg - did not show a beneficial effect of folic acid on FMD (-0.07%FMD; 95% CI: -0.37, 0.22%FMD), whereas the studies with a dose ≥5000 μg/d did (1.42%FMD; 95% CI: 1.25, 1.58%FMD). In a post hoc analysis, a dose-response effect became apparent when we created 3 strata: a low-dose stratum with folic acid doses between 400 and 800 μg/d (4 intervention groups), an intermediate stratum with studies using a folic acid dose of 5000μg/d (9 intervention groups), and a high-dose stratum with studies using a folic acid dose of 10 000μg/d (2 intervention groups). At folic acid intakes ≤800 μg/d, FMD did not change [-0.07%FMD (95% CI: -0.37, 0.22%FMD)]; at 5000 μg/d, it improved [1.37%FMD (1.12, 1.54%FMD)]; and, at 10 000 μg/d, it improved further [2.04%FMD (1.43, 2.65%FMD)].

Evaluation of the funnel plot showed little evidence for publication bias (*Figure 3*). In addition, the funnel plot was quite symmetric, as α = -0.05 and β = 1.09.[42]

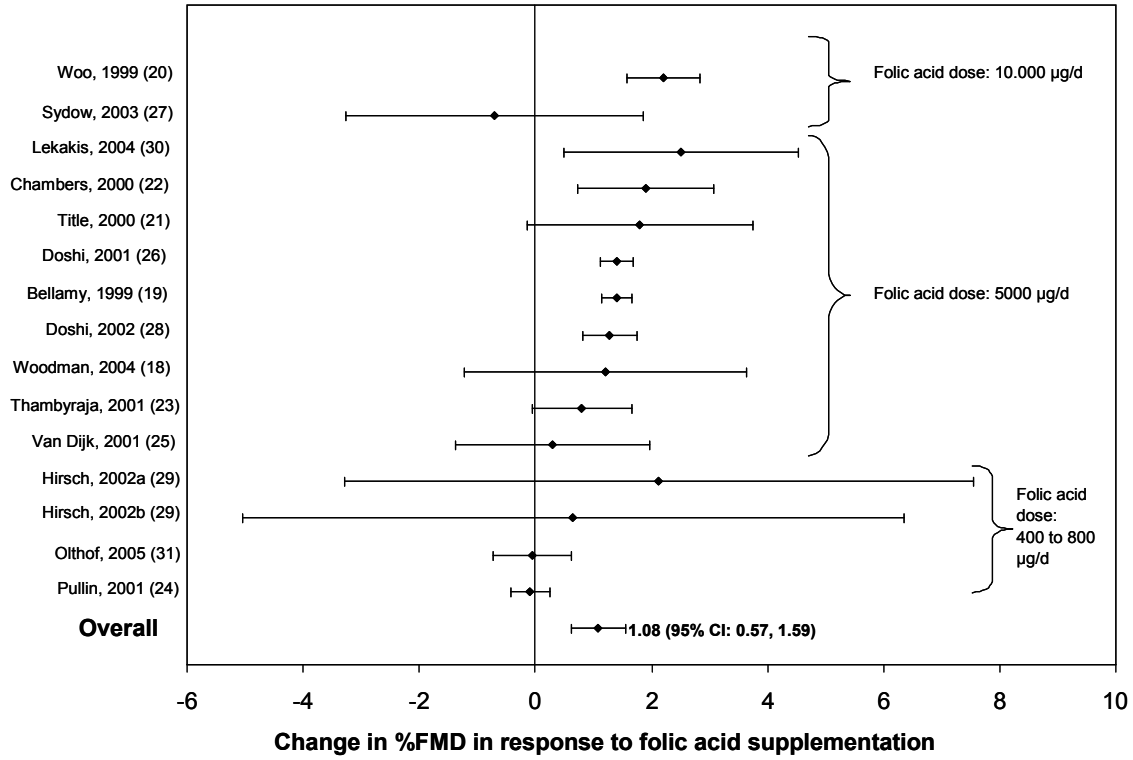

Figure 2 *Change (and 95% CIs) in the percentage flow-mediated dilatation (%FMD) due to folic acid supplementation per included intervention group and the overall estimated change (and 95% CI). Half of the participants in the study by Hirsch et al. had high concentrations of homocysteine.*

Table 2 *Effect of folic acid supplementation on flow-mediated dilation stratified by previously defined study characteristics.*

Characteristic		Intervention groups	Effect (95% CI)	P[1]
		n		
Design				
	Cross-over	6	0.99 (0.38;1.59)	0.6
	Parallel	9	1.21 (0.50;1.92)	
Mean Age (y)[2]				
	<56	7	1.01 (0.28;1.73)	0.8
	≥56	8	1.13 (0.53;1.74)	
Health				
	Generally healthy	8	0.84 (0.22;1.46)	0.3
	Chronic condition	7	1.34 (0.71;1.98)	
Folic acid dose (µg/d)				
	<5000	4	-0.07 (-0.37;0.22)	0.0001
	≥5000	11	1.42 (1.26;1.58)	
Study duration (wk)				
	<8	5	1.16 (0.49;1.84)	0.7
	≥8	10	1.01 (0.37;1.65)	
Addition of other B-vitamins				
	No vitamin B-6 and/or B-12	10	1.11 (0.60;1.62)	0.8
	Vitamin B-6, B-12, or both	5	0.93 (-0.25;2.12)	
Study quality				
	Delphi <8	5	1.34 (0.73;1.94)	0.2
	Delphi ≥8	10	0.81 (0.20;1.43)	

[1] Difference between strata. [2] One trial[19] did not provide data on this variable. We set the mean age in this trial at 56 y, which was the median age for all trials.

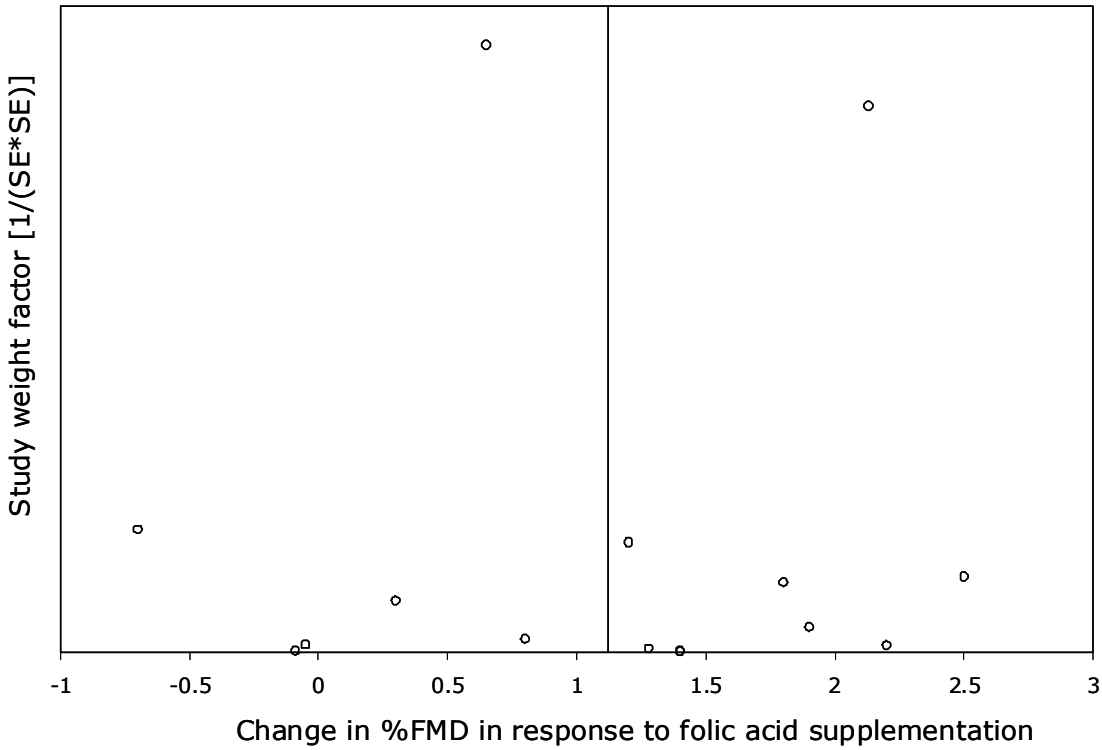

Figure 3 *Funnel plot of trial weight against change in percentage of flow-mediated dilatation (%FMD) in the 14 intervention trials included in the meta-analysis. The vertical line indicates a mean change in all studies.*

Discussion

This meta-analysis of randomized, double-blind, placebo controlled clinical trials showed that supplementation with high doses of folic acid for ≥4 wk improves FMD assessment of endothelial function. The result of a meta-analysis depends on the studies included. In the present review, we used a broad specified search and also contacted investigators for unpublished results to prevent any possible publication bias. In addition, we avoided the inclusion of studies on the basis of their outcome by defining inclusion and exclusion criteria. The use of these criteria led to 14 eligible trials. The number of studies is not large, but our methods ensured that the trials had a high internal validity and were reasonably comparable. To account for any heterogeneity, we used a random-effects model, and we assessed characteristics, as described in *Table 1*. To ensure comparability of the trials, we had to exclude some trials[42-51] even though they were randomized, double-blind, placebo-controlled trials; were not conducted in a specific patient

population; and assessed the effect of folic acid on endothelial function. All but one[50] of these studies support the findings of our meta-analysis.

