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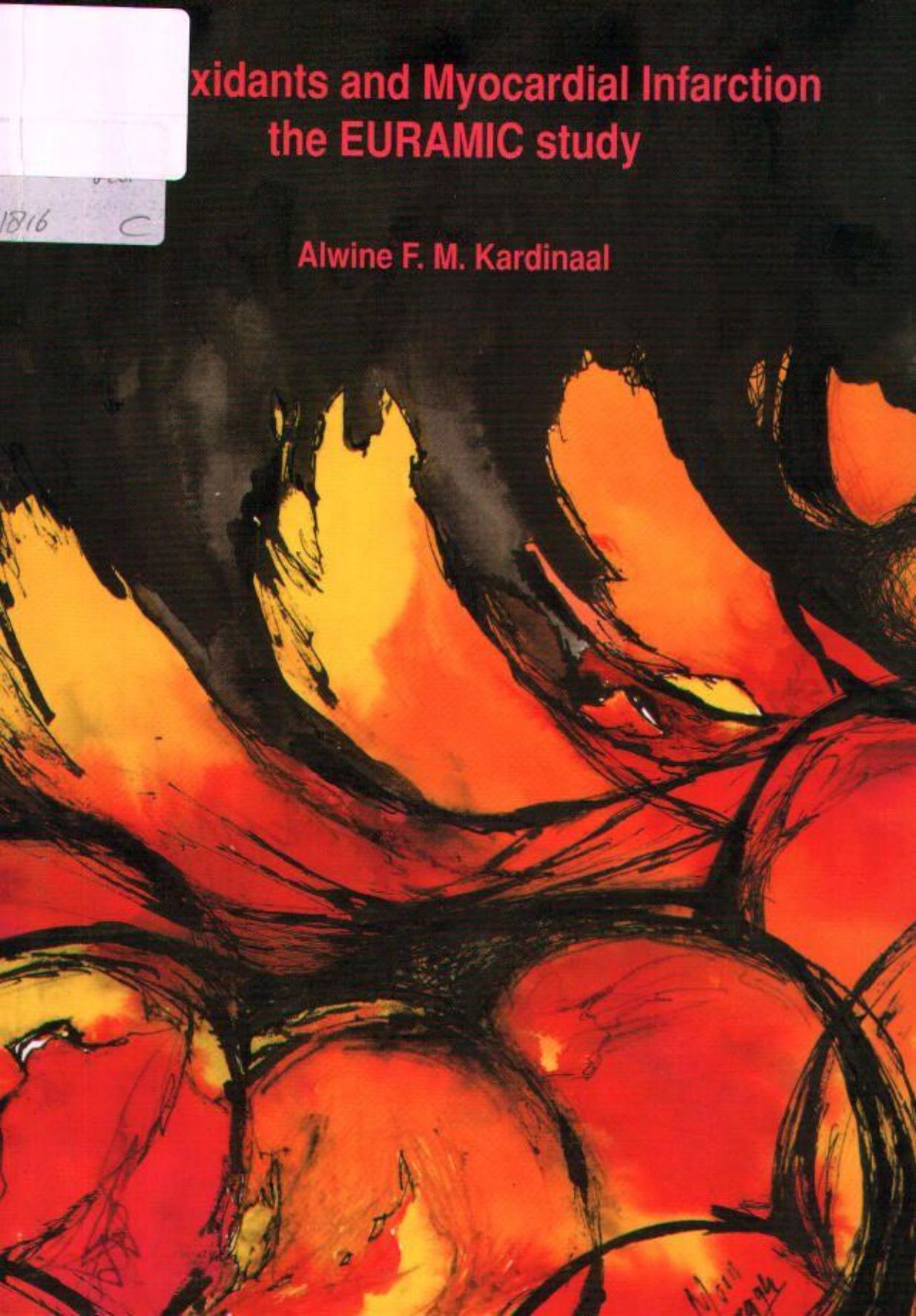
Alwine F. M. Kardinaal



Antioxidants and Myocardial Infarction: the EURAMIC Study

Alwine F.M. Kardinaal

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Antioxidants and Myocardial Infarction: the EURAMIC Study

Alwine Froukje Maria Kardinaal

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Abstract

Antioxidants and myocardial infarction: the EURAMIC study

PhD thesis, TNO Nutrition and Food Research Institute, Zeist, The Netherlands, 9 September 1994.

Alwine F.M. Kardinaal

This thesis reports the background, design and results of a multi-centre study on the relationship between diet-derived antioxidants and the risk of acute myocardial infarction (MI) in men. Levels of α-tocopherol and β-carotene in adipose tissue and of selenium in toenails were compared between almost 700 patients with first MI and a similar number of control subjects, recruited in 8 European countries and Israel. The concentration of β-carotene in adipose tissue, expressed in quintiles of the distribution in controls, was inversely associated with the risk of MI (p for trend 0.001), independently of other risk factors. This association was strongest in current cigarette smokers and in subjects with a high proportion of polyunsaturated fatty acids in the adipose tissue. The risk of MI was not related to α-tocopherol in adipose tissue. In persons with low vitamin E levels, low toenail selenium was associated with a 2.5-fold increased risk of MI, compared to high selenium levels. An additional study among 85 healthy, non-smoking volunteers, aged 50-70, showed only a modest correlation of adipose tissue concentrations with dietary intake of α-tocopherol and β-carotene, assessed by a food frequency questionnaire. Randomized controlled trials with varying doses and combinations of antioxidant nutrients should clarify whether the observed associations are causal. Until then, supplement use is not recommended, but a generous consumption of fruits and vegetables may be encouraged.

The EURAMIC Study was carried out at the following institutes: National Public Health Institute, Finland; Østfold Central Hospital, Norway; University of Edinburgh, Scotland; University of Ulster, Northern Ireland; Federal Health Office, Germany; University of Zürich, Switzerland; University of Granada, University of Malaga, Spain; Hadassah Medical Organization and Hebrew University, Israel; Research Centre for Preventive Medicine, Russia; TNO Nutrition and Food Research (Coordinating Centre).

The coordination of the EURAMIC Study was supported by grants from the European Union (Medical and Health Research Programme 4 and Europe against Cancer). National contributions were funded by the British Heart Foundation, Dutch Ministry of Welfare, Public Health and Cultural Affairs, Spanish FIS, German Federal Health Office, Norwegian Research Council, Russian Ministry of Science, Swiss NRF, and the Yrjö Jahnsson Foundation.

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Contents

Introduction

Although the incidence of coronary heart disease (CHD) is declining in countries with a western life-style, including the Netherlands, it still is the major cause of death among men and post-menopausal women[1,2]. Elevated serum cholesterol, hypertension and cigarette smoking have been identified as the prime risk factors for CHD, but many other clinical and lifestyle factors are associated with the risk of CHD. As yet, all these factors do not fully explain the variation in CHD risk. Moreover, little is known about the biological mechanisms by which specific factors may contribute to the expression of coronary heart disease. A major theory postulates that injury of the arterial endothelium leads to increased adhesion of monocytes and macrophages to the arterial wall[3]. These cells then migrate between the endothelial cells and become large foam cells due to lipid accumulation. The resulting fatty streak may progress to more severe lesions. However, it has been shown that high serum cholesterol in itself does not lead to foam cell formation, because the uptake of low-density lipoproteins (LDL) by cells is regulated[4]. This apparent contradiction is solved by a recent hypothesis, stating that cholesterol particles have to be modified, presumably by oxidation, to become atherogenic[5,6]. This hypothesis has led to intensified research into the potential protective role of antioxidants in atherogenesis[7].

Antioxidants are present in our daily diet. The main antioxidant nutrients are vitamin E (in particular its most active form, α-tocopherol), vitamin C and β-carotene, which can be metabolized to vitamin A in the body. Selenium is a trace element that is incorporated in the enzyme glutathione peroxidase, which also has a major antioxidant function in the body. Vitamin C and β-carotene are found primarily in fruits and vegetables, vitamin E mainly in vegetable oils. Important sources of selenium are cereals, meat and fish. Although evidence has accumulated that these nutrients are indeed good antioxidants and may protect LDL-cholesterol against oxidation in vitro, there are still few human studies to support their preventive role in atherogenesis. Firm evidence for such a role can be obtained from long-term intervention studies with antioxidant nutrients and coronary heart disease as end-point. Several of these studies are in progress, but definitive results will not be published for some time[8]. In the meantime, relatively short-term observational studies may yield information about which antioxidants or combination of antioxidants may be most effective.

In the EURopean study on Antioxidants, Myocardial Infarction and Cancer of the breast, in short the EURAMIC Study, we compared the antioxidant status of patients with acute myocardial infarction (MI) with that of controls without a history of MI. It was hypothesized that the antioxidant status would be lower in patients with acute MI than in control subjects. Moreover, we expected to find a combined effect of the antioxidants, especially for selenium and α-tocopherol. Because it was not expected that a very strong relationship would be found within any particular population, it was important to select a study population with a relatively large range of antioxidant status. Therefore, the study was conducted according to the same protocol in nine countries with varying dietary patterns and the data from these countries were pooled in the overall data analysis. Collaborating centres were found in Finland, Norway, Russia, Scotland, Germany, Switzerland, Spain and Israel. To overcome potential bias in the measurement of diet in different countries, the use of tissue concentrations of antioxidants was preferred. Since plasma levels represent relatively short-term intake and may be affected by acute infarction, other tissues were considered to measure exposure to antioxidant nutrients. For selenium, the concentration in toenails has been shown to be correlated to long-term dietary intake; for α-tocopherol and β-carotene, adipose tissue seemed to be suitable, since both antioxidants are fat-soluble and stored primarily in adipose tissue. Available evidence indicates a limited availability of the antioxidants from adipose tissue, which renders an acute effect of MI on tissue levels unlikely. However, little information is available on the relationship of adipose tissue concentrations with dietary intake of α-tocopherol and β-carotene. To clarify this relationship an additional study has been performed among healthy volunteers in the Netherlands.

Chapter 2 reviews the available evidence for the relation between antioxidants and coronary heart disease at the time the EURAMIC Study started. Details of the design and hypotheses of the EURAMIC Study are discussed in Chapter 3. The results regarding the main hypotheses are described in Chapter 4, 5 and 6; these results are based on the pooled data from all participating centers. Chapter 7 describes the additional methodological study, in which the antioxidant concentration in adipose tissue was compared with dietary and supplement intake. For our Dutch contribution to the EURAMIC Study, we linked the data on α-tocopherol and β-carotene in adipose tissue, as well as on dietary intake of these vitamins, to risk of MI. The results of these analyses are discussed in Chapter 8. Finally,

some methodological considerations, recent findings in experimental and epidemiological studies, and the possible consequences for public health are presented in Chapter 9.
A list of all EURAMIC collaborating centres is included.

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The protective role of antioxidants and coronary artery disease

Alwine FM Kardinaal, Geert van Poppel, Frans J Kok

Abstract

Free radicals may play a role in the development of atherosclerosis primarily by oxidizing polyunsaturated fatty acids in lipoproteins, especially low-density lipoprotein (LDL). The modified LDL is taken up rapidly by macrophages in the arterial wall, forming foam cells. Oxidation reactions are prevented by a complex antioxidant defense system, including the vitamins E, C and β-carotene, and the selenium dependent enzyme glutathione peroxidase. Experiments have shown that the resistance to oxidation of the LDL is influenced by the antioxidant concentration. However, a direct relation between antioxidants and cardiovascular disease has not been demonstrated conclusively in epidemiological studies. Partly this may be due to methodological shortcomings which can be overcome in the future by choosing the relevant parameters of antioxidant status and disease outcome in carefully selected populations.

Introduction

Free radicals are highly reactive molecules that can readily oxidize and damage essential biomolecules, such as lipids and proteins. In recent years, free radicals have been implicated in the development of many diseases, including atherosclerosis. Free radicals may be of exogenous origin, but also are constantly produced as a result of normal biological and metabolic processes. To prevent free-radical-induced damage, the human body possesses a number of free-radical-scavenging, or antioxidant, defense systems, involving vitamins E and C, β-carotene (the precursor of vitamin A), and enzymes such as the selenium-dependent protein glutathione peroxidase.

J Myocard Isch 1992;4:64-78

Recent experimental evidence indicates that a disturbed balance between free-radical production, or "oxidative stress," and these antioxidant defense mechanisms results in damage to biological molecules and lipoproteins and may contribute to the development of atherosclerosis. If an unambiguous association could be demonstrated among free radicals, antioxidant defense, and the incidence of cardiovascular (CV) disease, this would have a profound effect on preventive measures. In the following review, we will explain how free radicals may contribute to the initiation of atherosclerosis and will explore the possible protective role of antioxidants, as suggested by experimental and epidemiologic data.

Free radicals and lipid peroxidation

Free radicals are molecules or molecular fragments that have an unpaired electron. These molecules may have a heightened reactivity that leads to abstraction of a hydrogen atom from another molecule, thereby starting a chain reaction. The many known exogenous free radicals or radical precursors include ozone, nitrous oxides, various carcinogens and mutagens, ionizing radiation, cigarette smoke, and halogenated compounds. These stressors increase the generation of superoxide anion (O_2-) and hydrogen peroxide (H_2O_2). However, oxygen free radicals often are of endogenous origin. Many cell redox systems produce considerable quantities of the superoxide anion and hydrogen peroxide[1-3]; these molecules are not very reactive but may form the highly reactive hydroxyl radical (•OH) in the presence of metal catalysts:

$$O_2- + H_2O_2 \xrightarrow{Fe^{++}} \bullet OH + OH- + O_2$$

The hydroxyl radical reacts with most biological material, such as DNA, proteins, and lipids. Polyunsaturated fatty acids are especially vulnerable to this reaction. The hydroxyl radical can attack the polyunsaturated fatty acids (L) in biological membranes or lipoproteins:

$$LH + \bullet OH \longrightarrow L\bullet + H_2O$$

The carbon-centred radical of the fatty acid then takes up an O_2 molecule and forms a peroxyl radical:

$$L\bullet + O_2 \longrightarrow LOO\bullet$$

Subsequently, a hydrogen atom from another fatty acid molecule is taken up, thereby forming a lipid hydroperoxide, and a new radical:

LOO• + LH ----> LOOH + L• --> chain reaction

This chain reaction is known as lipid peroxidation[4]. The hydroperoxide finally decomposes to cytotoxic aldehydes, e.g., malonaldehyde or 4-hydroxynonenal.

Antioxidants: a complex defense system against oxidative stress

The human body has a complex mechanism for protection against oxidative stress. This defense mechanism includes a number of enzymes and non-enzymatic antioxidant micronutrients (Figure 1).

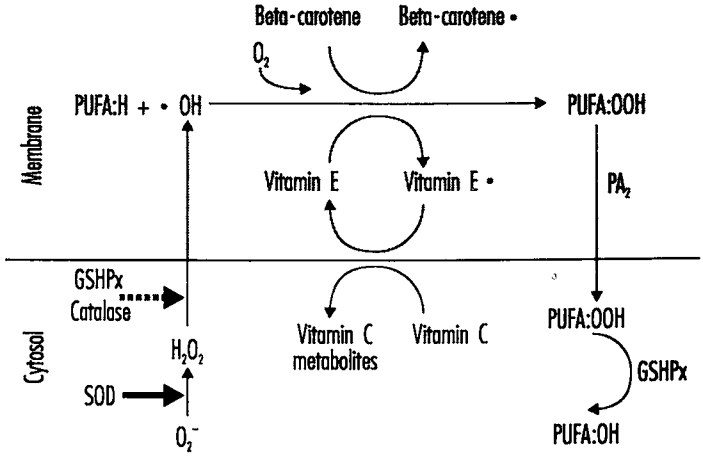

Figure 1. Major antioxidant defense mechanisms within the cell: β-carotene • = β-carotene radical, GSHPx = glutathione peroxidase, H_2O_2 = hydrogen peroxide, O_2 = oxygen, O_2- = superoxide anion, •OH = hydroxyl radical, PA_2 = phospholipase A_2, PUFA:H = polyunsaturated fatty acid, PUFA:OH = fatty acid hydroxide, PUFA:OOH = fatty acid hydroperoxide, SOD = superoxide dismutase, vitamin E• = vitamin E radical. Adapted, with permission, from Duthie et al.[2]

Intracellular defenses

Superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase constitute the primary enzymatic antioxidant defenses, which operate intracellularly. Zinc, copper and manganese are incorporated into the two types of SOD, which neutralizes the superoxide anion. Selenium is a component of GSHPx, which plays an important role in the catabolism of hydrogen peroxide

and lipid peroxides. Catalase contains iron and removes hydrogen peroxide from the cell.

Extracellular defenses

Extracellular free radicals must be inactivated by circulating antioxidants, such as vitamin E, vitamin C and β-carotene[5]. In plasma, vitamin C forms the first line of defense–this antioxidant is water-soluble and can neutralize free radicals as well as singlet oxygen. (Another reactive metabolite of oxygen but not a free radical, singlet oxygen contains an electron excited to a higher orbit by energy capture. This oxygen metabolite may cause tissue damage.) In membranes and lipoproteins, antioxidant protection is provided primarily by the fat-soluble vitamin E, especially the α-tocopherol vitamer, and β-carotene. Tocopherols and carotenoids are transported in the blood in plasma lipoproteins, predominantly LDL and high-density lipoprotein (HDL).

α-Tocopherol is one of the most efficient chain-breaking antioxidants available. Because it can compete for peroxyl radicals much faster than can polyunsaturated fatty acids, a small amount of α-tocopherol can protect a large amount of polyunsaturated fat. In biological membranes, approximately one α-tocopherol molecule is found per 1000 lipid molecules[6]. α-Tocopherol acts by quenching oxygen- or carbon-centred fatty acid radicals of membrane phospholipids, and may also inhibit peroxidation by quenching reactive oxygen species. The tocopheroxyl radical that is formed by this process is relatively unreactive, and tocopherol may be regenerated from this product, for example, by vitamin C[6,7].

β-Carotene is the most efficient naturally produced quencher of singlet oxygen and can also function as an antioxidant[5]. β-Carotene may act as a chain-breaking antioxidant at low levels of oxygen partial pressure, as found in many peripheral tissues[8,9]. As a chain-breaking antioxidant at high oxygen pressures, however, β-carotene is less efficient than vitamin E.

Oxidative modification of LDL

The fatty streak, the earliest lesion of atherosclerosis, contains a large number of foam cells, primarily macrophages, that are filled with cholesterol derived from plasma lipoproteins. Although a high level of low-density lipoprotein (LDL) in plasma is recognized as a risk factor for atherosclerosis, a number of observations indicate that native LDL, per se, does not induce the initial stages of atherosclerosis.

First, native LDL is taken up by the classic LDL receptor, which plays a significant role in cholesterol homeostasis. In the presence of high intracellular concentrations of cholesterol, however, the activity of this type of receptor is curbed–that is, uptake of native LDL is decreased[10]. Second, lesions rich in foam cells develop even in patients who are deficient in functional LDL receptors[11,12]. Finally, the incubation of macrophages with even high concentrations of native LDL does not result in the formation of foam cells[11-13].

The scavenger receptor

All these observations suggested that cholesterol must be taken up via an alternative receptor pathway. Goldstein and coworkers[14] discovered the receptor on monocytes and macrophages. This new receptor, called the "scavenger receptor", does not recognize native LDL, but does recognize specific chemically or biologically modified forms of LDL.

All three major cell types in the artery wall – endothelial cells, smooth muscle cells, and macrophages – can effect the peroxidation of polyunsaturated fatty acids in LDL by releasing oxygen free radicals[15-17]. Thus modified the LDL is recognized by scavenger receptors on macrophages and rapidly taken up (3 to 10 times faster than is native LDL), forming foam cells (Figure 2). This is the main hypothesis explaining the role of free radicals in the initial stages of atherosclerosis.

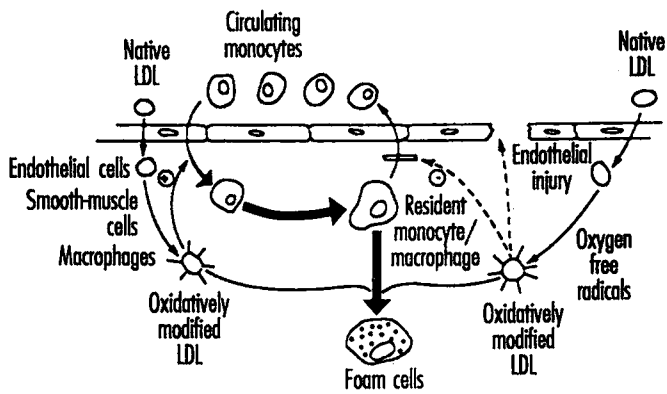

Figure 2. Proposed mechanisms by which the oxidation of low-density lipoprotein (LDL) may contribute to atherogenesis. Adapted, with permission, from Steinberg et al.[12]

Other atherogenic effects of oxidized LDL

Other characteristics of oxidized LDL also may contribute to atherosclerosis: First, this modified LDL is a chemoattractant that effects the migration of circulating monocytes into the intima. When, within the arterial wall, blood monocytes have differentiated into tissue macrophages, oxidized LDL also inhibits the mobility of these macrophages, which are then retained in the arterial wall. Furthermore, oxidized LDL is cytotoxic and may damage endothelial cells. This last property may account, at least in part, for the progression of relatively benign fatty streaks to more complex lesions composed of fibrous plaque[1,12]. In support of this hypothesis, oxidized LDL has actually been found in atherosclerotic lesions in humans[18,19].

Free radicals also appear to be involved in thrombogenesis. Lipid peroxides may stimulate the production of eicosanoids, which are potent vasoactive and platelet-reactive substances that play a role in thrombus formation[1,20]. At the same time, lipid peroxidation inhibits prostacyclin production, which may result in vasoconstriction and, in turn, hypertension and increased platelet aggregation. Moreover, as was mentioned above, free radicals and lipid peroxides may have a direct cytotoxic effect on the arterial endothelial cells.

All these different mechanisms are closely interrelated and appear to play some role in the development and progression of atherosclerotic lesions. However, the oxidized LDL hypothesis seems to be the most promising.

Do antioxidants prevent the oxidation of LDL?

If a causal relationship exists between oxidized LDL and atherosclerosis, then it would follow that antioxidants protect against atherosclerosis by preventing or retarding the oxidative modification of LDL. Because the lipid-soluble antioxidant vitamins are transported in plasma via LDL particles, LDL is somewhat immune to oxidation in plasma. However, in the process of protecting LDL against peroxidation, α-tocopherol, β-carotene, and other antioxidants eventually are consumed. The antioxidant levels present in LDL protect the lipoprotein for a relatively short time[12,21].

Studies have shown that the resistance of LDL to oxidation, indeed, is related to antioxidant levels[21-24]. In these experiments, LDL was oxidized in vitro. Only after investigators depleted levels of α-tocopherol and γ-tocopherol, β-carotene and other antioxidants contained in the LDL particle did they see the beginnings of LDL degradation, lipid hydroperoxide formation, and rapid uptake

of modified LDL by macrophages[25] (Figure 3). In two ex vivo studies, researchers increased the concentration of tocopherol in LDL by administering supplements to the volunteers from whom blood was taken. With the rise in tocopherol levels, a significantly increased resistance of the LDL to oxidation was demonstrated[22,26].

Most studies on antioxidant protection have focused on α-tocopherol, the most potent antioxidant compound. These investigations have revealed interindividual differences in the rates of lipid peroxidation that could not be entirely explained by variations in LDL-tocopherol concentrations[23]. Two explanations for this phenomenon are most likely: First, in addition to α-tocopherol, other antioxidants present in LDL, including γ-tocopherol, β-carotene, lycopene, and phytofluene (a recently discovered carotenoid), may have important protective effects. Second, different fatty acids vary in their sensitivity to oxidative metabolism. Polyunsaturated fatty acids, for example, are most vulnerable. Thus, variations in peroxidizable lipids in LDL also may determine the lipoprotein's susceptibility to oxidation. Indeed, Esterbauer[23] found that the fatty acid composition of LDL varied greatly from person to person.

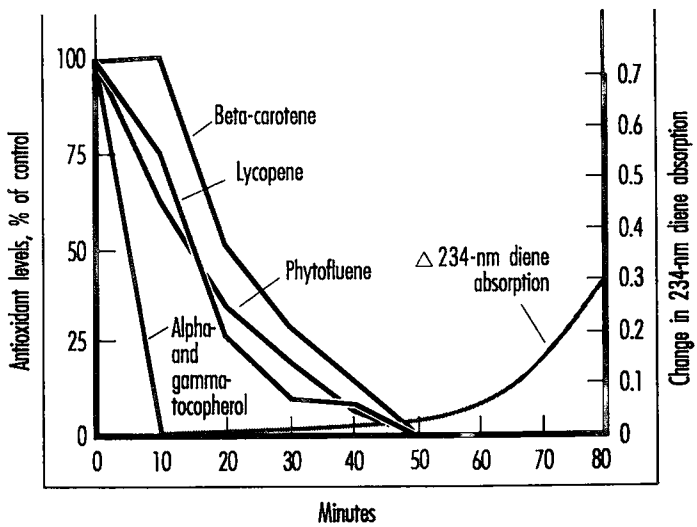

Figure 3. Relationship between consumption of antioxidants in LDL and the onset of lipid peroxidation. Adapted, with permission, from Esterbauer et al.[25]

Last, recent publications[23,27] indicate that ascorbate (a salt of vitamin C) strongly inhibits LDL oxidation in vitro, while simultaneously preserving levels of α-tocopherol, γ-tocopherol, and β-carotene in the LDL.

Epidemiologic evidence

Epidemiologic studies focusing on the possible protective effects of antioxidants not only must explore the association between absolute levels of single antioxidants and cardiovascular disease but also must take into account interrelationships among different antioxidants and the effects of known risk factors on antioxidant levels. For example, cigarette smokers have lower plasma vitamin C and carotene concentrations than do non-smokers[28]. Epidemiologic research into these associations has only just begun, and the results of long-term studies are still scant.

The concentration of antioxidant micronutrients in human tissues depends primarily on dietary intake. Antioxidant nutrients for which intake has been studied in relation to CV disease include vitamins E and C, β-carotene, and selenium. Table 1 lists important dietary sources of these nutrients and their recommended dietary allowances[29].

Table 1. Dietary sources of vitamins E and C, β-carotene, and selenium and the recommended dietary allowance (RDA) of each nutrient.

