DIETARY EFFECTS ON ANTIOXIDANTS, OXIDISED LDL AND HOMOCYSTEINE

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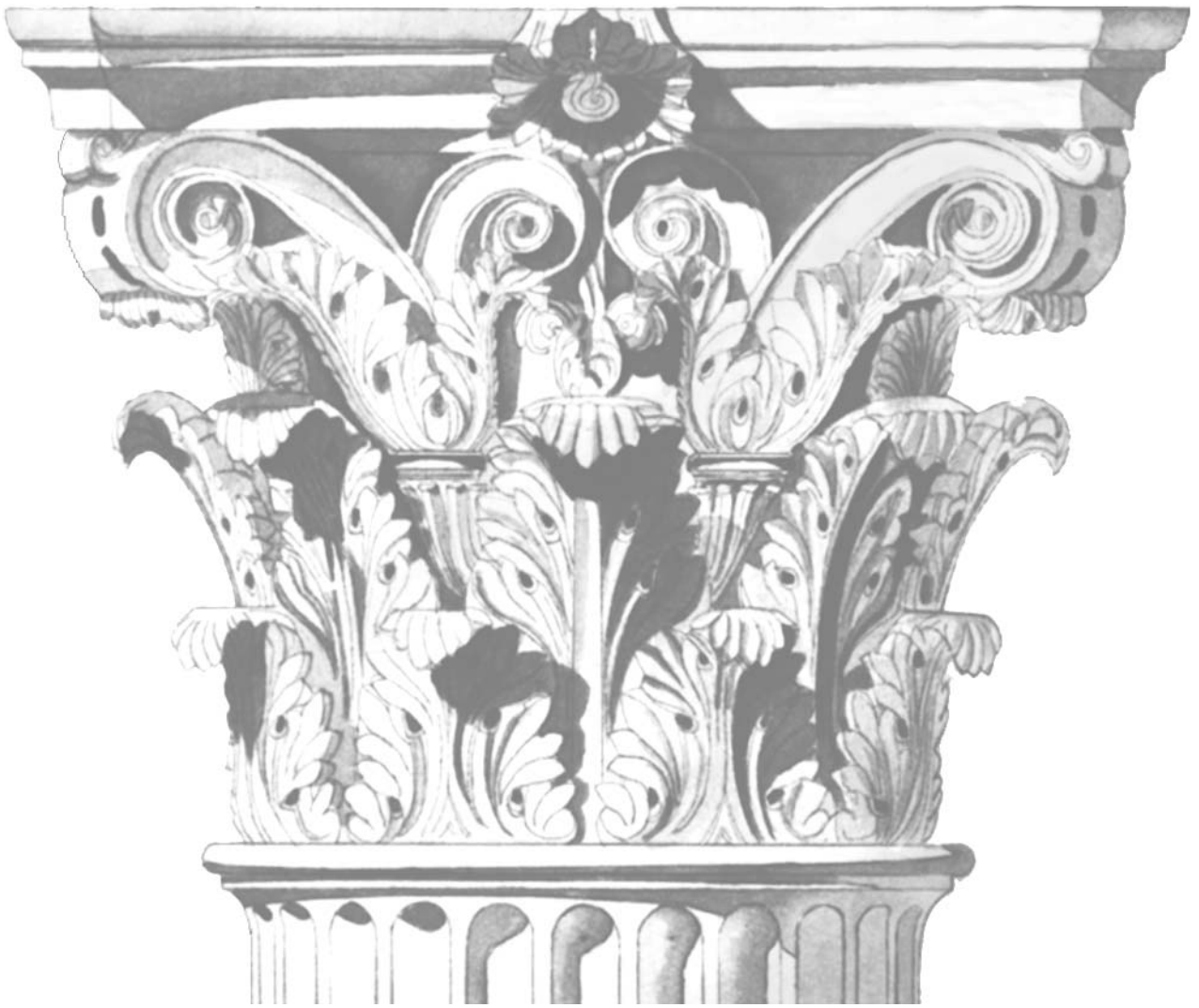

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Abstract

Dietary vegetables and fruit may play a significant role in atherosclerosis. We investigated the effects of a high intake of vegetables, berries, and citrus fruit along with a diet low in total and saturated fat on plasma concentrations of lipids, lipoprotein(a), antioxidants, oxidised LDL (OxLDL), folate, homocysteine, and on serum paraoxonase-1 activity. We also determined whether gene polymorphisms affect diet response of plasma homocysteine and serum paraoxonase-1 activity. Thirty-seven healthy females consumed two diets (low and high vegetable diets) in a controlled crossover intervention. The plasma measurements were determined at the baseline and at the end of diet periods.

The average plasma concentrations of total, LDL, and HDL cholesterol were 5.0 mmol/l, 2.8 mmol/l, and 1.7 mmol/l, respectively, on the low vegetable diet, and decreased by 8%, 8%,and 5%, respectively, in response to the high vegetable diet. The high vegetable diet increased the plasma concentrations of alpha-carotene, beta-carotene, lutein-zeaxanthin, beta-cryptoxanthin, and vitamin C by 133%, 134%, 107%, 65%, and 25%, respectively, compared with the low vegetable diet. There were no differences in the plasma concentrations of OxLDL between the low and high vegetable diets. The mean serum paraoxonase-1 activity was lower at the end of the high vegetable diet (226 U/l) than at the end of the low vegetable diet (240 U/l). Subjects having a genotype with high baseline paraoxonase-1 activity showed the most extensive reduction in their serum enzyme activities.

The high vegetable diet enhanced the serum and erythrocyte folate concentrations by 78% and 14%, respectively, and reduced the plasma homocysteine by 13% compared with the low vegetable diet. The dietary treatment was effective even among subjects homozygous for $C_{677}T$ mutation in methylenetetrahydrofolate reductase gene, who are susceptible to high homocysteine levels.

In conclusion, a high intake of vegetables, berries, and citrus fruit resulted in reduced plasma total and LDL cholesterol concentrations and enhanced plasma antioxidant levels. The high vegetable diet also effectively increased blood folate concentrations and reduced plasma homocysteine concentration.

Keywords: antioxidants, diet, folic acid, fruit, genetic polymorphism, homocysteine, lipids, lipoproteins, paraoxonase, vegetables

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Oulu, June 2003 Marja-Leena Silaste

Abbreviations

apoA-I	Apolipoprotein A-I
apoB	Apolipoprotein B
BMI	Body mass index
CBS	Cystathionine beta-synthase
CHD	Coronary heart disease
CI	Confidence interval
CV	Coefficient of variation
CVD	Cardiovascular diseases
E%	Percent of total energy intake
HDL	High-density lipoprotein
HPLC	High performance liquid chromatography
LDL	Low-density lipoprotein
Lp(a)	Lipoprotein(a)
MI	Myocardial infarction
MJ	Mega joule
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
MUFA	Monounsaturated fatty acids
NTD	Neural tube defect
ns	Not statistically significant
OxLDL	Oxidised low-density lipoprotein
OxLDL-EO6	Oxidised phospholipids of LDL recognised by antibody EO6
PCR	Polymerase chain reaction
PON1	Paraoxonase-1
PUFA	Polyunsaturated fatty acids
RR	Relative risk
SAFA	Saturated fatty acids
SD	Standard deviation
TBARS	Thiobarbituric acid-reactive substances
tHcy	Total homocysteine
VLDL	Very low-density lipoprotein

List of original articles

The thesis in based on the following articles, which are referred to in the text by their Roman numeral:

I Silaste M-L, Rantala M, Alfthan G, Aro A, Witztum JL, Kesäniemi YA & Hörkkö S. Changes in dietary fat intake alter plasma levels of oxidized low-density lipoprotein and lipoprotein(a). Manuscript.

II Rantala M, Silaste M-L, Tuominen A, Kaikkonen J, Salonen JT, Alfthan G, Aro A & Kesäniemi YA (2002) Dietary modifications and gene polymorphisms alter serum paraoxonase activity in healthy women. J Nutr 132: 3012–3017.

III Silaste M-L, Rantala M, Alfthan G, Aro A & Kesäniemi YA (2003) Plasma homocysteine concentration is decreased by dietary intervention. Br J Nutr 89: 295–301.

IV Silaste M-L, Rantala M, Sämpi M, Alfthan G, Aro A & Kesäniemi YA (2001) Polymorphisms of key enzymes in homocysteine metabolism affect diet responsiveness of plasma homocysteine in healthy women. J Nutr 131: 2643–2647.

Contents

1 Introduction

Cardiovascular diseases (CVD) are a leading cause of death in Finland and other westernised countries. Major risk factors for CVD are age, male sex, smoking, hypercholesterolemia, hypertension, family history of CVD, obesity, and physical inactivity. Three of these risk factors, namely plasma cholesterol concentration, blood pressure, and body weight, can be controlled and modified by diet. The effects of diet and dietary modifications on the plasma cholesterol and lipoprotein concentrations as well as on the blood pressure have been well documented. The amount and composition of dietary fat is the most important dietary determinant of plasma cholesterol concentrations. Other dietary factors influencing the plasma lipid concentrations include dietary fibre, dietary cholesterol, and alcohol. The knowledge of the dietary effects on the modifiable risk factors has been translated into specific dietary guidelines (Krauss *et al.* 2000, Nordic Nutrition Recommendations 1996). The current recommendations suggest that the total dietary intake of fat should be about 30 % of total energy intake. The dietary intake of saturated fatty acids should be below 10 % of total calories consumed, and the dietary intake of cholesterol should not exceed 300 mg per day. Importantly, the dietary guidelines encourage a high consumption of vegetables, fruit, and whole grains along with the fat-controlled diet for reducing and controlling the plasma cholesterol concentration. A similar diet rich in vegetables, fruit, and low-fat or fat-free milk products, together with limited alcohol consumption and dietary salt restriction, has been recommended for the treatment of hypertension and for maintaining normal blood pressure. (Krauss *et al.* 2000, Nordic Nutrition Recommendations 1996)

In addition to conventional risk factors, diet and dietary constituents may influence other factors that possibly play a role in the development of atherosclerosis and contribute to the CVD risk. One reason for searching for new determinants of CVD risk is related to ecological studies, which show that the different CVD rates between countries and within countries cannot be explained by the major established risk factors alone (The World Health Organization MONICA Project 1994). Several novel risk factors for atherosclerosis have been proposed (Ridker *et al.* 2001). One of them is homocysteine, an amino acid found in plasma, which has been suggested as an independent risk factor for atherosclerosis (Boushey *et al.* 1995). An important determinant of plasma homocysteine concentration is dietary intake of folate. The influence of dietary intake of fresh fruit and vegetables rich in natural folate on the plasma homocysteine concentration is an

interesting research area, but it has merited less consideration than the effects of folic acid supplementation. Dietary fruit and vegetables may also affect other aspects of atherosclerosis. Oxidative modification of low-density lipoprotein (LDL) is recognised to have an important role in the early development of atherosclerosis (Steinberg *et al.* 1989). The role of LDL oxidation in atherosclerosis and the possible protective effect of dietary antioxidants emphasise the impact of fruit and vegetable intake on the CVD risk. Because the oxidative modification of LDL is thought to occur primarily in the arterial wall, and thus beyond the reach of routinely available biological samples, the relationship between diet and LDL oxidation has to be investigated indirectly, for example by measuring the susceptibility of LDL to oxidation, other markers of oxidative stress, and the plasma levels and dietary intake of antioxidants. In addition to dietary antioxidants, the human body has several endogenous antioxidative systems which confer protection against oxidative stress. For example, the LDL may be protected by high-density lipoprotein (HDL) -associated enzymes, such as paraoxonase-1 (PON1). Interestingly, the activity of this enzyme may be modulated by diet (Mackness *et al.* 2000).

The present work was carried out to examine the effects of a high dietary intake of common vegetables, fruit, and berries rich in naturally occurring antioxidants and folate on the plasma levels of antioxidants, folate, and homocysteine. In order to study these effects we conducted a closely controlled dietary intervention with regular Finnish diets that were either low or high in vegetables, citrus fruit, and berries. The study diets were planned according to the current dietary guidelines for fat intake, and we were able to investigate how this “whole-diet approach” affected plasma lipid and lipoprotein concentrations, the plasma levels of oxidised LDL, and the activity of PON1. In addition, we investigated interactions between genes and diet by determining gene polymorphisms of PON1 and those of enzymes in homocysteine metabolism, and examining their influences on dietary response.

2 Review of the literature

2.1 Dietary antioxidants and cardiovascular diseases

2.1.1 Dietary antioxidants

An antioxidant has been defined as "a substance in foods that significantly decreases the adverse effects of reactive oxygen and nitrogen species, or both on normal physiological function on humans" (Food and Nutrition Board 2000). Generally, vitamin C, vitamin E, and carotenoids are considered the major dietary antioxidants. In addition, selenium is an essential constituent of a number of enzymes, some of which have antioxidant functions. Because the dietary intake of selenium in Finland is related to selenium supplementation of fertilisers rather than to dietary modifications (National Public Health Institute 2001), selenium has not been included in this thesis.

Carotenoids are natural pigments that are responsible for the bright colours of plants, flowers, fruit, and vegetables. In animals, carotenoids may serve as precursors of vitamin A and as colourants. More than 600 carotenoids exist, and about 50 of them can be converted into vitamin A in a variety of animal species. Human diet contains about 40 carotenoids (Beecher & Khachik 1992). The absorption of carotenoids in humans seems to be selective, because all ingested carotenoids cannot be detected in plasma. The proportionally most important carotenoids for humans are lycopene, alpha- and beta-carotene, lutein, zeaxanthin, and beta-cryptoxanthin. (Beecher & Khachik 1992) Provitamin A function is exhibited by beta-carotene and structurally related carotenoids, such as alpha-carotene and beta-cryptoxanthin, but not by lutein, lycopene, or zeaxanthin. In the human diet, carotenoids are mainly found in vegetables, legumes, fruit, and berries. According to analyses of Finnish foods, beta-carotene and lutein are the predominant carotenoids in most vegetables, berries, and fruit (Heinonen *et al*. 1989). Carrots, spinach, red sweet pepper, dill and parsley are particularly rich in beta-carotene. Alpha-carotene is found in carrots, beans, yellow sweet pepper, orange, mandarin, banana, avocado, cloudberries, and raspberries. Traces of alpha-carotene are also found in several other vegetables and berries. The major sources of dietary lycopene are watermelon, tomatoes,

and tomato products, such as ketchup. Cryptoxanthin appears in avocado, broccoli, peach, orange juice, kiwi-fruit, grapefruit, and canned peach and pineapple. (Heinonen *et al.* 1989)

Carotenoids are lipid-soluble substances, and need fat to be absorbed (Prince & Frisoli 1993). In the plasma, carotenoids are bound to lipoproteins, primarily LDL (Clevidence & Bieri 1993). The plasma concentrations of carotenoids are considered useful biomarkers of total dietary intake of vegetables and fruit (Campbell *et al.* 1994). To date, the provitamin A function is the only proven physiological function of carotenoids in humans. Thus, no specific recommendations exist for dietary carotenoid intake.

Vitamin C (ascorbic acid) is an essential nutrient for humans. It is a water-soluble vitamin, which humans and other primates cannot synthesise themselves. Vitamin C is involved in many metabolic functions, such as biosynthesis of collagen. Deficiency of vitamin C results in weakening of collageneous structures, causing tooth loss, joint pains, bone and connective tissue disorders, and poor wound healing, which are characteristics of scurvy (Burri & Jacob 1997). The major dietary sources of vitamin C are fresh fruit, berries, and vegetables, including potatoes (National Public Health Institute 2001). Citrus fruit and some berries, such as blackcurrants, are particularly rich in vitamin C. Vitamin C is easily destroyed due to oxidation during storage and cooking. The current recommended dietary intake of vitamin C for adults is 60 mg/day (Nordic Nutrition Recommendations 1996). On an average, this recommendation is well met in Finland, where the dietary vitamin C intake of adults is about 110 mg per day (National Public Health Institute 2001).

Vitamin E is a lipid-soluble vitamin that appears in vegetable oils, whole grains, seed, nuts, and some vegetables. The main food source of vitamin E in the Finnish diet is dietary fat, which provides about 40 % of total dietary vitamin E. Other sources include grains, dairy products, and eggs. (Heinonen & Piironen 1991) Vitamin E is composed of a family of tocopherols and tocotrienols. The most abundant form in food is alpha-tocopherol, which accounts for approximately 85 % of total dietary vitamin E in the Finnish diet (Heinonen & Piironen 1991). Alpha-tocopherol is also the form generally used in vitamin supplements. Alpha-tocopherol is the predominant lipid-soluble nutrient in the LDL with a concentration that is over 20 times higher than that of beta-carotene, for example (Chopra & Thurnham 1999). The current recommended dietary intake of vitamin E is 8 mg per day for adult females and 10 mg for males (Nordic Nutrition Recommendations 1996).