Although several review articles have indicated that folic acid could beneficially affect endothelial function as measured with FMD[11,52], a comprehensive meta-analysis that takes into account both within- and between-study variability was lacking. Thus, our analysis is the first that provides a quantitative estimate of the improvement in FMD after folic acid supplementation, in which the overall effect showed a favorable change of 1.08%FMD. A (statistically nonsignificant) larger improvement was seen in subjects at a higher risk of CVD (1.34%FMD) than in healthy subjects (0.84%FMD). Because the average FMD value in populations at greater risk of CVD was ~3.6%FMD and that in healthy populations was ~5.6%FMD, our findings indicate potential significant improvements of ~37% in subjects at CVD risk and ~9% in healthy populations.

An important question is the extent to which FMD can be used as a predictor of long-term CVD risk? It is clear that FMD is a predictor of this risk in a selected group of patients, such as those with coronary heart disease, heart failure, and hypertension.[53-57] However, the extent to which FMD can be used to predict the risk of CVD in the general population is less clear. Nevertheless, preliminary epidemiologic data showed a modest, positive correlation between FMD and the Framingham Study risk score in a general population sample of 1016 elderly persons.[58]

The FMD value indicates the bioavailability of endothelium derived NO, which is essential to cardiovascular health.[59] High homocysteine concentrations are postulated to reduce NO availability in several ways. Indeed, homocysteine may induce the formation of free radicals, as shown by in vitro studies.[60,61] A certain proportion of these free radicals can be neutralized by NO, but other free radicals may directly damage endothelial cells[60], and both processes would lead to a smaller amount of available NO. Oxidative stress may also increase as homocysteine inhibits glutathione peroxidase[61,62], a potent cellular defense mechanism against free radicals. Homocysteine can also reduce NO availability by forming *S*-nitrosohomocysteine complexes.[63,64] Finally, homocysteine may induce the formation of asymetric dymethyl arginine, which is a competitive inhibitor of enzymatic nitric oxide synthase (eNOS).[65,66] Therefore, we postulate that folic acid administration beneficially affects FMD by lowering the plasma homocysteine concentration.

It is conceivable that folic acid could also improve the FMD value independently of homocysteine lowering. This possibility is supported by 3 mechanisms that would result in a greater availability of NO: *1*) folic acid may act as an antioxidant[48]; *2*) it may regenerate the cofactor for eNOS [49, 54]; and 3) it may directly stimulate eNOS.[67] Two

indications from the present study support an independent effect of folic acid. First, post hoc analysis hinted toward a favorable effect with higher doses of folic acid (>5000 μg/d). A folic acid dose of 400 to 800 μg/d is typically required to achieve an almost-maximal homocysteine lowering effect over an 8-wk period[4], but the present analysis showed that such large homocysteine reductions apparently do not result in enhanced %FMD.[24,29,31] In contrast, this observation is based on only 3 studies involving a small number (ie, 185) of subjects. In addition, one of these studies may have been performed against the background of folic acid fortification[29], although the amount of folic acid provided by the supplements will have been much higher than that provided by the fortification program. Finally, all 3 studies involved healthy young (mean age: ~31 y) persons, who probably have the least to gain from supplementation with folic acid. Thus, before we discard a beneficial effect of low doses of folic acid on FMD, we would like to see large studies in healthy older persons or subjects with (reversible) vascular dysfunction (eg, overweight or smoking). Second, the combination of folic acid supplementation with vitamin B-12 results in an additional 7% reduction in homocysteine (4), yet our analysis indicates no additional benefit with vitamin B-12 and vitamin B-6. In addition, because the study duration had no significant effect on our study outcome, our result would point to an acute effect of folic acid. Taken together, the findings of the studies considered in this meta-analysis suggest that the effect of folic acid is largely independent of a homocysteine-lowering effect. Yet, we must be careful with this interpretation because of the small number of studies in the low and high-dose strata. Therefore, it would be worthwhile to investigate acute effects of several doses (low and high) of folic acid or dietary folate or both on FMD values in subjects with suboptimal folate status.

In conclusion, this meta-analysis indicates that a high dose of folic acid can improve endothelial function as measured with FMD after 4 wk of supplementation, and this effect would seem to be independent of a reduction in homocysteine. Restored endothelial function in subjects with CVD may, in the short term, not prevent another CVD event, as can be deduced from published secondary prevention trials.[5-8] However, an optimized FMD may be crucial to prevent a first-ever CVD event.

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Chapter 8

General Discussion

Blood pressure is an important risk factor for cardiovascular diseases (CVD). There is a graded increase in risk of CVD across the entire blood pressure range. The majority of people in Western populations have above optimal blood pressure levels. Modest reductions in blood pressure on the population level have a major impact on CVD incidence. Favorable lifestyle changes can play an important role on the individual as well as on the population level to improve blood pressure and reduce the burden of CVD (**Chapter 1**).

The studies in this thesis addressed two general aims. The first aim was to assess the importance of a selected number of minerals for population blood pressure levels. The second aim was to investigate the effect on blood pressure and endothelial function of other food components that have recently attracted much attention in the scientific literature. In this last chapter the main findings are summarized and interpreted, followed by a discussion on methodological aspects of the studies, interpretation of the findings and relevance for public health. Finally, suggestions for future research and strategies to improve current potassium and calcium intakes in populations are given.

Main findings

An overview summarizing the studies in this thesis is presented in *Table 1.* The first 3 chapters (**Chapter 2-4**) address the potential public health impact of minerals for population blood pressure levels. The results of a review of national surveys (**Chapter 2**) showed that current potassium intakes in countries are suboptimal (range: 1.7 – 3.7 gram per day). Increasing intake (to 4.7 gram per day) was estimated to result in reductions in population blood pressure of 2-3 mmHg in Western countries. The size of this estimated effect on population blood pressure is comparable to that which can be achieved with a reduction in salt intake (from 9 to 5 gram per day). In a meta-analysis of 40 randomized controlled studies the effect of calcium supplementation on blood pressure overall and in population subgroups was assessed. The results of this analysis indicated statistically significant reductions of 2 mmHg for systolic blood pressure and 1 mmHg for diastolic blood pressure per ~1200 mg per day of additional calcium intake. Blood pressure response to calcium supplementation tended to be stronger in populations with a low habitual calcium intake (<800 mg/day) (**Chapter 3**). In an 8-week randomized placebo controlled parallel study in 124 subjects, the effect on blood pressure of daily consumption of a skimmed milk enriched in potassium (either 1500 mg or 750 mg), combined with relatively low doses of calcium (446 mg), magnesium (100

mg), selenium (40 mg), vitamin C (180 mg), vitamin E (30 mg tocopherol equivalents) was studied. In this study we observed no significant effects on blood pressure (**Chapter 4**).

The last 3 chapters address the potential blood pressure lowering effect of other food components that have recently attracted much attention in scientific research for their possible effects on blood pressure and endothelial function (**Chapter 5-7**). Two 4-week randomized controlled cross-over studies, including 162 Scottish subjects in total with untreated elevated blood pressure, were performed to examine blood pressure response to intake of lactotripeptides in a European population (**Chapter 5**). No antihypertensive effect of daily consumption of a dairy drink with enzymatic lactotripeptides (5.8 mg Isoleucine-Proline-Proline (IPP) and 4.4 mg Valine-Proline-Proline (VPP) per day) or enzymatic lactotripeptides (2.7 mg IPP and 1.9 mg VPP) plus 350 mg added potassium per day was found. In a 4-week randomized placebo-controlled cross-over study in 35 healthy male subjects the effect of two different grape solids (wine-grape mix and grape seed), each containing 800 mg of polyphenols, on endothelial function and blood pressure were investigated. No improvements in flow-mediated dilation and/or blood pressure were observed (**Chapter 6**). Finally, the effect of folic acid on endothelial function was assessed based on a meta-analysis of 14 randomized controlled studies (**Chapter 7**). The results of this analysis showed that supplementation with folic acid (median dose 5000 μg per day) significantly improved flow-mediated dilatation by 8%. This effect was only found in studies administrating a high folic acid dose (≥5000 μg per day).

Table 1 *Overview of research findings of the studies presented in this thesis.*

Type of research	Exposure	Main findings
Chapter 2 *What is the impact of increased potassium intake on population blood pressure?*		
Systematic review of 21 national surveys	Potassium	Increasing current suboptimal potassium intakes (range 1.7-3.7 g per day) may result in a population BP reduction of 2-3 mmHg
Chapter 3 *What is the effect of increased calcium intake on blood pressure, overall and in population subgroups?*		
Meta-analysis of 40 randomized controlled studies	Calcium	Calcium supplementation (mean: 1200 mg per day) significantly reduces systolic BP by 2 mmHg, and diastolic BP by 1 mmHg, with a tendency towards a larger effect in populations with low calcium intakes (<800 mg per day)
Chapter 4 *Does potassium combined with other micronutrients at low doses have beneficial effects on blood pressure?*		
Randomized controlled study in 124 Dutch subjects with elevated BP	Minerals and vitamins enriched milk	After 8 weeks daily consumption of a skimmed milk with potassium (either 1500 mg or 750 mg) in combination with other minerals and vitamins at low dose, no significant BP reductions were observed compared to a water placebo
Chapter 5 *Do lactotripeptides improve blood pressure in a European population?*		
Two randomized controlled studies in 162 Scottish subjects with elevated BP	Lactotri-peptides	After 4 weeks daily consumption of a yogurt drink with lactotripeptides (study 1: 5.8 mg IPP and 4.4 mg VPP; study 2:2.7 mg IPP and 1.9 mg VPP plus 350 mg added potassium), no significant BP reductions were observed compared to a yogurt placebo
Chapter 6 *Does the polyphenolic fraction of grape extracts improve endothelial function and blood pressure?*		
Randomized controlled study in 35 healthy males	Grape polyphenols	After 2 weeks daily consumption of 2 grape solids (wine-grape mix and grape seed capsules), at a level of 800 mg polyphenols per day, no significant improvements in endothelial function or BP were observed compared to placebo capsules
Chapter 7 *Does folic acid improve endothelial function?*		
Meta-analysis of 14 randomized controlled studies	Folic acid	In studies applying high doses of folic acid (≥5000 μg per day), endothelial function as measured by flow-mediated dilation was improved by 8%

BP, blood pressure ; IPP, Isoleucine-Proline-Proline ; VPP, Valine-Proline-Proline.