		RDA	
Nutrient	Dietary sources	Men	Women
Vitamin E	Vegetable fats and oils, margarine, nuts, fish, meat, eggs	70-80 α-TE†	60-65 α-TE†
Vitamin C	Fresh fruits and vegetables, potatoes	60 mg	60 mg
β-carotene	Green-yellow and leafy vegetables	1,000 RE‡	800 RE‡
Selenium	Cereals, meat, fish	70 μg	55 μg

† 1 mg RRR-α-tocopherol = 1 α-TE (tocopherol equivalent).

‡ RDA for total vitamin A intake; 1 RE (retinol equivalent) = 1 μg retinol or 6 μg β-carotene.

Based on data from National Research Council[29].

Intake of foods can be assessed by interview or questionnaire, which may introduce a recall bias. The use of food composition tables may also introduce

errors, because there are large variations in the nutrient content of some foods. Furthermore, dietary intake of nutrients does not provide an accurate indicator of bioavailability, as variations in absorption and metabolism are not taken into account. Therefore, to study the association between micronutrients and CV disease, investigators primarily have used biochemical indicators.

Biochemical indicators

The biochemical indicators of nutrient status used in epidemiologic studies often are plasma or serum concentrations, which reflect ingestion during the past few days to weeks. In theory, the concentration of antioxidant vitamins in plasma may only reflect the consumption of specific foods, other constituents of which may be responsible for a protective or adverse effect on the risk of CV disease.

Plasma or serum concentrations of antioxidants may be adequate indicators of exposure, if such levels are fairly constant and dietary intake has been stable over a longer period. However, if dietary intake has been influenced recently as a consequence of disease, it is more appropriate to use a biochemical indicator reflecting intake before that change. For lipid-soluble antioxidants such as vitamin E, the concentration in subcutaneous adipose tissue is recognized as a valuable long-term indicator, whereas levels of a mineral such as selenium can be measured reliably in toenails. For other antioxidants, such as vitamin C, a long-term indicator is not available[30].

Vitamin E

Of all the antioxidants, vitamin E has been studied most extensively in relation to CV disease. In the Multinational Monitoring Project of Trends and Determinants of Cardiovascular Disease, or the World Health Organization (WHO)/MONICA study, plasma levels of antioxidants were compared among different groups of apparently healthy men from regions with a six-fold difference in age-standardized ischemic heart disease (IHD) mortality; each group consisted of about 100 men between the ages of 40 and 49. Gey and coworkers[31] reported results for 16 different populations. For most of the vitamins studied, these investigators found an inverse correlation between plasma antioxidant levels and IHD mortality; this relationship was strongest for vitamin E, independent of plasma lipid levels (Figure 4). These data should be interpreted cautiously, however. Since antioxidant exposure and IHD incidence were assessed at the same point in time, no conclusions can be drawn about the time course of this association and no adjustment can be made for possible confounding factors.

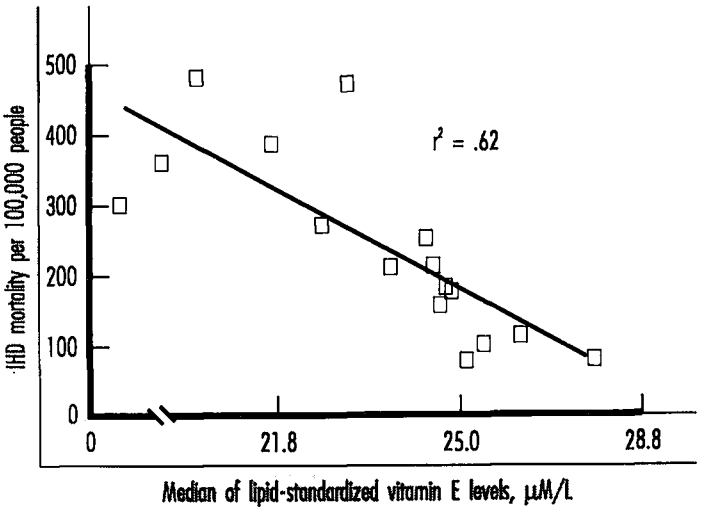

Figure 4. Relationship between plasma vitamin E levels and mortality due to ischemic heart disease (IHD), as determined in the Optional Vitamin Study, World Health Organization (WHO)/MONICA project. Adapted, with permission, from Gey et al.[31]

In a more recent study, Riemersma and colleagues[32] found an inverse relationship, which was independent of smoking behaviour, between plasma levels of vitamin E and angina pectoris. Their results indicated that individuals whose plasma vitamin E levels were in the lowest quintile had almost a threefold greater risk of angina than did those whose plasma vitamin E measurements were in the highest quintile. As in the MONICA study, vitamin intake and disease in the study by Riemersma et al. were measured at the same time. Another limitation of the latter investigation was the method of recruitment of cases, which was based on self-reported symptoms. Again, no inferences about a possible cause-and-effect relationship between antioxidant intake and angina pectoris can therefore be made from these data.

The effect of vitamin E intake on CV disease also has been examined in prospective cohort studies, in which antioxidant exposure was measured at a given point in time before the diagnosis of disease. In the three prospective studies reported to date[33-35], the mean baseline plasma vitamin E levels among patients who died from IHD during follow-up were not significantly lower than levels among controls.

In a prospective study from the Netherlands, Kok et al.[34] compared serum vitamin E levels in 84 individuals who died of CV disease during a 9-year follow-up period with levels in 168 matched controls; in both groups, the serum

samples were obtained at the baseline evaluation. Although the researchers observed no significant associations between serum vitamin E levels and CV mortality, the results may have been biased by loss of vitamin E during prolonged storage of the serum.

In a similar Finnish study that entailed a 5-year follow-up period[33], lengthy storage of serum also may have influenced the results. Furthermore, in this study, vitamin E levels were not adjusted for plasma cholesterol; such an adjustment may be appropriate in view of the strong association between these two measurements. In both the Dutch and Finnish studies, vitamin E levels were within normal ranges. Therefore, the possibility that marginal levels of vitamin E may influence CV risk cannot be excluded.

Several small-scale intervention studies have failed to demonstrate an effect of vitamin E on symptoms of angina pectoris[36]. Long-term intervention studies specifically designed to evaluate the effects of lipid-soluble antioxidants on CV disease have not yet been reported.

Vitamin C

In the United Kingdom, IHD mortality is high in areas where residents have relatively low vitamin C consumption[37]. The previously mentioned MONICA study also revealed a moderately strong inverse correlation between plasma vitamin C and IHD mortality[31]. In a prospective study from Basel[35], IHD mortality was 60% higher in individuals who had low plasma vitamin C levels than in those who had higher vitamin C levels; however, due to the small number of cases, this mortality difference did not reach statistical significance. No long-term studies of vitamin C as an intervention for CV disease have been reported.

β-Carotene

Few epidemiologic studies have addressed the possible protective role of β-carotene against CV disease. Nevertheless, the consumption of fresh fruits and vegetables containing β-carotene has been shown to be inversely correlated with mortality due to coronary disease (CAD) and with the risk of acute myocardial infarction (MI)[38,39]. The results of cross-sectional or prospective studies evaluating the association between plasma levels of β-carotene (or any other biological indicator) and CAD have yet not been published[40]. Some early data suggest, however, that β-carotene supplementation may reduce morbidity and mortality due to CV disease. A preliminary analysis of data from the US Physicians' Health Study showed a 44% reduction in major coronary events among physicians with angina pectoris who took 50 mg of β-carotene on

alternate days[41]. This analysis was carried out in a relatively small subgroup, and, hence, the results do not suffice as evidence for a protective effect of β-carotene.

Selenium

Selenium, a cofactor of glutathione peroxidase, has been widely investigated for its effect on CV disease. After several cross-sectional studies were performed in the early 1970s, the first case-control study on serum selenium concentration and risk of cardiovascular disease was published in 1982[42]. This study revealed that the risk of CAD mortality in individuals with low serum selenium concentrations (≤ 45 μg/l) was more than twice as high as in those with higher serum selenium levels (> 45 μg/l).

In the decade that followed, several other studies focusing on the association between selenium and CAD were published[43-48]. As Table 2 shows, the findings from different studies are conflicting. In fact, the number of studies showing a statistically significant inverse relationship between serum selenium concentrations and CV disease is as large as the number showing no association.

Table 2. Epidemiologic evidence for a relationship between serum or plasma selenium concentration and cardiovascular disease.

Reference	Patients	Controls	Relative risk (95% CI)
Salonen et al.[42]	51.8 ± 14.2	55.3 ± 14.2	2.2 (1.2-4.0)
Miettinen et al.[43]	71.6 ± 13.8	72.9 ± 14.4†	–
Salonen et al.[33]	62	68	1.3 (0.3-7.0)
Virtamo et al.[44]	–	–‡	1.6 (1.1-2.3)
Kok et al.[34]	125.1 ± 3.1	126.5 ± 2.2	1.6 (0.8-3.2)
Ringstad et al.[45]	1.57 ± 0.21	1.61 ± 0.27¶	1.0 (0.4-2.3)
Kok et al.[46]	100.8 ± 3.0	106.8 ± 2.6†	–
Beaglehole et al.[47]	82.8 ± 2.9	87.9 ± 1.8	1.6 (1.1-2.2)
Kok et al.[48]	95.1 ± 2.0	108.8 ± 29.3§	–

† $p > 0.05$.

‡ Cohort analysis, not a case-control analysis; selenium levels for total cohort were analyzed and relative risk calculated for individuals with selenium levels ≤ 45 μg/l vs > 45 μg/l.

¶ in μmol/l.

§ $p \leq 0.05$.

Experimental observations suggest that selenium intake must be below a threshold level before a relationship between selenium status and CV risk becomes evident. Selenium supplementation increases the activity of glutathione peroxidase in platelets of selenium-deficient humans, and this increase levels off when a plasma selenium concentration of about 100 to 135 μg/l is achieved[49]. The earliest investigation of selenium and CV disease in Finland was conducted when the average dietary intake of selenium was still very low[42]. In later years, the soil in Finland was seeded with selenium, and intake has, therefore, increased.

It may be argued that, in case-control studies, low serum selenium concentrations are difficult to interpret, because the serum measurement may be affected by the disease and, on the other hand, a single serum measurement may not reflect the selenium status over years. As mentioned above, an alternative to calculating selenium levels in plasma is to measure levels of the antioxidant in toenails, which reflects long-term intake. Kok and colleagues found significantly lower toenail selenium levels in patients who had suffered an MI than in healthy population controls[46]. This finding indicates that low selenium levels were present before the infarction, which may be of etiologic relevance.

Directions for future research

Animal and in vitro experiments, for the most part, have investigated the influence of oxidative stress on the LDL particle and the preventive effect of antioxidant compounds. In contrast, epidemiologic studies have focused on the relationship between plasma antioxidant levels and CV disease, the eventual endpoints of oxidative stress. The populations in which most of these epidemiologic studies have been performed have had a high intake of vitamins and minerals, but positive results have been found in some populations with a relatively low intake of antioxidants. Still, the sum of these studies has failed to produce conclusive evidence of a protective effect of any single antioxidant nutrient.

Considering the complex relationships among the various antioxidant nutrients, a more sophisticated research approach might be more successful. Future studies should be aimed at identifying subgroups that may be at increased risk of CV disease due to factors such as low vitamin intake or to high exogenous oxidative stress, such as might be caused by cigarette smoking. Experimental studies have shown a synergistic effect of certain antioxidants; this possible synergy should be studied in epidemiologic studies as well.

In addition to antioxidant status, epidemiologic studies should assess intake of polyunsaturated fatty acids (PUFAs). For example, Kok et al.[48] recently evaluated the combined association of blood levels of α-tocopherol, PUFAs, and selenium with coronary atherosclerosis. These investigators found a significantly lower ratio of plasma selenium to plasma PUFAs in patients with atherosclerosis who had relatively low α-tocopherol levels but not in those who had high α-tocopherol levels. This potential interrelationship among plasma PUFAs, selenium, and α-tocopherol needs to be studied further.

Moreover, recent findings suggest that increased consumption of monounsaturated fatty acids (MUFAs) may decrease the susceptibility of LDL to oxidation and uptake by macrophages[50]. MUFAs seem to have a cholesterol-lowering effect equivalent to that of PUFAs but do not decrease HDL levels[51,52]. In view of the susceptibility of PUFAs to peroxidation, a diet that is rich in MUFAs may afford greater protection than a diet high in PUFAs.

Epidemiological studies need not be limited to addressing the direct association between antioxidant status and risk of CV morbidity and mortality. Observational studies in humans could serve to elucidate earlier steps in the LDL oxidation hypothesis. For example, in vivo demonstrations of this inhibition of LDL oxidation by antioxidants (which, up to now, has been studied primarily in vitro) and of the subsequent retardation or regression of atherosclerosis would provide strong confirmation of this hypothesis.

Measurement of the resistance of LDL to oxidation and levels of lipid peroxides may prove to be valuable parameters in future epidemiologic studies. Mobarhan et al.[53] showed that the level of circulating lipid peroxides in healthy subjects can be reduced by β-carotene supplementation. Stringer[54] reported a significantly higher plasma lipid peroxide concentration in patients hospitalized for occlusive arterial disease than in control patients hospitalized for other conditions. In relation to CV disease, however, the significance of lipid peroxide concentrations in plasma is not clear. High lipid peroxide levels may be an indicator of the disease, but they may be a result rather than a cause. Furthermore, the relevant process of LDL oxidation most likely takes place in the subendothelial space in the arterial wall, rather than in plasma. The oxidative stress in the subendothelium may be quite different from that in plasma. Therefore, the resistance of the LDL particle to oxidative stress may be more relevant to CV disease than are levels of peroxidation products in plasma. However, other mechanisms, such as thrombogenesis may also play a pathological role in which plasma lipid peroxides could be involved. Clearly, additional research is needed to clarify these issues.

Conclusions

A large amount of experimental evidence shows that the oxidation of LDL in the intima of the arterial wall may play an important role in the initial phase of atherosclerosis. In vitro studies indicate that oxidation can be inhibited by various antioxidant agents, of which vitamin E seems to be the most effective. Whether dietary antioxidant intake actually influences the course of atherosclerosis and the incidence of CV events has not been proven in epidemiologic studies, however.

The inconsistency between the results of in vitro studies and the findings of epidemiologic investigations may be attributed, at least in part, to methodological weaknesses in the study designs. Relatively small studies in homogeneous populations suffer from too little variation in the intake of relevant nutrients. Misclassification of exposure or disease status also may be a source of bias. Therefore, it is essential to use an adequate method of diagnosis and a relevant indicator of nutrient intake.

Of course, researchers must also control for known CV risk factors such as smoking, high plasma cholesterol levels, and hypertension. And finally, since the body's antioxidant defense is a complex system in which the function of different antioxidants is closely interwoven, the combined effect of nutrient antioxidants should be studied.

Fortunately, analytical tools for the measurement of nutrient biologic markers in epidemiologic studies are now available. The use of these biologic markers in methodologically sound studies will permit the comprehensive evaluation of the antioxidant hypothesis in human populations.

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EURAMIC Study: Antioxidants, myocardial infarction and breast cancer. Design and main hypotheses

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Abstract

Epidemiological studies have not given sufficient evidence yet for the role of antioxidant nutrients in the prevention of cardiovascular disease. As regards cancer, an inverse association between β-carotene intake and specific types of cancer, especially lung cancer, has been shown. For other cancer sites and other antioxidants, the association is less clear. The EURAMIC Study, an EC Concerted Action, is a case-control study conducted in 10 countries, in which the combined effect of vitamin E, β-carotene and selenium, in relation to fatty acid intake, will be examined. The disease endpoints are acute myocardial infarction and early stage breast cancer. The broad range of antioxidant intake, the use of biomarkers of exposure, and the analysis of pooled data will allow an estimate of the strength of the putative beneficial effect. In this paper the background and design of the study will be introduced.

Introduction

In recent years the deleterious effects of free radicals of exogenous and endogenous origin in the human body have been implicated in the development of age-related disorders, such as cardiovascular and malignant disease[1,2]. Free radicals initiate chain reactions that may eventually lead to adverse effects including the formation of toxic lipid peroxides and genomic

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alterations. To prevent free-radical damage, the human body possesses a number of free-radical-scavenging or antioxidant defense systems, including the vitamins E and C, β-carotene, and enzymes such as selenium-dependent glutathione peroxidase[3,4]. The potential preventive role of antioxidants in the development of cardiovascular disease (CVD) and malignant diseases has been the subject of a number of epidemiological studies. Cross-cultural comparisons have shown correlations between lower serum vitamin E levels and higher age-standardized CVD mortality[5]. Three prospective studies[6-8] showed no correlation between plasma vitamin E levels and CVD mortality, whereas in a recent study Riemersma et al.[9] found an inverse relationship between plasma vitamin E levels and angina pectoris. Few studies have addressed the role of β-carotene status in CVD prevention[10]. Consumption of fresh fruits and vegetables has been shown to be inversely correlated with mortality from coronary heart disease and acute myocardial infarction[11,12]. The inverse relation found between plasma carotene levels and angina pectoris[9] disappeared when the confounding influence of cigarette smoking was taken into account. Preliminary findings from the Physicians' Health Study, a large intervention trial with β-carotene, suggest that patients with a history of stable angina pectoris who received β-carotene had a significantly reduced risk of further cardiovascular events[13]. Regarding selenium, observational data on its possible protective effect on CVD are also conflicting. An early case-control study in Finland showed a significant higher risk for CVD mortality in persons with low serum selenium levels[14]. In some later studies these results were repeated[15,16], while in others no association was found[6,8,16].

Regarding cancer, the antioxidant nutrient that has been studied most is β-carotene. Since Peto et al.[18] hypothesized that carotenoids may reduce cancer rates, a large number of epidemiological studies has addressed this topic. The association between intake of fruits and vegetables rich in carotenoids, or plasma carotene levels, and a decreased risk of cancer appears to be most consistent for lung cancer and stomach cancer[7,19-22] and less consistent for cancers of the breast and prostate[23-28]. Serum or plasma vitamin E levels in relation to cancer have been studied in several prospective investigations[23,27-31]. Wald et al.[23] observed a significant inverse association between plasma vitamin E levels and subsequent risk of breast cancer. Comstock et al.[28] reported a protective association of pre-diagnostic serum vitamin E levels with lung cancer, but with none of the other sites studied. Early epidemiological studies[32,33] suggested that dietary selenium compounds might protect against cancer at several sites. In general, studies on selenium

and total cancer risk have shown slightly lower selenium levels in cases than in controls, suggesting an inverse association[8,36-38].

A lack of association in some studies may be attributed, at least in part, to methodological weaknesses. Most studies have focused on the effect of single antioxidants. However, since the body's antioxidant defense is a complex system in which the function of different antioxidants may be closely intertwined, it is the combined effect of nutrient antioxidants that is of interest. It is difficult to obtain accurate information of dietary vitamin intake in epidemiological studies. Intake of foods can be assessed by interview or questionnaire, but may be recalled differentially between cases and controls. The use of food composition tables may also introduce errors, because of large variations in nutrient content of some foods. Furthermore, when measuring intake, individual variation in absorption and metabolism is not taken into account. Therefore, relevant biochemical indicators of intake are often preferred. These indicators are often plasma or serum concentrations, which mostly reflect intake during the past few days or weeks. If levels are fairly constant and intake has been stable over a longer period of time, these plasma/serum concentrations may be adequate indicators of exposure. However, if dietary intake has been influenced recently as a consequence of disease, it is more appropriate to use a biochemical indicator reflecting intake before that change. In summary, for CVD the associations with antioxidant nutrients found in several, but certainly not all studies, warrant further observational studies to be followed by human intervention studies. Results from cancer studies indicate that β-carotene may affect carcinogenesis, but not necessarily at all cancer sites. The study described in this paper was designed to address the association between vitamin E, β-carotene and selenium status prior to disease occurrence and well-defined endpoints of cardiovascular and malignant disease. The field work of this multi-centre study was conducted (during the 1990-1992 period) in nine European countries and in Israel, and is known as the EURopean Study on Antioxidants, Myocardial Infarction and Cancer of the Breast (EURAMIC).

Research questions

The specific hypothesis that is to be tested in the EURAMIC study relates to levels of the fat-soluble antioxidant nutrients and selenium in tissues with a low turn-over, as biomarkers of the intake of these nutrients, integrated over a longer period of time. The hypothesis, formulated in such a way that it

applies to both disease endpoints, is as follows: The concentration of α-tocopherol and β-carotene in adipose tissue, and of selenium in toenails, is lower in cases as compared to healthy population controls. As polyunsaturated fatty acids are an important substrate for free radical peroxidation, and the intake of linoleic acid is positively associated with the intake of vitamin E, the vitamin status relative to fatty acid composition will also be compared. Moreover, the study design allows to evaluate the shape of the antioxidant-disease relationship, which is postulated to be non-linear, i.e. the increased risk may only be observed at the lowest antioxidant concentrations. Lastly, the synergistic effect of the different antioxidants, in particular α-tocopherol and selenium, on disease will be analysed. The putative protective effect of β-carotene may be particularly important at low α-tocopherol/selenium status or in smokers experiencing high oxidant stress.

Study design

The study design selected is a set of equally sized population-based case-control studies, conducted in nine European countries and Israel, thus including subjects with a large variation in antioxidant intake and background disease risk. The disease endpoints of first acute myocardial infarction in men and early stage-breast cancer in women have been chosen. These early stage, first occurrences protect against artefactual results due to secondary changes in life-style and exposure status. Moreover, both diseases are major contributors to morbidity and mortality in industrialized countries. The concentration of fat-soluble vitamins in adipose tissue and selenium in toenails is used as an indication of long-term dietary intake of these antioxidants. A common protocol, including identical case definition, standardized sampling and handling procedures and centralized laboratory analyses, is being used.

Subjects

Each of the participating study centres agreed to enrol 100 cases and an equal number of controls for the myocardial infarction and/or the breast cancer part of EURAMIC. Cases for the cardiovascular endpoint are defined as male subjects aged under 70 years, with diagnosis of first acute myocardial infarction (ICD-code 410), confirmed by specific abnormalities on the ECG, with elevated enzyme levels, who are admitted to hospital within 24 hours after beginning of symptoms. Breast cancer cases are defined as female

subjects aged 50-75 years, with first diagnosis of breast cancer (ICD-code 174), histologically classified as ductal carcinoma, with primary tumors less than 5 cm, axillary lymphe nodes stage ≤ N3, without any clinical indication of distant metastases at discharge. Cases are recruited from the Coronary Care Units and Surgical Units of participating hospitals. Informed consent is obtained in accordance with the ethical standards of the responsible committees on human experimentation. Controls are drawn to represent the exposure levels in the population giving rise to cases. The actual way in which controls are recruited differs between participating centres (Table 1). However, sufficient care is taken that differential exposure-related selection between cases and controls is being avoided. The sampling of controls is frequency-matched for age (5-year interval). The inclusion criteria applying to both cases and controls are listed in Table 2.

Table 1. Source populations for control recruitment.

Centre	study		population
	AMI	BC	
Berlin	AMI	BC	population register
Coleraine		BC	via general practitioners
Edinburgh	AMI		Health Board Register
Granada	AMI	BC	hospital controls
Helsinki	AMI		population register
Jerusalem	AMI		population register
Malaga	AMI	BC	hospital controls
Moscow	AMI		hospital controls
Sarpsborg	AMI		blooddonors
Zeist	AMI	BC	via general practitioner of case
Zürich	AMI	BC	population register

Biomarkers of exposure

Contrary to most studies so far, in which short or intermediate-term biomarkers (plasma concentration mainly) of antioxidant status have been determined, in this study biomarkers of long-term status were chosen. These biomarkers are believed to be an integrated measure of exposure over months rather than days. Tangney et al.[39] showed that large intraindividual day-to-day variations in diet and plasma vitamin E and β-carotene can constitute an important source of error in classifying subjects according to exposure.

Several independent measurements are required to distinguish even large differences between individuals. About 90% of vitamin E is contained in adipose tissue[40]. Schäfer & Overvad[41] showed that adipose tissue vitamin E is strongly associated with dietary intake (r = 0.76); these data support the use of adipose tissue vitamin E as a long-term measure of nutrition status.

Table 2. Inclusion criteria for cases and controls.

Acute Myocardial Infarction	Breast Cancer
men	women
< 70 year	50-74 year
	postmenopausal
no anamnesis of previous MI	no anamnesis of previous BC
Belonging to the indigenous population of the country and speaking the national language	
During the past year no changes in the use of dietary supplements containing either one or more of the studied antioxidants	
No new/altered dietary prescription by GP in the past year	
No weight loss over 5 kg in the past year	
No anamnesis with treatment for alcohol or drug abuse	
No major diagnosed psychiatric disorder	
No institutionalization	

For β-carotene the relation between diet and adipose tissue concentration has not yet been studied. However, it is known that adipose tissue and liver are the quantitatively most important sites of deposition of carotenoids, and the relative concentrations of the different carotenoids in adipose tissue are quite similar to those in plasma. It may be postulated that adipose tissue carotenoid levels reflect long-term intake, as has been shown for fatty acid composition[42]. Another reason not to use plasma levels is that the acute myocardial infarction event will influence antioxidant plasma levels[43], thereby precluding the feasibility of control comparison. Selenium exposure will be determined as the concentration in toenail clippings, which can time-integrate dietary exposure over intervals ranging from several weeks to 12 months[44]. The sampling procedures have been standardized. Toenail clippings from all

toes (giving an integrated measure of exposure to selenium), after cleaning and removing nail polish, are collected within eight weeks of diagnosis or surgery. This will be done either in hospital, or by the subjects themselves at home. The nails are stored in a small plastic bag at room temperature. Subcutaneous adipose tissue (20-50 mg) is taken from the buttock by needle aspiration as described elsewhere[45]. The adipose sample will be taken within four days after diagnosis for myocardial infarction, and within one week after surgery for breast cancer. Samples are immediately put on dry ice or in liquid nitrogen, and subsequently stored at −40°C or −80°C. To assist in acquiring the appropriate skills for sampling, and to assure standardized procedures, a videotape showing the technique was distributed to all participating centres.

Background variables

Information on socio-economic status, familial history, smoking habits, alcohol intake, and anthropometric measures is collected for all subjects. In addition, information on angina pectoris, diabetes, blood pressure, serum total and HDL cholesterol and triglycerides in both AMI patients and their controls is required. Time of diagnosis of diabetes and onset of angina are also taken into account, to control for information bias due to differential diagnosis for cases and controls. These data are assessed by questionnaire; cholesterol levels are determined in serum, prepared from a blood sample taken as soon as possible after hospital admission, but no later than 24 hours after start of symptoms. For the breast cancer study, age at menarche, age at pregnancies, parity, use of oral contraceptives, age at menopause and type of menopause are assessed. Finally, the fatty acid composition of adipose tissue will also be determined. The intake of vitamin E is positively associated with the intake of linoleic acid, while linoleic acid in adipose tissue is inversely related to the risk of MI[46]. Thus linoleic acid intake is a potential confounder of the association between vitamin E and the disease endpoints. Furthermore, Schäfer & Overvad[41] showed that the content of n-3 fatty acids in adipose tissue influences adipose tissue vitamin E negatively.