2.1.2 Role of LDL oxidation in atherosclerosis

Oxidation of LDL is thought to play an important role in the development of atherosclerosis (Steinberg *et al.* 1989). The oxidative modification of LDL is a series of complex reactions. The earliest step in the generation of oxidative modified LDL is peroxidation of polyunsaturated fatty acids (PUFA) in the LDL phospholipids. The PUFA undergo extensive breakdown yielding an array of reactive aldehydes, some of which can become covalently attached to apolipoprotein B (apoB) moiety of the LDL (Steinbrecher 1987, Ylä-Herttuala *et al.* 1989, Palinski *et al.* 1989). The modification and degradation

of apoB causes the LDL to be recognised by scavenger receptors of macrophages in the arterial wall.

The oxidative modification of LDL enhances atherogenesis by a number of different mechanisms, in particular by attracting the monocytes into the vascular intima and transforming them into foam cells (Steinberg *et al.* 1989). Several studies have supplied evidence that oxidised LDL (OxLDL) is present *in vivo*. For example, OxLDL can be extracted from atherosclerotic lesions (Ylä-Herttuala *et al.* 1989, Palinski *et al.* 1989), epitopes of OxLDL can be demonstrated immunohistochemically in atherosclerotic lesions in humans and animal models (Palinski *et al.* 1989, Rosenfeld *et al.* 1990), autoantibodies reactive with OxLDL are present in plasma and lesions of humans and animals (Palinski *et al.* 1994, Ylä-Herttuala *et al.* 1994), and small amounts of minimally oxidised LDL can even be demonstrated in plasma (Palinski *et al.* 1996). Consequently, there has been a vast amount of interest in evaluating factors that influence the LDL oxidation, as well as development of pharmacological agents and antioxidants that could reduce the oxidative modification of LDL.

The quantity and quality of dietary fat influences the susceptibility of LDL to oxidative modification by affecting the plasma LDL concentration and altering the fatty acid composition of LDL. Diets high in PUFA result in higher PUFA content of the LDL, higher tendency towards lipid peroxidation, and higher LDL oxidation in humans than diets high in monounsaturated fatty acids (Berry *et al.* 1991, Reaven *et al.* 1991). Antioxidants, on the other hand, can inhibit the oxidation of LDL. Administration of lipid-soluble antioxidants, such as probucol (Reaven *et al.* 1992) or high doses of alpha-tocopherol (Reaven *et al.* 1993, Jialal *et al.* 1995) is associated with their incorporation into the LDL and an increase in the resistance of LDL to oxidative modification *in vitro*. In contrast, supplementation with beta-carotene does not seem to protect the LDL from oxidation, despite its accumulation within the particle (Reaven *et al.* 1993, Gaziano *et al.* 1995a). As a water-soluble vitamin, vitamin C does not incorporate into the LDL; however, it may protect the LDL against oxidation *in vitro* (Jialal *et al.* 1990). Promisingly, in animal models antioxidants have been shown to prevent the progression of atherosclerosis *in vivo* (Kita *et al.* 1987, Williams *et al.* 1992).

Data on the effects of whole diets with a high intake of fruit and vegetables on the LDL oxidation are limited and conflicting. In a controlled dietary intervention, breath ethane, a marker of *in vivo* lipid peroxidation, was reduced among subjects who consumed a diet high in fruit and vegetables compared to the control group (Miller, III *et al.* 1998). Plasma thiobarbituric acid-reactive substances (TBARS), which are commonly used as a marker of lipid peroxidation, have been reported to remain unaffected (Miller, III *et al.* 1998) or to increase (Freese *et al.* 2002) in response to diets high in fruit and vegetables. Additional studies on the influence of dietary fruit and vegetables on the LDL oxidation are indicated.

In addition to dietary antioxidants, HDL can inhibit the oxidative modification of LDL *in vitro* (Mackness *et al.* 1993) and *in vivo* (Klimov *et al.* 1993). Several enzymes, such as paraoxonase-1 (PON1) and platelet activating factor acetylhydrolase, are present in the HDL, and some of the antioxidant activity of HDL is thought to be associated with its enzymes, particularly PON1 (Mackness & Durrington 1995). Interest has been focused on serum PON1 in the fields of both toxicology and atherosclerosis. PON1 is capable of detoxifying by hydrolysis a large number of organophosphate compounds, such as

paraoxon, a metabolic product of the pesticide parathion. The enzyme also breaks down nerve gases, such as sarin. At the moment, the physiologic substrate(s) of PON1 are not known. The activity of this enzyme in serum is usually measured towards paraoxon. Serum PON1 activities vary widely between individuals, but remain relatively constant in a given person (Heinecke & Lusis 1998). Polymorphisms of the PON1 gene are at least partly responsible for the interindividual differences in enzyme activities (Davies *et al.* 1996).

Based on experimental data, PON1 is believed to protect against the development of atherosclerosis. In the HDL, PON1 is responsible for HDL's ability to metabolise lipid peroxides (Mackness *et al.* 1991a, Mackness *et al.* 1993). PON1 has the ability to retard the oxidation of LDL by hydrolysing LDL-associated oxidised phospholipids and cholesteryl-ester hydroperoxides and by destroying the pro-inflammatory molecules involved in the initiation and progression of atherosclerotic lesions (Watson *et al.* 1995, Hedrick *et al.* 2000). Low serum PON1 activities have been observed in subjects at high risk of coronary artery disease, including those with hypercholesterolemia and diabetes (Mackness *et al.* 1991b, MacKness *et al.* 1998). However, a causal effect of PON1 in the development of atherosclerosis has not been confirmed. For example, a reported reduced serum PON1 activity and an increased CHD risk (Jarvik *et al.* 2000) may be related to the atherosclerosis-promoting abnormalities of lipid metabolism. More interestingly, dietary habits may regulate PON1 activity and may explain, at least partly, the reduced PON1 activities among CHD patients.

In animal studies, an atherogenic diet reduced serum PON1 activity, and this reduction was associated with a reduction in HDL-cholesterol concentration (Hedrick *et al.* 2000, Mackness *et al.* 2000). The effect of diet on the serum PON1 activity in humans has not been explored in detail. At the moment, data from only few dietary interventions on humans are available. In those studies, supplementation with pomegranate juice (Aviram *et al.* 2000) and a daily moderate alcohol consumption (van der Gaag *et al.* 1999) resulted in increased serum PON1 activities. The increase in the serum PON1 activity was strongly correlated with increases in the plasma HDL cholesterol and apolipoprotein A-I (apoA-I) concentrations (van der Gaag *et al.* 1999). On the other hand, serum PON1 activity and apoA-I concentration were reduced after a meal rich in oxidised fat used for deep-frying in fast-food restaurants (Sutherland *et al.* 1999). Because PON1 may play an important role in the development of atherosclerosis, studies of dietary and also genetic modifications on serum PON1 activity in humans are indicated.

2.1.3 Food-based studies of dietary antioxidants

The role of dietary antioxidants from fruit and vegetables in CVD has been considered in epidemiological studies, which generally support the theory that fruit and vegetables promote cardiovascular health (Ness & Powles 1997). In a Finnish cohort study with over 5,000 subjects and a 14-year follow-up, a high dietary intake of both fruit and vegetables was associated with reduced CVD mortality (Knekt *et al.* 1994). More recently, the positive effects of a high intake of vegetables and fruit were confirmed in large prospective cohort studies. The findings from the Nurses' Health Study (about 80,000

women) and Health Professionals' Follow-up Study (about 40,000 men) support the protective effect of the consumption on fruit and vegetables against ischaemic stroke (Joshipura *et al.* 1999) and coronary heart disease (Joshipura *et al.* 2001) in both men and women. An increment of one serving of vegetables and fruit per day was associated with a 6 % lower risk of ischaemic stroke (relative risk [RR] 0.94; 95% confidence interval [CI] 0.90–0.99) and a 4 % lower risk of CHD (RR 0.96; 95% CI 0.94–0.99) (Joshipura *et al.* 1999, Joshipura *et al.* 2001). The fruit and vegetable intake was also observed to reduce the risk of CVD and total mortality in the general US population (Bazzano *et al.* 2002). Consuming fruit and vegetables more than 3 times per day compared with once a day was associated with a 42 % lower stroke mortality (RR 0.58; 95% CI 0.22–1.02), a 27 % lower CVD mortality (RR 0.73; 95% CI 0.58–0.92), and a 15 % lower all-cause mortality (RR 0.85; 95% CI 0.58–1.00) (Bazzano *et al.* 2002). In addition to fruit and vegetables, a high dietary intake of vitamin E from food has been observed to associate with a lower CVD risk (Knekt *et al.* 1994, Kushi *et al.* 1996).

Reports from trials with diets high in fruit and vegetables support the possible cardioprotective effect of these foods. The Lyon Diet Heart study compared the effects of a Mediterranean-type diet high in fruit, vegetables, legumes, and cereals with a regular low-fat diet in the secondary prevention of coronary heart disease (CHD) (de Lorgeril *et al.* 1999). The Mediterranean diet provided a survival benefit which was maintained up to 4 years after first MI (de Lorgeril *et al.* 1999). In another trial, too, a diet rich in fruit, vegetables, grains, and nuts was associated with reduced complications and mortality for over one year after acute MI (Singh *et al.* 1992). Even though fruit and vegetables were an important part of these dietary trials, the trials were designed to test the effects of whole diets on the CVD. The designs included several dietary modifications, such as changes in the quality of dietary fat, and the isolated impact of fruit and vegetables, or dietary antioxidants, cannot be separated from the general effects of the study diets.

2.1.4 Antioxidant supplements and cardiovascular diseases in observational studies

Several observational studies have emphasised the role of antioxidant supplements in the prevention CVD. For example, a high dietary intake of vitamin C attributable to vitamin supplements was reported to associate with a low CVD mortality (Enstrom *et al.* 1992). Other large observational studies have observed that intake levels of vitamin E achievable only from vitamin supplements are associated with about 40 % reduction in the risk of CHD (Stampfer *et al.* 1993, Rimm *et al.* 1993). In those studies, the dietary intake of vitamin C was not associated with the reduced risk of CVD (Stampfer *et al.* 1993, Rimm *et al.* 1993), and carotenoid intake was associated with the reduced risk only among smokers (Rimm *et al.* 1993). In general, the effect of supplemental beta-carotene on CVD has been suggested to be of minor importance, because beta-carotene does not seem to inhibit the oxidation of LDL (Reaven *et al.* 1993, Gaziano *et al.* 1995a). However, some studies have indicated that a high dietary intake of carotenoids from food is associated

with lower CVD risk (Gaziano *et al.* 1995b, Klipstein-Grobusch *et al.* 1999), suggesting a possible protective role of these constituents or foods rich in them.

2.1.5 Effects of antioxidant supplements in clinical trials

The potential protective effect of antioxidant supplementation has been tested in clinical trials, which were first designed to study the effects of antioxidant supplementation on cancer incidence, but offer data on the effects of supplementation on the CVD, too. In the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC)-study nearly 30,000 male smokers were allocated to receive either alpha-tocopherol (50 mg per day), beta-carotene (20 mg per day), both, or placebo for about 6 years (The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994). The results of this trial were not encouraging. An 18 % increase (95% CI 3–36) in the incidence of lung cancer and an 8 % increase (95% CI 1–16) in total mortality were observed among the men who received beta-carotene as compared to those subjects who did not. There were more deaths from haemorrhagic stroke (66 versus 44), but fewer cases of prostate cancer (99 versus 151) among the men who were given alpha-tocopherol than among those who were not; no effect of vitamin E on the total mortality was observed. (The Alpha-Tocopherol Beta-Carotene Cancer Prevention Study Group 1994) In the Beta-carotene and Retinol Efficacy Trial (CARET), the combination of beta-carotene and retinyl palmitate increased the risk of lung cancer (RR 1.28; 95% CI 1.04–1.57), and there was a trend towards increased risk of cardiovascular death (RR 1.26; 95% CI 0.99–1.61) as compared with the placebo group (Omenn *et al.* 1996). In the Physicians' Health study no impact of beta-carotene supplementation on the incidence of coronary heart disease was observed (Hennekens *et al.* 1996).

The antioxidant supplementation has not been proven to be more beneficial in secondary prevention studies, i.e. in subjects with high risk of CVD. In the Cambridge Heart Antioxidant Study (CHAOS) high doses (400 IU or 800 IU per day) of alpha-tocopherol reduced the risk of non-fatal MI in patients with established ischaemic heart disease effectively by 77 % (RR 0.23; 95% CI 0.11–0.47) as compared with the placebo group, but caused no reduction in total cardiovascular mortality (Stephens *et al.* 1996). In fact, there seemed to be more cardiovascular deaths among patients that received alpha-tocopherol than among those who did not (27 versus 23) (RR 1.18; 95% CI 0.62–2.27) (Stephens *et al.* 1996). In the further analysis of the ATBC-study, the beta-carotene supplementation was observed to associate with an increased risk of CHD death among men who had previous MI, and were thus at high risk of coronary event (Rapola *et al.* 1997). There were more deaths from fatal CHD in the beta-carotene group (RR 1.75; 95% CI 1.16–2.64) and in the combined beta-carotene and alpha-tocopherol group (RR 1.58; 95% CI 1.05–2.40) as compared to the placebo group. In the alpha-tocopherol group, the relative risk of fatal coronary event was 1.33 (95% CI 0.86–2.05) as compared to the placebo group. (Rapola *et al.* 1997) The Heart Outcomes Prevention Evaluation Study (HOPE) observed no benefit from vitamin E supplementation (400 IU per day) on the CVD or all-cause mortality (Yusuf *et al.* 2000). In the Heart Protection Study (HPS), a combination of antioxidants (vitamin E, vitamin C, and beta-carotene) alone or with the

lipid-lowering drug simvastatin had no effect on mortality or cardiovascular events (Heart Protection Study Collaborative Group 2002). In those studies (Yusuf *et al.* 2000, Heart Protection Study Collaborative Group 2002), no significant adverse effects of alpha-tocopherol were observed.

Thus, in clinical trials, either beta-carotene or vitamin E supplementation has not provided any cardiovascular health benefits. Various explanations have been given for the different results from the observational studies and clinical trials with vitamin supplements. These include suggestions that the supplement trials did not use the correct isomer of beta-carotene, that the antioxidants were not the right ones, that the antioxidant doses were too low, and that the durations of trials were too short (Witztum & Steinberg 2001). Confounding is a potential explanation. This means that the protective effect of certain dietary patterns seen in the observational studies, which has been supposed to associate with the dietary antioxidants, may in fact associate with other compounds in fruit and vegetables, or even substitution of dietary fat and meat. The high dietary intakes of vitamin E, vitamin C, and beta-carotene in observational studies may reflect the overall dietary pattern that is protective. For example, a high intake of beta-carotene from food may be a marker of the consumption of foods that are rich in beta-carotene, but also rich in other beneficial carotenoids or other bioactive constituents. The dietary flavonoids in food may also contribute to the CVD risk (Hertog *et al.* 1993). Despite the contradictory results from the observational studies and supplementation trials, the consumption of foods naturally rich in antioxidants, such as fruit and vegetables, ameliorates cardiovascular health (Joshipura *et al.* 1999, Joshipura *et al.* 2001). Obviously, people are and will be consuming mixed meals containing different types of foods, rather than specific foods or nutrients alone. Therefore, controlled dietary interventions are needed to study the effects of whole diets and alterations in the dietary intakes of fruit and vegetables on the mechanisms involved in atherosclerosis and on intermediate markers of disease risk. These can be explored by determining the effects of dietary vegetables and fruit on the plasma lipid and lipoprotein concentrations, and on the plasma levels of naturally occurring antioxidants and oxidised LDL.

2.2 Folate, homocysteine and cardiovascular diseases

Several novel risk factors for atherosclerosis have been proposed (Ridker *et al.* 2001). One of them is homocysteine, an amino acid found in plasma. An important determinant of plasma homocysteine concentration is a dietary intake of folate.

2.2.1 Folate

2.2.1.1 Folate in diet

Folate is a B vitamin, which acts as a coenzyme in several single-carbon transfer reactions to synthesise components of DNA, RNA, and proteins. The term folate describes the naturally occurring folates in food, whereas folic acid is the form of folate used in supplements and fortified foods. Because mammals do not have the ability to synthesise folates, they require preformed folate in diet (Shane 1995). Dietary folate has a lower bioavailability than synthetic folic acid. Based on the response in plasma folate concentration, the overall bioavailability of natural folate from a mixed diet has been estimated to be about 50 % of the bioavailability of synthetic folic acid (Sauberlich *et al.* 1987).

Folate is present in a wide range of foods. Liver, green leafy vegetables, legumes, and citrus fruit are particularly rich in folate (Bailey 1995). Naturally occurring folate is easily destroyed by light, heat, and oxygen. In particular, the folate of fresh vegetables and fruit is destroyed by cooking and storage (Hurdle *et al.* 1968). Therefore fresh, uncooked vegetables, berries, and fruit are the best dietary sources of folate. In practice, vegetables, fruit, and whole grains are the major contributors to the dietary folate intake in Finland. The average dietary intake of folate is 303 µg and 240 µg per day for Finnish men and women, respectively. (National Public Health Institute 1998) In the European countries, the average daily folate intake is 291 µg for men and 247 µg for women (de Bree *et al.* 1997). The current recommended dietary intake of folate for adults is 300 µg per day in the Nordic countries (Nordic Nutrition Recommendations 1996) and 400 µg per day in the USA (Bailey & Gregory, III 1999). The recommended folate intake for pregnant women is 400 µg per day in the Nordic countries (Nordic Nutrition Recommendations 1996) and 600 µg per day in the USA (Bailey & Gregory, III 1999).