Methodological considerations

This paragraph addresses a number of the methodological aspects of the studies presented in this thesis that are relevant for the validity of our findings. These aspects are discussed separately for the systematic reviews and intervention studies.

Systematic reviews

For both meta-analyses in this thesis (**Chapter 3 and Chapter 7**) the Quality of Reporting of Meta-analyses (QUORUM)[1] guidelines were followed. A priori eligibility criteria were defined and only randomized placebo controlled studies were included in the meta-analyses. Because the validity of a meta-analysis depends not only on the quality of the data, but also on the comparability of data from the included studies[2], a statistical approach was used to analyze the data that accounted for both within- and between-study variability.[3] Funnel plots were constructed to examine the possibility of publication bias. In the folic acid meta-analysis there was little evidence for publication bias. In our calcium meta-analysis a non-parametric 'trim and fill' method revealed that small trials reporting large blood pressure reductions were overrepresented. To account for the possible bias toward overestimation we adjusted our effect estimate for putative missing data. For all studies included in the meta-analyses changes in blood pressure or flow-mediated dilation in the intervention group were extracted from changes in the placebo group the yield the net change resulting from the intervention.

Well-performed meta-analyses provide a more precise estimate of a treatment effect, but they also have some potential pitfalls that can compromise validity.[4, 5] An important methodological aspect of meta-analyses is heterogeneity across studies. Heterogeneity may result from differences in study characteristics as well as from differences in populations studied across studies.[6]

In our meta-analysis of folic acid and endothelial function, we included 14 randomized controlled studies with very different types of populations (e.g. healthy subjects and CVD patients) and study characteristics (e.g. dose of folic acid, with or without other B-vitamins). We explored why effects differed across studies in this meta-analysis, but because of the limited number of studies in each subgroup and the fact that we did not control for confounders, the results should be interpreted cautiously. An improvement in flow-mediated dilation was only found for intakes of folic acid higher than ≥5000 μg per day (P = 0.0001). It should be mentioned that studies in CVD patients used high doses of folic acid (5000 or 10.000 μg per day), whereas studies performed in healthy populations used high doses of folic acid (5000 or 10.000 μg per

day) as well as much lower doses of folic acid (400–800 µg per day). Therefore, it is not clear whether folic acid dose and/or type of population were responsible for this observed difference. Furthermore, it remains possible that in high risk populations lower doses of folic acid can result in reductions in flow-mediated dilation, but this could not be addressed in our meta-analysis. Another aspect relates to the fact that folic acid supplementation was combined with other B-vitamins (vitamin B_6 or vitamin B_{12} or both) in several studies. Therefore, it could be argued that the effects observed in our meta-analysis are not completely attributable to folic acid alone. However, this was not confirmed by stratified analysis (P for difference 0.8).

The large number of studies, 40 in total, in our meta-analysis on calcium and blood pressure allowed testing of the a priori hypothesis of differential effects in population subgroups.[5] In these stratified analyses, we used a multivariable model to adjust for potential confounders. Though, an important limitation is that these subgroup analyses were performed using aggregated rather than original data. This could have blurred the between-group differences in blood pressure response to calcium supplementation. Furthermore, findings from multiple subgroup analyses should be interpreted with caution since these are observational by nature and not based on randomized comparisons. For a better insight in differences in effects between population subgroups, data should be assembled for each participant within the separate studies.[7]

To estimate the impact of increasing potassium intakes to recommended levels on population blood pressure (**Chapter 2**) we relied on available data from national surveys, intervention and observational studies. No direct estimations of blood pressure effects for changes in potassium intake were made. Nevertheless, the dietary potassium intakes in our review are based on data from representative national surveys in >1000 participants using dietary assessment methods that reasonably well estimate dietary potassium intakes.[8-13] Furthermore, our estimated effect of increasing potassium intake on blood pressure is based on data from the INTERSALT study and supported by data from several other observational studies[14-17] and well-controlled intervention studies.[18-21] Therefore, we believe our estimates are reliable.

Intervention studies

The 4 intervention studies in this thesis were double-blind randomized placebo-controlled studies with low drop out rates. Compliance varied between 86-99% in these studies. In each study changes in blood pressure and flow-mediated dilation in the intervention group were extracted from changes in the placebo group to yield the net change

resulting from the intervention. A number of limitations, however, also need to be addressed. Our cross-over studies (**Chapter 5 and 6**), were of relatively short duration (2-4 weeks). Nevertheless, in other studies with similar duration, blood pressure lowering effects of lactotripeptides[22] and improvements in endothelial function for grape polyphenols[23] were found. Extending the intervention periods, particularly for studies with cross-over designs, might also have negative consequences, e.g. increase in variation in outcome due to seasonal variations, lower compliance and higher drop-out rates.

Doses of potassium in our intervention studies were relatively low, since we preferred a diet-based approach and expected an additive or maybe even a synergistic effect of potassium in combination with other micronutrients (**Chapter 4**) or lactotripeptides (**Chapter 5**) on blood pressure. However, the choice of these levels where somewhat arbitrary, and it can be debated whether higher doses would have been better. In our intervention study addressing the effect of combinations of minerals and vitamins participants consumed skimmed milk enriched in potassium at (either 1500 or 750 mg per day), in combination with relatively low levels of other minerals and vitamins (calcium 446 mg, magnesium 100 mg, selenium 40 mg, vitamin C 180 mg and vitamin E 30 mg tocopherol equivalents per day). In comparison, the amounts of the micronutrients tested in intervention studies addressing the effect of the individual micronutrients on blood pressure were much higher; median daily doses of 2933 mg for potassium, 500-2000 mg for calcium, 374 mg for magnesium, and 500 mg for vitamin C.[21, 24-26] In addition, our study was powered to find a reduction in systolic blood pressure of 5 mmHg. Considering the relevance for public health of small reductions in blood pressure, nutritional intervention studies of this type should aim more at detecting modest effects in the order of 2-3 mmHg. The confidence intervals of the changes observed in our study are still compatible with a possible 2-3 mmHg reduction in systolic blood pressure. For potassium combined with lactotripeptides (**Chapter 5**), we also hypothesized an additive effect. However, recent studies suggest that the lactotripeptides do not lower blood pressure.[27-31] As a result, the additional dose of 350 mg potassium in this study was too low for finding a significant blood pressure effect.

Concerning the study on grape polyphenols, it should be mentioned that the number of subjects (**Chapter 6**) was relatively small i.e. 35 normotensive men (mean systolic blood pressure 123 mmHg). The primary outcome in this study was endothelial function and not blood pressure. Therefore this study can not be taken as definitive evidence that grape polyphenols have no antihypertensive effect.

An important methodological issue in blood pressure research is statistical power; blood pressure shows large fluctuations within and between days[32], and diet has relatively modest effects on blood pressure. In our four intervention studies (**Chapter 4-6**), we used several approaches to reduce variation and increase accuracy of blood pressure. First, participants were randomly allocated to treatment.[33] Secondly, average blood pressure values were determined based on multiple standardized measurements of blood pressure on several days in an attempt to get a better estimate of each subject's true blood pressure at the time of measurement. Thirdly, in our analyses we adjusted for baseline blood pressure by means of Analysis of Covariance (ANCOVA)[33], except for the micronutrient intervention study. For this study we used ANOVA analysis. Furthermore, in the 2 lactotripeptide studies (**Chapter 5**) we used ambulatory blood pressure during screening and as the primary outcome. Several studies have shown that ambulatory blood pressure is a better predictor of CVD risk, and correlates more closely to hypertension-related organ damage.[32] In addition, studies show that ambulatory blood pressure has a much better reproducibility than office blood pressure.[32]

Interpretation of findings

This section describes the studies in this thesis in light of the scientific evidence in this area. We separately discuss the studies addressing the effect of minerals and the studies addressing the effect of other food components.

Minerals

Meta-analyses of >30 intervention studies showed that increasing potassium intake lowers blood pressure. In these studies an increase in potassium intake of ~2-3 gram per day resulted in a decrease in systolic blood pressure of ~3-4 mmHg.[18-21] A review of the scientific literature by the US Institute of Medicine showed that an adequate potassium intake, defined at 4.7 gram per day, blunts the age-related rise in blood pressure, reduces the adverse effects of high sodium chloride intake on blood pressure, reduces the risk of recurrent kidney stones, and possibly decreases bone loss.[8] The review in **Chapter 2** of this thesis assesses the impact of improving current potassium intakes on population blood pressure levels. Our findings show that improving current nation-wide potassium intake has the potential to result in reductions in systolic blood pressure of 2-3 mmHg at the population level, which is comparable to the estimated effects for reduced sodium intake.[34, 35] A number of independent organizations and authoritative bodies

support the link between increased potassium intake and the beneficial effect on blood pressure and/or CVD risk[36-39], and several recent publications highlighted a critical role for potassium in relation to blood pressure and CVD[40-42], but to the best of our knowledge no other studies assessed the impact of suboptimal potassium intake levels on population blood pressure levels in different countries.