Laboratory analysis

Crucial for the quality of the data is central analysis for the determination of the antioxidant vitamins and fatty acid composition in adipose, for selenium in toenails and cholesterol in serum. Concentrations of β-carotene, lycopene and single tocopherols in adipose tissue will be determined by reverse-phase HPLC[47,48]. Quality control is assured by analysing reference samples in each run and evaluating the within and between run analytical variation. Equal

numbers of samples from cases and controls will be analysed in each run. Storage and transport conditions will be monitored by analysis of pooled adipose tissue samples with known vitamin content, stored with the actual samples in each participating centre. The concentration of the vitamins is expressed on the basis of fatty acids, which are assessed by GLC[49,50] in an aliquot of the same extract. Toenail selenium is determined by Neutron Activation Analysis at the Interfaculty Reactor Institute in Delft, Netherlands[51]. References for quality control will be included in the analysis. Enzymatic methods are used for the determination of serum cholesterol and triglyceride levels at the National Public Health Institute in Helsinki (Finland). HDL-cholesterol is assayed after precipitation of VLDL and LDL by dextran sulphate and MgCl2.

Data analysis

In a study population of 100 cases and an equal number of controls, the power (at $\alpha = 0.05$, two-sided) to detect a twofold increased relative odds when comparing the lowest and highest quartile of exposure will be about 40% only, while a threefold increased risk can be detected with a power of 73% (Table 3;[52]). Thus, while moderate associations can be detected by individual centres, pooling of data from all study centres provides sufficient power to detect weaker associations (Table 3) and an opportunity to take multiple variables into account. These power calculations are based on comparisons of subjects in the extreme quartiles of antioxidant status within a single population. These estimations of the power may be too conservative, if a trend over all quartiles may be demonstrated. On the other hand, the estimate may be too optimistic, since covariates and potential confounders and effect modifiers have not been taken into account.

Table 3. Power of a case-control study in relation to study size and strength of association.

		Power to detect		
# cases	# controls	OR = 1.5	OR = 2	OR = 3
100	100	17%	40%	73%
200	200	29%	68%	95%
600	600	69%	99%	~100%
1000	1000	89%	99%	~100%

Each participating centre will analyse its own dataset. A pooled analysis, stratifying not only by confounders and interaction terms, but also by centre, will be performed by the coordinating centre. Mean levels of antioxidants among cases and controls will be compared for both disease endpoints. Risk analyses will be performed by calculating the relative odds of the disease endpoints for different categories and combinations of the antioxidants, using stratified analysis to identify potential confounders and/or effect modifiers. A model of relative risk will be constructed taking into account factors shown to confound the potential relationship between antioxidant status and disease outcome in the study population, using multiple logistic regression analysis. Heterogeneity of associations between study centres will be assessed and related to the differential prevalence of other disease risk factors between countries.

Discussion

Much of the recent experimental interest in antioxidants and cardiovascular disease has been focused on their role in the prevention of LDL oxidation and subsequent formation of foam cells and fatty streaks, early stages of atherosclerosis[53]. However, there is evidence that free radicals could also induce thrombogenesis, and that antioxidant vitamins may be beneficial[54-56]. Lipid peroxides may stimulate the production of thromboxane, which has a potent vasoactive and platelet aggregation stimulatory activity[2,57]. On the other hand, prostacyclin production is inhibited[58], which may result in unopposed activity of thromboxane and thus increased vasoconstriction, platelet aggregation and thrombus formation. These mechanisms may be important in later stages of atherosclerosis and in ischaemic heart disease. Antioxidant vitamins thus may act at the early as well as the late stages of CVD. The endpoint chosen in this study, acute myocardial infarction, allows a clear definition of cases and controls and minimizes the effect of lifestyle changes due to a longer history of cardiac complaints. It should be clear that this design does not enable to distinguish the effects on atherogenesis and thrombogenesis.

Breast cancer, the endpoint chosen for malignant disease, is a diet-related type of cancer; attention has been focused on the role of dietary fat. Since antioxidant nutrients, especially β-carotene, have been shown to affect cancers at other sites (e.g. lung cancer), it is of particular interest to study these nutrients in relation to breast cancer, the major cause of death in

women in industrialized countries. It should be realised, however, that only effects in the later stages of the malignancy may be detected. Because only recently diagnosed cases will be included, secondary changes in life-style and exposure status will be avoided as much as possible.

A multi-centre study, carried out in different countries, has unique advantages. We can expect a larger range of exposure to antioxidant vitamins and selenium, because of major differences in food consumption. By pooling the data from participating centres, smaller samples per centre still allow to detect weak associations with sufficient statistical power. This increases the cost-effectiveness of the local studies. Simultaneous data collection in many centres also makes it possible to recruit the required number of cases and controls in a relatively short period. The problem of reliable assessment of nutrient intake has been avoided by the use of biomarkers, which also takes into account the biological variability in absorption and metabolism. Systematic variation in data collection and biomarker analysis between centres could severely limit the results of a multi-centre study. Therefore, considerable effort was directed to the standardisation of clinical and analytical procedures.

Prospective studies, in which the individual exposure to antioxidant nutrients can be related to disease incidence, will provide valuable information in due time[59-61]. However, since relatively large populations are needed and the induction time of the diseases of interest is relatively long, these studies take years to complete and are very costly. The EURAMIC Study will provide data on the combined effect of tocopherols, carotenoids and selenium on manifestations of cardiovascular disease and cancer, within a relatively short period of time. It may present valuable information for the design of future intervention studies and support evaluations of dietary guidelines.

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Antioxidants in adipose tissue and risk of myocardial infarction: the EURAMIC Study

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Abstract

Laboratory and epidemiological studies suggest that the antioxidants, vitamin E and β-carotene, protect against coronary heart disease. In a European multi-centre case-control study α-tocopherol and β-carotene concentrations were measured in adipose tissue samples collected in 1991-1992 from 683 people with acute myocardial infarction and 727 controls.

Mean adipose tissue β-carotene concentration was 0.35 μg/g in cases and 0.42 μg/g in controls, with age-adjusted and centre-adjusted mean difference 0.07 μg/g (95% confidence interval [CI] 0.04-0.10). Mean α-tocopherol concentrations were 193 μg/g and 192 μg/g for cases and controls, respectively. The age- and centre-adjusted odds ratio for risk of myocardial infarction in the lowest quintile of β-carotene as compared with the highest was 2.62 (95% CI 1.79-3.83). Additional control for body mass index and smoking reduced the odds ratio to 1.78 (95% CI 1.17-2.71); other established risk factors did not substantially alter this ratio. The increased risk was mainly confined to current smokers: the multivariate odds ratio in the lowest β-carotene quintile in smokers was 2.39 (95% CI 1.35-4.25), whereas it was 1.07 for people who had never smoked. A low α-tocopherol concentration was not associated with risk of myocardial infarction.

Our results support the hypothesis that high β-carotene concentrations within the normal range reduce the risk of a first myocardial infarction. The findings for α-tocopherol are compatible with previous observations of reduced risk among vitamin E supplement users only. The consumption of β-carotene-rich

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foods such as carrots and green-leaf vegetables may reduce the risk of myocardial infarction.

Introduction

Oxidation of low-density lipoprotein (LDL) particles may play a part in the formation of foam cells, atherosclerotic lesions, and coronary heart disease (CHD)[1,2]. Evidence first came from pharmacological studies in animals, in which large amounts of antioxidants such as probucol, butylated hydroxytoluene[3], or α-tocopherol inhibited lesion formation or caused the regression of atherosclerotic plaques[4].

The importance of antioxidant vitamins is the possible protection they provide against CHD. The most important antioxidant vitamins are α-tocopherol (the most active and most abundant form of vitamin E) and β-carotene (pro-vitamin A). Large prospective diet studies have recently shown decreased risk of CHD at high intakes of vitamin E in men[5] and in women[6]. In Scotland, plasma vitamin E was found to be lower in newly-diagnosed angina than in controls[7]. However, prospective studies of the association between low α-tocopherol and CHD do not support causation[8,9]. An inverse association of β-carotene intake with CHD risk among current and former smokers has been observed[5], and serum β-carotene concentrations were found prospectively to be inversely associated with myocardial infarction[10] and ischaemic heart disease mortality[11]. Moreover, preliminary findings in patients with stable angina suggests that β-carotene supplements may reduce the risk of cardiovascular complications[12].

We studied the relation between α-tocopherol, β-carotene, and first acute myocardial infarction (MI) in a multi-centre case-control study in men aged 35-70. In order to obtain a time-integrated measure, antioxidant vitamin concentrations were measured in adipose tissue.

Methods

Design and subjects

Ten centres collaborated (Table 1). The study design has been described elsewhere[13]. Eligible subjects were men < 70 years, native residents, speaking the predominant or official local language, and without previously-reported MI. They had to have stable dietary patterns in the past year: no changes in the use of dietary supplements containing α-tocopherol, β-carotene, or selenium; no new

or altered dietary prescription or advice from a physician, except changes to low-sodium or energy-restricted diets; and no weight loss > 5 kg in the past year.

Cases were subjects recruited from the coronary care unit of participating hospitals with a first acute MI (ICD-code 410) – confirmed by specific abnormalities on the electrocardiogram (ECG) and elevated enzyme levels – and admitted within 24 hours of symptoms.

Subjects without a history of MI were recruited as controls from the population in the catchment area, frequency-matched for age according to 5-year intervals. Whenever possible, random samples from local population registries were used (Finland, Israel, Germany, Scotland, Switzerland). In some centres (Russia, Spain) population registries could not be used because of incomplete coverage or legal restrictions, so hospital controls were selected, with diseases not known to be associated with antioxidant status (renal colic, non-infectious prostatism, acute appendicitis, non-infectious ear disease, hernia, volvulus, rectal or anal disease except cancer, hemorrhoids, or chronic infection). Where it was thought that low response rates from population based samples would spoil the internal validity, controls were selected from the catchment area via a random sample by the patient's general practitioner (Netherlands) or by inviting friends and relatives of the case (Norway). In the Netherlands, Spain, and Russia, methods of subject recruitment were combined. Table 1 shows response rates of patients and controls between centres. Informed consent was obtained in accordance with the ethical standards of the responsible committees.

Subjects on antioxidant supplements or prescribed diets for more than one year were included. Subjects with a history of treatment for alcohol or drug abuse, psychiatric disorders that would interfere with their ability to give informed consent, and institutionalized subjects were excluded.

Information on smoking habits, blood pressure, angina pectoris, and diabetes was collected for all subjects by standard questionnaires[14]. Socio-economic status, family history, and alcohol intake were assessed through locally-developed questionnaires.

Adipose tissue and blood sampling

Subcutaneous adipose tissue was taken from the buttock by needle aspiration[15]. To standardise sampling, a videotape showing the technique was distributed to all centres. In cases, the adipose sample was taken within seven days of hospital admission. Samples were kept in the plastic adaptor, immediately placed on dry ice or in liquid nitrogen, and stored at –70°C. On average 29 mg (SD 16) material was obtained containing 19 mg (11) fatty acids.

Table 1. Population response rate and method of control recruitment.

Centre	Cases (response %)	Controls (response %)	Method of control recruitment†
Finland (Helsinki)	62 (97)	61 (51)	PR
Germany (Berlin)	77 (82)	97 (73)	PR
Israel (Jerusalem)	59 (60)	60 (53)	PR
Netherlands (Zeist)	72 (75)	63 (50)	GP, PR
Norway (Sarpsborg)	101 (96)	102 (98)	FR
Russia (Moscow)	100 (97)	100 (79)	GP, H
Scotland (Edinburgh)	58 (98)	43 (61)	PR
Spain (Granada)	57 (45)	54 (67)	H
Spain (Malaga)	100 (89)	102 (77)	H, GP
Switzerland (Zürich)	57 (93)	74 (26)	PR

† PR = population register; GP = general practitioners; H = hospital controls; FR = friends, relatives.

Vitamin concentrations were not associated with the time between infarction and biopsy (Pearson $r < 0.05$) or with storage time before analysis ($r = 0.02$ for β-carotene, $r = -0.04$ for α-tocopherol).

A non-fasting blood sample was drawn not later than 24 hours after onset of symptoms. Time between onset of symptoms and blood sampling was recorded. Pool samples were stored with study samples. In cases, cholesterol concentrations were inversely related to time from onset of symptoms (5.8 mmol/l at 2 hours, 5.2 mmol/l at 20 hours), probably due to the effect of the acute MI on serum cholesterol.

Laboratory procedures

Biological samples (except those from Scotland) were analyzed in a central laboratory. During the study, samples were transported to the coordinating centre on dry ice, at −56°C. Pilot studies showed this did not influence vitamin concentrations. To monitor transport and storage conditions, pool samples were included in shipments and processed in a similar manner. Samples from cases and controls were analyzed blind and simultaneously.

β-Carotene and α-tocopherol in adipose tissue were determined concurrently by reverse-phase high performance liquid chromatography[16] and spectrophotometric detection. The sample was saponified and quantitatively split for vitamin and fatty acid determination. The coefficients of variation for the analysis of β-carotene and α-tocopherol were 6.7% and 6.9% (at mean values of 2.13 μg/g and 84.1 μg/g in the quality control samples, respectively).

Detection limits were 0.02 μg/g for β-carotene and 2 μg/g for α-tocopherol, at mean sample weight. Samples from Scotland were analysed in a separate laboratory. 16 samples were analysed in both laboratories; the median (25-75% range) of α-tocopherol and β-carotene levels were not significantly different by paired t-test: 88 (47-137) vs 89 (52-126) and 0.49 (0.21-0.92) vs 0.30 (0.13-0.39) μg/g, respectively.

Concentrations of the vitamins were calculated on the basis of fatty acid concentration, which was assessed by gas-liquid chromatography[17] in an aliquot of the same extract as β-carotene and α-tocopherol, adding nonadecanoic acid as an internal standard to the sample before saponification. Vitamin concentrations were expressed in μg/g total fatty acids.

Serum total and HDL cholesterol were determined enzymatically at the National Public Health Institute in Helsinki (Finland).

Data analysis

Needle biopsy and questionnaire data were available for 1499 eligible subjects. Vitamin results were unavailable for 34 subjects (no adipose tissue found). 55 samples with very high (> 130%) or very low (< 30%) fat (weight of total fatty acids/total biopsy weight), indicating measurement errors in very small samples. were excluded.

Crude means for major risk factors and potential confounders were calculated; linear and logistic regression were used to obtain age- and centre-adjusted differences. Mean concentrations of antioxidants in the different centres were calculated, and the mean differences and 95% confidence intervals (CI), adjusted for age and centre, were estimated by linear regression. α-Tocopherol and β-carotene concentrations were log-transformed. Potential confounders and/or effect modifiers were identified with stratified analysis. For multivariate analysis, multiple logistic regression was used with maximum likelihood estimation of the regression coefficients and their standard errors. Relative risks were estimated as odds ratios for the lowest quintile as compared with the highest, based on the distribution among controls. Tests for trend were done by assigning each subject the median value for the category and modelling this value as a continuous variable. The fitted model included age, centre, smoking, and body mass index (BMI). Cigarette smoking was modelled in detail, given its strong association with acute MI in the data and its association with β-carotene. Smoking categories included never smokers, ex-smokers, pipe/cigar smokers, and current cigarette smokers–the last category further divided in subjects smoking < 6, 6-10, 11-20, and > 20 cigarettes per day. Finally, interactions between centre and exposure variables and effect

modification for smoking categories were examined. Data analysis was repeated without the Scottish samples, and because results were similar, these data were included in all analyses.

Results

Risk factors

Risk factors for CHD in MI cases and controls are shown in Table 2 and 3. Age, serum cholesterol, history of hypertension, smoking, angina pectoris, diabetes mellitus, family history for CHD, and BMI differed significantly. The lower concentrations of cholesterol in cases almost certainly reflects the cholesterol-lowering effect of the MI. Therefore, cholesterol was not further considered in the analyses.

Table 3. Risk factors for myocardial infarction in the EURAMIC Study.

Risk factor	Crude means		Age and centre adjusted difference (95% CI)	
	cases n = 683	controls n = 727		
Age	54.7	53.3	1.5	(0.6 - 2 .4)
Serum total cholesterol (mmol/l)	5.4	5.6	−0.16	(−0.28 - −0.03)
History of hypertension (%)	26	17	12	(7 - 17)
Smoking				
- % current smokers	56	33	29	(24 - 34)
- cigarettes/day (among smokers)	25	18	8	(6 - 10)
Angina pectoris (%)	14	5	6	(4 - 7)
Diabetes mellitus (%)	8	4	2.5	(1.5 - 4.0)
Family history of CHD (%)	57	43	13	(8 - 19)
Alcohol use (%)	80	82	−1	(−4 - 3)
BMI (kg/m^2)	26.5	26.0	0.5	(0.1 - 0.9)

Inter-centre variation

Mean adipose tissue concentrations of α-tocopherol and β-carotene varied between centres (Table 4): Switzerland had the highest α-tocopherol (305 μg/g) and β-carotene (0.59 μg/g); lowest were found in Israel for α-tocopherol (112 μg/g) and in Spain for β-carotene (0.18 μg/g).

Table 2. Distribution of cardiovascular risk factors by disease status and study centre: EURAMIC Study.

study centre	Age		Total cholesterol (mmol/l)		Hypertension (%)		Current smoking (%)		Diabetes (%)†		Body mass index (kg/m²)	
	cases	contr	cases	contr	cases	contr	cases	contr	cases	contr	cases	contr
Finland (Helsinki)	52.8	53.7	5.1	5.8	23	10	65	25	13	3	27.1	26.7
Germany (Berlin)	56.9	52.8	5.5	6.0	34	23	51	38	15	6	26.8	25.6
Israel (Jerusalem)	54.8	54.0	4.9	5.2	22	10	51	36	10	9	26.0	26.1
Netherlands (Zeist)	53.5	52.0	5.7	5.7	15	11	53	41	5	2	26.1	25.3
Norway (Sarpsborg)	55.3	55.5	5.8	6.3	29	13	58	23	7	1	26.0	25.0
Russia (Moscow)	54.0	47.8	5.6	5.1	37	38	60	39	3	2	26.2	26.0
Scotland (Edinburgh)	54.6	55.0	6.8¶	6.5¶	7	5	48	26	0§	0§	26.8	26.2
Spain (Granada)	54.5	55.0	5.2	5.2	31	9	77	52	8	0	26.1	26.3
Spain (Malaga)	54.6	55.2	5.3	5.2	25	16	74	33	10	6	27.6	27.3
Switzerland (Zürich)	56.3	53.5	5.6	5.5	33	18	38	21	8	4	26.4	25.9

† Diagnosed at least 6 months before myocardial infarction.

‡ Contr.: control subjects.

¶ Samples were not analyzed in central laboratory.

§ Subjects with diabetes were excluded in this centre.

The overall mean concentrations for cases and controls (adjusted for age and centre, transformed back from the log values) were 193 μg/g and 192 μg/g respectively for α-tocopherol and 0.35 and 0.42 respectively for β-carotene. Concentrations of α-tocopherol and β-carotene were correlated (r = 0.24 in cases and 0.37 in controls).

Table 4. Mean adipose tissue concentrations of α-tocopherol and β-carotene, by study centre.

	Number of		α-tocopherol†		β-carotene†	
	cases	controls	cases	controls	cases	controls
Finland (Helsinki)	60	60	217	165	0.46	0.53
Germany (Berlin)	67	96	195	204	0.46	0.45
Israel (Jerusalem)	51	59	173	112	0.32	0.29
Netherlands (Zeist)	60	54	201	194	0.42	0.58
Norway (Sarpsborg)	96	95	145	188	0.37	0.58
Russia (Moscow)	92	95	210	240	0.45	0.56
Scotland (Edinburgh)‡	58	42	135	181	0.37	0.49
Spain (Granada)	52	54	192	194	0.23	0.31
Spain (Malaga)	95	100	202	166	0.18	0.19
Switzerland (Zürich)	52	72	290	305	0.44	0.59
Overall mean¶			193	192	0.35	0.42
Mean difference¶ (95% CI)			1	(−12 - 14)	−0.07	(−0.10−−0.04)

† Calculated from $\log_e$ transformed values, in μg/gram fatty acids.
‡ Samples were not analyzed in central laboratory.
¶ Adjusted for age and centre.

Vitamins and risk factors

We examined the relation between adipose tissue vitamin concentrations and CHD risk factors in the controls. For β-carotene, negative associations were observed for the number of cigarettes smoked per day ($r = -0.15$, $p < 0.05$) and for BMI ($r = -0.35$, $p < 0.001$). Subjects with a positive family history of CHD had significantly higher β-carotene ($p = 0.009$). β-carotene was not associated with serum total cholesterol ($r = 0.010$, $p = 0.8$), but positively associated with HDL cholesterol ($r = 0.275$, $p < 0.001$).

α-Tocopherol concentration was also negatively associated with BMI ($r = -0.12$, $p = 0.001$) and with number of cigarettes smoked per day ($r = -0.13$, $p = 0.053$). There were no significant correlations with age, serum cholesterol,

alcohol use, angina pectoris, diabetes, history of hypertension, or family history of CHD.

Vitamins and MI

To assess the risk of MI for low vitamin concentrations, a logistic regression model was fitted, including age, centre, smoking, and BMI. The odds ratios for MI, relative to the highest quintile of the distribution of adipose tissue vitamin concentrations in controls, are shown in Table 5.

Table 5. Odds ratio of acute myocardial infarction, according to quintiles of adipose tissue α-tocopherol and β-carotene.

		Number of		Age & centre adjusted		Multivariate†	
Quintiles (μg/g)	median	cases	controls	OR	95% CI	OR	95% CI
α-tocopherol							
1 < 127	(103)	136	145	0.92	(0.65-1.33)	0.83	(0.57-1.21)
2 127-175	(149)	132	146	0.93	(0.66-1.31)	0.88	(0.61-1.28)
3 175-229	(198)	136	145	0.99	(0.70-1.39)	1.01	(0.70-1.46)
4 229-303	(253)	144	145	1.07	(0.76-1.49)	1.12	(0.78-1.60)
5 > 303	(385)	135	146	1.00		1.00	
χ^2 trend (p-value)				0.35	(0.55)	1.21	(0.27)
Continuous OR‡				1.00	(0.78-1.27)	0.88	(0.67-1.13)
β-carotene							
1 < 0.21	(0.15)	177	145	2.62	(1.79-3.83)	1.78	(1.17-2.71)
2 0.21-0.37	(0.30)	184	146	2.48	(1.73-3.55)	1.79	(1.21-2.64)
3 0.37-0.53	(0.45)	124	145	1.66	(1.15-2.41)	1.40	(0.94-2.10)
4 0.53-0.82	(0.65)	119	145	1.49	(1.03-2.16)	1.27	(0.85-1.88)
5 > 0.82	(1.11)	79	146	1.00		1.00	
χ^2 trend (p-value)				32.56	(< 0.0001)	10.72	(0.001)
Continuous OR‡				1.99	(1.50-2.65)	1.48	(1.09-2.03)

† Adjusted for age, centre, smoking, and BMI.

‡ Odds ratio of MI at 10th percentile level of antioxidant, compared to 90th percentile level.

For α-tocopherol no association with MI was observed. The age- and centre-adjusted odds ratio (OR) in the lowest quintile of the β-carotene distribution was 2.62 (95% CI 1.79-3.83, p for trend < 0.0001). After additional adjustment for smoking and BMI, the OR was 1.78 (95% CI 1.17-2.71; p for trend = 0.001). Adjustment for other CHD risk factors (including HDL

cholesterol) had only marginal effects on the estimate of the odds ratios. Including β-carotene concentration as a continuous variable, the OR for a contrast of the 10% point of distribution of β-carotene compared to the 90% point, was 1.48 (1.09-2.03). This OR ranged from 0.57 (0.21-1.59) in Israel to 5.07 (1.81-14.23) in Norway; in 7 of the 10 centres, there was an increased risk of MI at low β-carotene concentrations.

The odds ratios for MI at quintiles of β-carotene, (Table 5) were recalculated for different subgroups of centres, according to type of control selection. When centres with hospital controls (Spain, Granada, and Malaga; Russia) or centres using mixed methods of control recruitment (Netherlands; Spain, Malaga; Russia) were excluded, odds ratios were similar. When type of control selection was included in the logistic regression model the interaction terms for these models were not statistically significant.

Table 6. Odds ratio[†] of myocardial infarction, according to quintiles of adipose tissue β-carotene and smoking category.

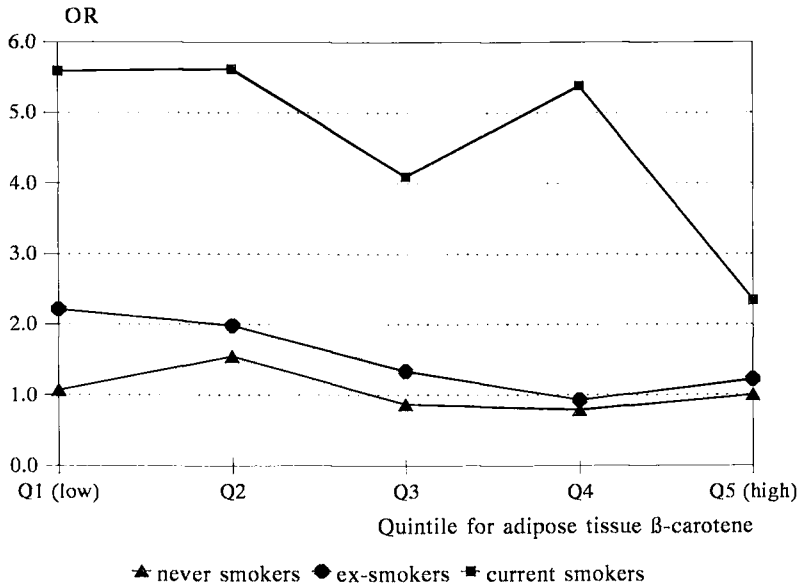
Quintile[‡]	Never smokers (n = 83/200)[¶] OR (95% CI)	Ex-smokers (n = 116/187) OR (95% CI)	Cigarette smokers (n = 435/272) OR (95% CI)
1	1.07 (0.44-2.57)	1.81 (0.81-4.06)	2.39 (1.35-4.25)
2	1.55 (0.67-3.58)	1.62 (0.74-3.55)	2.40 (1.38-4.17)
3	0.87 (0.40-1.90)	1.10 (0.50-2.43)	1.75 (0.97-3.17)
4	0.79 (0.36-1.70)	0.76 (0.32-1.84)	2.30 -, 4.15)
5	1.00 -	1.00 -	1.00 -
Continuous OR[§]	0.98 (0.52-1.87)	1.42 (0.77-2.61)	1.67 (1.12-2.49)

[†] Adjusted for age, centre, BMI.
[‡] Quintile cutpoints as in table 5.
[¶] Number of cases/controls.
[§] OR of MI at 10th percentile level compared to 90th percentile level of β-carotene.