2.2.1.2 Folate in human health

Folate has an important role in human health. Deficiency of dietary folate leads to impaired cell division and alterations of protein synthesis. Because folate is needed in the conversion of homocysteine to methionine (Fig. 1), a low dietary intake and serum concentration of folate are associated with an elevated plasma homocysteine concentration (Selhub *et al.* 1993). A more severe clinical consequence of dietary folate deficiency is megaloblastic anaemia (Lindenbaum & Allen 1995).

Folate is involved in the closure of the neural tube of a developing fetus (Hibbard & Smithells 1965), and a low folate status of a mother has been observed to associate with an increased risk for neural tube defects (NTDs) of a fetus or infant (Smithells *et al.* 1976). In clinical trials, folic acid supplementation has been shown to reduce the risk of NTDs (Milunsky *et al.* 1989, MRC Vitamin Study Research Group 1991). Therefore, health authorities in several countries have recommended that women in reproducible age should routinely consume 400 µg of folate or folic acid per day (Centers for Disease

Control 1992, Cornel & Erickson 1997). Also, in the United States, a food-fortification programme was initiated in 1998 to increase the folic acid intake of women and to reduce the rate of NTDs. All enriched grain products, such as flours and breakfast cereals, are now fortified by 140 µg of folic acid per 100 g, resulting in about 100 µg of extra folic acid daily for an average woman (Food and Drug Administration 1996). When consumed continually, the additional folic acid intake of 100 µg improves the erythrocyte folate concentration and may also prevent NTDs (Daly *et al.* 1997). Natural folate may also play a role in the prevention of NTDs (Werler *et al.* 1993, Shaw *et al.* 1995), but its effects have not been evaluated in clinical trials.

Dietary folate also seems to reduce the risk of CVD. Low blood folate levels have been observed to associate with an increased risk of CVD in a cross-sectional study (Schwartz *et al.* 1997), in a cohort study (Morrison *et al.* 1996), and in case-control studies (Verhoef *et al.* 1996, Robinson *et al.* 1998). Reports from the Nurses' Health Study (Rimm *et al.* 1998) and the Kuopio Ischemic Heart Disease Risk Factor Study (Voutilainen *et al.* 2001) showed that 20 % of the individuals with the highest consumption of folate or folic acid had less CVD than those with the lowest consumption. In the Nurses' Health Study, the relative risk of CHD among the women who were in the highest quintile of dietary folate intake was 0.69 (95% CI 0.55–0.87) as compared to the women with the lowest folate intake (Rimm *et al.* 1998). In the Kuopio Ischemic Heart Disease Risk Factor Study, the relative risk of acute coronary events was 0.45 (95% CI 0.25–0.81) among the men with the highest folate intake as compared to those with the lowest intake (Voutilainen *et al.* 2001). The possible mechanisms by which dietary folate may prevent CVD include antioxidant actions of folate, such as reducing intracellular endothelial superoxide (Doshi *et al.* 2001), and lowering plasma homocysteine concentration. The role of homocysteine in vascular diseases and the effects of folate/ folic acid on plasma homocysteine levels are discussed in chapter 2.2.2.

2.2.1.3 Interventions on dietary folate

Increased intake of folic acid by using either folic acid supplements or foods fortified with folic acid increases serum/plasma and erythrocyte folate concentrations (Cuskelly *et al.* 1996, Malinow *et al.* 1998). Folic acid supplementation is cheap and easy, and is considered to be safe (Campbell 1996). Folic acid also exhibits a higher bioavailability than natural folate (Sauberlich *et al.* 1987). In contrast to taking a vitamin supplement or consuming folic acid fortified flours or cereals, increasing the dietary intake of natural folate from vegetables and fruit requires a sustained change in dietary patterns. Consequently, increasing the dietary intake of natural folate effectively has commonly been thought to be difficult, or even impossible. However, there are a few interventions which have determined the influence of dietary folate on serum and/or erythrocyte folate concentrations (Table 1). The first study was carried out to compare the efficacy of folic acid supplements, fortified foods, and dietary folate in increasing erythrocyte folate concentrations of healthy women with respect to NTD prevention (Cuskelly *et al.* 1996). After a three-month intervention, the authors observed a non-significant increase of 11 % in erythrocyte folate concentration in the dietary folate group, and they concluded that

natural folate is practically ineffective in increasing the folate status (Cuskelly *et al.* 1996). Other investigators have also compared the effects of dietary folate and supplemental folic acid on serum and erythrocyte folate concentrations. Riddell and co-workers (2000) observed a 52 % increase in the serum folate concentration among the group that consumed only natural folate from food. In that study, too, folic acid was more effective than natural folate in increasing the serum and erythrocyte folate concentrations (Riddell *et al.* 2000). In contrast to these two studies, Brouwer and co-workers (1999b) reported that natural folate was almost as effective as folic acid in increasing plasma and erythrocyte folate concentrations. In a recent study (Venn *et al.* 2002), dietary counselling to consume foods naturally rich in folate proved to be effective in increasing serum folate levels.

In the previous dietary interventions, the increase in serum folate concentration in response to dietary folate has varied from 11 % to 52 % (Table 1). Three of the studies also determined the erythrocyte folate concentration, but in only one study was the change in the erythrocyte folate statistically significant. The results of previous interventions are modified by different dietary intakes of folate. In addition, varying bioavailabilities of food folate certainly influence the dietary response and affect the results of the interventions. For instance, the folate in citrus fruit and juices may be more available than the folate in vegetables and bread. The subjects' compliance may also influence the dietary response. A closely controlled dietary intervention including supervised meals (Brouwer *et al.* 1999b) may produce a more extensive increase in the folate concentration than an intervention carried out by a less controlled design (Cuskelly *et al.* 1996). In addition, individual factors, such as genetic variability, may play a role in folate metabolism, affecting the dietary response of serum folate concentration to the increased dietary folate intake. Therefore, additional studies on the impact of diet on the intake and blood levels of folate are indicated.

Table 1. Effects of natural folate on serum and erythrocyte folate concentrations in previous dietary interventions.

Study	Sub-jects*	Diet description	Study period (weeks)	Daily folate intake (µg)	Basal S-folate (nmol/l)	Relative increase in S-folate (%)	Basal E-folate (nmol/l)	Relative increase in E-folate (%)
Cuskelly *et al.* 1996	10	Folate-rich foods	12	410	not reported	not reported	366	11 †
Brouwer *et al.* 1999	23	High in vegetables and fruit	4	560	13.8	47	338	17
Appel *et al.* 2000	38	High in vegetables and low-fat dairy products	8	418	13.0	11	not reported	not reported
Riddell *et al.* 2000	15	High in vegetables and fruit	12	≈ 600	15.0	52	571	15 †
Venn *et al.* 2002	20	High in vegetables and fruit	4	618	18.0	37	not reported	not reported

S-Folate, serum folate concentration; E-Folate, erythrocyte folate concentration
*number of the subjects in the dietary intervention group
† not statistically significant change

2.2.2 Homocysteine

2.2.2.1 Homocysteine metabolism

Homocysteine is a sulphur-containing amino acid that is mainly derived from dietary methionine (Fig. 1). Under normal metabolic circumstances, about 50 % of the homocysteine formed is remethylated to methionine. The remethylation of homocysteine requires two key enzymes: methionine synthase (MS) (E.C.2.1.1.13) and methylenetetrahydrofolate reductase (MTHFR). The MS is found in almost all mammalian tissues. It uses vitamin B12 as a cofactor and 5-methyltetrahydrofolate as a methyl donor. The MTHFR is needed in the formation of 5-methyltetrahydrofolate. When there is an excess of protein or methionine, a larger proportion of homocysteine is metabolised by irreversible transsulfuration pathway, which degrades homocysteine to cysteine. In the transsulfuration, homocysteine is first sulfconjugated to cystathionine by cystathionine beta-synthase (CBS) (E.C.4.2.1.22), which has been isolated and characterised from human liver. Cystathionine is further cleaved into cysteine and alpha-ketobutyrate by cystathionine γ-lyase. Both enzymes need vitamin B6 as a cofactor. Cysteine may be utilised in the protein synthesis or as a precursor of the antioxidant glutathione.

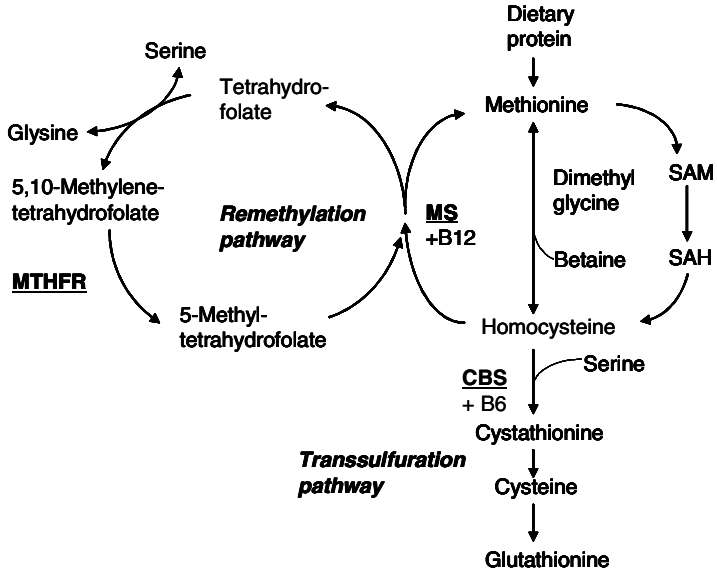

Fig. 1. Metabolism of homocysteine (CBS, cystathionine beta-synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine. Adapted from Fowler B (1997) Disorders of homocysteine metabolism. J Inherit Metab Dis 20: 270–285.).

If homocysteine accumulates and cannot be metabolised, it is excreted from the cells, which leads to elevated concentrations of homocysteine in the plasma or in the urine. (Ueland *et al.* 1993.)

In the plasma, approximately 70 % of homocysteine is bound to albumin. The rest combines with itself to form a dimer homocystine, or combines with other thiols to form disulphide, or circulates as a free thiol compound. (Ueland 1995) Total homocysteine (tHcy) refers to the combined pool of all four forms of homocysteine in plasma, and is a recommended measurement in a clinical setting (Ueland *et al.* 1993). In adults, the plasma tHcy concentration is about 10 µmol/l. The plasma levels of homocysteine are higher in men than in women, and tend to increase with age (Nygård *et al.* 1995). Elevated plasma homocysteine concentration, hyperhomocysteinaemia, has been divided into mild (15–30 µmol/l), intermediate (31–100 µmol/l), and severe (> 100 µmol/l) (Kang *et al.* 1992).

2.2.2.2 Nutritional control of plasma homocysteine

Plasma tHcy levels are regulated by several factors. Among the nutritional factors, deficiencies of vitamins B6, B12, and folate are associated with elevated plasma tHcy concentrations (Selhub *et al.* 1993). Supplementation with folic acid alone (Brattström *et al.* 1988, Jacques *et al.* 1999) and in combination with vitamins B6 and B12 (Ubbink *et al.* 1993, Brönstrup *et al.* 1998) reduces the plasma tHcy concentration. According to a meta-analysis of 12 randomised controlled interventions, a daily supplementation with 500 µg of folic acid and 500 µg of vitamin B12 decreases the plasma tHcy concentration by 25–30 % (Homocysteine Lowering Trialists' Collaboration 1998). However, some interventions indicated that a daily supplementation of only 200–250 µg of folic acid is as effective as higher amounts of folic acid in reducing the plasma tHcy concentrations (Ward *et al.* 1997, Brouwer *et al.* 1999a). The results from trials with low doses of folic acid encouraged investigators to test the impact of dietary folate on the plasma tHcy concentration. Indeed, a high intake of natural folate from vegetables and fruit has been observed to decrease plasma tHcy concentration (Brouwer *et al.* 1999b, Appel *et al.* 2000, Venn *et al.* 2002).

In addition to B vitamins, other dietary factors may influence the plasma tHcy concentration. Homocysteine is derived from the metabolism of the essential amino acid methionine, which is found at greatest concentration in animal protein. In humans, the plasma tHcy concentration increases after dietary intake of animal protein (Guttormsen *et al.* 1994), which may contribute to the development and progression of atherosclerosis (Chambers *et al.* 1999).

2.2.2.3 Genetic regulation of plasma homocysteine

The genetic factors affecting plasma tHcy levels include polymorphisms of three key enzymes participating in homocysteine metabolism (Fig. 1). Severe mutations, such as $G_{919}A$ and $T_{833}C$ in the CBS gene, are rare, affecting less than one percent of the general population (Kluijtmans *et al.* 1996, Folsom *et al.* 1998). Subjects with untreated CBS deficiency due to the homozygosity for the $T_{833}C$ mutation have very high plasma tHcy

concentrations and are at high risk of CVD (Gaustadnes *et al.* 2000). A 68-bp insertion (844ins68) of the CBS gene is fairly prevalent; it is present in the heterozygous state in 12 % of the USA population (Tsai *et al.* 1996). This insertion of the CBS gene may be associated with low plasma tHcy concentrations (Kluijtmans *et al.* 1997, Tsai *et al.* 1999). In the MS gene, the $A_{2756}G$ transition is highly prevalent, and the presence of the G_{2756} allele has been reported to be associated with lowered fasting levels of plasma tHcy (Tsai *et al.* 2000).

An important genetic determinant of plasma tHcy concentration is a common polymorphism of the MTHFR gene (Harmon *et al.* 1996). This defect results from a C to T point mutation at the nucleotide position 677 in DNA, leading to substitution of alanine by valine. The mutation is present in about 35 % of the alleles, and about 12 % of the population is homozygous for the mutation (TT genotype). The defect reduces the basal activity of the enzyme by about 50 %. (Frosst *et al.* 1995) Therefore, individuals homozygous for the mutation (TT genotype) have elevated plasma tHcy concentrations (Jacques *et al.* 1996, Nelen *et al.* 1998). One study found that the subjects homozygous for the T_{677} allele had an elevated plasma tHcy concentration when plasma folate concentration was in the lower range, but not when plasma folate was high (Jacques *et al.* 1996). Thus, homozygous individuals (TT genotype) may have a higher folate requirement for the regulation of plasma tHcy concentration. The $C_{677}T$ mutation of the MTHFR gene also influences the responses of plasma tHcy and folate concentrations to the supplemented folic acid. After consuming folic acid pills, subjects homozygous for the T_{677} allele (TT genotype) showed a more extensive decrease in the plasma tHcy concentration than subjects with the CT or CC genotypes (Malinow *et al.* 1997, Nelen *et al.* 1998). In fact, among the TT genotype, the folic acid supplementation results in a plasma tHcy concentration similar to that in the genotypes CT and CC (Nelen *et al.* 1998). The effects of MTHFR or other gene mutations on the response of serum folate and plasma tHcy to increased intake of vegetables and fruit rich in natural folate have not been determined in previous studies.

2.2.2.4 Illnesses and medications influencing plasma homocysteine

In end-stage renal disease, plasma tHcy concentrations increase two- to threefold, possibly because of reduced systemic clearance of homocysteine, lower blood folate concentration, and folate inhibition (Bostom & Lathrop 1997). Folate and vitamin B6 and B12 antagonists, such as methotrexate (Refsum *et al.* 1989), antiepileptic drugs (Schwaninger *et al.* 1999), and metformin (Carlsen *et al.* 1997) increase plasma tHcy concentration. A lipid-lowering drug, fenofibrate, also raises plasma homocysteine concentration by an unknown mechanism (Bissonnette *et al.* 2001).

2.2.2.5 Homocysteine and vascular diseases

An association between a high plasma homocysteine concentration and atherothrombotic vascular events in two patients with homocystinuria was first reported over 30 years ago (McCully 1969). Since then, several studies have reported that a high plasma homocysteine concentration is a risk factor for vascular diseases. The strongest evidence comes from cross-sectional and case-control studies, which generally support the association between a high plasma homocysteine concentration and a risk of CVD (Boushey *et al.* 1995, Christen *et al.* 2000). However, data from prospective cohort studies indicate weaker or no association between the plasma homocysteine concentration and the risk of CVD (Table 2). Five prospective studies have reported that an elevated plasma homocysteine concentration increases the risk of CVD (Stampfer *et al.* 1992, Arnesen *et al.* 1995, Wald *et al.* 1998, Bostom *et al.* 1999, Ridker *et al.* 1999), whereas an equal number of studies failed to show any association between plasma homocysteine and CVD (Alfthan *et al.* 1994, Chasan-Taber *et al.* 1996, Evans *et al.* 1997, Folsom *et al.* 1998, Knekt *et al.* 2001). Thus, data from prospective studies indicate little predictive ability of plasma homocysteine in CVD.