Comparison of our data on potassium intakes obtained from national surveys with other data sources, such as 24-hour urinary potassium excretion data from INTERSALT (1982-1985)[43] and INTERMAP (1990-1997)[44] suggests that potassium intakes in populations may have increased over time. The recently reported potassium intake data based on the EPIC study[45] suggest that intakes for most countries are similar to those based on national surveys. This comparison should be done with caution, because of essential differences in methodology of assessing potassium intake (urinary excretion versus dietary assessment) and in sampling of subjects (representative versus not representative for total population). Nevertheless, these different types of data together, are consistent in pointing to lower potassium intake levels in populations than the recommended intake as set by the Institute of Medicine.[8]

The meta-analysis in **Chapter 3** addresses the effect of calcium supplementation on blood pressure. Previous meta-analyses of intervention studies[24, 46, 47] showed reductions of 0.9-1.4 mmHg for systolic blood pressure and 0.2-0.8 mmHg for diastolic blood pressure after calcium supplementation. In these meta-analyses heterogeneity between studies was observed, but the effect in population subgroups was only partly addressed. In our meta-analysis of 40 randomized controlled studies, we tried to explain this heterogeneity and addressed the impact of calcium supplementation on blood pressure in various population subgroups based on pre-defined subgroup analyses (**Chapter 3**). Our results confirm a blood pressure lowering effect of 2 mmHg for systolic blood pressure and 1 mmHg for diastolic blood pressure after a mean increase in calcium intake of ~1.2 gram per day, in addition to intake from the diet. The effects in our updated meta-analysis were somewhat larger than the effects observed in previous meta-analysis.[24, 46, 47] The meta-analysis by Griffith *et al.* showed a reduction in systolic blood pressure of 1.4 mmHg and 0.8 mmHg for diastolic blood pressure.[24] Several methodological aspects might explain this difference in estimated effects between our meta-analysis and the meta-analysis by Griffith *et al.* For example, our exclusion criteria were stricter, especially with regard to co-intervention of calcium with other nutrients or as part of a dietary intervention, but also for other inclusion criteria, such as studies performed in populations with renal disease or hyperparathyroidism. In addition, we included 6 studies

that were not included in previous meta-analyses. Other methodological and statistical aspects that might explain differences in estimated effect are selection of blood pressure outcome to use in the meta-analysis when more than one different measurement method is reported for an individual study; the way data from studies with more than one intervention group were included in the meta-analysis; and the approach used to estimate the variance of the blood pressure change in the individual studies.

Our subgroup analyses showed that the blood pressure level of populations with lower calcium intakes (≤800 mg per day) may particularly benefit from an increased intake of calcium. This was not addressed in previous meta-analyses estimating the effect of calcium supplementation in the general population.[24, 46, 47] Several intervention studies in children and pregnant women, both population subgroups with increased calcium requirements[48], support our findings and also observed larger blood pressure reductions after calcium supplementation in individuals with lower initial calcium intakes.[49-52]

In our 8-week randomized controlled trial in 124 Dutch subjects we observed no significant blood pressure lowering effect of potassium (either 1500 mg or 750 mg per day) combined with these specific other micronutrients at low dose (calcium 446 mg, magnesium 100 mg, selenium 40 mg, vitamin C 180 mg, vitamin E 30 mg tocopherol equivalents per day) in a dairy matrix (**Chapter 4**). No other intervention studies have investigated the effect of intake of multiple minerals combined with vitamins on blood pressure. Few studies investigated the effect of multiple minerals on blood pressure.[53-57] In 2 large scale studies (study 1: 125 subjects with untreated mild or borderline hypertension; study 2: 321 normotensive women with low habitual intakes) conducted by Sacks *et al.*[55, 56] with long duration (16 weeks and 6 months) no indications for additive effects of combinations of potassium, calcium and magnesium supplements were found. Two relatively small studies (study 1: 53 normotensive female students on a low calcium diet; study 2: 38 participants) with shorter intervention periods (4 and 6 weeks), suggested possible blood pressure reductions of combinations of potassium, calcium and magnesium in a dairy matrix (see Introduction, **Chapter 1**).[54, 57] In our study we could not confirm a significant blood pressure lowering effect of a combination of micronutrients in a dairy matrix. We considered several explanations why studies investigating combinations of micronutrients did not show strong suggestions for a blood pressure lowering effect. As explained the levels of micronutrients in our and other studies on combinations of minerals were much lower compared to the levels tested in studies addressing the effect of individual minerals on blood pressure (median daily dose

for potassium: 2933 mg, for calcium range 500-2000 mg, for magnesium 374 mg, for vitamin C:500 mg).[21, 24-26] The differences in mineral content between the DASH diet and its control diet was also much larger (4415 versus 1752 mg for potassium, 1265 versus 443 mg for calcium, and 480 versus 176 mg for magnesium).[58] Another explanation for the small or absent effects that we observed might be that calcium and magnesium or other micronutrients interfere with the blood pressure lowering effects of potassium. This has also been suggested by Sacks *et al.*[56] Recent studies indicate that potassium bicarbonate, in contrast to potassium chloride, reduces urinary calcium excretion.[59-61] In the DASH combination diet, excretion of urinary calcium was not increased, despite the large increase in calcium intake in this diet, and the "Fruits and vegetables diet" induced a reduction in urinary calcium.[62] This might also explain why the small studies investigating the effect of combinations of minerals in a natural matrix did find indications for blood pressure lowering effects[54, 57], whereas the studies performed with mineral supplements, using potassium chloride, did not.[55, 56] However, this cannot explain why we did not observe blood pressure lowering effects in our study, as we used potassium lactate, which is a bicarbonate precursor.

In summary, our findings add to other evidence in underscoring the importance of increasing current potassium intakes for improving population blood pressure levels. Furthermore our data confirm a modest effect of increasing calcium intake on blood pressure levels in the general population, and show that this effect might have a larger impact in populations with low habitual calcium intakes. This hypothesis is supported by findings from other studies in population subgroups with increased calcium requirements, e.g. children and pregnant women. The intervention study in this thesis on blood pressure effects of potassium combined with other minerals and vitamins at low doses does not provide evidence for additive effects of these micronutrients. This is in line with a limited number of other blood pressure studies addressing combinations of minerals at low doses.

Other food components

The results of our two randomized controlled studies, which involved a total of 162 Scottish subjects, do not suggest a blood pressure lowering effect of lactotripeptides (**Chapter 5**). Dairy peptides, and particularly lactotripeptides, have been tested in many intervention studies for their possible blood pressure lowering effect. Most studies were performed in Japan.[63-67] Studies from Finland have also found blood pressure lowering effects, but these were smaller than in Japan.[68-71] Recently, several well-designed and

large scale intervention studies on lactotripeptides and blood pressure in other European populations have been published.[27-31] These data do not suggest beneficial effects on blood pressure of these peptides, which is in line with the results from our studies. The combined IPP and VPP intake tested in our studies (study 1: 10.2 mg per day and study 2: 4.6 mg per day) was comparable or higher than in most of the Japanese and Finnish studies (~3-5 mg per day).[72] Of note is that lactotripeptides, which are assumed to be the active ingredients, can be generated by different processes. In most of the recent studies[27, 31], including our own studies, the lactotripeptides were obtained via enzymatic hydrolysis. On the contrary, in most Japanese and Finnish studies fermented lactotripeptides were used, either in the form of fermented milk or fermented powdered milk.[31] One could argue that fermented lactotripeptides have different blood pressure lowering properties, because they contain in addition to IPP and VPP a significant amount of other peptides, which may also affect blood pressure.[31] Engberink *et al.*[28] investigated fermented powdered lactotripeptides, enzymatic lactotripeptides, and also synthetic lactotripeptides in an intervention study, in 135 Dutch subjects with elevated blood pressure, but did not confirm this hypothesis. The fermented powdered lactotripeptides tested in this study were gained from dried fermented milk after removing micro-organisms, casein and lactic acid. Fermented milk, however, was not tested in this study and contains in addition to lactotripeptides uncleaved protein, lactic acid, micro-organisms and minerals like potassium, calcium and magnesium. Therefore, it can not be excluded that other compounds in fermented milk might be responsible for the blood pressure lowering effects observed in Japanese and Finnish studies.

There are also several methodological shortcomings in the Japanese and Finnish studies that might explain the differences in effect sizes found between the recent lactotripeptide studies and the studies performed in Asian and Finnish populations. In a number of these intervention studies placebo effects were not taken properly into account and in other studies initial blood pressure levels tended to be higher in the intervention group than in the control group.[72] Furthermore, differences in genetic background and habitual diet might explain (part of) the heterogeneity.[73]

Two different polyphenol-rich solids in capsules, derived from a wine-grape mix or grape seed, were examined for their effect on flow-mediated dilation and compared to placebo capsules in a randomized crossover study (**Chapter 6**). No significant effects on endothelial function and blood pressure were observed. The total daily dose of grape polyphenols for both interventions was 800 mg, according to gallic acid equivalents, which is considerable and higher than the total polyphenol content in a glass of 100%

fruit juice (0.4-3.8 mg/ml) or a glass of red wine (2.2 - 3.5 mg/ml).[74, 75] The amount of polyphenols tested in our intervention study was comparable to that in the 4 other small intervention studies (15-40 participants) addressing the chronic effects (2-8 weeks) of the polyphenolic fraction of grape consumption (759-1664 mg polyphenols per day) on endothelial function and/or blood pressure.[76-79] Some of these studies suggested improvements in endothelial function or blood pressure, but the quality of these studies was relatively poor; only 2 studies included a control group (see Introduction, **Chapter 1**).