The lower relative risk after adjustment is largely due to the effect of smoking. Mean β-carotene concentrations in smokers differ between categories of smoking. In subjects who never smoked, β-carotene was significantly higher (0.49 μg/g) than in ex-smokers (0.41 μg/g) or heavy current smokers (0.37 μg/g). The interaction between smoking behaviour and β-carotene status was analyzed. The odds ratio of MI, at quintiles of the β-carotene distribution compared to the highest, is given for different categories of smoking behaviour (Table 6). In

current smokers the risk for MI is higher at low β-carotene concentrations (OR 2.39; 95% CI 1.35-4.25). For ex-smokers the increased risk is smaller (OR 1.81) and non significant. Never-smokers seem to have no elevated risk of MI at low β-carotene status (OR 1.07). The Figure gives the risk of MI in the quintiles of the β-carotene distribution, but also takes into account the higher risk by smoking (OR for former smokers as compared to never smokers was 1.22, whereas it was 2.34 for current smokers, in the highest β-carotene quintile).

Inclusion of α-tocopherol as a continuous covariable in the multivariate model strengthened the inverse association with MI for β-carotene; the OR in the lowest quintile was 1.99 (1.29-3.07). The association between β-carotene and MI was also examined multivariately in tertiles of α-tocopherol. The continuous OR for β-carotene (10% point vs 90% of distribution) at the lowest α-tocopherol tertile was 1.09 (95% CI 0.67-1.77); at the highest tocopherol tertile the OR for β-carotene was 2.28 (1.36-3.84). The risk at α-tocopherol concentrations in between was 1.86 (1.05-3.28).



[1] The risk in the highest ß-carotene quintile, in the category never smokers, has been taken as single reference value.

Figure 1. Odds ratio[1] of MI, according to quintiles of β-carotene and smoking category.

Discussion

Our results, based on adipose tissue concentrations in 1410 subjects, are consistent with the suggested protective effect of high β-carotene concentrations against acute MI, whereas high – but within the normal range – α-tocopherol concentrations were not protective. Increased risk of MI at low adipose tissue β-carotene levels, was most pronounced in current smokers. This is consistent with data indicating higher oxidative stress due to smoking[26,27], which may require relatively higher antioxidant concentrations for adequate protection. α-Tocopherol seemed to modify the effect of β-carotene; at high tocopherol levels, the inverse association of β-carotene with MI was strongest.

Control recruitment

This study, in 9 countries with substantially different diets, used several methods of control recruitment. Five centres used random samples of population registries, and others hospital controls for whom the diagnosis, according to current knowledge, was not related to antioxidant status. If, however, a control disease was related to antioxidants, it is most likely to have reduced anti-oxidants, and thus diminish the risk estimates for MI. To minimize potential bias if any one disease was related to exposure, controls were chosen with a variety of diseases. The use of friend or relative controls may lead to overmatching on lifestyle exposures; but this would also result in a decreased estimation of association between antioxidants and MI. Exclusion of centres using hospital controls or mixed methods of control recruitment did not affect the strength of association. Moreover, risk factors – cigarette smoking, hypertension and diabetes – were consistently more prevalent in acute MI cases than controls, which provides support for the overall validity of the case-control comparison.

Sample analysis

Standardized procedures were applied in each centre for the collection of questionnaire data and biological materials. Freezer problems were reported in two centres. In Israel (Jerusalem) the temperature increased to −17°C once, and one shipment from Spain, Granada reached the central laboratory thawed. However, to prevent differential misclassification of exposure, samples from cases and controls were stored in the same freezer and shipped in the same container. Moreover, the adipose tissue pool samples that were stored and shipped with the study samples had vitamin concentrations similar to other pool samples.

Vitamin and diet

Adipose tissue vitamin concentrations were chosen to represent long-term antioxidant status. Adipose tissue and liver are the most important sites of deposition of carotenoids; the relative concentration of different carotenoids in adipose tissue is similar to that in plasma, and an effect of high dose supplementation on adipose tissue concentrations has been observed[19]. For α-tocopherol, almost 90% of the body pool is concentrated in adipose tissue[20]. In a small experiment, adipose tissue α-tocopherol concentration showed a correlation both with dietary intake ($r = 0.56$) and plasma concentrations ($r = 0.59$) of α-tocopherol[21]. This suggests that adipose tissue β-carotene and α-tocopherol concentrations probably reflect long-term dietary intake.

Variation between centres

Concentrations varied between centres, as expected. Notable are the lower concentrations, especially of β-carotene, in Jerusalem and both Spanish centres, which is not in agreement with plasma concentrations reported for several European countries[22]. Furthermore, the relative odds of MI at low β-carotene status was not the same in different centres. The highest OR was found in Norway, and the lowest in Israel. Centres with low mean β-carotene levels seem to have the lowest relative risk (Jerusalem, Malaga). Although within-centre sample sizes were relatively small and chance effects could play a part, these findings might be explained by smoking, drinking, or dietary intake affecting antioxidant requirements, and resulting in altered adipose tissue antioxidant concentrations. Similarly, the unexpected finding of a stronger association between β-carotene and MI at high α-tocopherol concentrations might also be attributed to these country-specific exposure characteristics rather than to α-tocopherol as such.

Previous studies

Recent reports from prospective studies[5,6] support the protective role of food antioxidants. Women in the highest quintile of vitamin E intake had a relative risk of CHD of 0.66 (95% CI 0.50-0.87), after adjustment for age and smoking[6]; a benefit mainly found in users of vitamin E supplements (moderately high levels of dietary intake were not associated with a significant reduction in risk). Results were similar for vitamin E intake in men[5]; supplement users had a multivariate relative risk of 0.64 (95% CI 0.49-0.83). In Scotland[7] a significant relation between plasma vitamin E and angina pectoris was observed in a case-control study. Although an inverse association has been reported between plasma cholesterol-standardized vitamin E concentrations and age-specific CHD

mortality in 16 European countries[22,23], Gey et al.[11] did not find an association of plasma vitamin E with the risk of cardiovascular disease in a 12-year follow-up study. Two nested case-control studies in the Netherlands and Finland[8,9], also found no association between baseline vitamin E in frozen serum samples and cardiovascular disease mortality. The lack of association between α-tocopherol and risk of MI in the present study is compatible with the finding that such an association may be present only in supplement users[5,6].

The part played by carotene was also addressed in the US Health Professionals Study[6]. Carotene intake was not associated with a lower risk of CHD among those who never smoked, but among current smokers and former smokers, the relative risk in the highest quintile was decreased (current: OR 0.30 (0.11-0.82); former: OR 0.60 (0.38-0.94)). In a prospective cohort study[24], the age- and sex-adjusted relative risk of CHD at the highest quintile of intake of β-carotene-rich fruits and vegetables was 0.54 (95% CI 0.34-0.87; p trend = 0.044). Recently, it has been shown that low concentrations of plasma β-carotene and α-carotene (< 0.23 μmol/l) are associated with a significantly higher relative risk of CHD mortality, after adjustment for age and classical risk factors, as well as other antioxidant variables (RR 1.53, 95% CI 1.07-2.20)[11]. Street et al.[10] reported a protective association of β-carotene with myocardial infarction. Preliminary results from a β-carotene intervention study[12] showed a significant reduction of vascular events in the intervention group. Our findings for β-carotene are therefore also consistent with the available evidence, including those testing for effect modification by smoking. Although this type of study does not provide direct proof of a cause and effect relationship, the results are supported by experimental evidence.

Protective actions of antioxidants

Oral supplements with α-tocopherol protect low-density lipoproteins against oxidation in vitro[25]; although for β-carotene comparable studies have yielded conflicting results[25,26]. It is, however, possible that β-carotene is the more important in vivo. β-Carotene is an efficient quencher of singlet oxygen and a radical trapping antioxidant[27], and differs from the chain-breaking antioxidant α-tocopherol, in being more effective at low O_2 pressure, as may be found in the tissues[28]. Short-term, high-dose β-carotene supplements have been reported to lead to an increase in serum high-density lipoproteins, which affects cardiovascular risk. In this study we also found an association between β-carotene in adipose tissue and serum HDL-cholesterol, but it did not explain the association with MI. β-Carotene may have other biological functions,

possibly related to its pro-vitamin A activity, or perhaps via the immune system[30], which affect atherogenesis or thrombosis.

Our finding of an inverse association of adipose tissue β-carotene, derived from normal dietary intake, with acute MI, supports the hypothesis that antioxidants protect against CHD. The lack of association for α-tocopherol raises interest in possible alternative mechanisms for β-carotene. The results suggest that the consumption of β-carotene-rich foods, such as carrots and green leafy vegetables, may reduce the risk of myocardial infarction in men.

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Letter to the Editor

Sir-The EURAMIC study (Dec 4, p 1379) attempts to shed light on a potential protective role for antioxidant vitamins against myocardial infarction (MI). The study draws on cases and controls sampled in several populations with varying adipose tissue concentrations of β-carotene and α-tocopherol. The results are therefore potentially important. Unfortunately, the argument falls short of precision in places.

The cases were recruited from coronary care units up to 24 hours after onset of symptoms of MI. In view of the consistently high out-of-hospital case-fatality rate for MI[1] the EURAMIC study was of survivors rather than of all cases of MI. Unless an identical pathogenesis and no relation between vitamin status and early prognosis after acute MI are assumed, this point should have been explicitly addressed. The selection of controls seems to be a weakness too. Some of the control groups in EURAMIC deviate considerably in their vitamin concentrations and in established risk factors from standardised international population estimates[2,3]; this leads to striking differences between cases and controls. The vitamin quintiles may be based on controls who are unusually healthy selections from the populations studied, and this may have resulted in overestimations of vitamin-associated MI risks. The most prominent finding is a strong inverse relation of β-carotene with MI risk, which is modified by smoking, the effect being strongest in current smokers. No further analyses are presented on the impact of the number of cigarettes smoked. It may be that some of this presumed effect of β-carotene in current smokers is due to residual confounding. There was a strong inverse relation between β-carotene and smoking in this and other[4] studies; current smokers with low vitamin concentrations are expected to be predominantly heavy smokers, whereas light smokers are likely to have the higher vitamin concentrations. Likewise, the observed lesser risk gradient in ex-smokers could be accounted for by misclassifications (e.g., still smoking occasionally), or short time periods since quitting, and thus could also be related to numbers of cigarettes smoked.

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Authors' reply

Sir–The cases were survivors of acute MI and direct conclusions should be restricted to this group. Might survival be related to the antioxidants under study? The significance of free radicals in the process of myocardial injury and necrosis of reperfused tissue is recognised and treatment with antioxidants may slow down or inhibit these processes[1]. That would suggest that fatal cases have similar or perhaps lower levels of antioxidants than the survivors, so including them might have led to stronger associations with low antioxidants status. Theoretically, if vitamin E were an important determinant of case fatality, this could partly explain the lack of association between vitamin E and MI in our study.

Hense and Döring also ask about selection toward healthier controls, and they cite the plasma vitamin concentrations and prevalence of coronary heart disease risk factors from the MONICA study[2]. However, adipose tissue vitamin concentrations differ from plasma levels in units, range, and order of magnitude. We know of no standard international population estimates for adipose tissue levels and cannot tell whether the means in our controls are high or low. The ranking of countries according to adipose tissue vitamin levels may differ from that for plasma concentrations[3]. We did discuss the relatively low values in Spain and Israel, but lower than expected antioxidant levels do not suggest the selection of healthier subjects.

The prevalence of smoking and hypertension and mean values for body mass index (BMI) and serum total cholesterol might be compared between our study population and specific MONICA centres. Unfortunately, Norway, the Netherlands, the south of Spain, and Israel, countries that contribute half of the EURAMIC population, are not represented in MONICA, and for the remaining countries regional differences within countries may be as large as between-country differences, as shown in the MONICA report[1]. Anyway, we see only minor differences for BMI; the prevalence of smoking shows larger differences (e.g., for Moscow 46% in MONICA and 39% in our controls). Within-country means for EURAMIC are based on only 70 cases and 70 controls per centre, on average. The 95% confidence intervals (about ± 10%) are bigger than in MONICA

and values from the MONICA study tend to fall well within these intervals. Moreover, our population was studied in 1991-1992 whereas most of the MONICA data were collected between 1982 and 1986; a real decrease in smoking among men may explain lower prevalence figures among EURAMIC controls. A direct comparison of the prevalence of risk factors in MONICA and EURAMIC is not valid; nor would it support the view that our controls were biased by the inclusion of healthier individuals.

The issue of residual confounding by number of cigarettes smoked in the association between β-carotene and MI is important. In our statistical model to test whether the association between β-carotene and MI was dependent on smoking status, we did not include the number of cigarettes smoked since this is a redundant variable for never-smokers. However, we did account for some misclassification in ex-smokers by limiting this category to those who had stopped smoking at least 2 years before inclusion in the study. We have now done separate calculations for smokers and ex-smokers, with adjustment for number of cigarettes and maximum number of cigarettes smoked daily before stopping smoking, respectively. The 'continuous' odds ratio (OR) is reported here, because this is less susceptible to irrelevant fluctuations by small numbers in the smoking categories than ORs in quintiles. Among ex-smokers, the OR for MI at low versus high β-carotene (10th vs 90th percentile) reported in the paper was 1.42 (95% CI 0.77-2.61); the same model, applied to ex-smokers only, gives an OR of 1.65 (0.73-3.74). Additional adjustment for number of cigarettes changes the OR to 1.61 (0.71-3.66), a 2% decrease. Among current smokers, the respective ORs are 1.67 (1.12-2.49), 2.02 (1.23-3.32), and 1.66 (1.00-2.80) (18% decrease). These results indicate that some of the increased risk at low β-carotene was still due to smoking, but an independent association of β-carotene and MI remains.

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Interaction between low selenium and vitamin E status and risk of acute myocardial infarction: the EURAMIC study

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Abstract

To examine the association between selenium status and risk of acute myocardial infarction and interactions with vitamin E status, a multi-centre case-control study was conducted in ten centres from Europe and Israel. Selenium in toenails and vitamin E in adipose tissue were assessed for 683 non-fatal cases with first acute myocardial infarction (MI) and 729 controls under 70 years of age. Mean toenail selenium content was 0.575 μg/g for cases and 0.599 μg/g for controls ($p < 0.05$). After multivariate adjustment, the odds ratio for MI in the lowest quintile of selenium as compared to the highest was 1.55 (95% confidence interval (CI) 0.89-2.70, p for trend 0.12). Adipose tissue vitamin E was not associated with risk of MI (odds ratio (OR) 0.94, 95% CI 0.62-1.41). The relation between selenium and MI was most pronounced at low vitamin E levels (OR at the lowest selenium quintile: 2.62, 95% CI 1.16-5.89, p for trend 0.02). At medium vitamin E levels, the OR was 1.88 (95% CI 0.83-4.27), whereas no increased risk was observed at high vitamin E levels. An overall comparison of the risk of MI at combined low levels of selenium and vitamin E relative to combined high levels of both, showed no significantly increased OR. These results suggest that selenium may play a role in the etiology of myocardial infarction especially when vitamin E status is low.

Submitted

Introduction

Selenium is part of the enzyme glutathione peroxidase (GSHPx), which plays an important role in the body's antioxidant defense against the deleterious actions of free radicals and lipid peroxides. Evidence is accumulating that oxidation of low density lipoprotein particles and cytotoxic effects of lipid peroxides enhance the formation of foam cells and atherosclerotic lesions[1,2]. The question whether low selenium status predisposes to cardiovascular disease has been addressed in a number of studies in the past decade[3-10]. In several prospective[3-5] and case-control[6] studies, low serum selenium levels were associated with increased risk of coronary heart disease (CHD). However, in other studies, especially in populations with an intermediate selenium status, this association could not be confirmed[7-10].

Antioxidant defense is achieved by additive or synergistic action of enzymes and antioxidant nutrients[11,12]. Selenium and vitamin E in particular seem to have overlapping and partly compensative functions[13-17]. GSHPx is more effective as a free radical scavenger in the cytoplasm, while vitamin E prevents lipid peroxidation by breaking the oxidation chain reaction, primarily in biomembranes. However, vitamin E can protect against many of the symptoms of selenium deficiency and vice versa[12]. Therefore, it is conceivable that low selenium status is associated with risk of CHD especially at low vitamin E status. This notion is supported by a study in rabbits, in which the administration of both selenium and vitamin E markedly reduced atherosclerotic plaque formation induced by a high-fat diet, but an intensified effect was observed at the combination of both[18].

We have studied the association between selenium, separately and in combination with vitamin E levels, and acute myocardial infarction in a multi-centre case-control study. The large variation in selenium intake in these centres permits evaluation of a dose-response relation. Selenium and vitamin E levels were represented by long-term biomarkers of intake, not affected by the acute event of the disease: selenium was measured in toenail clippings[19-21] and vitamin E in subcutaneous adipose tissue[22].

Methods

Design and subjects

A total of 683 cases and 729 controls were recruited in 8 European countries and Israel, according to the same eligibility criteria. Table 1 shows the

numbers of cases and controls that were enrolled and the response rates in the different centres. The study design has been described elsewhere[23,24]. Briefly, eligible subjects were men under 70 years of age, without previously reported myocardial infarction, with stable dietary patterns in the past year. Cases were subjects diagnosed with a first acute myocardial infarction (MI) (ICD-code 410) - confirmed by specific abnormalities on the ECG and elevated enzyme levels[25] – and admitted within 24 hours of manifesting symptoms. They were recruited from the coronary care unit of participating hospitals.

Subjects without a history of myocardial infarction were eligible as controls, and were recruited from the population in the catchment area, frequency-matched for age according to 5-year intervals. If possible, random samples from local population registries were used (Finland, Israel, Germany, Scotland, Switzerland). In some centres (Russia, Spain) population registries could not be used due to incomplete coverage or legal restrictions.

Table 1. Population response rate and method of control recruitment.

Centre	Cases No. (response %†)		Controls No. (response %)		Method of control recruitment‡
Finland (Helsinki)	57	(97)	62	(51)	PR
Germany (Berlin)	75	(82)	97	(73)	PR
Israel (Jerusalem)	57	(60)	59	(53)	PR
Netherlands (Zeist)	63	(75)	57	(50)	GP,PR
Norway (Sarpsborg)	96	(96)	101	(98)	FR
Russia (Moscow)	92	(97)	99	(79)	GP,H
Scotland (Edinburgh)	41	(98)	32	(61)	PR
Spain (Granada)	55	(45)	53	(67)	H
Spain (Malaga)	94	(89)	100	(77)	H,GP
Switzerland (Zürich)	57	(93)	74	(26)	PR

† Percentage of cases and controls who consented to participate, from those who were eligible and invited to participate.

‡ PR = population register; GP = general practitioners; H = hospital controls; FR = friends, relatives.

Therefore, hospital controls were selected, with diseases that were not known to be associated with antioxidant status – e.g., renal colic, non-infectious prostatism, acute appendicitis, non-infectious otic pathology, hernia, volvulus, rectal/anal pathology (except cancer, hemorrhoids, chronic infections). Where

it was anticipated that low response rates from population-based samples would compromise internal validity, controls were selected from the catchment area via a random sample by the patient's general practitioner (Netherlands) or by inviting friends and relatives of the case (Norway). In three centres (Netherlands, Spain, Russia) several methods of subject recruitment were employed. Informed consent was obtained in accordance with the ethical standards of the responsible committees on human experimentation.

Information on smoking habits, blood pressure, angina pectoris, and diabetes was collected for all subjects by standard questionnaires[26]. Socio-economic status, family history, and alcohol intake were assessed through locally developed questionnaires.

A non-fasting blood sample was drawn not later than 24 h after onset of symptoms. Serum total and HDL cholesterol levels were determined enzymatically (Boehringer Mannheim Kit) at the National Public Health Institute in Helsinki (Finland). In cases, cholesterol concentrations were inversely related to time from onset of symptoms (Pearson $r = -0.18$, $p < 0.001$), which may be due to the effect of acute MI on serum cholesterol.

Selenium in toenails

Toenail clippings were collected, within 8 weeks of inclusion in the study, and stored in small plastic bags at room temperature. Nails were cleaned before clipping. The selenium content of the toenails was assessed by instrumental neutron activation analysis of the metastable-selenium-77 isotope (Interfaculty Reactor Institute (IRI), Delft University, Netherlands)[27]. Samples were irradiated for 17 seconds, in a thermal flux of 1.2×10^{13} neutrons.s^{-1}.cm^{-2}. After a decay time of 20 seconds, gamma radiation of ^{77m}Se was measured for 60 seconds. Mean level of selenium ($n = 87$) in certified bovine liver reference material (NBS-1577A) was 0.761 ± 0.043 ppm, against a certified value of 0.804 ± 0.036 ppm. Reproducibility of measurement was evaluated by repeated analysis of 19 samples; the coefficient of variation was 5%.

Vitamin E in adipose tissue

Subcutaneous adipose tissue was taken from the buttock by needle aspiration[28]. In cases, the adipose sample was taken within seven days of hospital admission. Samples were stored at −70°C; handling of the samples has been described previously[24]. In short, samples were analyzed in a central laboratory. Concentrations of vitamin E (determined as α-tocopherol, the major active form of vitamin E) in adipose tissue were determined by

reverse-phase HPLC[29] and spectrophotometric detection. The coefficient of variation for the analysis of vitamin E was 7% (at mean values of 84 μg/g in the quality control samples). Detection limit was 2 μg/g for vitamin E, at mean sample weight (29 mg).

The concentration of vitamin E was calculated on the basis of fatty acid content of the sample, which was assessed by GLC[30] in an aliquot of the same extract as vitamin E, adding nonadecanoic acid (C19:0) as an internal standard to the sample before saponification. Vitamin E concentration was expressed in μg/g of total fatty acids.

Data analysis

Questionnaire and selenium results were available for 1421 eligible subjects (Table 1). In 9 samples (4 cases, 5 controls), selenium values were found to be below the detection level; these were excluded from data-analysis. In analyses including data on vitamin E, another 34 subjects (30 cases, 4 controls) were excluded because no biopsy material was obtained and 51 subjects (27 cases and 24 controls) because the amount of fat collected was extremely small (below 5 mg), resulting in unreliable measurement of vitamin E.

Crude means for major risk factors and potential confounders among cases and controls were calculated and significance testing of the difference in means was performed with Student's t-test and chi-square analyses. Mean levels of selenium (along with the standard deviations) among cases and controls in the different centres were computed, and the mean difference and 95 percent confidence intervals (CI), adjusted for age and centre, were estimated by linear regression. Selenium and vitamin E values were log-transformed in all calculations; retransformed values are presented in the tables. Relation between selenium and vitamin E was assessed by calculating the Pearson correlation coefficient. Potential confounders and/or effect modifiers were identified with stratified analysis. Smoking categories included never smokers, ex-smokers, pipe/cigar smokers, and current cigarette smokers; the last category was further divided into subjects smoking < 6, 6-10, 11-20, and > 20 cigarettes per day. For multivariate analysis, multiple logistic regression was used with maximum likelihood estimation of the regression coefficients and their standard errors. Odds ratios were calculated for the lowest quintile as compared to the highest, based on the distribution among controls. Tests for trend were performed by assigning each subject the median value for the category and treating this value as a continuous variable in the model[31]. Interaction between selenium and vitamin E was evaluated

using a model including dummy variables for each combination of the categorical variables (Table 7). The same approach was used for the potential interaction of selenium and smoking status, the latter divided in three categories: subjects who never smoked, ex-smokers (who have stopped smoking for at least two years) and current smokers. All analyses were performed with the BMDP statistical software package.

Results

The prevalence of CHD risk factors in MI cases and controls is given in Table 2. Significant crude differences are observed for age, history of hypertension, smoking, angina pectoris, diabetes mellitus, family history of CHD and body mass index (BMI). The lower cholesterol concentration in cases may well be caused by the acute effect of the infarction.

Table 2. Risk factors for myocardial infarction in cases and controls.

Risk factor	Cases (n = 683)		Controls (n = 729)		
	Means	(SD)	Means	(SD)	
Age (years)	54.7	(8.9)	53.2	(9.2)	*
Serum cholesterol (mmol/l)	5.5	(1.1)	5.6	(1.1)	
History of hypertension (%)	26		18		**
Current smokers (%)	58		33		**
No. of cigarettes/day (among smokers)	25	(15)	18	(12)	**
Angina pectoris (%)	14		5		**
Diabetes mellitus (%)	8		4		**
Family history of CHD (%)	58		46		**
Alcohol use (%)	80		83		
Body mass index (kg/m^2)	26.5	(3.9)	25.9	(3.4)	*

* $p < 0.01$; ** $p < 0.001$.

Mean selenium concentrations in toenails for cases and controls in the different centres are shown in Table 3. The age- and centre-adjusted overall mean is 0.575 μg/g in cases and 0.599 μg/g in controls ($p < 0.05$). The relation of selenium concentration with CHD risk factors was examined in controls (Table 4). Subjects who currently smoked cigarettes had significantly lower selenium levels than non-smokers (0.555 μg/g and 0.621 μg/g, respectively). Other classical risk factors (including total, HDL and LDL cholesterol) were not associated with selenium concentration.

Table 3. Mean toenail selenium concentrations† by centre and disease status.

Centre	Number of		Selenium concentrations	
	cases	controls	cases	controls
Germany (Berlin)	75	96	0.424 (0.103)	0.460 (0.076)
Netherlands (Zeist)	62	56	0.466 (0.091)	0.479 (0.113)
Spain (Granada)	54	52	0.488 (0.076)	0.515 (0.105)
Spain (Malaga)	94	100	0.506 (0.097)	0.532 (0.107)
Scotland (Edinburgh)	41	32	0.507 (0.090)	0.553 (0.114)
Switzerland (Zürich)	57	74	0.561 (0.121)	0.616 (0.214)
Russia (Moscow)	91	98	0.630 (0.178)	0.643 (0.162)
Norway (Sarpsborg)	96	100	0.671 (0.258)	0.652 (0.115)
Finland (Helsinki)	57	62	0.798 (0.158)	0.833 (0.124)
Israel (Jerusalem)	56	59	0.845 (0.198)	0.866 (0.142)
Overall mean‡	683	729	0.575	0.599
Mean difference‡ (95% CI)			0.024 (0.012-0.037)	

† μg/g, retransformed $\log_e$-values.
‡ Age- and centre-adjusted.