The theory of homocysteine being an independent risk factor for vascular diseases is supported by experimental evidence of mechanisms by which homocysteine might cause vascular damage and disease. The possible mechanisms include endothelial dysfunction and injury, which is followed by platelet activation and thrombus formation. Homocysteine can exert a direct cytotoxic effect on endothelial cells, which is related to generation of potent reactive oxygen species (Blundell *et al.* 1996), impaired production of endothelium-derived nitric oxide and endothelial dysfunction (Stamler *et al.* 1993, Tawakol *et al.* 1997), and stimulation of smooth-muscle cell proliferation (Tsai *et al.* 1994). It has been postulated that homocysteine promotes atherosclerosis by increasing lipid peroxidation and oxidation of LDL, but this has not been confirmed in all studies (Blom *et al.* 1992, Halvorsen *et al.* 1996). Another suggested mechanism of the vascular damage associated with homocysteine relates to formation of oxygen free radicals, which cause vascular damage, proliferation of smooth-muscle cells, alteration of endothelial function and structure, and increased thrombogenicity that leads to atherothrombosis (Welch & Loscalzo 1998). However, an alternative explanation for an association between homocysteine and vascular damage has been proposed (Dudman 1999). It has been suggested that plasma homocysteine concentration increases after tissue damage, and the elevated levels of homocysteine further promote the endothelial damage. A high plasma homocysteine level would thus be an indicator of tissue damage and a promoter or enhancer of inflammatory thickening of vascular damage. (Dudman 1999) Findings from a Finnish study support the possibility that homocysteine is a consequence rather than a cause of vascular damage and disease (Knekt *et al.* 2001). In addition, some epidemiological studies have observed that low serum folate and B6 vitamin concentrations increase the risk for vascular diseases, and the elevated plasma tHcy would thus be a marker of the low vitamin concentrations (Folsom *et al.* 1998, Robinson *et al.* 1998, Rimm *et al.* 1998). These findings are strengthened by a recent study showing that folic acid supplementation improves endothelial function in patients with coronary artery disease independently of plasma tHcy reduction (Doshi *et al.* 2002a).

Table 2. Major prospective studies on plasma total homocysteine concentration and cardiovascular diseases in subjects free of disease at baseline.

Study (Reference)	Follow-up (years)	Out-come	Study population	Cases or events / controls	Sex	Age	Plasma tHcy cases / others (µmol/l)	Adjusted relative risk (95% CI)
Physicians' Health Study (Stampfer *et al*. 1992)	5	MI, CHD death	14916	271 / 271	M	40–84	11.1 / 10.5	3.4 (1.3–8.8) [a]
North Karelia Study (Alfthan *et al*. 1994)	9	MI, stroke	7424	265 / 265	M, F	40–64	10.0 / 9.8 (M)	1.06 (0.64–1.77) [b]
Tromso Study (Arnesen *et al*. 1995)	3.5	CHD	21826	122 / 478	M, F	12–61	12.7 / 11.3	1.32 (1.05–1.65) [c]
Physicians' Health Study (Chasan-Taber *et al*. 1996)	7.5	MI	14916	333 / 333	M	40–84	not reported	1.7 (0.9–3.3) [a]
Multiple Risk Factor Interven-tion Trial (MRFIT) (Evans *et al*. 1997)	<11 <11	MI CHD death	12866	93 / 186 147 / 286	M	35–57	12.6 / 13.1 12.8 / 12.7	0.82 (0.55–1.54) [d]
Atherosclerosis Risk is Commu-nities (ARIC) (Folsom *et al*. 1998)	3.3	CHD	15792	232 / 527	M, F	45–64	8.9 / 8.5	1.28 (0.5–3.2) [e]
British United Provident Asso-ciation Study (BUPA) (Wald *et al*. 1998)	8.7	Fatal CHD	21250	229 / 1126	M	35–64	13.1 / 11.8	2.9 (2.04–4.12) [d]
Framingham Study (Bostom *et al*. 1999)	10	CVD mortal-ity	1933	244 cases	M, F	59–91	not reported	1.52 (1.16–1.98) [f]
Women's Health Study (Ridker *et al*. 1999)	3	CVD	28263	122 / 244	F	post-menop.	14.1 / 12.4	2.3 (1.2–4.3) [d]
Finnish Mobile Clinic Health Examination Sur-vey (Knekt *et al*. 2001)	13	MI, CHD death	3471	272 / 524	M	45–64	10.8 / 11.2	0.90 (0.51–1.60) [e]

MI, myocardial infarction; CHD, coronary heart disease; IHD, ischaemic heart disease
[a] top 5 % compared with lowest 10 % of total homocysteine levels, [b] highest 10 % compared with lower 90 % of total homocysteine levels, [c] per 4 µmol/l increase in total homocysteine level, [d] highest compared with lowest quartiles of total homocysteine levels, [e] highest compared with lowest quintile of total homocysteine levels,
f highest compared with three lower quartiles of total homocysteine levels

Although the exact mechanisms are not fully understood, dietary folate and other B vitamins seem to be important for cardiovascular health. It has clearly been shown that supplementation with folic acid and other group B vitamins reduces the plasma tHcy

concentration, but other mechanisms may be involved, too (Verhaar *et al.* 2002). The effects of vitamin B supplementation may also translate into favourable clinical outcomes. According to reports of a recent clinical trial, supplementation with folic acid, vitamin B6, and B12 results in less restenosis of coronary artery after surgery (Schnyder *et al.* 2001), and decreases the incidence of major adverse events one year after successful coronary angioplasty (Schnyder *et al.* 2002). This randomised clinical trial is important because it is one of the very first showing that group B vitamin supplementation results in less clinical atherosclerotic disease. The next step for researchers will be to confirm this possible protective effect against CHD in longer-term studies and large randomised clinical trials, which are now ongoing (Table 3, Doshi *et al.* 2002b).

Vitamin B supplementation is not the only option to increase the dietary intake of folate and plasma folate levels, and to reduce plasma homocysteine concentration. Dietary vegetables and fruit are important contributors to dietary folate intake, which may influence the risk of CVD as well. It is, therefore, worthwhile to study in detail the effects of dietary factors and whole diets high in fruit and vegetables on the dietary folate intake and on the plasma levels of folate and homocysteine. In addition, the influence of genetic factors, such as the polymorphisms of key enzymes in the homocysteine metabolism, and the possible interaction between diet and genes on the dietary response of plasma homocysteine should be clarified.

Table 3. Ongoing clinical trials of homocysteine-lowering vitamin therapy.[1]

Study	Sample size	Folic acid (mg)
Vitamin Intervention for Stroke Prevention (VISP), USA	3600	2.5 vs 0.2
The Women's Antioxidant Cardiovascular Study (WACS), USA	6000–8000	2.5
The Study of Effectiveness of Additional Reduction in Cholesterol and Homocysteine (SEARCH), UK	12000	2
Cambridge Heart Antioxidant Study (CHAOS 2), UK	4000	5
Norwegian Vitamin Interventional Trial (NORVIT), Norway	3000	0.8
Western Norway B-vitamin Intervention Trial (WENBIT), Norway	2000	0.8
The Prevention with a Combined Inhibitor and Folate in Coronary Heart Disease (PACIFIC), Australia	10000	2
Heart Outcome Prevention Evaluation-2 (HOPE-2)	5000	2.5

[1] Adapted from Doshi *et al*. Atherosclerosis 2002;165:1–3

3 Aims of the study

The purpose of the present study was to investigate the influence of modifications in the vegetable, berry, and fruit intake and dietary fat on the plasma concentrations of antioxidants, lipids, lipoprotein(a), oxidised LDL, folate, and homocysteine (Fig. 2). The following specific questions were addressed:

1. Does a diet high in common vegetables, berries, citrus fruit, and PUFA enhance the plasma concentrations of carotenoids, vitamin C, and vitamin E?
2. Do the modifications in the dietary intake of vegetables, berries, and fruit influence the plasma concentrations of lipids, lipoproteins, and oxidised LDL?
3. How do the dietary modifications and gene polymorphisms affect the serum paraoxonase-1 activity?
4. Does a high intake of natural folate from food increase the serum folate concentration and decrease the plasma tHcy concentration?
5. Do the common gene polymorphisms alter the dietary response of plasma tHcy concentration?

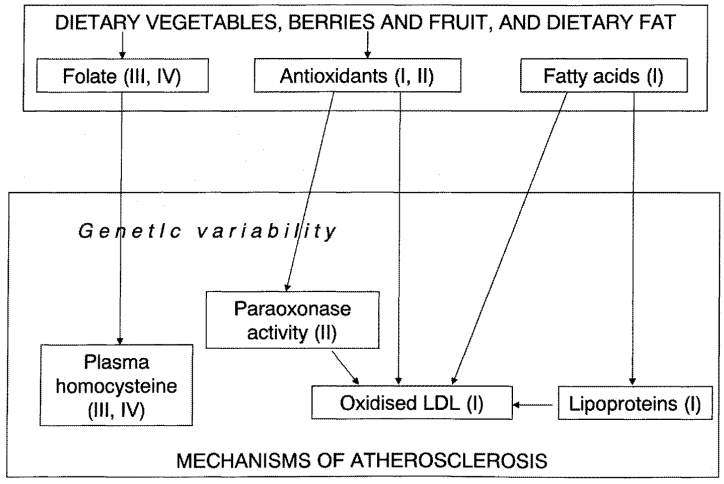

Fig. 2. Schematic presentation of the variables studied and their interrelationships.

4 Subjects and methods

4.1 Subjects

We interviewed and examined altogether 86 women, among whom 38 healthy volunteers were selected. The run-in examinations included laboratory tests and interviews by both a doctor and a nutritionist. The participants were either employees of the University Hospital of Oulu or medical students. The inclusion criteria for the study were: 1) body mass index (BMI) between 20 to 29 kg/m^2; 2) normal blood glucose (3.7 to 6.2 mmol/l); 3) normal plasma lipid concentrations (total cholesterol < 7.0 mmol/l, triglycerides < 3.0 mmol/l); 4) non-smoking, 5) no gastrointestinal, renal or hepatic diseases; 6) no food allergy; 7) no alcoholism; 8) no use of supplemental vitamins and/or minerals for at least 6 month prior to the onset of the study; 9) not pregnant or lactating. Thirty-eight subjects started the intervention, but one of them withdrew from the study in the first week because of a coming training period abroad. The remaining 37 women completed the study. According to the power calculation, this was an adequate number of subjects (chapter 4.6). The age of the women varied from 22 years to 57 years, the average age being 43 years. The average BMI of the women was 23.7 ± 2.2 kg/m^2 at the study entry. Six of the women used oral contraceptives and three were on postmenopausal hormone replacement therapy.

We carried out the study in accordance with the instructions of the Declaration of Helsinki. Informed consent was obtained from each participant. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

4.2 Study design

The dietary intervention was carried out in autumn 1998. The intervention consisted of a baseline (2 weeks) and two diet periods (low and high vegetable diets, 5 weeks each) with a wash-out period (3 weeks) in between (Fig. 3). An important feature of the study

was a crossover design, in which each individual served as her own control. The order of the study diets was randomly assigned for each subject.

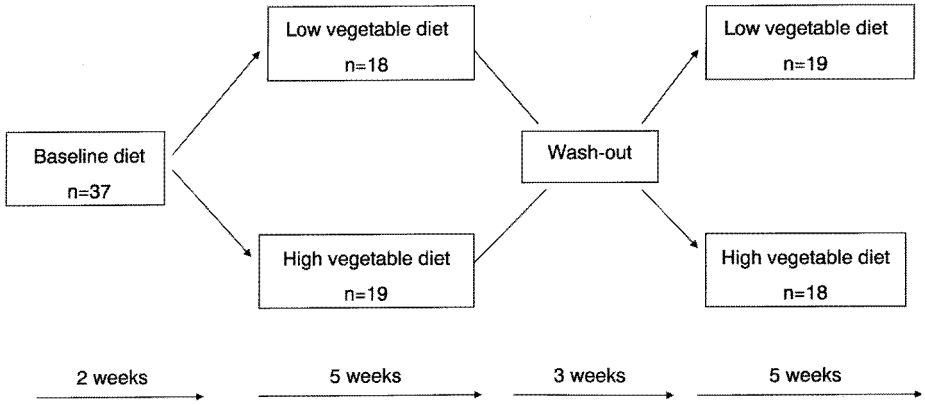

Fig. 3. Design of the study.

4.3 Study diets

The dietary intervention included the subjects' habitual diets and two intervention diets: a low vegetable diet and a high vegetable diet. At the baseline and the wash-out period, the subjects followed their habitual diets. The low and high vegetable diets were designed on the basis of regular hospital meals (a 5-week menu), and they contained conventional foods and beverages. The basic diet and foods were same for all participants. The foods were prepared, packaged, and delivered to the subjects by the hospital kitchen. The nutritionist planned the study diets and advised the staff at the kitchen how to choose the foods and prepare the meals. On working days, the lunches and dinners were served at the hospital cafeteria. The participants could also take the packaged dinner meals home. On weekends, the subjects were able to eat the lunches and dinners at the hospital cafeteria or take the packaged weekend meals home on Friday. Other foodstuffs, including bread, milk, and fruit, were delivered to the subjects twice a week. The study foods and meals were free to the subjects.

Both study diets were low in dietary cholesterol (< 200 mg per day) and SAFA (approximately 10 E%). The quantity and quality of dietary fat was controlled by using low-fat meat and dairy products, low-fat cooking methods, and vegetable oils and spreads.

At the baseline, the same experienced nutritionist interviewed all the participants concerning their eating and exercise habits and determined an isocaloric energy intake level for each. During the intervention, the participants weighed themselves daily before lunch, and their dietary energy intake was adjusted to maintain their body weight unchanged during the study. The nutritionist surveyed the study lunches daily and was

able to assess the subjects' compliance. In addition, the subjects submitted written reports of any deviations from their study diet. Alcohol consumption was determined at the baseline by interviewing the subjects, who were advised to restrict their use of alcohol to less than four drinks per week during the study. The amount of alcohol consumed was negligible, and alcohol was therefore not included in the calculations of diets.

Low vegetable diet. The low vegetable diet was designed to provide the subjects with the current recommended dietary intakes of vitamins and minerals (Nordic Nutrition Recommendations 1996). It contained about 200 g of vegetables and fruit per day. The fruit portions were consumed at breakfast and in the evening, and vegetables were served mainly as a side salad at lunch. Citrus fruit were avoided, and fruit such bananas, apples, pears, and canned fruit, were consumed. Rapeseed oil-based salad dressing and a soft vegetable oil spread (10 mg of vitamin E per 100 g) were used in the low vegetable diet. The bread was mainly mixed grain bread, dark wheat bread, and white wheat bread.

High vegetable diet. In the high vegetable diet, the subjects ate 600–800 g of fresh vegetables, citrus fruits, and berries daily in addition to the basic diet. The bread was mainly rye bread or other whole grain bread, which are rich in dietary folate. The vegetables, fruit, and berries were chosen based on their contents of alpha- and beta-carotene, vitamin C, and folate. At breakfast of the high vegetable diet, the subjects ate 30 g of fresh sweet red pepper and a piece of fruit, e.g. orange or kiwi, or 125 ml of juice in addition to the basic diet. The lunch included approximately 100–150 g of salad with carrots, cauliflower, cabbage, or other fresh vegetables, and approximately 100 g of steamed vegetables, e.g. broccoli, peas, carrots, or cauliflower, in addition to the basic diet. Fresh strawberries, black currants, or raspberries were served as a dessert. At dinner, approximately the same amounts of fresh and steamed vegetables were consumed. The dessert after dinner consisted of either fresh berries or a piece of fruit. At both lunch and dinner, 100 ml of orange juice was consumed. In the evening, the subjects ate 30 g of sweet red pepper and drank 125 ml of orange or pineapple juice. In order to increase the dietary intake of vitamin E, sunflower oil was used in the salad dressing and a soft vegetable oil spread containing 20 mg of vitamin E per 100 g was used with bread.

4.4 Blood tests

Overnight fasting blood samples were drawn into EDTA-containing tubes for the measurement of plasma lipids, lipoprotein(a) [Lp(a)], OxLDL, alpha-carotene, beta-carotene, lutein-zeaxanthin, beta-cryptoxanthin, lycopene, alpha- and gamma-tocopherols, vitamin C, tHcy, serum vitamin B12, serum and erythrocyte folate, and serum PON1 activity. The blood tests were performed at the baseline and at the end of both diet periods. Some measurements were also determined at the end of the wash-out period. At the baseline, overnight fasting blood samples were also drawn for the basic clinical chemistry tests including blood glucose, serum potassium and sodium, and for the isolation of DNA.

During the study, the plasma lipids (total and HDL cholesterol, and triglycerides), serum folate, and erythrocyte folate concentrations were analysed immediately after the blood was drawn. For all other measurements, the plasma and serum samples were stored

at –70 °C until analysed. The stored blood samples of an individual subject at different time periods (i.e. baseline, the low vegetable diet, and the high vegetable diet) were analysed at the same time.