Polyphenols, and particularly flavonoids, are increasingly being investigated in intervention studies for their effects on CVD risk factors.[80] Evidence for efficacy in humans varies between the different polyphenol subclasses. For some flavonoids sources, e.g. cocoa, effects on blood pressure and endothelial function are promising, but results are still based on a limited number of mainly small intervention studies.[80] It is important to note that research on polyphenols is complex. Not only because of the huge number of different polyphenols[81], but also for other reasons; the bioavailability of polyphenols is limited, polyphenols are extensively metabolized, and the polyphenol content of foods is dependent on many factors, including genetics, environmental and agronomic factors and processing techniques.[82, 83] In addition, most intervention studies on CVD risk factors focused on polyphenol-rich foods. As a result it is unclear whether observed effects are fully attributable to the polyphenols.[80, 81]

In **Chapter 7** the evidence for effects of folic acid on endothelial function as measured by flow-mediated dilation was reviewed in a meta-analysis of randomized controlled studies. Results indicate an improvement in endothelial function after consumption of folic acid, but only at high doses (≥5000 μg per day). Also, effects seemed to be larger in populations with CVD compared to healthy populations. Another recent meta-analysis, only including studies with high doses of folic acid (≥5000 μg per day), concluded that folic acid reduced systolic blood pressure by 2 mmHg, but not diastolic blood pressure, and increased flow-mediated dilation by 1.61%.[84] The majority of studies included in this meta-analysis were conducted in patients. Thus, available results suggest that folic acid can improve endothelial function when applied at high doses in patients, but effects at lower doses in the general population are not supported by current evidence.

It is important to mention here that several large-scale randomized controlled intervention studies, mainly secondary prevention studies, have tested effects of folic acid supplementation on CVD mortality and morbidity. These trials were set up because folic acid reduces plasma homocysteine levels and observational data showed a link

between elevated blood homocysteine and risk of CVD. Results of these intervention studies did not confirm a reduction in CVD mortality and/or morbidity after folic acid supplementation.[85, 86] The fact that many conditions and CVD risk factors, e.g. diabetes and smoking, are associated with increased homocysteine levels, and the discrepancy between the epidemiological data and results of intervention studies and suggest that homocysteine levels are a marker rather than a cause of CVD.[86] These data also indicate that if folic acid would have some effect on flow-mediated dilation, the relevance of increased folic acid intake for long-term CVD outcome is limited.

In conclusion, in line with other recent studies, our data confirm that the lactotripeptides IPP and VPP do not exert a beneficial effect on blood pressure in European populations. A blood pressure lowering effect for fermented milk products and in Asian populations cannot be excluded based on the data available, although we consider it unlikely. Only a few intervention studies in humans have addressed the effect of grape polyphenols on blood pressure and/or endothelial function. Based on these and our own data, we conclude that evidence on vascular health benefits is insufficient. Two meta-analyses, including our own, suggest that folic acid may have beneficial effects on endothelial function, but only at high doses (≥5000 μg per day). This evidence is based on a limited number of studies with substantial heterogeneity regarding study and population characteristics between the included studies.

Relevance for public health

Several aspects need to be considered when estimating the potential health impact of dietary changes in free-living populations based on effects measured in controlled intervention studies. These include differences in compliance, and uncertainties about the extent to which effects observed in short-term studies under experimental conditions and with different subjects can be translated to longer term effects in the general population. Participants in intervention studies are more motivated towards dietary changes than people in the general population. Furthermore, effects observed in most intervention studies are based on relatively short duration (weeks). It is conceivable that adherence to diets after longer follow-up periods will reduce. In addition, blood pressure effects of dietary changes tend to be stronger in (pre)hypertensives compared to normotensives.[20, 87-89] In intervention studies, participants are often selected on basis of elevated blood

pressure levels. As a result, the effects on blood pressure in the free-living population will likely be smaller compared to the effects observed in intervention studies.

Regardless of this possible overestimation of the true potential to affect blood pressure in intervention studies, the majority of people in populations in Western countries have high-normal blood pressure, and even small reductions in blood pressure on a population level will have a large public health impact.[90-94] For instance, a population-wide reduction in systolic blood pressure of 2 mmHg is estimated to reduce stroke mortality by ~10% and mortality from ischemic heart disease or other vascular causes by ~7%.[95] Thus, even though favorable effects of dietary interventions on a population level might be smaller than effects found in intervention studies, these small changes on population blood pressure level are still highly relevant.

Minerals

We estimated that a nation-wide increase in potassium intakes (to 4.7 gram per day) has the potential to reduce systolic blood pressure by about 2-3 mmHg in Western populations (**Chapter 2**). Intervention studies showed larger effects of increased potassium intake on blood pressure in subjects with a high salt intake[20, 21], which is the case in current societies.[96] Furthermore, the sodium to potassium intake ratio may be more strongly related to blood pressure[43] and CVD risk[42] than sodium and potassium alone. Therefore, a strategy combining an increase in potassium intake with reductions in sodium intake could be a more powerful strategy to reduce population blood pressure levels than salt reduction alone. Next to favorable effects of increasing potassium intake, potential adverse effects should also be considered. In the general population dietary potassium intake above recommended levels does not lead to a potential risk for adverse health effects as excess potassium is readily excreted in the urine.[97, 98] Some specific subgroups, such as people with severely impaired kidney function and patients on certain type of blood pressure lowering medication should avoid high potassium intake.[99-101] In practice, most of these patients are under medical supervision and follow specific guidelines to control mineral intake.[8, 98]

The results of our meta-analysis (**Chapter 3)** suggest that not only potassium, but also adequate calcium intake is relevant for population blood pressure levels. Increasing calcium intakes might be especially relevant for populations in which calcium intake is relatively low, as is indicated by our subgroup analysis. Regarding the source of calcium, data do not suggest a clear difference in efficacy between dietary and supplemental calcium sources for lowering blood pressure.[24] Public health guidelines could consider the

evidence for the blood pressure lowering potential of adequate calcium intakes when setting future recommended intakes for calcium. Current recommended intakes for calcium in adults are largely based on its favorable effects on bone health[102], and vary from 800-1000 mg per day.[103, 104] Calcium intakes in Europe are not optimal, especially females frequently consume less than the recommended intakes.[104] A report from the World Health Organization shows that calcium is one of the nutrients of greatest concern for public health, and that intakes in developing countries are even lower than in Europe.[102] High calcium intakes of 2500 mg from both diet and supplements were found to be well tolerated in many studies of long duration, and there does not seem to be reason to restrict calcium intake because of adverse effects [102, 105, 106]

Data from studies with combined potassium, calcium, and magnesium intake, including our own study (**Chapter 4**) do not support an additive effect on blood pressure. However, only a limited number of studies addressed the combined effect of these minerals, and the doses of the individual minerals in these studies were relatively low compared to those in studies addressing the effect of individual minerals. Furthermore, our study was not designed to detect relatively small reductions in systolic blood pressure, i.e. 2-3 mmHg, that are still relevant for public health.

Other food components

Current evidence, based on a large number of studies, does not support a antihypertensive effect of the lactotripeptides IPP and VPP (**Chapter 5**). However, a beneficial effect of fermented milk and in Asian populations cannot be excluded. For assessing the potential health impact of dietary effects on endothelial function, additional prospective studies should address the importance of endothelial function for predicting CVD risk. Without such data, effects on endothelial function as observed in intervention studies cannot be translated to relevance for public health. Regarding grape polyphenols and blood pressure, well-controlled intervention studies are scanty and findings cannot yet be translated into public health practice. In our meta-analysis on folic acid and endothelial function, we concluded that folic acid at high doses (≥5000 mg per day) may improve endothelial function. However, this finding is of limited value for public health. Apart from our considerations regarding endothelial function as a risk marker for CVD, the evidence was based on a limited number of studies, largely performed in patients, and at doses of folic acid intake that cannot be attained by every-day diets. Furthermore, results from recent intervention studies do not suggest that supplementation of folic acid and other B-vitamins has much relevance for reducing CVD risk.[85, 86]

Future research directions

The present thesis provides several directions for further research. Some recommendations for establishing the relevance of minerals, polyphenols and for improving blood pressure at the population level are given below.

Research may focus on both the practical aspects and feasibility of increasing the intake of potassium on a large, population-based scale, as well as assess the impact on population blood pressure under long-term, every-day conditions.

Furthermore, randomized controlled intervention studies of reduced sodium plus increased potassium intake compared to reduced sodium intake alone, are warranted. Such studies should provide additional evidence for the potential of a reduction in sodium intake combined with an increased potassium intake for population blood pressure levels.

Intervention studies in various population subgroups[49-52], including our own meta-analysis, suggest that calcium supplementation might be especially relevant for blood pressure in population subgroups with low habitual calcium intakes.[49-52] To test this hypothesis, a randomized placebo controlled intervention study could be performed in which the effect of increasing calcium intake on blood pressure in participants on a low calcium diet is compared with the effect on a normal or high calcium diet.

More insight in bioavailability and metabolism is needed to address which of the many individual polyphenols and/or combinations of polyphenols, are most promising for further study in the field of vascular function.

In meta-analyses, the options for addressing heterogeneity and investigating differences between different subgroups are often limited due to restrictions in the availability of the data from the individual studies. To allow systematic evaluation of subgroups, guidelines for reporting stratified data in future intervention studies would be useful.

An increasing number of studies, including two studies in this thesis, address endothelial function as primary outcome. Whether endothelial function predicts hypertension or CVD is not yet known. Therefore, additional prospective cohort studies in the general population should address the relevance of endothelial function as a reliable risk indicator for CVD (risk factors).

Strategies to improve mineral intake in populations

While adequate intakes of potassium and calcium are potentially important for population blood pressure levels, as supported by our and several other studies, many more studies have demonstrated the efficacy of decreasing sodium intakes. Since current intakes of sodium are too high and potassium and sodium together play an important role in regulating blood pressure and CVD risk[34, 43, 107], public health strategies should aim at adequate potassium and calcium intakes, combined with reductions in sodium intake. This paragraph discusses current guidelines and strategies in the area of mineral intakes and population blood pressure, and provides suggestions for governmental and food industry actions to improve current intakes in populations.