Table 4. Selenium concentration† in controls by CHD risk factor.

Risk factor	Present		Age- & centre-adjusted	
	Yes	No	difference	95% CI
Current smoking	0.555	0.621	0.052	(0.034 - 0.068)
History of hypertension	0.582	0.602	0.009	(−0.015 - 0.032)
Angina pectoris	0.578	0.593	0.026	(−0.022 - 0.071)
Diabetes	0.580	0.598	0.016	(−0.026 - 0.056)
Alcohol use	0.597	0.580	−0.000	(−0.025 - 0.024)
Family history of CHD	0.623	0.579	−0.012	(−0.030 - 0.007)
Body mass index > 25.6	0.591	0.603	0.004	(−0.013 - 0.021)

† μg/g, retransformed $\log_e$-values.

Vitamin E was not associated with selenium (r = −0.04, p = 0.25). Smokers and non-smokers had similar levels of vitamin E (189 μg/g and 196 μg/g respectively, p = 0.46).

To evaluate confounding by CHD risk factors, stepwise logistic regression was used. Age and centre were included in all models. Of the

classical risk factors (Table 2), only the addition of smoking significantly altered the odds ratio (OR) for MI. The age- and centre-adjusted OR in the lowest selenium quintile was 3.19 (95% CI 1.96-5.21, p for trend < 0.001). After additional adjustment for smoking (in 7 categories), the OR in the lowest quintile was 1.61 (95% CI 0.95-2.73, p for trend 0.07). Inclusion of all major risk factors (except serum cholesterol, because of dubious values in cases) in the logistic model only slightly further changed the estimates of the ORs: in the lowest quintile the OR was 1.55 (95% CI 0.89-2.70, Table 5). Subsequently, vitamin E was included in the model, but showed no confounding effect. Selenium was also included in the model as a continuous variable (Table 5); the adjusted OR of MI at the 10th percentile of selenium, compared to the 90th percentile, was 1.15 (95% CI 0.76-1.74).

Table 5. Odds ratio of acute myocardial infarction, according to quintiles of toenail selenium.

Quintiles		Cases	Controls	Age- & centre adjusted		Multivariate†	
Selenium (μg/g)		n = 683	n = 729	OR	95% CI	OR	95% CI
Q1	< 0.467	181	148	3.19	(1.96-5.21)	1.55	(0.89-2.70)
Q2	0.467-0.548	156	143	2.42	(1.52-3.85)	1.50	(0.89-2.52)
Q3	0.549-0.633	109	147	1.49	(0.95-2.33)	0.99	(0.60-1.64)
Q4	0.634-0.755	130	145	1.55	(1.04-2.32)	1.27	(0.81-1.98)
Q5	> 0.755	107	146	1.00		1.00	
χ^2 trend (p-value)				23.55	(< 0.001)	2.48	(0.12)
Continuous OR‡				2.00	(1.38-2.92)	1.15	(0.76-1.74)

† Adjusted for age, centre, smoking, BMI, diabetes, history of hypertension and family history of CHD.

‡ OR of MI at 10th percentage level (0.432 μg/g) of selenium compared to 90th percentage (0.862 μg/g) level.

To see whether the inverse association of selenium and MI was consistent among individual centres, the continuous OR was estimated for each centre separately. In 8 out of the 10 centres, adjusted ORs exceeded one, but had very wide confidence intervals including one. When centres using hospital controls (Russia, Spain) were excluded from the analysis, multivariate odds ratios from the lowest to the highest quintile were 2.19-1.78-1.25-1.61-1.00.

Smoking was the most important confounder of the association between selenium and MI; selenium levels were shown to be lower in current cigarette

smokers than in non-smokers (Table 4). Therefore, we examined the potential interaction of smoking behaviour and selenium status. Increased odds ratios for the lowest vs highest quintile of selenium were observed in ex-smokers (OR 2.35, 95% CI 1.04-5.34) and in current smokers (OR 1.70, 95% CI 0.89-3.25), respectively, but not in subjects who have never smoked (OR 0.98). However, the interaction between the three smoking categories and selenium (as a continuous variable in the model) was not significant (p = 0.23). Moreover, additional adjustment for the number of cigarettes smoked daily, in the category current smokers, resulted in a considerable decrease of ORs in the selenium quintiles (–30% in the lowest quintile). Therefore, the varying associations between selenium and MI among never, ex- and current smokers are most likely due to residual confounding by smoking.

Table 6. Odds ratio of acute myocardial infarction, according to quintiles of adipose vitamin E.

Quintiles		Cases	Controls	Multivariate†	
Vitamin E (μg/g)		n = 626	n = 701	OR	95% CI
Q1	< 127	122	143	0.94	(0.62-1.41)
Q2	127-175	134	140	0.98	(0.66-1.45)
Q3	175-229	126	142	1.08	(0.73-1.61)
Q4	229-303	127	139	1.25	(0.86-1.83)
Q5	> 303	117	137	1.00	
χ^2 trend (p-value)				0.20	(0.65)
Continuous OR‡				0.92	(0.70-1.22)

† Adjusted for age, centre, smoking, BMI, diabetes, history of hypertension and family history of CHD.

‡ OR of MI at 10th percentage level (105 μg/g) of vitamin E compared to 90th percentage (387 μg/g) level.

No association was observed between vitamin E and risk of MI when confounding was taken into account (Table 6). To test the a priori hypothesis that an inverse association of selenium and MI would be most pronounced at low levels of vitamin E, the risk of MI for quintiles of selenium was estimated at different levels (tertiles) of vitamin E (Table 7), by including all combinations of selenium quintiles and vitamin E tertiles in the logistic model. The OR at the lowest selenium quintile was 0.86 (95% CI 0.39-1.92)

in subjects with relatively high vitamin E status (highest tertile); 1.88 (95% CI 0.83-4.27) at intermediate vitamin E status; and 2.62 (95% CI 1.16-5.89) at low vitamin E status. A significant linear trend was observed at the lowest vitamin E level. However, this apparent interaction between selenium and vitamin E was not statistically significant when both were included as continuous variables in the logistic regression model (p = 0.72).

The largest increase in risk would be expected in the combined low selenium/low vitamin E category, when compared to the high selenium/high vitamin E category. To see whether this was true, the OR of MI at different combinations of vitamin E and selenium levels was calculated, with high selenium and high vitamin E as single reference category (Fig. 1). Surprisingly, there was an unexpected positive, although non-significant association between vitamin E and MI at all but the lowest selenium level. In the highest selenium quintile, the OR for MI at low compared to high vitamin E level was 0.50 (95% CI 0.23-1.05). Therefore, OR in the combined low selenium and low vitamin E level was only 1.29 (95% CI 0.58-2.92). The three-way interaction between selenium, vitamin E and smoking, was not evaluated, as the number of subjects was not sufficient for reliable risk estimates. Considering the lack of association between smoking and vitamin E, it is not likely that the interaction between selenium and vitamin E could be explained by differences in smoking status.

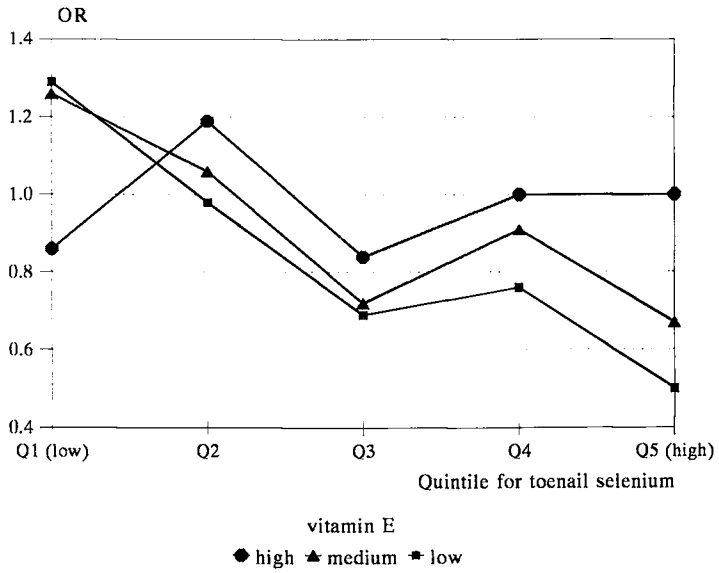
Table 7. Odds ratio[†] of MI at quintiles of selenium, and at tertiles of adipose tissue vitamin E.

	Vitamin E		
Selenium	Low OR (95%CI)	Medium OR (95%CI)	High OR (95%CI)
Q1 low	2.62 (1.16-5.89)	1.88 (0.83-4.27)	0.86 (0.39-1.92)
Q2	1.97 (0.89-4.38)	1.58 (0.71-3.54)	1.19 (0.54-2.59)
Q3	1.38 (0.61-3.17)	1.07 (0.48-2.36)	0.84 (0.40-1.80)
Q4	1.54 (0.74-3.19)	1.36 (0.64-2.87)	1.00 (0.48-2.09)
Q5 high	1.00	1.00	1.00
χ^2 trend (p-value)	5.42 (0.02)	2.23 (0.14)	0.02 (0.88)

† Adjusted for age, centre, smoking, BMI, diabetes, history of hypertension and family history of CHD.

‡ Quintile cutpoints as in Table 5.

¶ Tertile cutpoints: 161 and 244 μg/g.



a Odds ratio adjusted for age, centre, smoking, BMI, diabetes, history of hypertension, family history of CHD.
b Single reference category: high selenium/high vitamin (OR = 1.0).
c Quintile cutpoints as in Table 5.
d Tertile cutpoints as in Table 6.

Figure 1. Risk of MI[a] by joint[b] distribution of toenail selenium[c] and adipose tissue vitamin E[d]

Discussion

In this multi-centre case-control study we observed an inverse relation between toenail selenium levels and risk of acute myocardial infarction, dependent on vitamin E status. Selenium by itself was inversely associated with MI, although not significant, whereas vitamin E showed no association with MI at all. However, the adjusted odds ratio at low selenium was increased by a factor 2.6 compared to high selenium status, in subjects with low adipose tissue vitamin E. When compared to the category with high levels of both selenium and vitamin E, there was no increased risk of MI at the combined low selenium and low vitamin E levels.

To avoid changes in selenium status due to previous disease, the cases in this study were subjects presenting acute, first-time myocardial infarction. When subjects with a history of angina pectoris, who might have changed their diet previously, were excluded from data-analysis, the ORs increased somewhat in all quintiles of the selenium distribution.

Data collection was restricted to survivors of acute MI. It is conceivable that survival is associated with antioxidant status, independent of the role of antioxidants in the pathogenesis of MI. Indeed, the potential significance of free radical production in the process of myocardial injury and necrosis of reperfused tissue is recognized[32] and treatment with antioxidants may be a way to slow down or inhibit these processes. This would imply that fatal cases of MI may have lower levels of antioxidants than the survivors. Therefore, including them would have increased the difference with the control population and perhaps have led to stronger associations with low selenium status. If vitamin E were an important determinant of case fatality, this could partly explain the lack of association between vitamin E and MI in our study.

As the selection of controls is an important issue in the interpretation of the results, we evaluated whether source of controls might have affected our estimates. The classical risk factors – cigarette smoking, hypertension and diabetes – were consistently more prevalent in acute MI cases than controls. In our study, five centres used random samples of population registries. In some centres, population registries were inadequate or impossible to use because of legal restrictions (Russia, Spain). Hospital controls were used, for whom the diagnosis, according to current knowledge, did not affect and was not influenced by antioxidant status. To minimize potential bias if any one disease turns out to be related to exposure, controls were chosen from a variety of disease categories. If, however, a control disease would have been related to antioxidant status, it would most likely reduce antioxidant levels, and thus diminish the risk estimates for MI. Indeed, exclusion of centres using hospital controls or mixed methods of control recruitment increased the strength of association considerably. However, this is not necessarily caused by selection bias, but may also be due to centre-specific factors, interacting with the relation between selenium and MI. The use of friend/relative controls (in Norway) may lead to overmatching on lifestyle (including diet) exposures. This would also result in a decreased estimate of association between selenium and MI.

Toenail selenium represents an integrated measure of dietary intake over a period of 6-12 months[21], which will not be affected by the acute event of infarction. Major dietary sources are meat and cereal products. Apart from the diet, also smoking habits, gender and possibly age are predictors of toenail selenium levels[20,33]. Rich in vitamin E are vegetable oils, nuts and green leafy vegetables. About 90% of the body's vitamin E is stored in adipose tissue.

Evidence for an association between serum selenium and risk of CHD has not been conclusive so far. Two cohort studies[3,5] have found an inverse association. Virtamo et al.[3] reported an increased risk of CHD at serum selenium levels below 45 μg/l, which is extremely low. The more recent study by Suadicani et al.[5] shows a RR of ischemic heart disease of 1.70 (95% CI 1.14-2.53) at levels below 1 μmol/l (± 79 μg/l). These studies confirmed an earlier report of an inverse association between serum selenium and CHD[4]. Several nested case-control studies have found no association between serum selenium and CHD[7-10], but this may be due to relatively small numbers of cases. Toenail selenium levels of MI cases were found to be significantly lower than in controls in a study in the Netherlands[34]; the multivariate OR for MI at the lowest toenail selenium level compared to the highest was 4.5 (95% CI 1.3-15.7). A more recent case-control study from New Zealand[6] reported an increased risk of MI at low selenium levels which was confined to smokers. In the light of our findings, this might be due to insufficient adjustment for smoking behavior. In a prospective study, Kok et al.[8] reported no interaction between serum selenium and serum vitamin E and risk of cardiovascular death. However, in a case-control study[35], plasma selenium (relative to polyunsaturated fatty acids) was significantly lower in patients with severe as opposed to mild atherosclerosis, but only at low plasma vitamin E status. No other epidemiological studies have addressed this possible interactive effect. In rabbits, an enhanced effect of selenium and vitamin E administered simultaneously was observed, on induced hyperlipidemia and atherosclerotic lesions[18].

The best characterized function of selenium is that as a constituent of the enzyme glutathione peroxidase (GSHPx), which catalyzes the removal of hydrogen peroxide and lipid peroxides. Lipid peroxidation may involve modification of LDL cholesterol within the arterial wall, damage of cell membranes, and activation of cyclooxygenase, leading to increased thromboxane synthesis and platelet aggregation[1,36,37]. The exact function of selenium, in GSHPx or seleno-proteins, in relation to other nutrients with antioxidant properties, has not yet been elucidated[13,14]. Experimental studies have provided evidence for overlapping, though not identical roles for selenium and vitamin E[14,16,17], especially in their effect on the arachidonic acid cascade. The results of the present study are in line with such an antioxidant function for selenium. Theoretically, selenium status may be an indicator of other dietary or lifestyle factors that affect risk of MI; such a confounding effect is not likely to have caused our results, since the major factors known to be associated with risk of MI and selenium status have been adjusted for

in the data analysis.

The biological relevance of adipose tissue vitamin E needs further evaluation. It may be an indicator of dietary intake, but may also reflect the status after vitamin E has been used in defense against oxidative stress. The low levels of adipose tissue vitamin E that have been observed for Jerusalem, where intake of polyunsaturated fats is high and concomitant intake of vitamin E is expected to be high as well, support the latter view. In that case, lower vitamin E status in controls compared to cases could represent more effective antioxidant defense through vitamin E in the stage before storage in adipose tissue takes place.

In conclusion, our results suggest a protective role for selenium in the etiology of myocardial infarction, in men with low vitamin E status. Additional information about a possible causal relationship should be provided by preventive trials, to support the clinical relevance of this finding.

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Association between antioxidants and acute myocardial infarction depends on poly-unsaturated fatty acid status: the EURAMIC study

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Abstract

Dietary antioxidants may play a role in the prevention of coronary heart disease, by inhibiting free radical-induced lipid peroxidation. Experiments indicate that dietary polyunsaturated fatty acids (PUFA) should also be considered, as they are susceptible to peroxidation.

The combined association between diet-derived antioxidants and PUFA with acute myocardial infarction (MI) was investigated in a multi-centre case-control study in eight European countries and Israel, including 674 patients and 725 control subjects. Fatty acid composition, α-tocopherol and β-carotene were determined in adipose tissue, selenium in toenails, as time-integrated measures of intake. For α-tocopherol no association with MI was observed at any level of PUFA. The overall odds ratio (OR) for low (10-percentile) versus high (90-percentile) β-carotene, adjusted for age, centre, smoking and body mass index was 1.98 (95% CI 1.39-2.82). The strength of this inverse association with MI was dependent on PUFA levels (tertiles): at low PUFA, the OR for low versus high β-carotene was 1.79 (95% CI 0.98-3.25), at medium PUFA the OR was 1.76 (95% CI 1.00-3.11) and at high PUFA 3.47 (95% CI 1.93-6.24). For selenium increased risk was observed only at the lowest PUFA tertile (OR 2.49, 95% CI 1.22-5.09). This interaction between selenium and PUFA was not significant and may at least partly be explained by a higher proportion of smokers at the low PUFA level.

These findings support the hypothesis that β-carotene plays a role in the protection of PUFA against oxidation, and subsequently in the protection

Submitted

against myocardial infarction. No evidence was found that α-tocopherol or selenium may protect against MI at any level of PUFA intake.

Introduction

Experimental as well as epidemiological studies have accumulated evidence that antioxidants may play a part in the prevention of cardiovascular disease[1]. Micronutrients such as vitamin E, β-carotene and selenium may protect the LDL cholesterol particle against oxidation in the vascular subendothelium[2] and thereby prevent the enhanced uptake of cholesterol by macrophages, leading to the formation of foam cells[3]. In vitro studies have shown that the resistance of LDL against oxidation is dependent not only on antioxidant levels[4,5], but also on its fatty acid composition. Increasing the ratio of oleic to linoleic acid in LDL by dietary intervention reduces the uptake of the LDL by macrophages in vitro[6,7].

Several studies[8-11] have shown an inverse association of dietary antioxidant intake or plasma or serum concentrations with risk of cardiovascular disease. However, other studies[12-15] did not find such an association. This lack of association may be attributed to relatively high levels of antioxidants in these populations or to deterioration of vitamin E during prolonged storage. It is also possible, that the balance between antioxidants and polyunsaturates is the more important factor. Kok et al.[16] have reported lower selenium to polyunsaturated fatty acid ratios in patients with severe versus mild atherosclerosis.

Here we present the results of a case-control study on the combined association of antioxidants and polyunsaturated fatty acids with the risk of acute myocardial infarction in nine different countries, with varying dietary habits. Concentrations of α-tocopherol, β-carotene and fatty acids in subcutaneous adipose tissue and selenium in toenails were compared between 674 cases with acute myocardial infarction and 725 controls without a history of infarction.

Methods

Design and subjects

Eligible subjects in this study, conducted in 1991-1992, were men under 70 years of age, from ten study centres in nine countries. They were native residents of respective countries, with stable dietary patterns in the past year

i.e., no changes in the use of dietary supplements containing α-tocopherol, β-carotene, or selenium; no new or altered dietary prescription or advice for health reasons and no weight loss over 5 kg in the past year. Cases were subjects diagnosed with a first acute myocardial infarction (MI) (ICD-code 410) - confirmed by specific abnormalities on the ECG and elevated enzyme levels - and admitted within 24 hours of manifesting symptoms. They were recruited from the coronary care unit of participating hospitals.

Controls were subjects without history of myocardial infarction and frequency-matched for age according to 5-year intervals. The controls were recruited from the population in the catchment area, from population registers or other appropriate sources. In some centres, hospital controls were selected, with diseases that were not known to be associated with antioxidant status (renal colic, non-infectious prostatism, acute appendicitis, non-infectious otic pathology, hernia, volvulus, rectal/anal pathology (except cancer, haemorrhoids, chronic infections)). Where it was thought that low response rates from population based samples would affect the internal validity, controls were selected from the catchment area via a random sample by the patient's general practitioner (Netherlands) or by inviting friends and relatives of the case (Norway).

Excluded from both groups were subjects with a history of treatment for alcohol or drug abuse, those diagnosed with major psychiatric disorders that would interfere with their ability to give informed consent, and institutionalized subjects. Informed consent was obtained from all subjects and the study protocol was approved the institutional committees on human experimentation. The study design has been reported in detail elsewhere[17].

Biochemical analyses

Subcutaneous adipose tissue was taken from the buttock by needle aspiration[18]. In cases, the adipose sample was taken within seven days of hospital admission. Samples were stored at −70°C; handling of the samples has been described previously. In short, samples were analyzed in a central laboratory. Concentrations of α-tocopherol and β-carotene in adipose tissue were determined by reverse-phase HPLC[19] and spectro-photometric detection. The coefficient of variation for the analysis of β-carotene and α-tocopherol was 7% (at mean values of 2.1 μg/g and 84 μg/g in the quality control samples, respectively). Detection limits were 0.02 μg/g for β-carotene and 2 μg/g for α-tocopherol, at mean sample weight (29 mg). Vitamin concentration was expressed in μg/g of total fatty acids.

The fatty acids were assayed centrally at the National Public Health

Institute, Helsinki, Finland. The saponified sample was acidified with HCl and the free fatty acids were extracted with hexane and methylated with acidic methanol. Fatty acid composition was determined by a gas chromatograph (HNU Nordion Oy, Finland, HRCG 412) with a 60 m long SP-2380 column, I.D. 0.32 mm, phase layer 0.20 μm with split injector and helium as carrier gas. Fatty acid peaks from C12:0 (fatty acid with 12 carbon atoms:0 double bonds) to C22:6 were identified by an SC-workstation (Sunicom Oy, Finland) in a temperature-programmed run. Polyunsaturated fatty acids (PUFA) include linoleic acid (C18:2), alpha-linolenic acid (C18:3) and arachidonic acid (C20:4); monounsaturated fatty acids (MUFA) are C14:1, palmitoleic acid (C16:1), oleic acid (C18:1) and eicosaenoic acid (C20:1); saturated fatty acids (SFA) include lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). All fatty acids are expressed as proportion of total fatty acids. Minor fatty acids are not included in these aggregated categories; therefore, proportions of PUFA, MUFA and SFA do not add up to 100%.

Serum total cholesterol levels were determined enzymatically (Kits of Boehringer-Mannheim GmbH, Mannheim, Germany) in Helsinki (Finland), HDL cholesterol after precipitation with dextran sulphate and magnesium chloride. LDL cholesterol was calculated by the Friedewald formula. In cases, cholesterol concentrations were inversely related to time from onset of symptoms (Pearson $r = -0.18$, $p < 0.001$), which may be due to the effect of acute MI on serum cholesterol.

Toenail clippings were collected, within 8 weeks of inclusion in the study, and stored in small plastic bags at room temperature. Nails were cleaned before clipping. The selenium content of the toenails was assessed by instrumental neutron activation analysis of the metastable-selenium-77 isotope (Interfaculty Reactor Institute (IRI), Delft University, Netherlands)[20]. Samples were irradiated for 17 seconds, in a thermal flux of 1.2×10^{13} neutrons.s^{-1}.cm^{-2}. After a decay time of 20 seconds, gamma radiation of ^{77m}Se was measured for 60 seconds. Mean level of selenium ($n = 87$) in certified bovine liver reference material (NBS-1577A) was 0.76 ± 0.04 ppm, against a certified value of 0.80 ± 0.04 ppm. Reproducibility of measurement was evaluated by repeated analysis of 19 samples; the coefficient of variation was 5%.

Data analysis

Questionnaire data were available for 1499 eligible subjects. Vitamin results were unavailable (no adipose tissue in adaptor) for 34 subjects (30 cases, 4 controls). Extreme values, caused by measurement error due to very small

sample size, were excluded (n = 38 cases, 28 controls), leaving 674 cases and 725 controls for data analysis. With respect to selenium, for 79 out of 1499 subjects no toenail samples had been collected (55 cases and 24 controls); another 9 subjects (4 cases, 5 controls) had values below the detection level, handled as missing values as well. Of the remaining subjects, 28 cases and 4 controls had provided no biopsy, leaving 655 cases and 724 controls for the data analysis of selenium in combination with fatty acid composition.

Crude means for major risk factors and potential confounders were computed; the difference between cases and controls was tested with Student's t-test and chi-square analysis. Mean centre-adjusted levels of antioxidants and fatty acids (as proportion of total fatty acids) were calculated for cases and controls. As the distribution of PUFA and MUFA was skewed, the $\log_e$-transformed values were used. Potential confounders of the association between the antioxidants and MI were identified with stratified analysis. Pearson correlations between antioxidants and fatty acids were calculated. The odds ratio of MI was estimated for the 10th-percentile (p10) level of the antioxidants, relative to the 90th-percentile (p90) level, based on the distribution among controls, by multiple logistic regression, with maximum likelihood estimation of the regression coefficients. This "continuous" odds ratio was preferred to calculating odds ratios in quintiles of the antioxidant distribution to avoid irrelevant fluctuations of odds ratios which may occur due to small numbers when examining interactions. The fitted model included age, centre, smoking, and body mass index (BMI), for the relationship with α-tocopherol and β-carotene; the model for selenium and MI included only age, centre and smoking. Smoking categories included never smokers, ex-smokers, pipe/cigar smokers, and current cigarette smokers, the last category further divided in subjects smoking less than 6, 6-10, 11-20, and more than 20 cigarettes per day. The significance of the interaction of antioxidants and fatty acid composition was tested (with the loglikelihood-ratio test) by including an interaction term of the continuous, $\log_e$-transformed variables in the logistic regression model. Subsequently, risk of MI of the p10 compared to the p90 level of the antioxidants was estimated at tertiles of PUFA, MUFA and SFA (in nine separate models, for each combination of the three antioxidants and the three types of fatty acids).

Results

The prevalence of risk factors of CHD among cases and controls is

summarized in Table 1. Age, serum total and HDL cholesterol, history of hypertension, smoking, angina pectoris, diabetes mellitus, family history for CHD, socio-economic status and BMI differed significantly between the groups. The reduced concentrations of serum cholesterol in cases may reflect the cholesterol-lowering effect of the acute event.

Table 1. Prevalence of risk factors for cardiovascular disease among MI cases and controls.