The analyses of plasma lipids, Lp(a), apoB, OxLDL, and the determination of all gene polymorphisms were carried out in the Research Laboratory of the Department of Internal Medicine, University of Oulu, Finland. The serum vitamin B12 and serum and erythrocyte folate were determined in the laboratory of the Oulu University Hospital, Oulu, Finland. The plasma carotenoids, tocopherols, vitamin C, and tHcy were analysed in the National Public Health Institute, Helsinki, Finland. The serum PON1 activity was measured in the Research Institute of Public Health, University of Kuopio, Finland.

4.4.1 Plasma lipids and lipoprotein(a)

To measure plasma lipids, plasma was separated by centrifugation at 1200g (2600 rpm) for 15 minutes (4 C°). The total plasma triacylglycerol and cholesterol concentrations were determined enzymatically using Specific Clinical Chemistry Analyser (Kone OY, Espoo, Finland). Kits were provided by Boehringer Mannheim, GmbH, Germany, cat. n:s 236691 and 701912. The HDL cholesterol concentration was determined after precipitation of the plasma sample with heparin-manganese chloride. The LDL cholesterol concentration was calculated by the Friedewald formula (Friedewald *et al.* 1972). For the plasma total cholesterol concentration, coefficient of variation (CV) for intra- and interassay was 3.7 % (n=13) and 3.5 % (n=7), respectively. For the plasma HDL cholesterol concentration, the interassay CV was 7.3 % (n=16); the intra-assay CV was not determined.

Plasma Lp(a) concentrations were determined by a commercial enzyme-linked immunosorbent assay method (Biopool Ltd. Cat#610221), which has been shown to correlate well with the other Lp(a) assay methods. The intra- and interassay CVs were 1.4 % (n=4) and 8.2 % (n=4), respectively.

4.4.2 Oxidised LDL

A sandwich chemiluminescent immunoassay utilising a well characterised murine monoclonal antibody EO6, which binds specifically to oxidised phospholipids, was used to measure plasma oxidised LDL (Hörkkö *et al.* 1999). The data are expressed as EO6 epitopes per apoB-100 and we refer to this as OxLDL-EO6. First, a monoclonal anti-apolipoprotein B-100 antibody, MB47, was plated in microtitre wells at 5 µg/ml overnight at 4°C. After blocking the non-specific binding sites with PBS buffer containing 1% bovine serum albumin, the plasma samples at 1:50 dilution were incubated for one hour, and the amount of oxidised phospholipid epitopes in ApoB-100 particle (primarily LDL in those subjects with low VLDL levels), was measured with biotinylated murine monoclonal antibody EO6. In parallel wells, the amount of LDL bound to the plates was detected with polyclonal biotinylated anti-apoB antibody. The antibody bound

was detected with alkaline-phosphatase labelled Neutravidin and LumiPhos 530 substrate using a Dynex chemiluminescent microtitreplate reader. The results are expressed as amount of EO6 bound divided by the amount of anti-apoB bound into the wells, yielding the relative amount of oxidised phospholipid detected by EO6/apoB-100, i.e. OxLDL-EO6. The intra- or interassay CVs were not determined for this method.

4.4.3 Antioxidants

Carotenoids and tocopherols. Plasma concentrations of tocopherols and carotenoids were analysed separately by using high performance liquid chromatography (HPLC). To analyse the plasma concentrations of tocopherols and carotenoids, 0.2 ml of plasma, a solution (50% ethanol) containing 1% ascorbic acid and tocol and echinenon as internal standards for tocopherols and carotenoids were added, respectively. After vortexing, 4 ml of n-hexane was added and extracted. To facilitate separation of the phases, 2 ml of 2% NaCl was added, and the mixture was centrifuged for 5 min. Three ml of hexane was evaporated under vacuum, and the residue was dissolved in 120 µl of ethanol and transferred to a vial. The tocopherols were separated with a Supelco (Bellefonte, PA) C_{18} column (15 x 4.6 mm, 5 um) using methanol/water (99.5/0.5) as the mobile phase at a flow rate of 1.0 ml/min. The injection volume was 15 µl. Alpha- and gamma-tocopherols were detected by fluorescence at 290/324 nm. The carotenoids (alpha-carotene, beta-carotene, lutein-zeaxanthin, beta-cryptoxanthin, and lycopene) were separated with a Waters (Millford, MS) Nova-Pak column (15 x 3.9 mm, 4 µm) using acetonitrile/ dichlormethanol/methanol (72.5/2.5/25) as the mobile phase at a flow rate of 1 ml/min. Carotenoids were detected at 450 nm. Peak height/internal standard ratios were compared with the ratios of a reference plasma whose values were traceable to NIST-certified serum standards, 968b (National Institute of Standardization and Technology, Gaithersburg). The lycopene isomers were combined and calculated as area/internal standard ratios. The apparatus consisted of a Shimadzu Model 10A HPLC, Japan. The interassay CVs for the alpha- and gamma tocopherol concentrations were 4.5 % and 4.3 %, respectively (n=10). For the alpha-carotene, beta-carotene, lutein-zeaxanthin, beta-cryptoxanthin, and lycopene the interassay CVs were 6.6 %, 5.7 %, 18%, 16 %, and 6 %, respectively (n=10).

Vitamin C. For the measurement of plasma vitamin C, 0.5 ml of plasma was added to 4.5 ml of 5% metaphosphoric acid within 1 h after venipuncture and stored at –70°C. Total ascorbic acid was determined with an automated fluorimetric method using orthophenylenediamine and standardised against daily prepared ascorbic acid in 5% metaphosphoric acid (Brubacher & Vuilleumier 1974). The interassay CV for the plasma vitamin C was 6 % (n=10).

4.4.4 Homocysteine, vitamin B12, and folate

The concentrations of serum and erythrocyte folate and serum B12 were determined using the Quantaphase II B12 and Folate Radioassays (Bio-Rad Laboratories, Inc., 1996). For folate, the intra- and interassay CVs were less than 8.6 %. For vitamin B12, the intra- and interassay CVs were less than 3.3 %. For folates and vitamin B12, the mean recoveries from analyses of three different quality assurance sera (Labquality Ltd, Finland) were 107% and 97%, respectively. The plasma tHcy concentration was analysed by the immunofluorometric IMX method (Abbott Laboratories, IL) (Shipchandler & Moore 1995). The interassay CV was 3.2 %. Accuracy was ascertained by participating in a Nordic quality assurance system on plasma tHcy, in which the mean bias for 7 sera was 3.5 % (Möller *et al.* 1997).

4.4.5 Serum paraoxonase-1 activity

Serum PON1 activity was measured from whole serum based on its capacity to hydrolyse paraoxon. The formation of *p*-nitrophenol was monitored at 405 nm in Tris-HCl buffer, pH 8.0, in the presence of Ca^{2+} (Mackness *et al.* 1991b). The intra- and interassay CVs for the PON1 activity measurement were 2 % (n=10) and 3 % (n=9), respectively.

4.4.6 Gene polymorphisms

Genomic DNA was isolated from peripheral leukocytes isolated from anticoagulated blood (EDTA) by using a salting-out method according to Miller and co-workers (Miller *et al.* 1988).

Gene polymorphisms in homocysteine metabolism. The analysis of the $C_{677}T$ (alanine $\rightarrow$ valine) polymorphism in the MTHFR gene was investigated by polymerase chain reaction (PCR) of a DNA fragment, followed by restriction enzyme digestion with *Hinf* I (Frosst *et al.* 1995). The presence or absence of the 844ins68 of the CBS gene was tested using PCR amplification and digestion with the restriction enzyme *Bsr I* (Tsai *et al.* 1996). The $A_{2756}G$ mutation of the MS gene was detected using PCR amplification and *Hae III* restriction analysis (Harmon *et al.* 1999). The enzymes were provided by Finnzymes (Espoo, Finland). The fragments of all the gene polymorphisms were visualised on an ultraviolet transilluminator after electrophoresis on a 3 % low-melting-point agarose gel (3:1 NuSieve, BioWhittaker Molecular Applications, Rockland, ME) containing nucleic acid gel stain (GelStar®, BioWhittaker Molecular Applications, Rockland, ME).

PON1 gene polymorphisms. $PON1_{55}$ and $PON1_{192}$ genotyping was performed using PCR-restriction fragment length polymorphism (RFLP) analysis as previously described (Humbert *et al.* 1993) with slight modifications. Two sets of primers were designed to flank the polymorphic sites. The primers used for the amplification of the 169 bp DNA fragment for $PON1_{55}$ polymorphism were 5'-GAAGAGTGATGTTATAGCCCCAG-3'

and 5'-ACTCACAGAGCTAATGAAAGCCA-3'. The 99 bp DNA fragment encompassing the $PON1_{192}$ polymorphism was obtained using the primers 5'-TATTGTTGCTGTGGGACCTGAG-3' and 5'-CACGCTAAACCCAAATACATCTC-3'. Twenty-five µL of PCR mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 0.05 mM dNTPs for the $PON1_{55}$ polymorphism and 0.4 mM dNTPs for the $PON1_{192}$ polymorphism, 0.25 µM of each primer, 1U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 125 ng of DNA template. The PCR reaction for the amplification of both polymorphic regions was carried out with initial denaturation at 95 °C for 5 minutes, followed by 35 cycles, each consisting of three one-minute steps: denaturation at 95 °C, annealing at 61 °C and extension at 72 °C. The reaction was completed with a final extension at 72 °C for 10 minutes. The $PON1_{55}$ (169 bp) PCR product was digested with 5U of NlaIII (New England Biolabs Inc., Beverley, MA) at 37 °C for three hours. Digestion resulted in 127 bp and 42 bp fragments for the $PON1_{55}$ M (methionine) allele and a non-digested 169 bp fragment for the $PON1_{55}$L (leucine) allele. The $PON1_{192}$ (99 bp) PCR product was digested with 2U of AlwI (New England Biolabs Inc., Beverley, MA) at 37 °C for five hours. Digestion with AlwI resulted in 63 bp and 36 bp fragments for the $PON1_{192}$ R (arginine) allele and a non-digested 99 bp fragment for the $PON1_{192}$Q (glutamine) allele. The digested fragments were separated and visualised on an ultraviolet transilluminator after electrophoresis on a 5 % low-melting-point agarose gel (4:1 NuSieve; NuSieve, BioWhittaker Molecular Applications, Rockland, ME) containing nucleic acid gel stain (GelStar®; BioWhittaker Molecular Applications, Rockland, ME).

4.5 Dietary analyses

We collected four-day food records during the baseline diet period. The food records included three weekdays and one weekend day, and were completed after the first visit to our laboratory. The nutritionist advised the subjects on how to complete the food diaries and also checked the diaries when they were returned. The subjects reported the intake of foods using food portion pictures and household measures. The nutritionist calculated the nutrient content of the baseline diet using the Nutrica computer programme (Social Insurance Institution, Helsinki, Finland) based on the Finnish nutrient database.

The nutrient contents of the intervention diets were analysed from identical portions in the Agricultural Research Centre of Finland (Jokioinen, Finland). We collected identical food portions of both diets every day during the intervention at one energy level (7.5 MJ), which we chose based on the baseline energy intake of the women. Each evening, the collected foods of both diets were homogenised with a blender, and a sample (about 500 ml) of this food mass was frozen. The food samples were stored at –20ºC until analysis. In the Agricultural Research Center of Finland, the frozen food samples were thawed and combined to pooled samples of each diet. The nutrient analysis of the pooled food samples included total energy, total fat, carbohydrate, fibre, fatty acids, dietary cholesterol, potassium, sodium, calcium, iron, alpha-carotene, beta-carotene, vitamin C, and alpha-tocopherol.

The fatty acid composition of the diets was analysed by gas chromatography. The lipids of freeze-dried samples were extracted using chloroform-methanol. The sample was filtered to a separation funnel and 5 ml of water was added to the filtrate. The organic phase was separated and evaporated in a rotary evaporator to near dryness. The residual organic solvent was evaporated by a flow of nitrogen. Saponification of lipids was accomplished by 0.5 M sodium hydroxide in methanol (85 C°, 7 min). To the cooled sample 2 ml of 10 % boron trifluoride in methanol (Fluka, Milwaukee, WI) was added for preparing methyl esters of fatty acids. After incubation for 12 min at 85 C° the cooled-down sample was extracted using 1 ml of hexane (Metcalfe & Schmitz 1961). Fatty acid methyl esters were analysed by a Hewlett-Packard (Avondale, PA) 5890 series gas chromatograph equipped with 5970 series mass selective detector used in the scan mode. For quantification relative response ratios of different fatty acids were determined using Nu-Chek Prep (Elysian, MN) standards GLC-68A and GLC-85. The analytical column used was Hewlett-Packard´s HP-5MS (30 m x 0.25 mm) with helium as a carrier gas (0.5 ml/min).

Mineral and trace elements (potassium, sodium, calcium, iron) were determined by inductively coupled plasma emission spectrometry (ICP). 3–5 g of sample was dry-ashed in a muffle furnace in 500 ºC overnight. The ash was treated with hydrochloric acid and evaporated in dryness. 15 ml of hydrochloric acid was added, heated 30 min and filtered into the volumetric flask. Ashed again in 700ºC, ml of HF was added and evaporated into dryness. 10 ml of HCl was added, heated 30 min and filtered into the same volumetric flask with the previous filtrate. Elemental concentrations were measured by high resolution ICP (Thermo Jarrel Ash, IRIS Advantage). The accuracy of the analytical method was tested by determining certified reference materials in every batch of samples. The method is accredited to the minerals and trace elements studied except sodium.

Vitamin C was determined as dehydroascorbic acid according to the method of Speek and co-workers (1984). Vitamin C was analysed using Hewlett Packard´s 1090 Series HPLC (Waldbronn, Germany) equipped with a fluorescent detector. The analytical column was Waters Spherisorb (125 x 4.0 mm, 5 µm, Waters, MA, USA) operated at 35ºC. Isocratic mobile phase consisted of methanol and 0.08 M phosphate buffer (pH 7.8).

The contents of alpha- and beta-carotene in the diets were determined according to Hägg and co-workers (1994). Briefly, the method consisted of acetone extraction, filtration and concentration followed by liquid-liquid partitioning with hexane:diethyl ether (7:3). After evaporation of organic phase the samples were dissolved in acetonitrile:dichloromethane:methanol (7:2:1). Determination of the analytes was accomplished by Hewlett Packard´s 1090 Series HPLC (Waldbronn, German) with diode array detector set at 410 nm. A 201 TP54 column (250 x 4.4 mm, 5 µm, Vydac, CA,USA) was used with acetonitrile:methanol (1:9) as a mobile phase.

The alpha-tocopherol content was determined from the samples according to Piironen and co-workers (1984). The method included overnight saponification, liquid-liquid partitioning with hexane and evaporation followed by redissolvation in methanol. Tocolpherols were quantitated by Hewlett Packard´s 1090 Series HPLC (Waldbronn, Germany) with fluorescence detector set at 292 nm exitation and 324 nm emission. A Lichrosphere Si60 column (250 x 4 mm, 5 µm, Hewlett Packard, USA) was used with hexane:isopropanol (98.5 : 1.5) as a mobile phase.

4.6 Statistical tests

The estimation of group size was based on a 15 % decrease (about 1.2 μmol/l) in the plasma homocysteine concentration (about 8 μmol/l) after a four-week daily supplementation with 400 μg of folic acid and 6 μg of vitamin B_{12} in healthy, young women (Brönstrup *et al.* 1998). On the basis of these data, 33 subjects were needed for detecting a change of 1.2 μmol/l in the plasma tHcy concentration with a power of 80 % and an α of 0.05.

We used the Saphiro-Wilk test to test whether the variables had normal or skewed distributions. Several measurements (plasma triglycerides, OxLDL-EO6, Lp(a), plasma tHcy, serum folate, erythrocyte folate, carotenoids, and serum PON1 activity) were not normally distributed, and therefore we used non-parametric tests in most statistical analyses of these parameters. In some cases, logarithmic corrections and parametric tests were used. Regarding all non-parametric variables, the differences in the plasma concentrations during the intervention (the baseline, the low vegetable diet, and the high vegetable diet) were first tested by the Friedman test for repeated measurements. We used Wilcoxon signed ranks test to further test the differences in each variable between the diet periods. For those plasma measurements that were normally distributed (vitamin C, alpha-tocopherol, serum vitamin B12, and total, HDL, and LDL cholesterol), we used Student's t-test for paired samples to test the difference between the diet periods.

The diet-induced changes in the serum folate and plasma tHcy concentrations and the effect of the genotypes of MTHFR, CBS, and MS were tested with respect to the intra-individual variation during the diet intervention. This was done by using a layered design in the form of repeated measurements across time (ANOVA of repeated measurements). In this test, logarithmic corrections of serum folate and plasma tHcy concentrations were used. The top layer in the model was the between-subject layer, in which the effect of having a certain genotype was tested with respect to the inter-individual variation. The bottom layer was the within-subject layer, in which the repeated-measures for the diet periods (the baseline, low vegetable diet, and high vegetable diet) were tested with respect to the variation from one dietary period to another. In addition, we detected the differences in the concentrations of serum folate and plasma tHcy between the different genotypes of each gene by using either Student's t-test for independent samples (CBS and MS genes) or one-way ANOVA (MTHFR gene).