Current guidelines and strategies

Many guidelines and publications have addressed the importance of reducing salt intakes worldwide and in some countries strategies and programs to reduce salt intake have been implemented. However, even in countries in which salt intake has been reduced over the past years, the actual intake is still higher than recommended.[108, 109] Research from the World Health Organization has shown that salt reduction strategies can be implemented at low cost and result in large reductions in mortality.[110]

A number of independent organizations and authoritative bodies also acknowledge that increasing potassium intake lowers blood pressure and reduces the risk of stroke.[32, 36, 111] In the United States of America and Canada a review by the US Institute of Medicine showed that an adequate potassium intake, defined as 4.7 gram per day, blunts the age-related rise in blood pressure, reduces the adverse effects of high sodium chloride intake on blood pressure, reduces the risk of recurrent kidney stones, and possibly decreases bone loss.[8] In addition, several recent publications have highlighted a critical role for potassium in relation to blood pressure and CVD risk.[40-42] Current recommendations for calcium are primarily directed towards the prevention of osteoporosis.[102]

Suggestions for improvement of current strategies

Improving compliance to general dietary guidelines which recommend an increased intake of fruit and vegetables and low-fat dairy products would be an effective way to increase intake of both potassium and calcium. Both nutrients are abundantly available in these foods, but education programs aimed to optimize dietary intakes take a relatively long time to be effective. For example, in the United Kingdom (UK), despite many efforts

to promote the simple, clear, positive, quantified and food-based "five a day" campaign, there has been little improvement in fruit and vegetable intake.[112, 113] These data are in line with general findings in Europe showing that nutrition policies are in place, but strong support for the implementation and evaluation of those policies is lacking.[114]

In order to successfully improve dietary mineral intakes, two complementary approaches can be considered for which collaboration between governmental bodies and the private sector is crucial. First, the availability, affordability and accessibility of foods with a healthy mineral composition could be increased to encourage individuals to improve their mineral intakes.[114] Preferably, these changes need to affect the whole food range, from cheap to more expensive foods and not only in supermarkets, but also in restaurants.[109, 114] Secondly, dietary modifications that do not depend on individual choice might be implemented by modifying the food supply.[112] To improve the potassium and calcium content in foods, several options are available. One option is to use more potassium-rich and calcium-rich foods in processed foods. Other options are to restore losses in healthy minerals during food processing and/or to (partly) replace regular sodium chloride salt by potassium chloride mineral salts. In terms of health this last option will provide a double benefit. Implementation of these changes in the food environment combined with improvements in the mineral composition of foods that are habitually consumed can have a substantial impact on longer-term overall healthier mineral intake in the population.

To summarize, the main conclusions of this thesis are:

1. Adequate potassium and calcium intakes may play an important role in the prevention and control of hypertension at the population level.
2. Evidence for blood pressure lowering effects of lactotripeptides and for improvements in endothelial function by grape polyphenols and folic acid is limited.

Box

What was already known

- A healthy diet can substantially lower BP. Small reductions in BP on the population level have a large impact on CVD morbidity and mortality.
- Minerals play an important role in BP regulation. Decreasing sodium intake and increasing potassium intake, and to a lesser extent increasing calcium intake can reduce BP. An additive or synergistic effect of minerals has been tested in a few studies, with conflicting results.
- Lactotripeptides may lower BP, but evidence is mainly based on studies performed in Japan and Finland. Grape polyphenols have been suggested to improve BP and endothelial function, but evidence from randomized controlled studies is limited. The effect of folic acid on endothelial function is studied in several intervention studies, but effects over a wide dose range, including lower dose levels, have not been systematically reviewed.

What this thesis adds

- Current potassium intakes in countries are below the recommended level. A nation-wide increase in potassium intake can result in substantial reductions in BP levels in Western populations.
- We confirmed a BP lowering effect of calcium intake that may be especially relevant for populations with low habitual intakes.
- We could not establish a BP lowering effect of potassium combined with other micronutrients at low doses in Dutch subjects with untreated elevated BP levels.
- Lactotripeptides did not lower BP in Scottish subjects with untreated elevated BP.
- Grape polyphenols had no effect on endothelial function and BP in healthy male Dutch subjects.
- Folic acid could improve endothelial function, but only at high doses, that can not be attained by every-day diets.

Public Health implications

- Public health measures to encourage potassium intake (preferably from foods) are warranted, next to reductions in sodium intake. In populations or population subgroups with low calcium intakes, BP may benefit from increased calcium intake.
- Fruit, vegetables and low-fat dairy are good sources of potassium and calcium. Consequently, increased intakes of these minerals can be achieved by strategies improving compliance to existing dietary guidelines. In addition, the government and food industry should act in concert to improve mineral intakes in populations, thereby reducing the prevalence of hypertension and burden of CVD.

BP, blood pressure.

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Summary

Summary

Blood pressure levels show a graded independent relationship with risk of cardiovascular diseases (CVD). The majority of people in Western populations have blood pressure levels above optimal (i.e. ≥120/80 mmHg) and modest reductions in blood pressure on the population level can substantially reduce CVD incidence. Established lifestyle changes that lower blood pressure include weight loss, increased physical activity, moderation of alcohol intake, increased dietary potassium intake and reduced dietary sodium intake. Blood pressure is regulated by several physiological mechanisms, which also involve endothelial function.

The studies in this thesis have been initiated to investigate the effect of micronutrients and other bio-active food components on blood pressure and endothelial function, with two main objectives:

1. To assess the potential impact of selected minerals intake on population blood pressure levels.
2. To investigate the effects of 'emerging' food components on blood pressure and endothelial function.

Impact of minerals on population blood pressure

Three studies described in this thesis assess the effects of mineral intakes on population blood pressure levels. The first two chapters (**Chapter 2 and 3**) focus on potassium and calcium and the third (**Chapter 4)** on milk enriched with a combination of minerals and vitamins.

A systematic review of national dietary surveys estimates the potential impact of increasing potassium intake on population blood pressure (**Chapter 2**). Several meta-analyses of intervention studies have shown that potassium supplementation of 2–3 grams per day reduced systolic blood pressure by 3-4 mmHg, but the potential impact of improving potassium intakes on population blood pressure levels has not been estimated in previous studies. Our review of data from 21 countries shows that current potassium intakes are lower (range: 1.7–3.7 gram per day) than the current recommendation of 4.7 gram per day. We estimated that increasing intake to this level has the potential to reduce population systolic blood pressure by 2-3 mmHg in Western countries. The size of this projected effect is comparable to that which can be achieved with a reduction in salt intake from current intakes of about 9 gram per day to the recommended level of 5 gram per day.

Chapter 3 describes a meta-analysis of intervention studies on calcium supplementation and blood pressure. Previous meta-analyses of such intervention studies

showed reductions of 0.9-1.4 mmHg for systolic blood pressure, and 0.2-0.8 mmHg for diastolic blood pressure with calcium supplementation. These analyses only partly addressed the importance of increasing calcium intake in different population subgroups. We included 40 randomized controlled intervention studies, and investigated the importance of calcium supplementation on blood pressure for different population subgroups. Our analysis showed that calcium supplementation (mean dose 1200 mg per day) for at least two weeks significantly reduced systolic blood pressure by 1.9 mmHg and diastolic blood pressure by 1.0 mmHg. In addition, the data suggest that response of blood pressure to calcium supplementation tends to be stronger in populations with habitual calcium intakes <800 mg per day than in populations with intakes ≥800 mg per day.

The blood pressure effects of individual minerals have repeatedly been studied, but only a limited number of studies investigated the effects of combinations of minerals on blood pressure. Our 8-week double-blind randomized placebo controlled parallel study in 124 Dutch subjects examines the effect on blood pressure of a combination of minerals and vitamins in a dairy matrix (**Chapter 4**). Participants daily consumed either one of two types of skimmed milk enriched in potassium (either 1500 mg or 750 mg), combined with calcium (446 mg), magnesium (100 mg), selenium (40 mg), vitamin C (180 mg) and vitamin E (30 mg tocopherol equivalents) or placebo (water). Reductions in office systolic blood pressure after 8 weeks intervention were not significantly different between groups (P = 0.94). However, our study was not sufficiently powered to detect reductions in systolic blood pressure of 2-3 mmHg or less that can be considered relevant at the population level.

Other food components and blood pressure (related) effects

Three studies in this thesis address other food components that recently received much attention for their possible beneficial effects on blood pressure and/or endothelial function (**Chapter 5, 6 and 7**).

Chapter 5 presents the effect of lactotripeptides on blood pressure based on the results of two double-blind, randomized, placebo controlled, cross-over studies. Other intervention studies, mainly studies performed in Japanese and Finnish populations, showed promising blood pressure lowering effects of dairy peptides, in particular for the lactotripeptides Isoleucine-Proline-Proline (IPP) and Valine-Proline-Proline (VPP). However, more recent studies in non-Finnish European populations found no significant blood pressure lowering effects of lactotripeptides. In our two 4-week intervention studies, we investigated the effect on blood pressure of lactotripeptides (mainly IPP and

VPP) obtained by enzymatic hydrolysis (study 1: 10.2 mg per day; study 2: 4.6 mg plus 350 mg per day added potassium) in the form of a yoghurt drink in 162 Scottish subjects. No antihypertensive effects of the lactotripeptides were found; the mean difference in 24-h ambulatory systolic blood pressure response between intervention and placebo were 0.6 mmHg (P = 0.55) in study 1 and -0.9 mmHg (P = 0.42) in study 2.