	Mean (SD)		
	Cases n = 674	Controls n=725	
Age	54.7 (9.0)	53.4 (9.1)	**
Total serum cholesterol	5.4 (1.1)	5.6 (1.1)	*
LDL cholesterol	3.7 (1.0)	3.7 (1.0)	n.s.
HDL cholesterol	0.95 (0.3)	1.04 (0.4)	***
Current smokers (%)	59	33	***
Cigarettes/day (among smokers)	25 (15)	18 (12)	***
History of hypertension (%)	26	17	***
Family history of CHD (%)	58	45	***
Diabetes (%)	7.6	3.6	**
Angina pectoris (%)	14	5	***
Alcohol use (%)	79	82	n.s.
Low social class (%)	25	20	**
BMI	26.5 (3.8)	26.0 (3.5)	*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Mean centre-adjusted values for the antioxidants and the fatty acids are shown in Table 2 for cases and controls. β-Carotene and selenium concentrations are significantly lower in cases as compared to controls. Mean proportions of the saturated fatty acids (SFA) myristic acid (C14:0) and stearic acid (C18:0) are 6% and 7% lower in cases, respectively; monounsaturated fatty acids (MUFA), in particular palmitoleic acid (C16:1) and C18:1 (n-7) are higher in cases (both 6%), whereas polyunsaturated fatty acids (PUFA) are similar for cases and controls.

We examined the relationship between antioxidant concentrations and CHD risk factors in the controls. For β-carotene, negative associations were observed for the number of cigarettes smoked per day ($r = -0.17$, $p < 0.05$) and for BMI ($r = -0.36$, $p < 0.001$).

Table 2. Centre-adjusted means of antioxidants (μg/g) and fatty acids (% of total fatty acids) in adipose tissue by disease status.

	Cases	Controls	Difference	95% CI
α-tocopherol	192	193	−1	(−14 - 13)
β-carotene	0.34	0.42	−0.08	(−0.11 - −0.05)
selenium (in toenails)	0.575	0.600	−0.025	(−0.038 - −0.012)
saturated fatty acids†	28.4	29.0	−0.6	(−1.0 - −0.3)
C12:0	0.32	0.36	−0.04	(0.06 - −0.03)
C14:0	2.71	2.89	−0.18	(−0.24 - −0.11)
C16:0	21.5	21.6	−0.1	(−0.4 - 0.1)
C18:0	3.86	4.16	−0.30	(−0.40 - −0.19)
monounsaturated fatty acids†	52.7	52.1	0.6	(0.1 - 1.0)
C14:1	0.36	0.37	−0.01	(−0.02 - 0.01)
C16:1 n-7	5.86	5.53	0.33	(0.16 - 0.50)
C18:1 n-9	43.8	43.7	0.1	(−0.2 - 0.5)
C18:1 n-7	2.25	2.13	0.12	(0.07 - 0.17)
C20:1 n-9	0.68	0.69	−0.01	(−0.02 - 0.01)
polyunsaturated fatty acids†	14.2	14.2	0.0	(−0.3 - 0.4)
C18:2 n-6	13.0	12.9	0.1	(−0.2 - 0.5)
C18:3 n-3	0.77	0.80	−0.03	(0.05 - 0.00)
C20:4 n-6	0.42	0.41	0.01	(0.00 - 0.02)

† Proportions do not add up to 100%, due to exclusion of some minor fatty acids.

Subjects with a positive family history of CHD and subjects with higher socio-economic status had significantly higher β-carotene levels ($p < 0.001$). α-Tocopherol concentration was also negatively associated with BMI ($r = -0.12$, $p < 0.001$) and number of cigarettes smoked per day ($r = -0.13$, $p = 0.06$) and positively with socio-economic status ($p < 0.05$). There were no significant correlations with other risk factors. With respect to selenium, subjects who currently smoked cigarettes or had a negative family history of CHD, had significantly lower levels than non-smokers ($p < 0.001$). Other classical risk factors were not associated with selenium concentration. The association between antioxidants and proportion of fatty acids in adipose tissue is shown in Table 3.

To evaluate whether an association between antioxidants and MI

depends on the fatty acid composition of the adipose tissue, we tested the significance of the interaction for each of the fatty acid categories (PUFA, MUFA and SFA) with each antioxidant (β-carotene, α-tocopherol and selenium).

Table 3. Correlation between antioxidant concentrations and proportion of fatty acids in adipose tissue (Pearson r).

Fatty acids	α-tocopherol	β-carotene	toenail selenium
SFA	0.10 *	0.32 **	0.12 **
MUFA	–0.04	–0.21 **	–0.41 **
PUFA	0.03	–0.05	0.30 **

* $p < 0.01$; ** $p < 0.001$.

These parameters were included in a logistic regression model as continuous variables, with adjustment for age, centre, smoking and BMI (i.e. nine separate models for all possible interactions). A significant interaction was observed for α-tocopherol and SFA ($\chi^2 = 6.68$, $p < 0.01$). The interactions for β-carotene and PUFA ($\chi^2 = 3.20$, $p = 0.07$) and for selenium and SFA ($\chi^2 = 3.51$, $p = 0.06$) were borderline significant. Subsequently, the multivariate risk of MI at low (10th-percentile value) versus high (90th-percentile value) antioxidant concentrations was calculated, overall and for tertiles of PUFA, MUFA and SFA (Table 4). For β-carotene the association with MI appeared to be dependent on fatty acid composition. At all levels of PUFA an increased risk for low β-carotene as compared to high β-carotene was observed. The highest odds ratios were seen at the highest PUFA level (OR for low vs high β-carotene: 3.47, 95% CI 1.93-6.24) and at the lowest MUFA and SFA levels. Since PUFA, MUFA and SFA are expressed as proportions of total fatty acids, this interrelation is to be expected. Similar calculations were done for tertiles of the ratio between monounsaturated and polyunsaturated fatty acids (m/p-ratio). The OR for MI at low versus high β-carotene at low, medium and high m/p-ratio was 2.86 (95% CI 1.61-5.11), 2.76 and 1.38, respectively. For α-tocopherol, a significant positive association with MI was observed at the low SFA level, and a (non-significant) negative association at the high SFA level. This relationship was not reflected in the associations at different levels of PUFA or MUFA. For

selenium, an inverse association with MI was seen at low PUFA (OR 2.95, 95% CI 1.22-5.09) and high SFA levels (OR 1.75, 95% CI 0.89-3.42).

The association between β-carotene and MI at different levels of PUFA was recomputed, excluding persons with angina pectoris; the same trend was observed (ORs were 1.46, 2.03 and 3.20 for low, medium and high PUFA tertiles, respectively). To evaluate the contribution of centres with very high PUFA (Israel) or MUFA (Spain) levels to the observed interaction between PUFA and β-carotene, ORs were also computed with exclusion of these centres, which did not considerably change the estimates (results not shown).

Table 4. Risk of myocardial infarction at low (10-percentile) compared to high (90-percentile) levels of antioxidants, at different fatty acid levels.

	β-carotene	α-tocopherol	Selenium
	OR[†] (95% CI)	OR[‡] (95% CI)	OR[¶] (95% CI)
Overall	1.98 (1.39-2.82)	0.87 (0.67-1.12)	1.12 (0.75-1.68)
Polyunsaturated fatty acids[§]			
Low	1.79 (0.98-3.25)	0.77 (0.47-1.25)	2.49 (1.22-5.09)
Medium	1.76 (1.00-3.11)	1.23 (0.79-1.89)	0.76 (0.41-1.41)
High	3.47 (1.93-6.24)	0.95 (0.61-1.48)	1.11 (0.61-2.06)
Monounsaturated fatty acids[#]			
Low	2.68 (1.49-4.84)	1.07 (0.68-1.69)	1.00 (0.54-1.83)
Medium	1.89 (1.02-3.47)	1.12 (0.71-1.77)	0.99 (0.54-1.85)
High	1.66 (0.94-2.96)	0.68 (0.45-1.02)	1.21 (0.63-2.31)
Saturated fatty acids[@]			
Low	2.51 (1.39-4.56)	0.63 (0.41-0.96)	0.84 (0.46-1.53)
Medium	1.63 (0.87-3.03)	0.89 (0.60-1.33)	0.97 (0.55-1.73)
High	2.15 (1.19-3.90)	1.44 (0.86-2.42)	1.75 (0.89-3.42)

[†] 10-percentile: 0.15 μg/g, 90-percentile: 1.11 μg/g. Adjusted for age, centre, smoking and BMI.
[‡] 10-percentile: 103 μg/g, 90-percentile: 385 μg/g. Adjusted for age, centre, smoking and BMI.
[¶] 10-percentile: 0.431 μg/g, 90-percentile: 0.861 μg/g. Adjusted for age, centre and smoking.
[§] Cutpoints for tertiles: 12.32 %, 15.60 %.
[#] Cutpoints for tertiles: 50.27 %, 54.53 %.
[@] Cutpoints for tertiles: 26.86 %, 30.53 %.

The association of proportion of PUFA with risk of MI, without accounting for antioxidant levels, was examined in a logistic regression model including tertiles of PUFA, with adjustment for age, centre, smoking and BMI. A positive association was observed, with an OR of 1.26 (95% CI

0.92-1.71) in the middle tertile as compared to the lowest, and 1.76 (95% CI 1.24-2.50) for the highest tertile.

The most important predictor of MI in all multivariate models was smoking. The odds ratios for the smoking categories, in the model estimating coefficients for the interaction between β-carotene and PUFA, increased from 1.72 (95% CI 1.20-2.45) in ex-smokers and 1.74 (95% CI 0.86-3.51) in subjects smoking less than 6 cigarettes per day, to 9.63 (95% CI 6.03-15.4) in subjects smoking over 20 cigarettes daily, compared to those who had never smoked. Smoking was also associated with PUFA; the mean proportion of PUFA was 14.4% in non-smokers and 13.7% in smokers ($p = 0.06$). To test whether the interaction between selenium and PUFA was perhaps the reflection of an interaction with smoking, we compared the fit of a no-interaction model with the fit of a model with the selenium-PUFA interaction (both as continuous variables) and with a model including the selenium-smoking interaction (smoking in 7 categories). The relative improvement with the smoking interaction was larger ($\chi^2 = 12.30$ [6 degrees of freedom], $p = 0.06$) than with the PUFA interaction ($\chi^2 = 1.64$ [1 degree of freedom], $p = 0.20$). A similar approach was used for β-carotene: improvement of the model with the β-carotene-PUFA interaction was, as said before, borderline significant ($\chi^2 = 3.20$, $p = 0.07$); an interaction of β-carotene and smoking did not improve the model ($\chi^2 = 2.30$, $p = 0.89$).

Discussion

In this multi-centre case-control study, the strongest association of low adipose tissue β-carotene with increased risk of MI was observed at simultaneous high proportion of PUFA in adipose tissue. Due to the mathematical relation that exists between proportions of different types of fatty acids, opposite results were seen for levels of MUFA and SFA. Since the correlation between adipose tissue levels and dietary intake is best for PUFA[21], levels of MUFA and SFA may be considered derived values, not directly related to intake. For α-tocopherol no association with MI was observed, at neither of the PUFA levels. An inverse association of selenium with MI was observed at low PUFA and high SFA levels, which may be attributed to the larger proportion of smokers in the low PUFA category.

To avoid changes in antioxidant status due to previous disease, the cases in this study were subjects presenting acute, first-time myocardial infarction. Excluding subjects with a history of angina pectoris, who might have

changed their diet previously, did not change the overall trend for the β-carotene-PUFA interaction. Hospital controls, or friends and relatives, in some of the participating centres, might not have represented the distribution of the antioxidant status in the population from which the cases had originated, although due care was taken to avoid such a bias. If, however, a control disease would have been related to antioxidant status, it is most likely to reduce the antioxidant status, and thus diminish the risk estimates for MI. The use of friend/relative controls may lead to overmatching on lifestyle (including diet) exposures. This would also result in a decreased estimate of association between antioxidants and MI, thus a conservative bias. The classical risk factors - cigarette smoking, hypertension and diabetes - were consistently more prevalent in acute MI cases than controls.

Smoking habits and BMI were found to be confounders of the relationship between β-carotene and MI, and smoking habits alone for selenium and MI; therefore, we adjusted for these factors in the multivariate analysis. At low PUFA levels we observed an inverse association of selenium and MI. As smoking is also associated with PUFA status (lower PUFA in smokers compared to non-smokers), the interaction between selenium and PUFA may also be explained by an interaction between selenium and smoking, although we cannot say to what extent. For β-carotene, the differential risk of MI at different proportions of PUFA could not be explained by an interaction with smoking. Even if there were such an interaction, it would affect the odds ratios in the opposite way, as observed for selenium; better adjustment for smoking could only enhance our findings for β-carotene and PUFA. The interaction between α-tocopherol and SFA cannot easily be interpreted, since it is not reflected in any interaction with PUFA. Adipose tissue SFA in itself have a poor correlation with dietary fat intake.

Levels of β-carotene, α-tocopherol, selenium and fatty acid composition were determined in a central laboratory. Selenium in toenails has been shown to be a time-integrated measure of selenium intake[22]. For adipose tissue β-carotene and α-tocopherol, a relationship with intake has been shown, but it is not very strong[23]. However, most of the body's α-tocopherol is stored in the adipose tissue[24]. The fatty acid composition of adipose tissue, especially of essential fatty acids[21], has been shown to be a reliable indicator of dietary intake over a longer period of time. The absence of a relation between adipose PUFA and α-tocopherol is surprising, in view of their common food sources.

It should be remarked that the overall findings of an inverse association

of β-carotene with MI, especially at high PUFA levels, need not necessarily hold for all the populations contributing to this study. The number of subjects in the different centres are too small to allow these calculations for the separate centres, but the main conclusions did not change when the centres with extreme PUFA and MUFA levels (Israel and Spain) were excluded from analysis.

Experimental studies indicate that the balance between antioxidant status, oxidative stress and PUFA as the main substrate for oxidation, may determine the amount of damage to cells and tissues and eventually the occurrence of disease. The oxidation of LDL is thought to be a major factor in atherogenesis, mainly by causing an increased uptake of lipids in the macrophages in the arterial wall and by its cytotoxic effects on the endothelial cells[3]. Increasing the amount of vitamin E in the LDL particle by oral supplementation increases the resistance against in vitro oxidation[4,5]. However, the initial concentration of vitamin E in the LDL is not related to oxidation resistance[25,26]; this has been attributed to other components of the LDL, such as other antioxidants, but also to the fatty acid composition. Long-term supplementation with β-carotene resulted in increased β-carotene levels in the LDL, but these enriched LDL particles were not more resistant to in vitro oxidation[4,5]. Oxidation of the LDL results in the formation of hydroxylated derivatives of both oleic and linoleic acid[27]. However, a more modest increase in amounts of hydroxy derivatives is seen for oleic acid, as compared to linoleic acid[27]. Moreover, diets rich in oleic acid relative to PUFA, reduce the uptake of LDL by macrophages[6,7,28].

There is also a growing body of epidemiological evidence for an inverse relationship between antioxidant nutrients and CHD-risk[8-11,29,30]. However, not all studies have found this association[12-15]. For vitamin E, possibly only very high intake through supplementation is associated with decreased risk of CHD[8]. The findings for β-carotene indicate an inverse association at normal dietary intake levels. Population-based studies have scarcely addressed the combined effect of antioxidants and dietary fatty acids on CHD risk. Kok et al.[16] observed lower selenium to PUFA ratios in cases with severe versus mild coronary atherosclerosis, but these results were not adjusted for smoking status. The ratio of α-tocopherol to PUFA did not differ between these groups. Riemersma[9] reported no significant interaction between adipose linoleic acid and plasma vitamin E in the relationship with angina pectoris. This is in agreement with our findings for α-tocopherol. The interaction between β-carotene and PUFA has not been addressed in other studies, to our knowledge. As Reaven et al.[5] have remarked, from the fact that β-carotene

does not confer direct protection to the LDL in in vitro experiments, it should not be concluded that it has no role as an antioxidant in the prevention of coronary heart disease. The oxidation of LDL by different types of cells in the artery wall may well be influenced by the β-carotene content of these cells. Our finding of an interaction between β-carotene and PUFA supports such an alternative role of β-carotene as an antioxidant, whereas the protective role of α-tocopherol may be less important at normal dietary intake levels in the in vivo situation.

In conclusion, the association of low β-carotene levels in adipose tissue with increased risk of MI is modified by adipose tissue PUFA levels. At high PUFA, the risk of MI of low compared to high β-carotene status, is twice as high as at low PUFA levels. Since adipose tissue fatty acid composition and β-carotene concentration are reported to be indicators of long-term dietary intake, it may be concluded that high intake of PUFA may increase the risk of MI when β-carotene intake is inadequate (supposedly to protect the PUFA against oxidation). Low β-carotene intake may increase the risk of MI in men, particularly when they consume large amounts of PUFA. Increased consumption of yellow fruits and green leafy vegetables may improve the CHD risk-profile of middle-aged men. In the evaluation of PUFA, relative to MUFA and SFA, both the susceptibility to oxidative stress and the cholesterol-lowering potential should be taken into account. It is too early to make firm recommendations in favour of MUFA over PUFA on the basis of available evidence.

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Relationship between antioxidant vitamins in adipose tissue, plasma and diet

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Abstract

To evaluate fat-soluble vitamin concentrations in adipose tissue as biomarkers of intake, estimates of usual intake of β-carotene, total vitamin A and α-tocopherol assessed by food frequency questionnaire, were compared with plasma and adipose tissue β-carotene, retinol and α-tocopherol concentrations, respectively. Data were collected in 1992 for 38 male and 47 female healthy, non-smoking volunteers, aged 50-70. For α-tocopherol a significant age- and sex-adjusted partial correlation ($r = 0.24$, $p < 0.05$) was observed between adipose tissue levels and intake, which was mainly present in women ($r = 0.30$). For β-carotene, the partial r was 0.20 ($r = 0.30$ in men, 0.12 in women). Adipose tissue retinol did not reflect intake (partial $r = 0.08$). Correlations of adipose tissue vitamin levels with plasma vitamin levels were overall higher ($r = 0.34$ for α-tocopherol, $r = 0.56$ for β-carotene, $r = 0.17$ for retinol) than correlations with intake. Plasma concentrations of α-tocopherol, β-carotene and retinol were not associated with dietary intake (partial $r = 0.05$, 0.17 and −0.12 respectively). Pearson correlations of repeated measurements in adipose tissue (after 4 months) were 0.24 for retinol, 0.50 for β-carotene and 0.78 for α-tocopherol. Adipose tissue β-carotene was shown to increase 6-fold after 6 months supplementation with 30 mg β-carotene daily. Although gender differences may exist, it is concluded that, especially in case-control studies, adipose tissue vitamin concentrations may be preferable to plasma levels.

Introduction

Antioxidant vitamins may have an important role in the prevention of certain

Submitted

types of cancer and cardiovascular disease[1,2]. In the epidemiological studies, antioxidant intake is often assessed by means of a food frequency questionnaire. In case-control studies, however, this type of assessment is liable to differential recall by cases and controls and therefore may bias the outcome of the study; in multi-country studies, important to achieve larger ranges in intake, diet questionnaires are difficult to standardize. To overcome these problems, biomarkers of intake, e.g. plasma β-carotene, may be used as an alternative to diet assessment. However, dietary intake is not the only determinant of biomarker status. Antioxidant levels in blood or tissue are affected by genetic and lifestyle factors, as well as intake of other nutrients. In case-control studies, a useful biomarker of intake should be related to intake over a relatively long period of time, as the onset of chronic diseases is usually related to long-term exposures. Levels of vitamin E and β-carotene in plasma are related to intake, but represent a relatively short time period. To assess fatty acid composition of the diet, especially for polyunsaturated fats, the composition of adipose tissue has been shown to be a reliable indicator[3,4]. As vitamin E, β-carotene and retinol are fat-soluble compounds, which are stored in subcutaneous adipose tissue, concentrations in adipose tissue may be a better indicator of intake than plasma levels are. Information on the relationship between intake and adipose tissue levels of these vitamins in free-living human populations is very limited[5,6].

Therefore, we studied the relationship between intake, plasma and adipose tissue levels of vitamin E, β-carotene and retinol; linoleic acid content was included as well, to provide an independent means of comparison with other studies. The interrelation of these parameters was studied in a cross-sectional design. Moreover, reproducibility was assessed by repeated measurement and for β-carotene the tissue response to supplementation was examined.

Materials and methods

Study population

Subjects in this study were men and women aged 50-70 years, recruited by an advertisement in a local newspaper. All 85 volunteers (38 men and 47 women) were apparently healthy non-smokers, did not use vitamin supplements or prescribed medication, had not lost weight in excess of 5 kg and had had stable food habits during the previous year. The protocol was approved by an independent medical-ethical review board.

Design

A group of 30 men and 26 women (group 1) was invited to our institute for two visits, 4 months apart (in May and September). Both times a fat aspirate from the gluteal region and a blood sample were obtained; a food frequency questionnaire was taken home and returned by mail. Alcohol intake was assessed with a separate questionnaire during the first visit, and anthropometric measures were taken. A second group (8 men, 21 women) was asked to take two capsules with β-carotene (15 mg each) daily, for a period of 6 months (from April to October), before the second visit. The same data were collected as in the first group. Data for group 1 were used to assess reproducibility of measurements. Results from group 2 informed us on the responsiveness of adipose tissue concentrations to dietary intake of β-carotene. Data from the first visit of both groups were used to assess correlations between dietary intake, plasma and adipose tissue concentrations.

β-Carotene supplementation

At their first visit, subjects in group 2 were given instructions in writing, to take two β-carotene capsules daily with their main meal. The capsules used were commercially available (Lamberts Natural Beta Carotene, supplied by Adviesbureau Orthomoleculaire Voeding Den Haag) and contained 15 mg β-carotene, encapsulated with gelatine, glycerine, soya bean oil and water. Analysis in our lab indicated a mean value of 17.8 mg β-carotene per capsule. Participants received capsules in two-monthly batches and were instructed to store them in a cool, dry place and protected from light.

Fat aspirate and blood collection

Subcutaneous fat aspirates were obtained by a needle biopsy from the lateral buttock, as described by Beynen and Katan[7], using a 16-gauge needle, attached to a plastic container in which the tissue (mean 39 ± 15 mg) was collected by connecting a vacuum tube. The plastic container was immediately put on dry ice and stored at −80°C until analysis.

A venous blood sample was drawn in a 10 ml heparin tube from nonfasting participants and refrigerated until plasma was separated. Blood was centrifuged (10 min at 1500 g) within 2 hours and plasma was stored at −80°C.

Biochemical analyses

Samples taken at the first and second visit for each subject were determined in the same run. Concentrations of retinol, β-carotene and

α-tocopherol in adipose tissue and plasma were determined by reverse-phase HPLC[8,9] and spectrophotometric detection. Samples were protected from light during the analysis. The adipose tissue sample was saponified, and quantitatively split for vitamin and fatty acid determination. The coefficients of variation for the analysis of retinol, β-carotene and α-tocopherol in adipose tissue were 4.3%, 7.1% and 4.9% (at mean values of 4.66 μg/g, 2.36 μg/g and 85.3 μg/g respectively, in the quality control samples). For determination in plasma, coefficients of variation were 4.3% for retinol (mean 1.78 μmol/l), 7.3% for β-carotene (mean 0.26 μmol/l) and 3.2% for α-tocopherol (mean 25.4 μmol/l). Vitamin concentrations in adipose tissue were expressed in μg/g of total fatty acids (mean fatty acid content of aspirates 14.5 ± 9.1 mg).

Fatty acid content was assessed by GLC in an aliquot of the same extract as the vitamins, adding heptadecanoic acid (C17:0) as an internal standard to the sample before saponification. Linoleic acid (C18:2) was quantitated as the percentage of the peak area of total fatty acids. The coefficient of variation for linoleic acid was 3.4%.

Total plasma cholesterol was enzymatically determined (CHOD-PAP method, Boehringer, Mannheim, Germany).

Food frequency questionnaire

Usual dietary intake of retinol, β-carotene and vitamin E during the previous year was assessed by a 95-item self-completed semi-quantitative food frequency questionnaire (FFQ), adapted from a questionnaire on retinol and β-carotene intake, developed and validated by Stiggelbout et al.[10] Additional items representing vitamin E intake were selected on the basis of a high vitamin E content and regular use according to the Dutch National Food Consumption Survey 1987[11]. Frequency of consumption was reported in eight categories, never, seldom, 1 day per month, 1 day per 2-3 weeks, 1-2 days per week, 3-4 days per week, 5-6 days per week, 7 days per week. Portion sizes were quantified by respondents in terms of household measures (slices, spoons, cups) to which standard weights were assigned. For some foods (e.g., milk and dairy products), individual portion sizes were calculated from the number of glasses obtained from one litre. Consumption of a number of vegetables and fruits was asked separately for summer and winter. The use of fats and oils was specified according to type and brand.

Questionnaires were entered and processed using an automated processing system. Completeness, credibility of reported number of servings and consistency in reported consumption frequencies were checked. If necessary, participants were contacted for further information. Data on mean

daily consumption of food products in grams were converted to nutrient intake using the computerized Dutch Food Composition Table for retinol equivalents and linoleic acid. Information on vitamin E and β-carotene content was mainly derived from recent analyses of Dutch food products[12], but did not provide complete coverage of all foods in the questionnaire (75% for vitamin E, 97% for β-carotene). Additional information was obtained from the English food composition table. Intake of linoleic acid was calculated, although the questionnaire was not specifically designed to measure this nutrient. However, since the important sources of linoleic acid and vitamin E are largely the same foods, the questionnaire is considered to measure linoleic acid well enough to obtain adequate ranking of subjects according to intake.

Statistical analysis

In the group of 56 non-supplemented subjects, the fat aspirate was missing for three persons at the first measurement. For two others the vitamin values were outside three standard deviations of the mean; these values were considered unreliable because of very little fat tissue in the fat aspirate and therefore were excluded. For three subjects, the FFQ was excluded from the analysis, because their food pattern largely differed from the usual Dutch diet, for which the questionnaire was designed. Three other subjects had missing plasma data for the second measurement. In the group of 29 supplemented subjects, three subjects had missing fat aspirate data for the first measurement. For the second measurement, aspirate data were missing for two subjects, plasma data for two other subjects, and FFQ data for one person.

Skewed distributions of all dietary, plasma and adipose nutrients, except for dietary retinol equivalents and relative intake of linoleic acid (percentage of total fat intake), were normalized by natural logarithm. All correlation analyses were performed on the $\log_e$-transformed data. In tables 1 and 3, untransformed mean levels are presented; in table 5, values are re-transformed from the loge-value, for ease of interpretation. Plasma α-tocopherol values were strongly correlated with total plasma cholesterol ($r = 0.64$, $p < 0.001$), but plasma β-carotene was not ($r = 0.15$, $p = 0.16$). Therefore, absolute plasma α-tocopherol values as well as the ratio of α-tocopherol to total plasma cholesterol are presented where necessary.