For the serum PON1 activity, the differences between the genotype groups were tested by Mann-Whitney test. We did not use the ANOVA of repeated measurements, because the serum PON1 activity was so skewed that logarithmic corrections did not normalise the distribution.

Spearman’s correlation coefficient was used to determine the associations between the variables.

The SPSS software version 9.0 (SPSS Inc., Chicago) was used in the statistical analyses. All the differences were considered significant at a 5 % level. The values are expressed as mean ± standard deviation (SD), unless otherwise stated.

5 Results

5.1 Baseline characteristics

The subjects of the different arms of the cross-over study did not differ from each other with respect to the initial concentrations of plasma total cholesterol, serum folate, plasma beta-carotene, and plasma vitamin C (Table 4). There were some differences in the age, BMI, and plasma tHcy concentration between the groups; only the difference in the average BMI of the groups was significant (Table 4). The mean BMI of all the women was 23.7 ± 2.2 kg/m^2 at the baseline. At the end of the low and high vegetable diets, the average BMI of the subjects was 23.3 ± 3.2 kg/m^2 and 23.3 ± 3.3 kg/m^2, respectively.

Table 4. Baseline characteristics of the women (n=37) according to individual arms of the cross-over study [1]

Variable	Low vegetable diet first n=18	High vegetable diet first n=19	p-value
Age, years	39.4 ± 11.0	45.5 ± 8.5	0.07 *
Body mass index	23.0 ± 1.8	24.4 ± 2.0	0.04 *
Plasma total cholesterol, mmol/l	5.1 ± 0.8	4.9 ± 0.8	0.41 *
Plasma tHcy, µmol/l	7.6 ± 1.4	8.7 ± 2.2	0.07 †
Serum folate, nmol/l	10.9 ± 4.1	11.0 ± 3.3	0.88 †
Plasma beta-carotene, µmol/l	0.68 ± 0.32	0.79 ± 0.47	0.33 †
Plasma vitamin C, µmol/l	80 ± 17	80 ± 15	0.90 *

[1] Values are mean ± SD
* Statistical significance of the difference between groups; Student's t-test for independent samples
† Statistical significance of the difference between groups; Mann-Whitney test

5.2 Dietary intake

The dietary intake at the baseline diet period was calculated from the food records, whereas the contents of most nutrients of the low and high vegetable diet were analysed from identical food portions collected every day. The identical food portions were collected according to an energy intake level of 7.5 MJ per day, and the results represent an average daily dietary composition of the study diets.

The high vegetable diet resulted in marked increases in the dietary intake of carotenoids, vitamin C, folate, and dietary fibre compared to the low vegetable diet and the subjects' habitual diet (Table 5). The dietary intake of vitamin E was doubled on the high vegetable diet compared to the low vegetable diet. Compared with the subjects' baseline diet, the proportion of fat was lower and that of protein and carbohydrate somewhat higher on the low and high vegetable diets (Table 5). In addition, there were differences between the baseline diet and both study diets in the quality of dietary fat (Tables 5 & 6). The amounts of saturated fatty acids (SAFA) were lower and the amounts of polyunsaturated fatty acids (PUFA) were higher in both study diets compared to the baseline diets of the subjects. In particular, the proportion of linoleic acid increased during the high vegetable diet compared to the baseline (Table 6).

Table 5. The average daily nutrient intake of the women (n=37) at the baseline period and the average nutrient contents of the study diets [1]

	Baseline	Low vegetable diet	High vegetable diet
Carbohydrate (E%)*	46 ± 7	49	50
Protein (E%)	17 ± 2	20	20
Total fat (E%)	36 ± 6	31	31
Saturated fat (E%)	15 ± 3	11	9.5
Monounsaturated fat (E%)	14 ± 3	13	11
Polyunsaturated fat (E%)	6 ± 1	7	9.5
Dietary fibre (g)	21 ± 6	25	40
Calcium (mg)	1086 ± 326	1210	1280
Potassium (mg)	3538 ± 640	4200	5720
Iron (mg)	11 ± 3	10.0	13.5
Carotenoids (mg)	3.4 ± 2.3	4.6	18
Vitamin C (mg)	128 ± 60	147	430
Vitamin E (mg)	10 ± 4	8	17
Folate (µg)	284 ± 56	221 ± 24	596 ± 66
Vitamin B6 (mg)	1.8 ± 0.4	1.6 ± 0.3	2.7 ± 0.3
Vitamin B12 (µg)	5.7 ± 2.3	7.1 ± 3.6	6.4 ± 3.5

[1] The intakes at the baseline are calculated from the 4-day food records. The nutrient contents of the low and high vegetable diets are analysed from the identical food portions, except for folate, vitamin B6 and vitamin B12, which intakes are calculated from the study menus. Values are mean ± SD for calculated nutrients and the average daily intakes for analysed nutrients.

* E%, percent of total energy intake

Table 6. Fatty acid composition of the study diets [1]

	Baseline	Low vegetable diet	High vegetable diet
SAFA	43.3	35.0	31.9
C16:0 (Palmitic)	21.1	22.1	21.6
C18:0 (Stearic)	9.2	8.5	7.4
MUFA	39.3	41.2	35.7
C18:1 (Oleic)	– [*]	40.4	35.1
PUFA	17.3	23.8	32.4
C18:2 (Linoleic)	13.7	20.6	30.2
C18:3 (Linolenic)	2.4	3.2	2.2
Sum	99.9	100.0	100.0

[1] Values are expressed as percent (%) of total fat. The intakes at the baseline are calculated from the 4-day food records, and the fatty acid contents of the low and high vegetable diets are analysed from the identical food portions. SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[*] Oleic acid was not available from the nutrient calculation software

5.3 Plasma antioxidants (I, II)

Compared with the low vegetable diet, the high vegetable diet resulted in significant increases in the plasma concentrations of carotenoids and vitamin C (Fig. 4). The subjects' average plasma concentrations of alpha-carotene, beta-carotene, lutein-zeaxanthin, beta-cryptoxanthin, and vitamin C were 133 %, 134 %, 107 %, 65 %, and 25 % higher (P<0.001), respectively, after the high vegetable diet than at the end of the low vegetable diet. The plasma alpha-carotene, beta-cryptoxanthin, and vitamin C concentrations were higher also at the end of the low vegetable diet than at the baseline (Fig. 4). The average plasma concentration of lycopene decreased (P<0.001) in response to both the low and high vegetable diets compared with the baseline. The changes in the dietary intake of vitamin E (Table 5) did not affect the plasma concentration of alpha-tocopherol, which was practically unchanged during the intervention (Fig. 4).

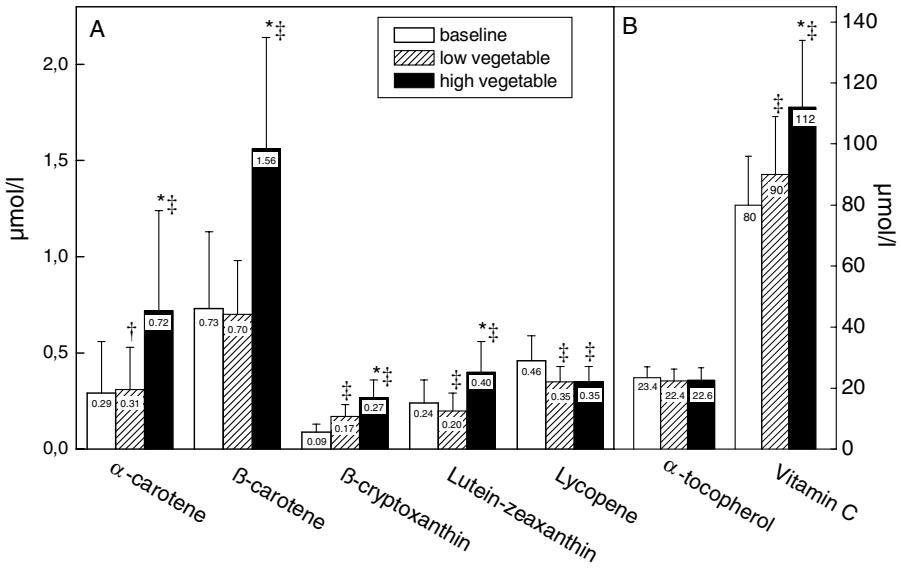

Fig. 4. Plasma concentrations of carotenoids, alpha-tocopherol and vitamin C of all the study subjects (n=37) during the study diets. Values are mean ± SD.
*** *P*<0.001; statistical significance for the difference between the low and high vegetable diets (Wilcoxon signed ranks test and Student's t-test for paired samples).**
† *P*<0.01; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test). ‡ *P*<0.001; statistical significance of the difference between the baseline diet period and the study diet period (Wilcoxon signed ranks test).

5.4 Plasma lipids and lipoprotein(a) (I)

Modest changes were observed in the lipid concentrations of the subjects during the dietary intervention (Fig. 5). On the low vegetable diet, the plasma total cholesterol was 5.0 mmol/l, and it was decreased by 8 % (*P*<0.001) in response to the high vegetable diet. The plasma LDL cholesterol decreased from 2.8 mmol/l to 2.5 mmol/l (–8%, *P*<0.001) in response to the high vegetable diet as compared to the low vegetable diet. There was also a slight reduction in the plasma HDL cholesterol concentration from the low vegetable diet (1.7 mmol/l) to the high vegetable diet (1.6 mmol/l). (Fig. 5)

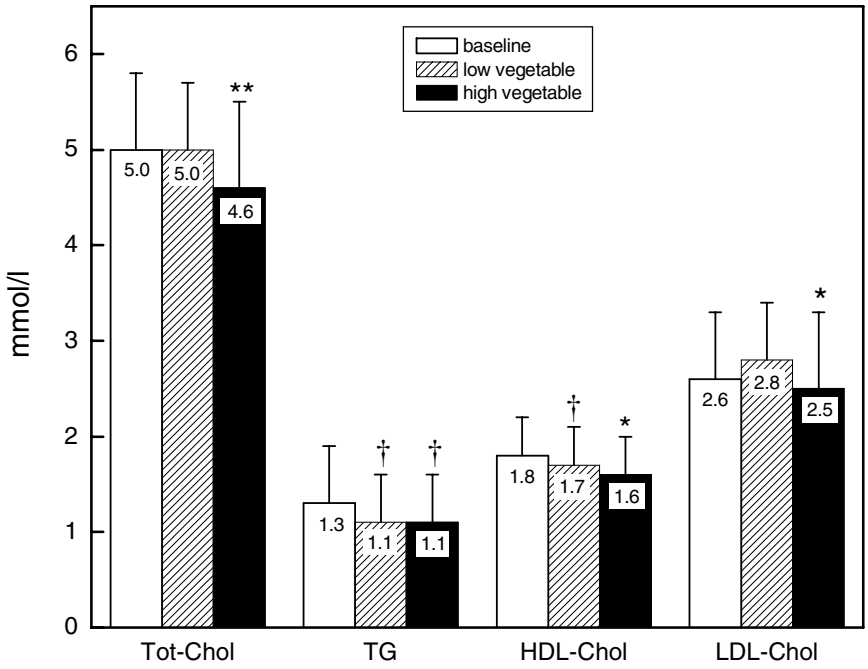

Fig. 5. Plasma concentrations of total, HDL, and LDL cholesterol, and triglycerides of all the study subjects (n=37) during the study diets.
Tot-Chol, total cholesterol; TG, triglycerides; HDL-Chol, HDL cholesterol; LDL-Chol, LDL cholesterol.
*** *P*<0.05, ** *P*<0.001; statistical significance of the difference between the low and high vegetable diets (Student's t-test for paired samples and Wilcoxon signed ranks test)**
† *P*<0.05; statistical significance of the difference between the study diet period and the baseline (Student's t-test for paired samples and Wilcoxon signed ranks test)

The average Lp(a) concentration was 185 mg/l at the baseline, 213 mg/l on the low vegetable diet, and 207 mg/l on the high vegetable diet. According to the Friedman test, there was a difference ($P<0.05$) in the Lp(a) concentrations between these diet periods. According to the Wilcoxon signed ranks test, the plasma Lp(a) concentrations were similar on the low and high vegetable diets ($P=0.48$ for the difference). However, the Lp(a) concentration of both the low and high vegetable diets was higher than that of the baseline period (Fig. 6). The average increase in the plasma Lp(a) concentration of the women was 15 ± 5 % ($P<0.01$) in response to the low vegetable diet compared to the baseline (Fig. 6A). At the end of the high vegetable diet, the mean plasma Lp(a) concentration was 11 ± 4 % higher ($P<0.05$) compared to the baseline diet (Fig. 6B). The median increases of the Lp(a) concentrations were 7 % (baseline vs. low vegetable diet) and 9 % (baseline vs. high vegetable diet).

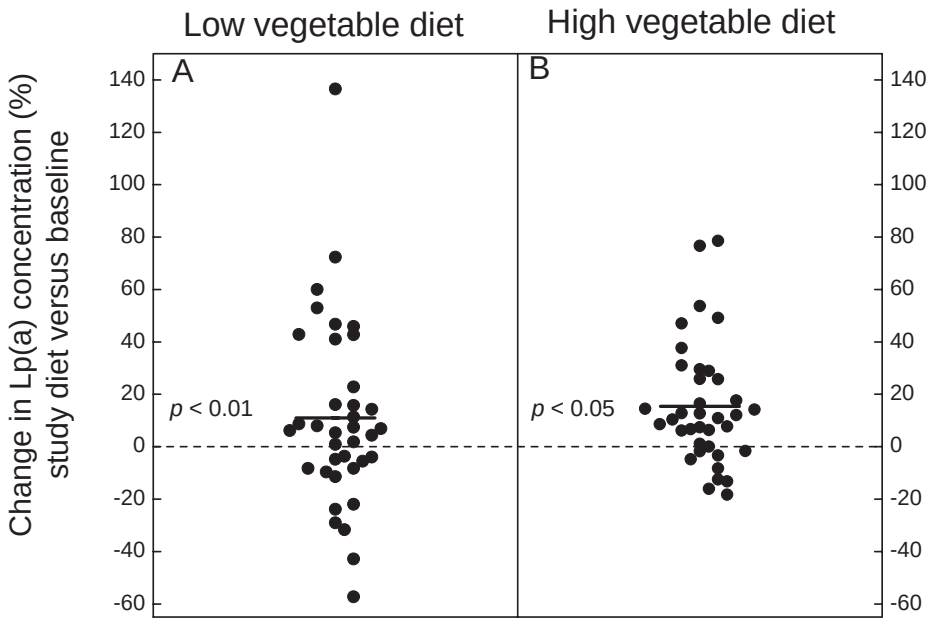

Fig. 6. Changes in the plasma concentration of lipoprotein(a) of all the study subjects (n=37) from baseline to low vegetable diet and from baseline to the high vegetable diet. Wilcoxon signed ranks test.

5.5 Plasma oxidised LDL (I)

According to the Friedman test, there was a difference (P<0.01) in the plasma OxLDL-EO6 concentrations between the baseline, the low vegetable, and the high vegetable diet periods. The plasma levels of OxLDL-EO6 were equal on the low and high vegetable diet period (0.090 and 0.089 EO6 epitopes per apoB-100 molecule, respectively). However, the average plasma OxLDL-EO6 was 87 ± 51 % higher (P<0.01) at the end of the low vegetable diet and 77 ± 46 % higher (P<0.01) at the end of the high vegetable diet than at the baseline diet period (Fig. 7). The median increases of the plasma OxLDL-EO6 were 27 % (baseline vs. low vegetable diet) and 19 % (baseline vs. high vegetable diet).

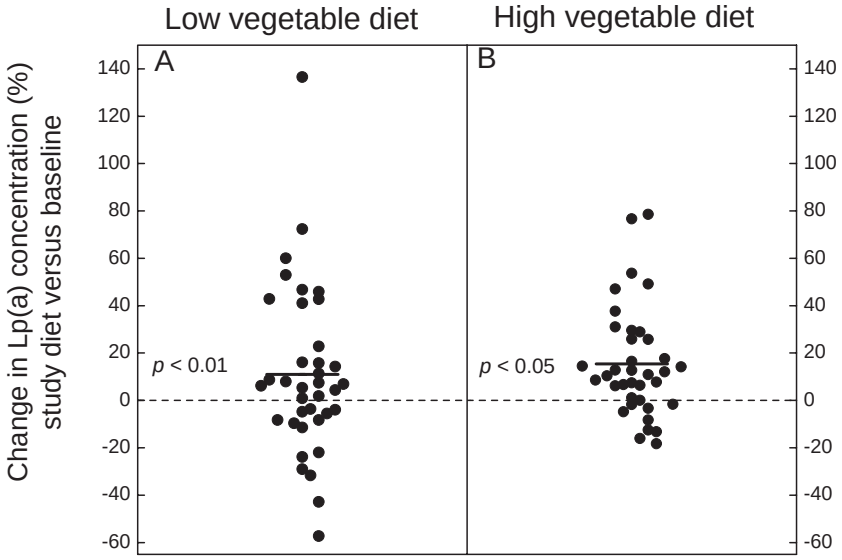

Fig. 7. Changes in the plasma concentration of oxidised LDL of all the study subjects (n=37) from baseline to low vegetable diet and from baseline to the high vegetable diet. Wilcoxon signed ranks test.