Chapter 6 addresses the effect of the polyphenolic fraction of two different solid grape extracts on endothelial function and blood pressure, in a randomized double-blind placebo controlled crossover study. Experimental data have suggested that red wine and its constituents can induce vasodilatation by increasing production of nitric oxide, but evidence from controlled human intervention studies is still limited. Earlier studies mostly addressed acute effects on endothelial function after administration of red wine, and were performed in patients with CVD, rather than in healthy subjects. In our study in 35 healthy male subjects, with 2-week intervention periods we tested the effects on endothelial function and blood pressure of two polyphenol-rich solids at a dose of 800 mg of polyphenols per day. One solid extract was derived from a wine-grape mix, representing a broad range of polyphenols (monomeric anthocyanins, catechins, flavonols, procyanidins and stillbenes and unidentified oligomers and polymers). The grape seed primarily consists of monomeric catechins and unidentified oligomers and polymers. Compared to placebo, both the wine-grape mix and grape seed extract did not significantly affect endothelial function as measured with flow-mediated dilation (-0.4%; P=0.77 and 0.2%; P=0.94, respectively) or office blood pressure (systolic blood pressure: -1.3 mmHg; P=0.70 and -0.6 mmHg; P=0.92, respectively).

Chapter 7 presents a meta-analysis of intervention studies on folic acid and endothelial function. Our combined estimate of 14 randomized, placebo-controlled intervention studies indicated that folic acid supplementation at a median dose of 5000 μg per day for at least 4 weeks significantly improved flow mediated dilatation by 1.1% over placebo. However, effects were confined to higher doses of folic acid (≥5000 μg per day). In addition, results indicated a larger effect in populations with CVD than in healthy populations.

Conclusions

With regard to dietary minerals, we conclude that increasing the intake of potassium, which is generally below the recommended level in Western countries, and calcium, especially in populations with intakes <800 mg per day, has the potential to improve population blood pressure levels. Future population-based intervention studies should

bear out the blood pressure lowering potential of potassium, preferably in combination with sodium reductions.

Our well-controlled studies refute that lactotripeptides in the form of isolated IPP and VPP can lower blood pressure. Although we consider it unlikely, beneficial blood pressure effects of fermented milk and of lactotripeptides in Japanese and Finnish populations can not yet be fully ruled out. We were not able to detect any effect of grape polyphenols on blood pressure or endothelial function. More insight in bioavailability and metabolism of polyphenols is needed to address which specific species or combinations of the many polyphenols in foods are most promising for further studies. Folic acid can improve endothelial function at high doses (≥5000 μg per day), but we consider this of limited value for public health as such intakes are far beyond what can be attained by diet.

For improving blood pressure at the population level, adherence to dietary guidelines is primarily recommended to achieve more optimal intakes of potassium and calcium from fruits, vegetables, and low-fat dairy products. In addition, the government and food industry should act in concert to improve mineral intakes in populations, thereby reducing the prevalence of hypertension and burden of CVD.

The main conclusions of this thesis are:

1. Adequate potassium and calcium intakes may play an important role in the prevention and control of hypertension at the population level.
2. Evidence for blood pressure lowering effects of lactotripeptides and for improvements in endothelial function by grape polyphenols and folic acid is limited.

Samenvatting

Samenvatting

Bloeddruk is rechtevenredig en onafhankelijk gerelateerd aan het risico op hart- en vaatziekten. De bloeddruk van de meeste mensen in Westerse landen is hoger dan optimaal (≥120/80 mmHg). Kleine verlagingen van de bloeddruk in de bevolking kan de incidentie van hart- en vaatziekten aanzienlijk verminderen. Leefstijlveranderingen met een bewezen bloeddrukverlagend effect zijn onder andere gewichtsverlies, meer bewegen, verminderen van de alcoholconsumptie, een hogere kaliuminname en een lagere natriuminname. Bloeddruk wordt gereguleerd door verschillende fysiologische mechanismen, onder andere door de functie van het endotheel (de binnenbekleding van de bloedvaten).

In dit proefschrift hebben we het effect van micronutriënten en andere voedingscomponenten op de bloeddruk en de functie van het endotheel onderzocht, met twee belangrijke doelstellingen:

1. Het schatten van de invloed van de inname aan mineralen op de bloeddruk in de bevolking.
2. Het onderzoeken of een aantal andere 'veelbelovende' voedingscomponenten een gunstig effect op de bloeddruk en endotheelfunctie hebben.

Mineralen en bloeddruk in de bevolking

In de **hoofdstukken 2, 3 en 4** van dit proefschrift onderzoeken we hoe belangrijk de inname aan mineralen is voor de bloeddruk in de bevolking. **Hoofdstuk 2 en 3** richten zich op kalium en calcium. **Hoofdstuk 4** richt zich op melk verrijkt met een combinatie van mineralen en vitamines.

De mogelijke effecten van een hogere kaliuminname op de bloeddruk in de bevolking worden berekend in een systematisch overzichtsartikel van nationale voedselconsumptiegegevens (**Hoofdstuk 2**). Verschillende eerdere meta-analyses van interventie-onderzoeken laten zien dat het verhogen van de kaliuminname met 2-3 gram per dag de systolische bloeddruk (bovendruk) met 3-4 mmHg kan verlagen. Eerdere onderzoeken hebben echter niet onderzocht wat het mogelijke effect is van het verhogen van de kaliuminname op de bloeddruk in de bevolking. Uit ons review, waarin we voedingsgegevens van 21 landen hebben meegenomen, blijkt dat de huidige inname aan kalium (range: 1,7-3,7 gram per dag) lager is dan de aanbeveling van 4,7 gram per dag. We schatten dat het verhogen van de kaliuminname tot het aanbevolen niveau leidt tot een verlaging van de systolische bloeddruk in de populatie met 2-3 mmHg in Westerse landen. Dit effect is vergelijkbaar met het effect dat bereikt kan worden met een

verlaging van de huidige consumptie van zout (natrium chloride) van ~9 gram naar het aanbevolen niveau van 5 gram per dag.

Hoofdstuk 3 beschrijft het effect van het verhogen van de calciuminname op de bloeddruk. Dit is gedaan middels een meta-analyse van interventie-onderzoeken. Eerdere meta-analyses laten een verlaging zien van de systolische bloeddruk met 0,9-1,4 mmHg en van de diastolische bloeddruk (=onderdruk) met 0,2-0,8 mmHg. In deze analyses is de grootte van het effect in verschillende groepen in de bevolking slechts beperkt onderzocht. In onze meta-analyse, gebaseerd op 40 gerandomiseerde en gecontroleerde interventie onderzoeken, onderzochten we het effect van het verhogen van de calciuminname voor verschillende groepen in de bevolking. De resultaten laten zien dat het verhogen van de calciuminname (gemiddelde dosering 1200 mg per dag), voor minimaal twee weken, leidt tot een significante verlaging van de systolische bloeddruk met 1,9 mmHg, en van de diastolische bloeddruk met 1,0 mmHg. Tevens suggereren onze data een sterkere relatie tussen het verhogen van de calciuminname en bloeddruk in populaties met een calciuminname <800 mg per dag vergeleken met populaties met een inname ≥800 mg per dag.

De bloeddrukverlagende effecten van individuele mineralen zijn veelvuldig onderzocht. Over het bloeddrukeffect van een combinatie van mineralen is echter weinig bekend. We hebben een dubbel-blinde gerandomiseerde en placebo-gecontroleerde parallelle studie in 124 Nederlandse proefpersonen uitgevoerd. In deze studie hebben we het effect van magere melk verrijkt met een combinatie van mineralen en vitaminen op de bloeddruk bestudeerd (**Hoofdstuk 4**). Proefpersonen consumeerden dagelijks één van de twee soorten magere melk verrijkt in micronutriënten of een placebo (water). De melkproducten bevatten kalium (1500 mg of 750 mg), calcium (446 mg), magnesium (100 mg), selenium (40 mg), vitamine C (180 mg) en vitamine E (30 mg tocoferol equivalenten). De dalingen in systolische bloeddruk na een interventieperiode van 8 weken waren niet verschillend tussen de groepen (P = 0,94). Onze studie was echter niet opgezet om relatief kleine effecten op systolische bloeddruk (2-3 mmHg) aan te tonen, hoewel deze wel relevant zijn op bevolkingsniveau.

Andere voedingscomponenten en bloeddruk (gerelateerde) effecten

In de **hoofdstukken 5, 6 en 7** onderzoeken we het effect van voedingscomponenten die in de belangstelling staan omdat ze een mogelijk gunstig effect op bloeddruk en/of endotheelfunctie hebben.

Hoofdstuk 5 behandelt het effect van lactotripeptiden op de bloeddruk, gebaseerd op de resultaten van twee dubbel-blinde, gerandomiseerde en placebo-

gecontroleerde cross-over onderzoeken. Andere interventie onderzoeken, voornamelijk gebaseerd op onderzoeken in Japanse en Finse populaties, hebben veelbelovende bloeddrukverlagende effecten van zuivelpeptiden gevonden, in het bijzonder voor de lactotripeptiden Isoleucine-Proline-Proline (IPP) en Valine-Proline-Proline (VPP). Recentere onderzoeken in niet-Finse Europese populaties konden echter geen significante bloeddrukverlagende effecten van lactotripeptiden aantonen. In onze twee gecontroleerde onderzoeken met 4-weekse interventieperiodes hebben in totaal 162 Schotse proefpersonen deelgenomen. In deze studies onderzochten we het effect van lactotripeptiden, voornamelijk IPP en VPP, verkregen door enzymatisch hydrolyse, op de bloeddruk. De proefpersonen consumeerden de lactotripeptiden in de vorm van een yoghurt drankje. De dagelijkse dosering lactotripeptiden was 10,2 mg in onderzoek 1 en 4,6 mg en 350 mg toegevoegd kalium in onderzoek 2. We vonden geen bloeddrukverlagende effecten van de lactotripeptiden. Het gemiddelde verschil in 24-uurs ambulante systolische bloeddruk tussen de actieve behandeling en placebo was 0,6 mmHg (P = 0,55) in het eerste onderzoek, en -0,9 mmHg (P = 0,42) in het tweede onderzoek.