Mean and standard deviations of vitamin levels were calculated. Reproducibility of all three independent measurements (in diet, plasma, adipose tissue) was assessed by calculating Pearson correlation coefficients. Association between adipose tissue vitamin levels and age, sex, body mass

index, waist-hip ratio, alcohol intake and socioeconomic status was tested by linear regression analysis. The change in adipose tissue and plasma antioxidant concentrations in the suppleted group was adjusted for changes in the non-suppleted group with use of linear regression of the second measurement on group and first measurement. Simple Pearson and age- and sex-adjusted correlations between adipose tissue and plasma levels, and intake assessed by FFQ were calculated. To account for variability in the plasma and adipose tissue measurements, correlation coefficients were deattenuated, using the within- to between-person variance ratios[13], calculated with ANOVA from the repeated measurements in the non-supplemented group. The deattenuated correlation (r_t) is calculated from the observed correlation (r_o) as follows:

$r_t = r_o$ SQRT$\{(1 + \lambda_x/2)(1 + \lambda_y/2)\}$, where λ is the variance ratio, x are plasma measurements and y adipose tissue measurements.

Finally, age- and sex-adjusted mean dietary intake and plasma levels are presented across quartiles of adipose tissue concentrations. Tests for trend over quartiles were performed by assigning the median value of the quartile to each category. Analyses were performed with the BMDP software package.

Results

Characteristics of the study population at the start of the study are presented in Table 1. Men and women differed with respect to socio-economic status and waist-hip ratio. Usual intake of vitamin E ranged from 5.5 to 37.3 mg per day; intake of β-carotene varied from 0.64 to 4.05 mg. Plasma levels of α-tocopherol ranged from 20.1 to 55.6 μmol/l (tocopherol:cholesterol from 2.8-8.0), β-carotene from 0.09 to 4.7 μmol/l. Adipose tissue concentration of α-tocopherol varied 11-fold, between 87 and 950 μg/g; the range for β-carotene was about 20-fold, from 0.33 to 6.9 μg/g.

Pearson correlation coefficients were calculated for repeated measurements of vitamin and linoleic acid content in adipose tissue and plasma, and intake assessed by FFQ, in the non-supplemented group (Table 2). The highest reproducibility was found for plasma values. It should be noted that the correlation for cholesterol-standardized α-tocopherol is lower than for absolute levels of α-tocopherol. For adipose tissue, the reproducibility of retinol levels is low ($r = 0.24$). The highest correlation in adipose tissue is observed for α-tocopherol ($r = 0.78$, $p < 0.001$). Reproducibility of adipose tissue β-carotene values was considerably better

Reproducibility of adipose tissue β-carotene values was considerably better for women than for men (r = 0.62 and 0.39, respectively). The same could be said for linoleic acid content (r = 0.91 for women, r = 0.36 for men). In the smaller samples, containing below 10 mg fatty acids, the correlation for α-tocopherol was 0.69, for β-carotene only 0.21 (p = 0.3) and for linoleic acid 0.44 (p = 0.02); corresponding correlation coefficients in samples containing over 10 mg fatty acids were 0.90, 0.90 and 0.89, over the same range. These data point to larger analytical variation in smaller samples. Repeated assessment of intake by FFQ showed somewhat higher correlation coefficients for linoleic acid (r = 0.80) as compared to the vitamins.

Table 1. Characteristics of the study population, mean (± SD) dietary intake, plasma and adipose tissue levels of fat-soluble vitamins and linoleic acid among 38 men and 47 women in the Netherlands, 1992.

	Men (n = 38) mean ± SD	Women (n = 47) mean ± SD
Age	59.5 ± 6.3	58.3 ± 5.9
BMI (kg/m²)	24.4 ± 2.7	24.7 ± 3.3
Waist/hip ratio	0.90 ± 0.06	0.83 ± 0.10
Serum cholesterol (mmol/l)	5.9 ± 1.2	5.7 ± 0.9
Alcohol use (%)	95	94
Socioeconomic status (%)		
low	32	52
high	68	48
Dietary intake		
β-carotene (mg)	1.73 ± 0.66	1.75 ± 0.72
retinol (RE)	0.83 ± 0.23	0.79 ± 0.24
α-tocopherol (mg)	17.3 ± 6.5	13.1 ± 5.9
linoleic acid (% of total fat)	19.0 ± 7.0	13.8 ± 7.2
Plasma		
β-carotene (μmol/L)	0.41 ± 0.26	0.63 ± 0.67
retinol (μmol/L)	2.47 ± 0.59	2.11 ± 0.68
α-tocopherol (μmol/L)	35.5 ± 8.6	32.6 ± 7.4
α-tocopherol/cholesterol	6.0 ± 1.0	5.7 ± 1.0
Adipose tissue		
β-carotene (μg/g)	1.08 ± 0.66	1.81 ± 1.20
retinol (μg/g)	2.99 ± 3.08	2.35 ± 1.30
α-tocopherol (μg/g)	240 ± 106	281 ± 152
linoleic acid (%)	15.9 ± 4.8	13.0 ± 4.4

Table 2. Pearson correlations between repeated measurements of vitamin and linoleic acid content in adipose and plasma, and intake measured by FFQ: the Netherlands, 1992.

	Adipose			Plasma			FFQ		
	Men n = 26	Women n = 25	Overall n = 51	Men n = 28	Women n = 25	Overall n = 53	Men n = 29	Women n = 24	Overall n = 53
β-carotene	0.39*	0.62***	0.50***	0.90***	0.95***	0.93***	0.60***	0.74***	0.67***
retinol	0.24	0.27	0.24	0.53**	0.79***	0.74***	0.38*	0.47*	0.43
α-tocopherol	0.77***	0.86***	0.78***	0.82***	0.89***	0.86***	0.68***	0.55**	0.63***
α-tocopherol/cholesterol	-	-	-	0.72***	0.67***	0.69***	-	-	-
linoleic acid (%)	0.36	0.91***	0.58***	-	-	-	0.81***	0.76***	0.80***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

To evaluate whether an increased β-carotene intake resulted in enhanced adipose tissue β-carotene levels, concentrations of the vitamins in adipose and in plasma were compared before and after the 6-month supplementation period. The difference was corrected for any changes observed in the non-supplemented group (Table 3). In adipose tissue a sixfold increase of mean β-carotene levels was measured. In plasma we observed an (eightfold) increase in β-carotene as well, on average with 3.28 μmol/l (95% CI 2.75-3.81). For α-tocopherol and retinol, no change was seen. There appeared to be no correlation between the increase in plasma β-carotene and the increase in adipose tissue β-carotene ($r = 0.09$); however, when two extreme values were excluded, a significant correlation was observed ($r = 0.56$, $p < 0.005$).

Table 3. Effect of β-carotene supplemention on β-carotene, α-tocopherol and retinol levels in adipose tissue and plasma: the Netherlands, 1992.

Vitamin	Group	Baseline mean (SD)	End mean (SD)	Diff mean (SD)	Δ Diff	(95% CI)
Adipose (μg/g)†						
β-carotene	Suppl	1.47 (0.83)	9.31 (9.74)	7.84 (9.42)	7.56	(4.81 - 10.3)
	Ref	1.54 (1.18)	1.84 (2.45)	0.31 (2.43)		
α-tocopherol	Suppl	278 (134)	327 (155)	49 (135)	22	(–35 - 80)
	Ref	258 (138)	286 (184)	28 (114)		
retinol	Suppl	2.21 (1.25)	3.07 (2.12)	0.86 (2.27)	–0.00	(–1.76 - 1.76)
	Ref	2.81 (2.60)	3.07 (4.23)	0.26 (5.02)		
Plasma (μmol/l)‡						
β-carotene	Suppl	0.46 (0.28)	3.77 (2.01)	3.31 (1.95)	3.28	(2.75 - 3.81)
	Ref	0.58 (0.65)	0.63 (0.67)	0.04 (0.14)		
α-tocopherol	Suppl	34.2 (8.6)	35.0 (9.6)	0.8 (6.2)	–0.4	(–2.7 - 1.9)
	Ref	33.1 (7.3)	34.3 (8.3)	1.2 (4.2)		
retinol	Suppl	2.32 (0.79)	2.43 (0.98)	0.11 (0.70)	0.10	(–0.14 - 0.34)
	Ref	2.21 (0.59)	2.26 (0.50)	0.04 (0.46)		

† supplemented group: n = 25; reference group: n = 51.

‡ supplemented group: n = 27; reference group: n = 53.

The difference in the supplemented group, after correction, was 7.56 μg/g (95% CI 4.81-10.3). Adipose tissue α-tocopherol and retinol did not change

significantly.

The association of adipose tissue α-tocopherol and β-carotene with sex, age, socioeconomic class, alcohol intake, body mass index and waist-hip ratio was tested by linear regression analysis, using the first measurement for all subjects. None of these factors were associated with adipose α-tocopherol; β-carotene levels were significantly higher in women compared to men, and were inversely associated with waist-hip ratio ($p = 0.01$), but not with body mass index ($p = 0.25$). When sex was taken into account, waist-hip ratio was no longer associated with adipose β-carotene. The intake of vitamin E was significantly associated with the intake of β-carotene ($r = 0.34$, $p < 0.05$); as this may be caused by the association of both nutrients with energy intake, we also looked at correlation of vitamin-intake, relative to total fat intake. The significant association remained: $r = 0.45$ ($p < 0.001$). Plasma β-carotene and cholesterol-standardized plasma α-tocopherol were not significantly associated ($r = 0.06$), but adipose β-carotene and tocopherol were ($r = 0.29$, $p = 0.01$).

Correlations between measurements in adipose, plasma and FFQ are displayed in Table 4. Plasma and FFQ measurements of retinol were not correlated ($r = 0.06$). Intake of α-tocopherol was weakly correlated with cholesterol-standardized plasma-tocopherol ($r = 0.22$), but not with absolute plasma levels. Overall, adipose tissue levels were stronger correlated to intake than plasma levels are; for β-carotene crude correlation was 0.16 (not significant), the age- and sex-adjusted partial correlation 0.20 ($p = 0.09$). For α-tocopherol similar correlations were calculated (crude $r = 0.19$, partial $r = 0.24$, $p = 0.05$). Linoleic acid content of adipose tissue was significantly related to intake ($r = 0.44$, $p < 0.001$). Mutual correlations for plasma and adipose levels were higher than with intake. Especially for β-carotene, correlation was relatively high (partial $r = 0.56$, $p < 0.001$). Standardization of plasma α-tocopherol to cholesterol did not affect the correlation with adipose α-tocopherol. For men a higher correlation between plasma and adipose α-tocopherol was observed than for women (crude $r = 0.53$ and 0.21, respectively).

From the repeated measurements in the non-supplemented group, the within- to between-person variance ratio was calculated. The ratio varied between 0.08 for β-carotene and 0.41 for cholesterol-standardized α-tocopherol in plasma, and between 0.31 for α-tocopherol and 1.7 for retinol in adipose tissue. Crude correlation coefficients for the interrelations of intake, plasma and adipose tissue levels were deattenuated for this variance ratio (Table 4), which did not change the results substantially.

Table 4. Pearson correlation between vitamin and linoleic acid levels in adipose, plasma and FFQ: the Netherlands, 1992.

Overall	Adipose vs Plasma (n = 77)			Adipose vs FFQ (n = 74)			Plasma vs FFQ (n = 82)		
	crude	age- and sex adjusted	deattenuated†	crude	age- and sex adjusted	deattenuated†	crude	age- and sex adjusted	deattenuated†
α-tocopherol	0.31**	0.34**	0.34**	0.19	0.24*	0.20	0.11	0.05	0.11
men	0.53**			0.16			0.18		
women	0.21			0.30*			–0.06		
β-carotene	0.62***	0.56***	0.77***	0.16	0.20	0.20	0.15	0.17	0.15
men	0.53**			0.30			–0.07		
women	0.59***			0.12			0.33*		
retinol	0.21	0.17	0.31**	0.12	0.08	0.14	0.06	–0.12	0.07
men	0.25			0.14			–0.02		
women	0.16			0.08			–0.12		
linoleic acid	-	-	-	0.44***	0.42***	0.51***	-	-	-
men				0.40*					
women				0.40*					
α-tocopherol/ cholesterol	0.30**	0.34***	0.35**	-	-	-	0.22*	0.19	0.24*
men	0.60***						0.33*		
women	0.18						0.09		

† For within- to between person variance ratio.

* $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 5 shows the age- and sex-adjusted mean plasma values and intake for β-carotene, vitamin E and linoleic acid, by quartile of adipose tissue concentration. Mean intake of β-carotene changed little across adipose quintiles. Intake of vitamin E was 27% higher in the highest adipose quartile than in the lowest quartile. For relative intake of linoleic acid the difference was 42%. Mean plasma levels of β-carotene in the highest adipose quartile (0.63 μmol/l) were more than twice as high as in the lowest quartile (0.30 μmol/l). For absolute plasma tocopherol, the difference between quartiles was not significant ($p = 0.11$); for the tocopherol:cholesterol ratio, there was a 15% increase over adipose quartiles and a significant trend ($p < 0.05$).

Table 5. Age- and sex-adjusted mean vitamin and linoleic acid concentrations in plasma and intake assessed by FFQ, by quartiles of adipose tissue concentration, among 38 men and 47 women, the Netherlands, 1992.

	Adipose quartiles†				
	1	2	3	4	p-trend
Plasma					
β-carotene (μmol/l)	0.30	0.32	0.51	0.63	< 0.01
α-tocopherol (μmol/l)	30.3	33.5	33.6	34.6	0.11
α-tocopherol:cholesterol	5.27	5.89	5.90	6.08	< 0.05
FFQ					
β-carotene (mg)	0.90	0.89	0.98	1.00	0.35
vitamin E (mg)	13.1	12.5	12.7	16.6	< 0.05
linoleic acid (g)	13.8	15.0	16.4	19.6	0.01

† Quartile cutpoints for β-carotene: 0.83, 1.14, 2.01 μg/g; α-tocopherol: 180, 238, 309 μg/g; linoleic acid: 11.7, 13.1, 16.4 %.

Discussion

Aim of the present study was to evaluate the use of fat-soluble vitamins in adipose tissue as biomarker of dietary intake in epidemiological studies. Comparison of dietary intake of β-carotene and α-tocopherol with plasma and adipose levels among 85 healthy men and women has shown a moderate correlation of adipose tissue levels with intake assessed by FFQ, only somewhat higher than the correlation observed between plasma levels and

intake. This is consistent with the notion that adipose tissue better reflects long-term intake and is less subject to short-term fluctuations. However, within-person variability of adipose tissue levels was higher than of plasma levels. Adipose tissue β-carotene was shown to respond to prolonged oral supplementation.

Strength of correlations partly depend on analytical and intra-individual variation of measurements. We were able to adjust for those, because repeated measurements were available. Repeated measures of adipose tissue levels showed relatively larger differences than plasma levels, although less fluctuation was expected. A possible explanation is the less homogeneous texture of the adipose tissue compared to plasma, which may cause an uneven distribution of the vitamins in the tissue, or the larger analytical variation observed in smaller samples, rather than biological variation. Overall analytical variation was within acceptable ranges. However, unlike plasma levels, adipose tissue values were expressed as μg per gram fatty acids. This means that the analytical variation of both the vitamin and the fatty acid analysis contribute to the error in the vitamin concentration.

Intake of α-tocopherol in the present study is similar to intake assessed in other studies, using different food consumption methods[5,14-16]; several other studies have reported lower intakes[17-20]. Mean intake of α-tocopherol in a Dutch total diet study among 18-year old men was 13.9 mg[21]. β-Carotene intake, however, is considerably lower than in other studies[16,18,22,23]. This may be due to the method of calculation of intake: in our study, as well as in that of Albanes et al.[22] (with a mean intake of 2.1 mg/day closest to our results), recently analyzed food composition data were used. Other studies determined β-carotene values by partitioning total vitamin A activity into β-carotene, retinol and other carotenoids. Perhaps this latter method leads to an overestimation of intake of β-carotene. Our results agree with those of a total diet study in the Netherlands[21], in which a mean daily intake of 1.3 mg/day for β-carotene was found for male adolescents. Relative intake of linoleic acid was similar to reports of the Dutch Food Consumption Survey[11].

Vitamin E content of adipose tissue has been reported by few authors[5,8,24-27]; mean α-tocopherol levels vary from 141 to 402 μg/g triglycerides. Parker[8] indicates a range of 61-811 μg/g adipose tissue. Concentrations cannot simply be compared between studies, because of the different ways to express these concentrations. In all studies relatively large between-person variation has been observed; in the present study levels varied 11-fold for α-tocopherol and 20-fold for β-carotene. Mean β-carotene content of adipose tissue in our study was of the same order as reported

by Parker[8].

The correlation of levels of β-carotene in adipose and plasma is much higher than the correlation of α-tocopherol levels. This may be explained in part by the larger range of adipose tissue β-carotene as compared to α-tocopherol. On the other hand, it might indicate a quicker response of adipose tissue β-carotene to fluctuations in plasma levels; if so, this would disagree with the notion that adipose tissue β-carotene levels reflect long-term intake.

Correlation between intake and biomarkers is always weakened by inter-individual differences in absorption and metabolism, and the effect of other, mostly unknown determinants of the biomarker levels. For β-carotene the sensitivity of plasma concentrations to intake has been shown previously[22,28]. Plasma vitamin E concentration as such is a questionable index of intake, as it is strongly correlated with plasma lipid concentration. Therefore, the use of tocopherol/lipid ratios is recommended[29] as indicator of nutritional status. In our study, plasma α-tocopherol (relative to plasma cholesterol) is weakly correlated with intake; of the other plasma parameters that were assessed, only β-carotene concentration was significantly associated with intake, among women. Others have found similar low correlations between intake of vitamin E and plasma α-tocopherol, among non-supplement-users[17,19]. Some other studies[15,18,20] have observed significant correlations a little over 0.3. Correlation between intake of carotenoids and plasma or serum β-carotene varies between 0.21[14,23] and 0.49[19], among non-supplement-users. In the present study, for women we observed a similar correlation (0.33), but for men there was no correlation between intake and plasma levels at all. These findings may be due to some extent to the larger range of intake among women. However, metabolic differences between men and women may also play a part.

As yet, little information is available on the relationship between intake and adipose tissue levels. Kayden et al.[25] reported increased adipose tissue tocopherol levels in two subjects taking supplemental vitamin E for more than a year; in one of them, who discontinued the supplement 20 months prior to the study, plasma tocopherol levels had returned to normal, but the adipose tissue level remained elevated. The only human study, to our knowledge, in which the effect of vitamin E supplementation on adipose tissue levels has been studied systematically, dates from some time ago[30]. Two week supplementation with 1 g of vitamin E daily resulted in a doubling of adipose tissue levels. Experimental studies in animals have shown that concentrations in adipose tissue reflect the intake over a period of time[31-33].

Accumulation of β-carotene in adipose tissue was observed 5 days after a single dose of 120 mg β-carotene[34]. In the present study an increased level of adipose tissue β-carotene in response to long-term supplementation has been observed. Since only one measurement was performed, six months after supplementation, no information can be derived on the time it takes before adipose levels start to increase, whether a steady state has been reached after six months, or the time required to return to normal levels, after the end of supplementation. The increase in adipose tissue appears to be correlated with the increase in plasma levels, suggesting that an equilibrium had been reached; both show large differences between individuals.

For linoleic acid, the correlation between intake and adipose tissue levels was in the same range as reported by others[3,35]. Aro et al.[6] recently reported a correlation of 0.46 ($p = 0.015$) between β-carotene intake assessed by five-day food records and β-carotene levels in adipose tissue, sampled immediately thereafter. A lower correlation ($r = 0.33$) was found between three days double portions and a biopsy 6 months later. The conclusion that adipose tissue levels reflect recent dietary intake may be valid, but it could also be attributed to this particular population which may have had relatively stable dietary habits. In our study, adipose tissue α-tocopherol correlated better with intake than plasma levels and intake, among women; for β-carotene, adipose tissue concentrations correlated well with intake among men, but not among women. An explanation is hard to give on the basis of these findings. However, it seems possible that metabolism and the distribution of fat-soluble vitamins over different body compartments may differ between men and women; this suggestion is supported by the finding that concentrations of β-carotene in plasma and in adipose tissue in women are substantially higher than in men, although intake is similar.

Overall, adipose tissue levels of linoleic acid enable us to rank subjects adequately according to intake, and high adipose tissue α-tocopherol was related to relatively high dietary intake. Adipose tissue β-carotene seemed to provide a less direct indication of intake as assessed by FFQ. Whether this is caused by a larger dependence on plasma concentrations, and thus recent dietary intake, or perhaps an insufficient ability of our FFQ to rank subjects according to intake adequately, for the limited range observed in this population, remains to be clarified.

In conclusion, adipose tissue concentrations of β-carotene in men and α-tocopherol in women, respectively, seem to be feasible biomarkers of usual dietary intake. Overall, the association with intake is somewhat, but not much better than of plasma levels with intake. The advantage of biomarkers in

adipose tissue over plasma is evident in case-control studies; in persons who may have changed their diet recently, or who have endured the effects of an acute event, such as a myocardial infarction, plasma levels will be affected, whereas adipose tissue levels will most likely be constant for a longer period of time. In prospective studies, the choice of biomarker may be determined by factors such as feasibility and cost; in most cases, plasma levels will then be preferable. Finally, even if these biomarkers are not perfect indicators of dietary intake, they may still be relevant predictors of disease risk, as they account for the individual differences of absorption and metabolism that affect nutritional status.

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Comparison of biomarkers and dietary intake of antioxidants in relation to myocardial infarction

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Abstract

In theory, antioxidant biomarkers may be more relevant predictors of disease risk than dietary intake assessment, which disregards individual variation in absorption and metabolism.

Concentrations of α-tocopherol and β-carotene in adipose tissue were compared with usual dietary intake, in 66 patients with first acute myocardial infarction (MI) and 58 population controls. Adipose tissue β-carotene was lower in cases (0.49 μg/g ± 0.37) than in controls (0.67 μg/g ± 0.45, $p = 0.08$), whereas dietary intake was similar. No significant differences were observed for α-tocopherol in adipose tissue or in diet. There was a weak positive correlation between intake and adipose tissue levels among controls ($r = 0.10$ for α-tocopherol; $r = 0.24$, $p = 0.08$ for β-carotene). An inverse correlation was observed among cases ($r = -0.27$, $p < 0.05$ for α-tocopherol; $r = -0.16$ for β-carotene). Other determinants of adipose tissue antioxidant levels were smoking status, body mass index and serum HDL cholesterol. The adjusted odds ratio (OR) of MI in the lowest tertile of adipose tissue β-carotene was 2.5 (95% CI 1.0-6.6). There was no significant association with dietary intake of β-carotene. For adipose tissue α-tocopherol also no association with MI was observed (OR in lowest tertile 0.7, 95% CI 0.3-1.7); dietary intake of α-tocopherol was inversely associated with MI (OR 1.8, 95% CI 0.8-4.2), though not statistically significant.

Adipose tissue antioxidants do not reflect dietary intake in the same way for MI patients and control subjects. This may indicate different absorption and metabolism, or a relation of the biomarkers with the disease status.

Submitted

Introduction

Dietary antioxidants may play a role in the prevention of coronary heart disease (CHD)[1,2]. Observational studies in humans have shown inverse associations between vitamin E, β-carotene and CHD[3,4,5]. The use of dietary intake assessment in case-control studies on antioxidants and CHD may introduce recall bias and disregards metabolic variation between individuals. Therefore, antioxidant biomarkers may be more relevant predictors of disease risk, as they account for individual differences of absorption and metabolism[6]. It is, however, necessary to know the relationship between intake and biomarker, for a better interpretation of diet studies and the support of dietary recommendations.

We have studied the exposure to the antioxidants α-tocopherol and β-carotene in male patients with acute myocardial infarction and population controls, by assessing usual dietary intake and by determining the level of these antioxidants in adipose tissue. Moreover, the proportion of linoleic acid in adipose tissue, which has been shown to be a good marker of intake[7,8], and in diet was determined for purpose of comparison. The relationship of the biomarkers with dietary intake and other determinants was investigated in patients and controls. Subsequently, to evaluate the two different approaches, the association of both exposure measures and risk of myocardial infarction was examined.

Methods

Study population

This study was conducted as part of the multi-centre EURAMIC Study on antioxidants and myocardial infarction[9]. Eligible subjects were men under 70 years of age, without previously reported myocardial infarction, with stable dietary patterns and body weight in the past year. Cases were subjects diagnosed with a first acute myocardial infarction (MI) (ICD-code 410) –confirmed by specific abnormalities on the ECG and elevated enzyme levels– and admitted within 24 hours of manifesting symptoms. They were recruited from the coronary care unit of participating hospitals. Of the 94 eligible subjects that were invited, 83 consented to participate.

Subjects without a history of myocardial infarction were eligible as controls. For each patient, four control subjects were randomly selected from his general practitioner's patient registry, according to a standard protocol,

within a 5-year age interval. They were sent a questionnaire addressing the exclusion criteria and, if eligible, were invited to participate. During the study it became apparent that this method of control recruitment resulted in a underrepresentation of the age category of 45-60 years. Therefore, another random sample was obtained from the municipalities in which the patients in this age category lived; these persons were invited in exactly the same way as the controls recruited via the general practitioner. Overall, 117 control subjects were invited to the study, of whom 99 returned the questionnaire. Five subjects were excluded because of previous MI, weight loss over 5 kg, age over 70 and alcohol abuse. 75 Subject could be enrolled in the study.

Biochemical analyses

Subcutaneous adipose tissue was taken from the buttock by needle aspiration[10]. In cases, the adipose sample was taken within seven days of hospital admission. Samples were stored at −70°C; handling of the samples has been described previously[9]. Concentrations of α-tocopherol and β-carotene in adipose tissue were determined by reverse-phase HPLC[11] and spectrophotometric detection. The coefficient of variation for the analysis of β-carotene and α-tocopherol was 7% (at mean values of 2.1 μg/g and 84 μg/g in the quality control samples, respectively). Detection limits were 0.02 μg/g for β-carotene and 2 μg/g for α-tocopherol, at mean sample weight (29 mg). Vitamin concentration was expressed in μg/g of total fatty acids.

Fatty acid composition of the samples was assessed by GLC[12] in an aliquot of the same extract as the vitamins, at the National Public Health Institute in Helsinki (Finland). The saponified sample was acidified with HCl and the free fatty acids were extracted with hexane and methylated with acidic methanol. Fatty acid composition was determined by a gas chromatograph (HNU Nordion Oy, Finland, HRCG 412) with a 60 m long SP-2380 column, I.D. 0.32 mm, phase layer 0.20 μm with split injector and helium as carrier gas. Fatty acid peaks were identified by an SC-workstation (Sunicom Oy, Finland) in a temperature-programmed run. Linoleic acid (C18:2) was expressed as proportion of total fatty acids.