The baseline plasma OxLDL-EO6 correlated with the plasma Lp(a) concentration (r=0.77, $P<0.001$). The were also strong correlations between the plasma OxLDL-EO6 and Lp(a) on the low vegetable diet (r=0.83, $P<0.001$) and on the high vegetable diet (r=0.84, $P<0.001$).

5.6 Serum paraoxonase-1 activity (II)

The $PON1_{192}$ R (arginine) allele frequency was 0.37 and the $PON1_{55}$ M (methionine) allele frequency 0.32, and the genotype distributions were not significantly different from the Hardy-Weinberg prediction. The genotype groups did not differ significantly from each other with respect to age, BMI and the concentrations of plasma lipids and lipoproteins at the baseline (data not shown). Because only five subjects were homozygous for the mutation glutamine → arginine of the $PON1_{192}$, all the subjects with the $PON1_{192}$ R allele were treated as one group. Also, the subjects with the $PON1_{55}$ MM genotype (n=2) were included in the group of PON_{55} LM genotype for statistical analyses.

The subjects with the $PON1_{192}$ R allele had a higher ($P<0.001$) serum PON1 activity at the baseline than the subjects with the $PON1_{192}$ QQ (glutamine/glutamine) genotype (Table 7). Also, subjects with the $PON1_{55}$ LL (leucine/leucine) genotype had a higher ($P<0.001$) serum PON1 activity at the baseline than those with the $PON1_{55}$ M allele (Table 7). Table 7 presents the medians for the serum PON1 activities. The mean serum PON1 activities of all subjects were 244 U/l at the baseline, 240 U/l at the end of the low

vegetable diet, and 226 U/l at the end of the high vegetable diet, and, according to the Friedman test, there was a difference (P=0.001) in the activities of these diet periods. When further tested by the Wilcoxon signed ranks test, the serum PON1 activity was lower (P<0.05) at the end of the high vegetable diet than the low vegetable diet (Table 7). There was no difference (P=0.31) in the serum PON1 activities between the baseline period and the low vegetable diet (Table 7). The average reduction of the serum PON1 activity (the low vegetable diet versus the high vegetable diet) was small, 14 U/l (4 %), but significant. The relative reduction of plasma HDL-cholesterol in response to the high vegetable diet correlated with the reduction of serum PON1 activity (r=0.35, P<0.05). Within the genotype groups, the decrease in the serum PON1 activity between the diets was significant (P<0.05) among the subjects who had the highest serum PON1 activities, i.e. among those with the $PON1_{55}$ LL genotype and those with the $PON1_{192}$ R allele (Table 7).

Table 7. Serum paraoxonase-1 activity of the women (n=37) on the low and high vegetable diets according to their $PON1_{192}$ and $PON1_{55}$ genotypes. [1, 2]

Genotype	n	Baseline PON1 activity (U/l)	Low vegetable diet PON1 activity (U/l)	High vegetable diet PON1 activity (U/l)
All	37	246 (54, 587)	240 (49, 617)	230 (52, 622) *
$PON1_{192}$				
Gln/Gln	15	91 (54, 146)	85 (49, 135)	79 (52, 144)
Gln/Arg, Arg/Arg	22	308 (225, 597) †	290 (206, 617) †	279 (209, 622) * †
$PON1_{55}$				
Leu/Leu	15	336 (54, 395)	331 (89, 617)	288 (81, 622) *
Leu/Met, Met/Met	22	113 (54, 395) §	109 (49, 399) §	105 (52, 377) §

[1] PON1; paraoxonase-1. [2] The values are median (minimum, maximum). Gln, glutamine; Arg, arginine; Leu, leucine; Met, Methionine. * P<0.05; different from the low vegetable diet concentration (Wilcoxon signed ranks test). † P<0.001; different from the $PON1_{192}$Glu/Glu genotype (Mann-Whitney test). § P<0.01; different from the $PON1_{55}$Leu/Leu genotype (Mann-Whitney test)

5.7 Serum folate and homocysteine (III, IV)

The high vegetable diet significantly increased the subjects' average serum and erythrocyte folate concentrations and decreased plasma tHcy concentration compared with the low vegetable diet. The average serum and erythrocyte folate concentrations of all subjects increased by 78 ± 49 % (P<0.001) and 14 ± 19 % (P <0.001), respectively, in response to the high vegetable diet compared with the low vegetable diet (Table 8). The mean plasma tHcy concentration decreased by 13 ± 13 % (P<0.001) in response to the high vegetable diet compared with the low vegetable diet (Table 8). The absolute change of the plasma tHcy concentration between the study diets correlated positively with the plasma tHcy concentration of the low vegetable diet (r=0.50; P<0.01) and negatively with the serum folate concentration of the low vegetable diet (r = –0.34; P<0.05). The change of plasma tHcy concentration correlated with the change in the erythrocyte folate

concentration (r = –0.42; $P<0.05$), but not with the change in the serum folate concentration (r = 0.05; $P=0.755$).

Table 8. Concentrations of serum and erythrocyte folate, serum vitamin B12 and plasma total homocysteine of the women (n=37) during the study. [1]

	Baseline	Low vegetable diet	High vegetable diet
Serum folate (nmol/l)	11.0 ± 3.7	11.0 ± 3.0	19.3 ± 6.4 *
Erythrocyte folate (nmol/l)	389 ± 122	412 ± 120	464 ± 138 *
Serum vitamin B12 (nmol/l)	328 ± 87	366 ± 98	351 ± 104
Plasma total homocysteine (µmol/l)	8.1 ± 1.9	8.0 ± 1.4	6.9 ± 1.5 *

[1] Values are mean ± SD
* $P < 0.001$; different from the low vegetable diet concentration (Wilcoxon signed ranks test)

A carry-over effect on the erythrocyte folate concentration was observed among the women who consumed the high vegetable diet first (Fig. 8). Among those women, the 3-week wash-out followed by the 5-week low vegetable diet was too short a period for the erythrocyte folate concentration to return to the baseline level.

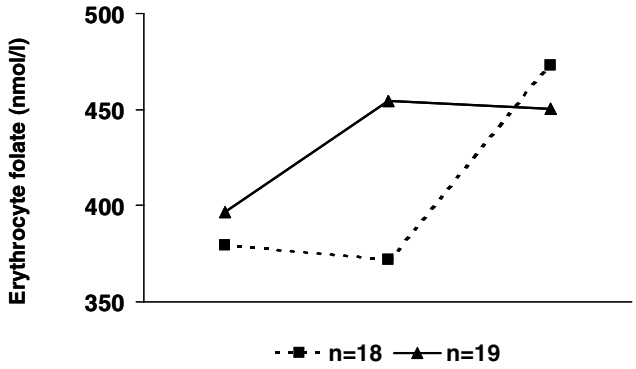

Fig. 8. Erythrocyte folate concentration according to individual arms of the cross-over study. Values are means.

5.7.1 Effects of genetic variation on serum folate and plasma tHcy (IV)

The frequencies of the CC, CT, and TT genotypes of the MTHFR gene among the subjects were 51 %, 35 %, and 14 %, respectively. The serum folate concentrations at the baseline did not differ between the subjects with different genotypes of the MTHFR gene (Table 9). The basal tHcy concentration tended to be non-significantly higher among the subjects homozygous for the T677 allele in the MTHFR gene than in those with the genotypes CT and CC (Table 9). The average serum folate increased and the plasma tHcy concentration decreased among all genotype groups of the MTHFR gene in response to

the high vegetable diet compared to the low vegetable diet (Table 9). When tested with ANOVA of repeated measurements across time, no MTHFR genotype effect was found in the diet-induced changes in the serum folate and plasma tHcy concentrations.

Table 9. Concentrations of serum folate and plasma total homocysteine (tHcy) of the women (n=37) according to their $C_{677}T$ genotypes of the methylenetetrahydrofolate reductase gene [1, 2]

	CC	CT	TT
Serum folate, nmol/l	n=19	n=13	n=5
Baseline	11.4 ± 4.4	10.6 ± 2.7	10.4 ± 3.6
Low vegetable diet	11.2 ± 3.1	10.7 ± 2.6	11.0 ± 3.8
High vegetable diet	19.9 ± 4.6	19.3 ± 8.3	17.1 ± 7.5
Percentage change from low to high vegetable diet	85 ± 51 ***	77 ± 50 ***	55 ± 33 *
Plasma total homocysteine, µmol/l			
Baseline	8.0 ± 1.6	8.0 ± 2.2	9.1 ± 2.6
Low vegetable diet	7.8 ± 1.3	8.2 ± 1.6	8.6 ± 0.8
High vegetable diet	6.9 ± 1.0	7.0 ± 2.2	7.0 ± 0.8
Percentage change from low to high vegetable diet	–11 ± 9 ***	–15 ± 18 **	–18 ± 9 *

[1] Values are mean ± SD
* P <0.05, ** P =0.01, *** P <0.001: statistical significance for changes within the genotype group (Student's t-test for paired samples)

The genotype frequencies of the AA (wild type), AG, and GG genotypes of the $A_{2756}G$ mutation of the MS gene were 59 %, 38 %, and 3 %, respectively. Because only one subject was homozygous for the G2756 allele, the genotypes AG and GG were treated as one group in the statistical analyses. According to the ANOVA of repeated measurements, the MS genotype and dietary modifications affected the plasma tHcy concentration during the study (P<0.05). The subjects with the allele G_{2756} had a lower plasma tHcy (P<0.01) at the end of the high vegetable diet and a more extensive decrease in plasma tHcy (P<0.05) in response to the high vegetable diet than those without the mutation (Table 10).

Six subjects (16 %) were heterozygous for the 844ins68 of the CBS gene. There were no differences in the plasma tHcy concentrations of the subjects with respect to this gene variant. According to the ANOVA of repeated measurements, there was no CBS genotype effect in the diet-induced changes of the serum folate and plasma tHcy concentrations.

Table 10. Plasma total homocysteine concentration of the women (n=37) according to their $A_{2756}G$ genotypes of the methionine synthase gene.[1]

	AA	AG and GG
Plasma total homocysteine, µmol/l	n=22	n=15
Baseline	8.6 ± 2.2	7.6 ± 1.3
Low vegetable diet	8.2 ± 1.3	7.7 ± 1.5
High vegetable diet	7.5 ± 1.5	6.2 ± 1.0 ††
Percentage change from low to high vegetable diet	–9 ± 11 *	–19 ± 14 * †

[1] Values are mean ± SD
* P <0.001: statistical significance for changes within the genotype group (Student's t-test for paired samples)
† P <0.05, †† P <0.01: statistical significance for differences between the genotype groups (Student's t-test for independent samples)

6 Discussion

6.1 Methodological considerations

6.1.1 Design

We conducted our diet study by using a closely controlled intervention with two diet periods. We provided all the food and beverages of the study diets to the subjects, and the study also included daily supervised meals. We chose this method because provision of meals and foods has been shown to increase dietary compliance and maximise physiologic responses in dietary interventions, and even to reduce between-person variability in dietary response (Wing 1997, Metz *et al.* 1997). It is also a suitable method in dietary interventions including multiple dietary modifications, as in our study.

An essential feature of the current study was a cross-over design, in which each individual served as her own control. The study design allowed us to study the dietary effects independently of the order in which the study diets were consumed. The duration of the diet periods was 5 weeks. We observed that for some variables, such as the erythrocyte folate concentration, the 5-week diet period was clearly too short. Among the women who consumed the high vegetable diet first, the erythrocyte folate concentration did not decline at all during the following 8 weeks (the wash-out period plus the low vegetable diet). This so-called carry-over effect is a potential confounding factor with respect to the dietary response of plasma homocysteine concentration. It is also possible that the 5-week diet periods may have been too short for the plasma homocysteine concentration to respond to the increased dietary intake of vegetables, berries, and fruit. On the other hand, relatively short diet periods can be suitable to explore the changes in the plasma carotenoids and vitamin C. In a previous dietary intervention with vegetables and fruit, the changes in the plasma alpha- and beta-carotene and vitamin C levels occurred within the first two weeks of the intervention and remained practically unchanged for the duration of the trial (Zino *et al.* 1997). Regarding the plasma antioxidants, the duration of our study diet periods can thus be considered sufficient.

6.1.2 Diets

The study diets were based on regular Finnish foods and meals. They differed from the average Finnish diet by the quantity and quality of dietary fat, which were modified according to the current recommendations (Nordic Nutrition Recommendations 1996) and in order to alter the dietary intake of vitamin E. It should be emphasised that the low vegetable diet was not planned to be a depletion diet. It was rather intended to provide the subjects with the currently recommended intakes of vitamins and minerals. For example, the low vegetable diet provided the subjects with the recommended intakes of vitamin E and dietary fibre, and higher amounts of vitamin C, calcium, vitamin B6, and vitamin B12 daily than is suggested in the current dietary guidelines (Nordic Nutrition Recommendations 1996). Moreover, the nutrient contents of the low vegetable diet and the subjects' habitual diet were fairly similar.

The main difference between the low and high vegetable diets was the amount of vegetables, berries, and fruit in the diet. We chose the vegetables, berries and fruit based on their contents of alpha- and beta-carotene, folate, and vitamin C. Without these qualifications of nutrient contents, the selection of vegetables, berries, and fruit consumed might have been more extensive, and the results might have been somewhat different. In addition, we wanted the high vegetable diet to contain more dietary vitamin E than the low vegetable diet, and chose different vegetable oils and spreads for the study diets. On the low vegetable diet, salad dressing was made from rapeseed oil and a spread containing 10 mg of vitamin E per 100 g was used. On the high vegetable diet, we used sunflower oil in salad dressing and a spread with 20 mg of vitamin E per 100 g. These changes affected the composition of dietary fat. Sunflower oil is higher in PUFA than rapeseed oil, which contains more MUFA. These changes in the fat quality might have influenced the plasma lipid concentrations and their changes, which are discussed in the chapters 6.2 and 6.3.

In addition to the vegetables and dietary fat, different breads were consumed on the study diets. Whole grains and rye bread, for example, are good sources of dietary folate (National Public Health Institute 2001) and vitamin E (Heinonen & Piironen 1991), and therefore we chose mainly rye bread for the high vegetable diet. On the low vegetable diet, mixed grain bread, as well as dark and white wheat bread were consumed. Thus, the bread may have also contributed to the dietary folate and vitamin E intake during the study. The changes in the bread quality may have also affected the contents of other B vitamins and dietary fibre of the study diets.

6.1.3 Compliance

We confirmed the compliance with the study diets by the food and meal provision, by the supervised meals, and by controlling the body weights 5 times per week. In addition, the subjects reported in writing any deviations in their diets. According to the surveyed meals and the subjects' reports, they followed the study diets without major exceptions. Moreover, the changes in the plasma levels of carotenoids and vitamin C, and the changes

in the blood folate concentrations indicated that the compliance with the study diets was good.

6.1.4 Dietary analyses

During the intervention, the nutrient composition of the low and high vegetable diets was analysed from identical food samples. This duplicate meal method is a reliable, yet expensive and time-consuming method for assessing dietary intake. In the present study, we collected identical food samples including drinks every day by one energy intake level (7.5 MJ) for each study diet. This energy intake level was chosen because it was close to the average dietary intake of the women at the baseline period and because most women started the intervention with this energy intake. In the Agricultural Research Centre of Finland the samples were combined to pooled food samples that represent the average daily dietary intake of the study diet. By this method we obtained reliable data on the average amounts of nutrients consumed, but lacked the data of individual intakes of energy and nutrients. The individual dietary intake data during the intervention could have been obtained from the food records, for example. Because the foods and meals were provided to the subjects according to the written instructions, which were also given to the subjects, and the nutritionist supervised the main meals, we collected no food diaries during the intervention. The subjects consumed the same amounts of vegetables, berries, and fruit regardless of the energy intake level, and we feel that food diaries would not have provided more accurate information of the study diets or compliance. Because we did not obtain data of individual intake of nutrients, we were not able to detect associations between the intake and biochemical determinants. However, assessing correlations between the dietary intake and biochemical variables was not the aim of our study.

6.2 Dietary effects on plasma lipids and lipoprotein(a)

The intervention resulted in moderate changes in the plasma lipid concentrations. The high vegetable diet decreased the plasma total, LDL and HDL cholesterol concentrations compared to the low vegetable diet. Increased dietary intake of PUFA on the high vegetable diet possible reduced the plasma lipid concentrations. In addition, the high intake of dietary fibre (40 g per day on an average) on the high vegetable diet may have contributed to the changes in the plasma lipid levels (Anderson *et al.* 1992).