Hoofdstuk 6 beschrijft het effect van twee verschillende polyfenolrijke druivenextracten op endotheelfunctie en bloeddruk. Dit is gebaseerd op een dubbel-blinde, gerandomiseerde en placebo gecontroleerde cross-over studie. Experimentele data lieten zien dat (bestanddelen van) rode wijn vaatverwijding kunnen induceren door het verhogen van de productie van stikstofmonoxide in het endotheel. Voor het optreden van dit effect bij mensen is echter weinig bewijs uit gecontroleerde interventie-onderzoeken. De meeste onderzoeken onderzochten acute effecten van rode wijn consumptie op endotheelfunctie. Bovendien zijn deze onderzoeken veelal uitgevoerd in patiënten met hart- en vaatziekten en niet in gezonde deelnemers. In onze studie, met interventieperiodes van 2 weken, in 35 gezonde mannen, onderzochten we de langere-termijneffecten op endotheelfunctie en bloeddruk van twee polyfenolrijke extracten (800 mg polyfenolen per dag). Eén van de extracten was afkomstig van een wijn-druivenmix en bestaat uit veel verschillende soorten polyfenolen (monomere anthocyanines, catechines, flavonolen, procyanidines en stillbenen en ongeïdentificeerde oligomeren en polymeren). Het andere extract was afkomstig van druivenpitten, en bestaat voornamelijk uit monomere catechines en ongeïdentificeerde oligomeren en polymeren. We vonden geen significante effecten van zowel het wijn-druivenmixextract als het

druivenpitextract op flow-gemedieerde dilatatie* (respectievelijk -0,4%; P = 0,77 en 0,2; P = 0,94) of bloeddruk (systolische bloeddruk respectievelijk: -1,3 mmHg; P = 0,70 en -0,6 mmHg; P = 0,92) vergeleken met placebocapsules.

Hoofdstuk 7 beschrijft een meta-analyse naar het effect van foliumzuur op endotheelfunctie. Onze gecombineerde schatting laat zien dat foliumzuursuppletie, bij een hoge mediane dosering van 5000 μg per dag, voor tenminste 4 weken, significant flow-gemedieerde dilatatie verbetert met 1,1% ten opzichte van placebo. Deze schatting is gebaseerd op 14 gerandomiseerde, placebo-gecontroleerde interventie-onderzoeken. De significante verbeteringen werden alleen bij de hogere doseringen van foliumzuur (≥5000 μg per dag) gevonden. Tevens suggereren de resultaten een groter effect in populaties met hart- en vaatziekten dan in gezonde populaties.

Conclusies

Het verhogen van de kaliuminname in Westerse landen, welke in het algemeen lager is dan de aanbevolen hoeveelheid, en de calciuminname, met name in populaties met een lage inname, kan de bloeddruk in de bevolking verbeteren. In onze goed gecontroleerde onderzoeken vinden we geen bloeddrukverlagende effecten van lactotripeptiden in de vorm van geïsoleerd IPP en VPP. Hoewel we het onwaarschijnlijk achten, kunnen bloeddrukverlagende effecten van gefermenteerde melk en van lactotripeptiden in Japanse en Finse populaties niet volledig worden uitgesloten. Wij vinden geen effect van druivenpolyfenolen op de bloeddruk en endotheelfunctie. Meer inzicht in de bio-beschikbaarheid en het metabolisme van polyfenolen is nodig om vast te stellen welke specifieke soorten polyfenolen of combinaties van de verschillende polyfenolen in voeding veelbelovend zijn voor toekomstig onderzoek. Foliumzuur kan endotheelfunctie verbeteren bij hoge doseringen (≥5000 μg per dag), maar deze bevinding is van beperkte waarde voor de volksgezondheid, omdat deze inname niet bereikt kan worden met een normale voeding.

De beste manier om de bloeddruk in de bevolking te verbeteren is door een betere naleving van de huidige voedingsrichtlijnen. Een verbetering in de inname van kalium en calcium kan bereikt worden door een hogere consumptie van fruit, groenten en magere zuivel. Daarnaast kan de overheid en de voedingsmiddelenindustrie samenwerken om de inname van mineralen in de bevolking te verbeteren en daarmee de

*flow-gemedieerde dilatatie is een techniek waarmee de stroomsnelheid van het bloed gemeten wordt. Het meet de elasticiteit van het endotheel, de bekleding van de vaatwand.

prevalentie van hypertensie en ziektelast ten gevolge van hart- en vaatziekten te verminderen.

De belangrijkste conclusies van dit proefschrift zijn:

1. Een adequate inname van kalium en calcium kan een belangrijke bijdrage leveren aan de preventie en het onder controle houden van hoge bloeddruk in de bevolking.
2. Het bewijs voor een bloeddrukverlagend effect van lactotripeptiden en voor het verbeteren van endotheelfunctie met druivenpolyfenolen en foliumzuur is beperkt.

Dankwoord

Dankwoord

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About the author

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Curriculum Vitae

Linda van Mierlo was born on 29th February 1980. After completing secondary school in 1998 (Bisschoppelijk College Weert), she started studying 'Nutrition and Health' at Wageningen University. As part of this MSc program she did her internship in the field of 'Sensory Perception' at the University of Helsinki, Finland. In 2003, Linda graduated with Majors in 'Epidemiology' and 'Marketing' and started working as a Nutrition Scientist in the field of Cardiovascular Health at Unilever Research & Development in Vlaardingen, the Netherlands. In the evening hours she completed the internationally recognized Netherlands Institute of Marketing (NIMA)-B training program. As part of her work at Unilever she was responsible for several human intervention studies in the area of nutrition and blood pressure which formed the basis of this thesis. In 2007 she supported the Unilever Spreads business in the United States during a six-month secondment, which provided her the opportunity to work in a business-environment, and to broaden her knowledge in the field of dietary fat. Back in the Netherlands she was the main science contact person for the launch of a new range of blood pressure products and decided to formally start her PhD program. As part of this PhD program she participated and presented at several national and international conferences and followed courses within the framework of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences). This PhD program was combined with her work at Unilever, where she will remain working in the Cardiovascular Health area.

About the author

List of publications

Original research papers

- **van Mierlo LA**, Greyling A, Zock PL, Kok FJ, Geleijnse JM. *Suboptimal potassium intakes and potential impact on population blood pressure*. Arch Intern Med; in press.
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- **van Mierlo LA**, Greyling A, Zock PL, Kok FJ, Geleijnse JM. *Suboptimal potassium intakes and potential impact on population blood pressure*. J Hypertens 2010;28:E240.
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- Grobbee DE, **van Mierlo LA**, Kok FJ, Geleijnse JM. *Blood pressure response to calcium supplementation: a meta-analysis of randomised controlled trials* J Hypertens 2003;21:S20.

About the author

Educational program

Discipline specific activities

- 20th European Society of Hypertension meeting, Oslo, Norway, *oral presentation*, 2010
- Meeting of the Netherlands Epidemiology Society, Nijmegen, NL*, oral presentation*, 2010
- *Nutritional and lifestyle epidemiology (9th edition advanced course)*, VLAG, Wageningen, 2009
- *History of Epidemiologic Ideas, Methods of Public Health Research*, Erasmus Summer Programme, Rotterdam, 2009
- 19th European Society of Hypertension meeting, Milan, Italy, *poster presentation*, 2009
- Wageningen Nutritional Sciences Forum, Arnhem, NL, *poster presentation*, 2009
- Symposium Zuivel en Bloeddruk: theorie en praktijk, Ede, NL, 2008
- Congres Lang Leven Hart en Vaten! Nijkerk, NL, 2008
- 48th Cardiovascular Disease Epidemiology and Prevention – and - Nutrition, Physical Activity and Metabolism, Colorado Springs, USA, 2008
- Food and Nutrition Conference & Expo, Philadelphia, USA, 2007
- World Congress of Cardiology – ESC Congress, Barcelona, Spain, 2006
- 16th European Society of Hypertension meeting, Madrid, Spain, 2006
- Centrum Voor Voeding en Gezondheid, RIVM, Bilthoven, NL, *oral presentation*, 2005
- Functional food ingredients: tools for improving health, Chipping Campden, UK, 2005
- Blood Pressure Monitoring Hypertension Symposium, Maastricht, NL, 2005
- 15th European Meeting on Hypertension, Milan, Italy, *poster presentation*, 2005
- Joint meeting of the NHG and MiVaB, Veldhoven, NL, *oral presentation*, 2005
- NWO 'meeting of the Dutch Nutrition Society', Papendal, NL, *oral presentation*, 2004

General courses

- Influencing Skills, Hemsley Fraser, 2010
- Statistical Issues in Drug Development, Unilever, 2009
- Master Trainer Course, Unilever, 2008
- Workshop 'Statistical Issues in Human Intervention Trials', Unilever, 2008
- Career Framework Workshop, Unilever, 2008
- Patent course, Unilever, 2008
- Coaching, Tack International, 2008
- Training 'Advising and convincing for researchers' Routs Laeven & Partners, 2006
- Mini symposium 'Statistics in clinical trials/consumer trials', Unilever, 2005
- Spoken English, Horizon Interlingua, 2004
- Lectures on Statistics, Unilever, 2004
- Carrousel course, Unilever, 2004

Optional courses and activities

- Preparing PhD research proposal
- Skillbase meetings, Expertise team meetings, Project team meetings, Science forum, Nutrition and Health updates, 2002-2010
- Secondment Unilever United States, May – October 2007

Colophon

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