Lipoprotein(a) is suggested to be an independent risk factor for coronary artery disease (Luc *et al.* 2002). Therefore, factors affecting its concentration are of major importance. We observed no differences in the Lp(a) concentrations between the low and high vegetable diets. However, compared to the baseline diet period, both the low and high vegetable diets resulted in equally elevated plasma Lp(a) concentrations. We do not know the exact reason for the increase in Lp(a) levels in response to the intervention diets. The

basal levels of Lp(a) are primarily genetically determined, but some data suggest that Lp(a) may act as an acute-phase reactant under some situations (Hobbs & White 1999). Dietary manipulations of Lp(a) levels in humans, on the other hand, have been mainly unsuccessful (reviewed in Berglund 1995). Some dietary treatments have been observed to influence the Lp(a) concentrations. For example, dietary n-3 fatty acids from fish oil reduced the Lp(a) concentrations in hypertriglyceridemic patients (Beil *et al.* 1991). Also, a diet high in SAFA (16 % of total energy) was shown to produce approximately 10 % lower plasma Lp(a) concentration than diets high in oleic acid or *trans*-fatty acids (Clevidence *et al.* 1997). This observation is consistent with our study in that both the low and high vegetable diets led to lower SAFA intake and consequently increased Lp(a). In addition, dietary *trans*-fatty acids may have an adverse effect on the plasma Lp(a) concentration, in particular in subjects with high plasma Lp(a) concentration (Clevidence *et al.* 1997). We were not able to analyse the *trans*-fatty acid contents of the study diets, but based on the food choices and the cooking methods used, the study diets were likely to be very low in dietary *trans*-fatty acids. For example, the intervention diets did not contain the major dietary sources of *trans*-fatty acids, such as cakes, biscuits, sweets, or hard margarines (Elias & Innis 2002). It is thus most probable that the plasma Lp(a) levels were not increased by *trans*-fatty acids in the present study.

The average elevation in the Lp(a) concentration in our study was 13 %. High Lp(a) concentrations increase the risk of CVD, but the threshold for the increased risk has most frequently been set at 300 mg/l (Armstrong *et al.* 1986), which is considerably higher than the average plasma Lp(a) level of our study subjects. Thus, the increase in the Lp(a) levels in our study would be rather insignificant with respect to CVD. On the other hand, the Lp(a) concentration was strongly associated with the plasma OxLDL-EO6 levels, suggesting that Lp(a) may play a role in atherosclerosis related to oxidised LDL. This hypothesis is discussed further in the next chapter.

6.3 Dietary effects on markers of oxidation

6.3.1 Plasma antioxidants

The high vegetable diet resulted in marked increases in the plasma concentrations of several carotenoids and vitamin C. These findings are in line with previous studies (Zino *et al.* 1997, Broekmans *et al.* 2000). The plasma concentration of alpha-tocopherol, however, did not change in response to the changes in the dietary intake of vitamin E. Based on previous interventions, the plasma alpha-tocopherol levels do not respond to the changes in the dietary intake as readily and strongly as those of plasma beta-carotene (Willett *et al.* 1983). In the ATBC-study, for example, a daily dose of 50 mg of alpha-tocopherol, which was 3 times the daily dietary intake of the subjects, resulted in a 50 % increase in the serum alpha-tocopherol concentration (Leppälä *et al.* 2000). Increasing the plasma alpha-tocopherol concentration through modification of the diet rather than supplements seems to be difficult. It would require a more extensive consumption of

foods rich in vitamin E, such as nuts and seeds, and perhaps a higher fat intake than in our intervention.

In contrast to other plasma carotenoids, the average plasma lycopene concentration decreased in response to both study diets, probably because the study diets were low in dietary lycopene or because of a lower bioavailability. Other investigators have reported similar findings. In previous controlled trials, the plasma lycopene concentrations decreased in response to a high vegetable diet (Broekmans *et al.* 2000) or were unaffected by increased consumption of vegetables (Miller, III *et al.* 1998, Freese *et al.* 2002). The sparse dietary sources of lycopene make it different from other carotenoids, which are more widely distributed in different vegetables and fruit in the human diet. Considering the sources of lycopene in diet and the fact that the bioavailability of this carotenoid is highest from processed and cooked foods (Stahl & Sies 1992), it is no wonder that the plasma lycopene concentration does not associate with a high intake of common vegetables and fruit in general. Moreover, lycopene is a good example how modifying diets in order to alter the intake of certain nutrients may affect the intake and plasma concentrations of other potentially beneficial dietary components in a less desirable way.

6.3.2 Plasma oxidised LDL

We found no differences in the plasma OxLDL-EO6 levels between the low and high vegetable diets. A diet high in vegetables, berries and fruit, and thus high in naturally occurring antioxidants did not reduce the plasma levels of oxidised LDL-EO6 compared to the low vegetable diet. Compared to the baseline diet, however, the plasma concentration of OxLDL-EO6 was equally elevated during the low and the high vegetable diets, yet the plasma antioxidant levels were particularly increased in response to the high vegetable diet. Thus, the explanation for the increased OxLDL-EO6 may be found from other dietary factors than dietary vegetables, berries, and fruit, or natural antioxidants, of the intervention diets.

The intervention included also modifications in the quantity and quality of dietary fat. We modulated the dietary fat intake according to current guidelines (Nordic Nutrition Recommendations 1996), and in order to alter the dietary intake of vitamin E. Consequently, as compared to the baseline diet of the subjects, the low and high vegetable diets were lower in total and saturated fat, and somewhat higher in PUFA. The proportions of PUFA and linoleic acid were particularly high in the high vegetable diet. Previous experiments have shown that diets high in linoleic acid lead to LDL particles enriched with linoleic acid, which are more susceptible to lipid peroxidation (Parthasarathy *et al.* 1990, Berry *et al.* 1991, Bonanome *et al.* 1992). In our study, the high linoleic acid intake could explain the increased plasma OxLDL-EO6 on the high vegetable diet. However, there was practically no change in the PUFA intake between the baseline diet and the low vegetable diet, which does not support the role of linoleic acid intake in increasing the plasma OxLDL-EO6 levels.

It is probable that the increased plasma OxLDL-EO6 concentrations in our study are related to lipoprotein(a) metabolism. We observed that the plasma Lp(a) concentration also increased in response to the intervention diets compared to the baseline, and that the

OxLDL-EO6 and Lp(a) were strongly correlated. A similar correlation has recently been reported by Tsimikas and co-workers (Tsimikas *et al.* 2003). They suggested that Lp(a) might be acting as a preferential acceptor that binds tightly oxidised phospholipid from tissues or lipoproteins (Tsimikas *et al.* 2003). It is thus possible that in our study the plasma OxLDL-EO6 increased in response to the increase in the Lp(a) concentration. Moreover, it can be hypothesised that Lp(a) is acting as a plasma transporter of oxidised phospholipids, and that both study diets resulted in favourable changes in the artery wall or other tissues that decreased oxidation, which resulted in transport of oxidised phospholipid away from the tissues, such as the artery wall. In turn, these oxidised phospholipids are preferentially bound by Lp(a) in plasma, which is reflected in the enhanced OxLDL-EO6 levels in plasma. Thus, it can be postulated that with respect to this parameter, both the low and high vegetable diets would be more beneficial than the baseline diet of the subjects. This interesting hypothesis needs to be explored in more detail before we are able to conclude whether the changes observed in the present study are pro- or anti-atherogenic.

6.3.3 Paraoxonase-1 activity

We investigated the effects of a high intake of vegetables, berries, and citrus fruit rich in natural antioxidants on the serum PON1 activity, and observed that consuming the high vegetable diet slightly reduced the average serum PON1 activity of all the subjects compared to the low vegetable diet. Because PON1 is inactivated by lipid peroxides, the presence of dietary antioxidants has been previously suggested to preserve serum PON1 activity (Aviram *et al.* 1999). Indeed, supplementation of pomegranate juice high in antioxidants to the habitual diets of healthy men not only preserved, but significantly enhanced their serum PON1 activities. In the same study, serum PON1 activity increased and lipid peroxidation decreased in a sample of three subjects, although their plasma lipids and lipoproteins did not change significantly. (Aviram *et al.* 2000) In another study (Jarvik *et al.* 2002), the serum PON1 activity of male subjects was positively associated with the dietary intakes of vitamin C and E, supporting the theory of PON1 preserving functions of dietary antioxidants. Our results are not in line with those previous findings (Aviram *et al.* 2000, Jarvik *et al.* 2002). According to our study, the natural antioxidants from vegetables, berries, and fruit do not seem increase or preserve the serum PON1 activity. This finding is in line with a recent dietary intervention, in which the serum PON1 activity decreased in response to all dietary regimens, either low or high in vegetables and fruit, while in the control group the activity was increased (Freese *et al.* 2002). Thus, the dietary vegetables, berries, and fruit rich in antioxidants do not seem to enhance the serum PON1 activity in humans. We also observed a correlation between the relative reductions of serum PON1 activity and plasma HDL-cholesterol concentration in response to the high vegetable diet, which is in line with a previous study (van der Gaag *et al.* 1999). These findings suggest that HDL-cholesterol metabolism is important in the dietary regulation of serum PON1 activity.

An important question is whether the relatively small reduction in the serum PON1 activity in our study could have any physiological significance or impact on

atherosclerosis. At the moment, it is still unclear whether the activity of PON1 towards paraoxon is a good marker of the physiologic function of this enzyme. In fact, the ability of PON1 to hydrolyse paraoxon seems to be inversely related to its capacity to hydrolyse lipid peroxides, and thus to its antiatherogenic action (Durrington *et al.* 2001). Information about the capacity of PON1 to prevent atheroma, for example, would be valuable for evaluating the clinical significance of PON1 activity.

The genetic variance of PON1 influences the enzyme activity (Davies *et al.* 1996). According to our results, the genetic variance may affect the dietary response of PON1 activity as well. The dietary response of serum PON1 activity was genetically regulated in such a way that those having a genotype with a high baseline PON1 activity ($PON1_{192}$ R allele and $PON1_{55}$ LL) had the most extensive reduction in their serum enzyme activities. Our findings with a relatively small number of subjects suggest that the serum PON1 activity in humans is modulated by both dietary factors and genetic variability. Whether this possible interaction of genes and diet in the serum PON1 activity plays a role in the development of atherosclerosis has to be explored in future studies.

6.4 Dietary and genetic effects on serum folate and plasma homocysteine

We observed enhanced serum and erythrocyte folate concentrations as well as reduced plasma total homocysteine concentration of the women in response to the increased intake of vegetables, berries, and citrus fruit rich in natural folate. The high vegetable diet increased the serum folate concentration by 78 % and the erythrocyte folate by 14 % compared to the low vegetable diet. The average increase in serum folate is extensive compared with previous diet interventions (Brouwer *et al.* 1999b, Riddell *et al.* 2000, Appel *et al.* 2000, Venn *et al.* 2002), and the increase in the erythrocyte folate concentration is comparable with the previous findings (Brouwer *et al.* 1999b). We observed these significant changes even though the carry-over effect of erythrocyte folate concentration might have diluted the changes. Good compliance with the high vegetable diet may have attributed to the extensive increases of serum and erythrocyte folate concentrations.

The high intake of vegetables, citrus fruit and berries resulted in a 13 % (1.1 µmol/l) decrease in the average plasma tHcy concentration. This reduction compares well with the previous observations. In a controlled dietary intervention (Brouwer *et al.* 1999b) the plasma tHcy decreased by 14 % (1.5 µmol/l) in response to increased intake of vegetables and fruit. In another study (Riddell *et al.* 2000), a non-significant reduction of 9 % in the plasma tHcy was observed among a dietary folate group. In a recent dietary counselling study (Venn *et al.* 2002), the plasma tHcy concentration decreased by 10 % during a 4-week intervention period. Interestingly, the plasma tHcy continued decreasing during the follow-up period, when no target for the dietary folate was set (Venn *et al.* 2002). This finding suggests that the plasma tHcy concentration may respond more slowly to the natural folate from food than to folic acid supplementation. It is possible that in our study and in previous interventions, the diet periods have been too short to observe the

maximum dietary response in the plasma tHcy concentration. Thus, the results of dietary interventions, including our study, may have underestimated the efficacy of dietary folate in reducing the plasma tHcy levels.

One of the major findings of our study was that the high vegetable diet resulted in similar concentrations of plasma tHcy among the subjects with the different genotypes of MTHFR gene. In other words, those who are genetically susceptible to higher plasma tHcy concentrations and may also be at higher risk for cardiovascular disease (Wald *et al.* 2002), may benefit from an increased intake of dietary folate and achieve a lower plasma tHcy concentration by increasing the consumption of foods rich in natural folate. Our finding seems to be in line with a previous study conducted with synthetic folic acid (Malinow *et al.* 1997). In that study, the subjects homozygous for the T_{677} allele had a more extensive decrease in plasma tHcy after folic acid supplementation than the subjects heterozygous for the mutation or with a wild type of the MTHFR gene (Malinow *et al.* 1997). In addition to the MTHFR gene polymorphism, we detected the 68-bp insertion of the CBS gene and the $A_{2756}G$ transition of the MS gene. Both the presence of the 68-bp insertion of the CBS gene and the G_{2756} allele of the MS gene are known to be associated with lower fasting levels of plasma tHcy (Tsai *et al.* 2000), but the associations of these gene mutations with the diet responsiveness of plasma tHcy were not defined in previous interventions. We observed no effect of the insertion in the CBS gene on the concentration or dietary response of plasma tHcy. The G_{2756} allele of the MS gene, on the other hand, was associated with a lower plasma tHcy at the end of the high vegetable diet and a more extensive decrease in the plasma tHcy in response to the high vegetable diet when compared with genotype AA. The findings of our dietary intervention with a relatively small number of subjects should be considered as preliminary data. However, our finding suggests that the diet response of plasma homocysteine may be genetically regulated.

In addition to folate, vitamins B6 and B12 are known to be important regulators of plasma tHcy (Mason & Miller 1992). The serum concentration of vitamin B12 is likely to be regulated more by absorption than intake, and we did not observe any major changes in the serum levels of vitamin B12. However, the calculated dietary intake of vitamin B6 from food was about 1 mg higher on the high vegetable diet than on the low vegetable diet, which might have influenced the plasma tHcy concentration. Beyond group B vitamins, other dietary factors may also affect the plasma tHcy concentration. A reduced plasma tHcy concentration has been achieved with a diet high in vegetables, but relatively low in dietary folate (228 µg per day) (Broekmans *et al.* 2000). This finding suggests that other components than folate in vegetables and fruit might affect the plasma tHcy concentration. Furthermore, the findings of dietary interventions emphasise the role of a whole diet with a high intake of common vegetables, berries, and fruit in reducing plasma homocysteine levels. The consumption of these foods has an important advantage over folic acid supplementation and fortification by increasing the intake of other beneficial nutrients as well.

7 Conclusions

In the present study, we investigated the effects of a daily consumption of common vegetables, berries, and citrus fruit (altogether about 700 g per day) along with a diet that was low in total and saturated fat and relatively high in polyunsaturated fat, on the plasma levels of antioxidants, folate, and homocysteine, on the serum paraoxonase-1 activity, and on the plasma oxidised LDL of healthy women. We also determined whether gene polymorphisms affected the diet response of plasma homocysteine and serum paraoxonase-1 activity.

The consumption of increased amounts of vegetables, berries, and citrus fruit resulted in enhanced plasma levels of antioxidants, such as alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein-zeaxanthin, and vitamin C. The high vegetable diet decreased the plasma total and LDL cholesterol concentrations, but did not result in lower plasma levels of Lp(a) and circulating oxidised LDL compared to the low vegetable diet. In fact, both the plasma levels of Lp(a) and oxidised LDL were increased in response to the low and high vegetable diets compared to the subjects' baseline diet.

The diet high in vegetables, berries, and fruit resulted in a slightly decreased serum paraoxonase-1 (PON1) activity, and the dietary response of PON1 was genetically regulated in such a way that those having a genotype with a high baseline PON1 activity ($PON1_{192}$ R allele and $PON1_{55}$ LL) had the most extensive reduction in their serum enzyme activities. Our findings suggest that the serum paraoxonase-1 activity in humans is modulated by both dietary factors and genetic variance. The correlation between the relative reductions of serum PON1 activity and plasma HDL-cholesterol concentration in response to the high vegetable diet suggests that HDL-cholesterol metabolism may be important in the dietary regulation of serum PON1 activity.

Increased consumption of common vegetables, berries, and citrus fruit high in natural folate effectively increased serum and erythrocyte folate concentrations and decreased plasma homocysteine concentrations. Notably, the plasma homocysteine concentration of subjects genetically predisposed to higher plasma homocysteine (TT genotype of the methylenetetrahydrofolate reductase gene) responded well to the dietary folate. The $A_{2756}G$ transition of the methionine synthase gene was associated with the dietary response of plasma homocysteine in such a way that the subjects carrying the G_{2756} allele showed a more extensive reduction in the plasma homocysteine in response to the increased dietary folate than those with the genotype AA. Our findings clearly show that

a diet high in common vegetables, citrus fruit, and berries decreases plasma homocysteine concentration and suggest that the diet response of plasma homocysteine may be genetically regulated.

In conclusion, the observed changes in response to the high vegetable diet were mainly positive: reduced plasma homocysteine and total and LDL cholesterol concentrations and enhanced plasma levels of several antioxidants and folate. Thus, our findings are in line with the current dietary recommendations to increase the consumption of vegetables, berries, and fruit.

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