Non-fasting blood samples were to be drawn as soon as possible after admission, and not later than 24 hours after the manifestation of symptoms for cases. Serum total and HDL cholesterol levels were determined enzymatically (Boehringer Mannheim Kit) at the National Public Health Institute in Helsinki (Finland).

Dietary questionnaire

Usual dietary intake of β-carotene and vitamin E during the previous year was assessed by a 95-item self-completed semi-quantitative food frequency questionnaire (FFQ)[13]. Frequency of consumption was reported in eight categories, never, seldom, 1 day per month, 1 day per 2-3 weeks, 1-2 days per week, 3-4 days per week, 5-6 days per week, 7 days per week. Portion sizes were quantified by respondents in terms of household measures (slices, spoons, cups) to which standard weights were assigned.

Completeness, credibility of reported number of servings and consistency in reported consumption frequencies were checked. Information on vitamin E and β-carotene content was mainly derived from recent analyses of Dutch food products[14], but did not provide complete coverage of all foods in the questionnaire (75% for vitamin E, 97% for β-carotene). Additional information was obtained from the English food composition table. Intake of linoleic acid was calculated, although the questionnaire was not specifically designed to measure this nutrient. However, since the important sources of linoleic acid and vitamin E are largely the same foods, the questionnaire is considered to measure linoleic acid well enough to obtain adequate ranking of subjects according to intake.

Data analysis

From 72 out of 83 patients and 61 out of 75 control subjects a fat aspirate could be obtained. Vitamin and fatty acid results from 6 patients and 3 controls were excluded from data analysis, because of too little sample material, resulting in unreliable measurements. Dietary intake data were available for 64 patients and 68 controls.

Crude means for major risk factors and potential confounders, as well as mean levels of the antioxidants and fatty acid composition, in adipose tissue and from the diet, among cases and controls were calculated. Significance testing of the difference in means was performed with Student's t-test and chi-square analyses. The distributions of α-tocopherol and β-carotene, both in adipose tissue and from the diet, were positively skewed; values were log-transformed for testing procedures. The relationship between the antioxidants and fatty acids from the diet and in adipose tissue was assessed by calculating Pearson correlation coefficients. Determinants of concentrations in adipose tissue were evaluated by linear regression analysis. The association between risk of MI and antioxidants and fatty acids, both from the diet and in adipose tissue, was estimated with logistic regression analysis. Odds ratios were calculated for the lowest tertile compared to the highest, based on the

distribution among controls. Linear trend of the association was tested by including the tertiles as a continuous variable in the model.

Results

The prevalence of risk factors for coronary heart disease among patients and controls is shown in Table 1.

Table 1. Prevalence of risk factors for coronary heart disease among cases and controls.

	Cases (n = 66)	Controls (n = 58)
Age (yr)	53.0 ± 9.9	52.3 ± 9.8
Total cholesterol (mmol/L)	5.7 ± 0.9	5.6 ± 1.0
LDL cholesterol (mmol/L)	3.9 ± 0.8	3.6 ± 0.9
HDL cholesterol (mmol/L)	1.0 ± 0.3	1.1 ± 0.3**
Current smokers (%)	55	46
Number of cigarettes	27 ± 16	18 ± 12*
Hypertension (%)	15	10
Family history of CHD (%)	49	29*
Diabetes (%)	4.6	3.4
Angina pectoris (%)	11	7
Low socioeconomic status (%)	50	43
Alcohol use (%)	91	90
BMI (kg/m^2)	25.9 ± 3.6	25.4 ± 2.8

* $p < 0.05$; ** $p < 0.01$.

Serum HDL cholesterol, smoking habits and a family history of CHD were significantly different between patients and controls. Since HDL concentration was dependent on the time-lag between the infarction and the blood sampling ($r = -0.34$, $p = 0.02$) in cases, this variable could be evaluated only in the control group. Table 2 presents the mean values (± SD) for the concentrations of α-tocopherol, β-carotene and linoleic acid in adipose tissue, as well as their mean daily intake, as assessed by the food frequency questionnaire. Adipose tissue β-carotene levels were lower in cases (0.49 μg/g ± 0.37) than in controls (0.67 μg/g ± 0.45, $p = 0.08$). Dietary intake of β-carotene was similar for cases and controls; vitamin E intake tended to be higher in controls, although the difference is not significant ($p = 0.11$).

The crude correlation between dietary intake and the adipose tissue biomarkers among cases and controls was assessed.

Table 2. Mean (± SD) concentration of α-tocopherol, β-carotene and linoleic acid in adipose tissue and mean dietary intake among cases and controls.

Antioxidant vitamins	Cases	Controls	p-value†
Biomarkers	n = 66	n = 58	
α-tocopherol (μg/g)	225 ± 98	221 ± 131	0.26
β-carotene (μg/g)	0.49 ± 0.37	0.67 ± 0.45	0.08
linoleic acid (% of total fatty acids)	14.6 ± 3.4	15.3 ± 3.7	0.36
Dietary intake	n = 64	n = 68	
α-tocopherol (mg/day)	15.3 ± 8.1	18.1 ± 11.7	0.11
β-carotene (mg/day)	0.86 ± 0.41	0.90 ± 0.42	0.54
linoleic acid (% of total fat)	15.9 ± 7.1	16.5 ± 6.3	0.42

† student's t-test performed with $\log_e$-transformed values where necessary.

For α-tocopherol, a correlation of 0.10 was found among controls, but an inverse correlation (Pearson $r = -0.27$, $p < 0.05$) among patients. For β-carotene the crude correlation was 0.24 ($p = 0.08$) among controls and −0.16 among cases. A higher correlation was observed for linoleic acid in adipose tissue and from the diet: 0.49 among controls and 0.48 among patients. Other determinants of the biomarker concentrations were evaluated by univariate linear regression analysis (Table 3), separately for cases and controls. Smoking status, body mass index (BMI) and serum HDL cholesterol appeared to be relevant predictors of the antioxidant biomarkers. These variables were included in a multivariate model, together with dietary intake (Table 4; HDL only in controls). The resulting regression equations were significantly different for patients and control ($p < 0.05$). BMI was inversely associated with adipose tissue α-tocopherol and β-carotene in controls, but not in patients. The effect of smoking was similar in patients and control subjects. HDL was an important predictor of α-tocopherol and β-carotene in controls, which, in the multivariate model, slightly lowered the coefficients for dietary intake. Dietary intake of linoleic acid was the main predictor of the proportion of linoleic acid in adipose tissue (among controls $\beta = 0.295$, $SE = 0.072$). None of the other variables measured in this study significantly improved the fit of the linear model (data not shown).

Next, we investigated the association between the biomarkers and the risk of MI on the one hand, and the association of dietary intake and risk of MI on the other (Table 5).

Table 3. Simple regression coefficients for predictors of adipose tissue α-tocopherol and β-carotene.

Predictors	α-tocopherol		β-carotene	
	patients β ± SE	controls β ± SE	patients β ± SE	controls β ± SE
Dietary intake	−0.256 ± 0.110 *	0.170 ± 0.169	−0.170 ± 0.194	0.339 ± 0.199
Age	0.006 ± 0.006	0.001 ± 0.008	−0.002 ± 0.008	0.006 ± 0.008
Total cholesterol	0.050 ± 0.069	−0.006 ± 0.091	0.183 ± 0.099	−0.097 ± 0.083
LDL cholesterol	0.131 ± 0.080	0.031 ± 0.104	0.145 ± 0.121	−0.150 ± 0.094
HDL cholesterol†	−0.371 ± 0.226	1.121 ± 0.369 **	0.139 ± 0.345	1.468 ± 0.306 **
Smoking	−0.246 ± 0.110 *	−0.214 ± 0.163	−0.267 ± 0.158	−0.429 ± 0.147 **
Hypertension	0.196 ± 0.157	0.039 ± 0.268	−0.111 ± 0.224	0.159 ± 0.254
Family history	0.128 ± 0.113	−0.313 ± 0.175	−0.270 ± 0.157	−0.046 ± 0.171
Diabetes	−0.304 ± 0.263	−0.155 ± 0.447	−0.580 ± 0.382	−0.113 ± 0.426
Angina	0.044 ± 0.185	−0.186 ± 0.322	0.117 ± 0.261	−0.329 ± 0.304
Low SES	−0.082 ± 0.113	−0.100 ± 0.164	0.083 ± 0.160	−0.036 ± 0.157
Alcohol use	−0.074 ± 0.198	−0.012 ± 0.268	0.290 ± 0.277	0.101 ± 0.255
BMI	0.602 ± 0.428	−1.699 ± 0.714 *	0.172 ± 0.622	−1.305 ± 0.690

* $p < 0.05$; ** $p \leq 0.01$.

† Serum HDL in patients probably affected by acute MI.

Table 4. Multivariate linear regression coefficients for predictors of α-tocopherol and β-carotene in adipose tissue among MI cases and control subjects.

		Linear model			
Biomarker	disease status	dietary intake β ± SE	BMI β ± SE	smoking status β ± SE	HDL β ± SE
α-tocopherol	patient (n = 52)	−0.246 ± 0.118	0.351 ± 0.439	−0.182 ± 0.120	-
	control (n = 51)	0.126 ± 0.166	−1.942 ± 0.805	−0.289 ± 0.183	-
	control (n = 42)	0.092 ± 0.180	−1.904 ± 0.903	−0.151 ± 0.201	0.876 ± 0.423
β-carotene	patient (n = 52)	−0.077 ± 0.203	0.124 ± 0.675	−0.400 ± 0.188	-
	control (n = 51)	0.261 ± 0.185	−1.810 ± 0.682	−0.377 ± 0.154	-
	control (n = 42)	0.256 ± 0.169	−1.555 ± 0.657	−0.276 ± 0.147	1.172 ± 0.302

The crude odds ratio of MI for subjects in the lowest tertile of the adipose tissue β-carotene distribution was 2.7 (95% CI 1.1-6.6). Adjustment for smoking (in five categories) and BMI lowered the odds ratios (OR 2.5 in lowest tertile, 95% CI 1.0-6.6). Both the crude and adjusted odds ratios showed a linear trend over tertiles. There was a slight tendency for a positive association of adipose tissue α-tocopherol and MI, and an inverse association of linoleic acid and MI, but both were not significant, nor was the trend over tertiles. Adjustment for BMI and smoking did not substantially affect the estimated odds ratio for α-tocopherol. Because of the apparent effect of the infarction on serum HDL levels, adequate adjustment for confounding was not possible. However, inclusion of HDL in the logistic regression model decreased the estimated odds ratio by 29% for α-tocopherol and 36% for β-carotene. Part of this effect is due to the artefactual low HDL among cases.

With respect to dietary intake, the results for α-tocopherol were suggestive of an inverse association with MI, but the odds ratios did not differ significantly from unity (OR 1.8, 95% CI: 0.8-4.2). For intake of β-carotene and linoleic acid no association with MI was observed.

Table 5. Odds ratios of MI in tertiles of the biomarker distribution and dietary intake among controls.

Antioxidant	Assessment method	tertile 1-low OR (95% CI)	tertile 2 OR (95% CI)	tertile 3-high OR (95% CI)	p for trend
α-tocopherol	biomarkers [a]	0.7 (0.3-1.7)	0.9 (0.4-2.2)	1.0	0.42
	diet [b]	1.8 (0.8-4.2)	1.4 (0.6-3.4)	1.0	0.18
β-carotene	biomarkers [c]	2.7 (1.1-6.6)	0.8 (0.3-2.3)	1.0	0.02
	diet [d]	1.2 (0.5-2.8)	0.8 (0.4-2.0)	1.0	0.59
linoleic acid	biomarkers [e]	1.6 (0.7-3.8)	1.4 (0.6-3.6)	1.0	0.30
	diet [f]	1.2 (0.5-2.7)	1.3 (0.5- 2.9)	1.0	0.75

Tertile cutpoints: [a] 143, 240 μg/g; [b] 12.9, 19.0 mg; [c] 0.44, 0.63 μg/g; [d] 0.68, 0.93 mg; [e] 13.6%, 16.4%; [f] 12.2%, 17.9%.

Discussion

The relationship between dietary intake and biomarkers of α-tocopherol and β-carotene was found to be different for patients with acute myocardial infarction as compared to population controls, whereas for linoleic acid, there was a similar association for patients and controls. In controls, we observed a weak positive correlation between intake and adipose tissue levels of both α-tocopherol and β-carotene. In cases, there was no correlation between both assessments for β-carotene and an inverse correlation for α-tocopherol. This was reflected in the finding of different risk estimates for MI, for low adipose tissue antioxidants as compared to low dietary intake. These results suggest that the use of dietary intake data in the study of antioxidants and coronary heart disease may yield different conclusions than the use of biomarkers, in the same population.

Of course, in view of the relatively small numbers of patients (n = 66) and controls (n = 58) in our study, it cannot be excluded that some of our observations are due to chance variation. However, the associations observed between the antioxidant biomarkers and risk of MI were very similar to the results of a pooled data analysis in a larger study population, of which the present study was a part[9].

Only a modest correlation between intake and biomarkers may be expected, because of inter-individual differences in absorption and metabolism, and the effect of other, mostly unknown determinants of the biomarker levels. Moreover, intra-individual variation and independent random errors in both assessment methods[13] weaken the correlation. The questionnaire used in this study has recently been validated in a non-smoking, but otherwise comparable population[13]. The correlation between intake and adipose tissue concentration was 0.30 for β-carotene, 0.16 for α-tocopherol and 0.40 for linoleic acid, comparable to what we observed in the present study in controls.

Absence of correlation between intake and adipose tissue levels may be explained in different ways. The use of a questionnaire to assess dietary exposure in a case-control study is liable to differential recall by cases and controls, introducing recall bias. It is conceivable that cases have reported higher intakes of fruits and vegetables in retrospect, as compared to true long-term intake, which could have diminished the correlation with adipose tissue levels in cases and underestimated a possible association between β-carotene intake and risk of MI. If we, however, assume that the

measurement of dietary intake was adequate, the different correlations with the biomarkers for patients and controls might be attributed to an effect of the disease, which relates to the validity of the biomarker. It is possible, although not supported by clinical evidence, that the level of β-carotene is affected by the process of atherosclerosis. This may indicate an increased requirement of β-carotene as a consequence of atherosclerosis. It does not exclude a possible role of β-carotene in the prevention of progression of atherosclerosis or an effect on hemostasis and thrombosis. Another possible explanation of the lower association between β-carotene intake and adipose tissue levels in patients is a restricted absorption capacity for β-carotene in patients, leading to the low tissue levels, associated with MI. To examine a potential acute effect of MI on adipose tissue levels in cases, we looked at the correlation between the number of days after the infarction, when the fat aspirate was obtained, and the concentration of β-carotene and α-tocopherol. We found no correlation at all ($r = 0.06$ for β-carotene, $r = -0.02$ for α-tocopherol). Comparison of vitamin concentrations in adipose tissue sampled immediately after hospitalization and after four days, in the same patients, did also not show a decrease (data not published).

An inverse correlation, as observed for α-tocopherol, is hard to explain by any biological mechanism and therefore may be attributed to chance. For α-tocopherol, patients tend to have higher adipose tissue concentrations than controls, whereas their dietary intake appears to be lower. Apart from the performance of the dietary questionnaire, this may also be due to differences in absorption (but this time higher in patients) or to a different distribution over body compartments. The latter is supported by the finding that BMI is a predictor of adipose tissue vitamins in controls, but not in cases. The fact that serum HDL is an important predictor of adipose tissue vitamin levels suggests a relation with lipid metabolism.

The determinants of adipose tissue β-carotene in our study (smoking and BMI) are also the main determinants of plasma β-carotene[15,16,17] apart from gender. No consistent determinants of plasma vitamin E have been identified, apart from supplement use[15,18]. The inverse association with BMI which we observed for adipose tissue α-tocopherol in controls was not reported by others.

Biomarkers of dietary antioxidants, taking into account the individual differences of absorption and metabolism, may be more relevant predictors of disease risk than dietary intake in itself. However, it is important to know how the disease may affect the biomarker levels, to evaluate what is cause and what is consequence. Moreover, an understanding of the relationship

between dietary intake and biomarker levels, and of the influence of lifestyle factors on this relationship, is necessary to support dietary recommendations.

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General discussion

The purpose of the EURAMIC Study, as described in Chapter 3, was to provide evidence for the potential role of diet-derived antioxidants in the prevention of coronary heart disease. It was designed as a multi-centre case-control study of the association of α-tocopherol and β-carotene in adipose tissue, and selenium in toenails, with risk of myocardial infarction (MI).

Table 1. Results of the EURAMIC Study in relation to the antioxidant hypothesis.

Type of evidence	Antioxidant	Observation	Chapter
Supportive	β-carotene	inverse association with MI	4
		strongest in smokers	4
		and at high PUFA-level	6
	selenium	inverse association	5
		strongest at low α-tocopherol level	5
Non-supportive	α-tocopherol	no association with MI	4
		association not dependent on PUFA level	6
Inconsistent	α-tocopherol	inverse association with MI at lowest selenium quintile; trend towards positive association at all other levels of selenium	5
	β-carotene	strongest inverse association at high α-tocopherol level	4
	selenium	inverse association at low but not at high PUFA level	6

First, the advantages and limitations of the study design will be summarized. Next, the findings, which are summarized in Table 1, will be evaluated in the light of results from recent experimental and epidemiological studies. Finally, the public health implications of our findings and those of others will be discussed.

Methodological considerations

Disease end-point

Ischaemic heart disease is the major cause of death among men in affluent

societies; in the Netherlands it accounts for about 20% of total mortality[1]. The most important diagnosis in this category of diseases is acute myocardial infarction. Atherosclerotic lesions in the coronary arteries form the underlying cause. In acute myocardial infarction, a rupture or fissure of the endothelium, covering a lipid-rich plaque, takes place, followed by haemorrhage into the plaque, acutely increasing stenosis. Additionally, a clot may develop, leading to total or partial occlusion of the vessel[2].

First-time acute myocardial infarction was a suitable end-point for the EURAMIC Study because of its high incidence, relatively straightforward diagnosis and acuteness. The latter is important, because a history of cardiac complaints may lead to a change of dietary habits, thereby biasing the exposure information obtained in a retrospective study. Subjects with a history of angina pectoris, who may have done just that, were not excluded from the EURAMIC Study, but they were present among cases as well as controls, and a possible confounding effect was evaluated in the data analysis.

A large proportion of persons suffering from acute MI die before reaching the hospital. These victims of 'sudden death' form a subgroup, whose pathology may be different. In the EURAMIC Study, only survivors of MI were enrolled. Theoretically, survival may depend on antioxidant status, and therefore any observed association with MI may be different in 'sudden death' cases. However, there is no reason to assume that these fatal cases have higher antioxidant levels than survivors. Moreover, current knowledge indicates that antioxidants may restrict the damage inflicted by free radicals in ischaemia and reoxygenation. Therefore, it is possible that patients with a relatively high antioxidant status are more likely to survive. This may have weakened the observed associations between the antioxidants and MI, leading to a conservative risk estimate.

Furthermore, it should be realized that the pathogenesis of MI is not identical to that of atherosclerosis, since thrombosis may play a crucial role as well. The choice of MI as end-point does not permit any conclusions about the specific mechanism in which antioxidants may play a part. Although the antioxidant levels in adipose tissue may not fluctuate as much as plasma levels, it is not likely that they reflect the antioxidant status at the time when atherosclerosis started, which is already at a very early age. Therefore, any association we have found with MI may tell us something about a relatively short-term mechanism, possibly related to thrombosis, or to progression of atherosclerosis. On the other hand, it cannot be excluded that the process of atherosclerosis, prior to the myocardial infarction end-point, has influenced

antioxidant levels, although we do not know theories with regard to this effect.

Study population

The use of different methods of control recruitment in the different centres is discussed in Chapters 4 to 6, as it is likely to be a point of criticism. The main criterion in selecting controls is that they should represent the population from which the cases are recruited (the study base principle), with respect to exposure distribution[3]. It can be argued that the best way to do this may vary among countries, depending on the determinants of hospital admission, completeness of case ascertainment, non-response or inadequacies of the sampling frame. It is not by definition true that population controls are preferable over hospital controls or controls from a medical practice[4]. Internal validity within a centre was considered more important than a common method of recruitment.

The EURAMIC Study population was confined to men. It is likely that the antioxidants have a similar biological function in men and women. However, the metabolism of fat-soluble antioxidants may well be different for men and women. In our additional study on the relationship between diet, plasma and adipose tissue levels of antioxidants, more than half of the study group were female. Women had higher antioxidant concentrations in both plasma and adipose tissue, at similar levels of intake. Considering the larger fat mass of women, lower levels in adipose tissue would have been easier to explain. Higher levels in adipose tissue were also observed in the female study population of the breast cancer part of the EURAMIC Study[5] (Chapter 3). Moreover, the relationship between dietary intake and tissue levels was not consistent for men and women (Chapter 7). It is possible that the association between α-tocopherol and β-carotene and MI is dependent on these differences. Therefore, our results may need to be confirmed in a female population.

Multi-centre design

The multi-centre approach was chosen for several reasons. The first, pragmatic, reason was that involvement of more than one centre would facilitate the recruitment of the required number of subjects within a limited period of time. This argument appeared to be valid; although most centres did not attain the aimed number of 100 cases and 100 controls within a period of 2 year, the power of the pooled study was adequate to detect the expected associations.

Second, the variation in exposure to antioxidant vitamins within a country is limited because of more or less homogeneous consumption patterns. The use of populations with larger variations in exposure would theoretically increase the chance of finding an existing association between exposure and disease. In the individual countries, the 10th and 90th percentiles differed on average by a factor of 3.7 for α-tocopherol, 6.3 for β-carotene and 1.6 for selenium. The overall variation between the 10th and 90th percentiles were 3.7, 7.5 and 2.0, respectively. To calculate the odds ratio of MI for low versus high levels of the antioxidants, categories were based on this overall distribution of antioxidants in the control population. However, it was considered necessary to adjust for centre in all data analyses, thereby again limiting exposure variation. We did this for two reasons: because controls were frequency-matched for centre and because the inclusion of many different centres adds not only to the heterogeneity of the exposure, but also to variation in potential confounders. Of course, the study design involved standardized measurement of potential confounders as well as exposure. However, lack of cross-culturally validated methods did not permit the use of a standard questionnaire for all variables. In theory, adequate adjustment for confounders may render additional centre-adjustment superfluous, but we have chosen for consistent centre-adjustment in our analysis strategy.

A third advantage of a multi-centre approach is the possibility to calculate centre-specific odds ratios which can be interpreted as replications of the study under different circumstances. Consistency of the results among the different centres provides arguments for the generalizability of the outcomes. In the EURAMIC Study, odds ratios were not consistent among countries, but, since the numbers in each centre were quite small and the variation was not statistically significant, this may be attributed to chance.

Biomarkers versus dietary intake

In general, biomarkers may be defined as "cellular, biochemical or molecular alterations that are measurable in biological media, such as human tissues, cells, or fluids"[6]. If an exogenous compound, such as a nutrient, is identified in body tissues or fluids through a laboratory assay, it becomes a marker of internal dose[6], an indication of the amount absorbed in the gut. This marker may better represent concentrations in the biological relevant target tissues than dietary intake assessment may. In the EURAMIC Study, the exposure to antioxidants was assessed by determining their concentration in adipose tissue and toenails.

Another advantage of the use of biomarkers in general is the comparability among countries, in case of centralized laboratory analysis. Even more important are the objectivity and comparability of the information obtained from cases and controls, provided that sample collection and handling takes place under similar conditions and samples from cases and controls are equally divided over analytical runs.

Although the use of biomarkers was preferred for the reasons mentioned above, it is relevant to have information about their relationship with dietary intake. The concentration of selenium in toenails has been shown to correlate well with dietary intake over a period of about 6-12 months[7]. Concentrations of α-tocopherol and β-carotene in adipose tissue and toenails were preferred to concentrations in plasma, for several reasons. First, plasma levels are affected by the event of an acute MI, precluding a valid case-control comparison. Second, a regulatory mechanism for α-tocopherol in plasma has recently been postulated[8], which probably limits the range of plasma values and hence its use as an indicator of intake. Third, concentrations in adipose tissue were believed to provide a more time-integrated measure of dietary exposure, as has been shown for the proportions of essential fatty acids in adipose tissue[9]. However, at the start very little information was available about the relation of adipose tissue levels to dietary intake. Our own comparisons of dietary intake, plasma and adipose tissue levels (Chapters 7 and 8) have shown that there is at most a very modest linear correlation between intake and biomarkers. This is partly due to the limitations of dietary assessment methods. Moreover, differences in absorption, regulation of plasma levels and influences of other dietary or life-style factors affect this correlation. Perhaps there is even a non-linear relationship. Surprising was the finding that β-carotene levels in Spain and Israel were the lowest among all centres, whereas comparative data on consumption of fruit and vegetables[10] show a relatively high use in these countries. It may be speculated that the intake of retinol (vitamin A) from animal sources in Spain and Israel is relatively low, causing an efficient conversion of β-carotene into retinol. In summary, the results, based on the "internal dose" of α-tocopherol and β-carotene in adipose tissue, cannot be easily extrapolated to conclusions about dietary intake, except in general terms.

Adipose tissue sampling

The EURAMIC Study was the first to use the α-tocopherol and β-carotene concentration in adipose tissue as biomarker. In practice, we had to face a number of technical difficulties, related to the amount of material obtained. It

is difficult to assess the amount during the biopsy procedure, because tissue other than fat may be part of the biopsy. Moreover, in some subjects the procedure was not successful and no material could be obtained at all. Due to small amounts of adipose tissue, no reliable vitamin concentrations could be assessed for about 6% of our population, although a biopsy was taken. Other procedures may yield more sample material, but may not be performed without applying local anaesthetics, which also has drawbacks.

Absolutely as well as relatively small amounts of fat tissue in the biopsy adversely affect the precision of vitamin concentrations: absolute amounts, because a minimum amount of tissue is necessary to determine especially β-carotene, which is present in a much lower concentration than α-tocopherol; relative amounts, or the proportion of fat tissue in the biopsy, because a correction for this proportion needs to be made in the calculation of the vitamin concentration. In a biopsy in which only 40% of the tissue is fat, the concentration of fat-soluble vitamins per mg biopsy is multiplied by 2.5 to obtain the concentration per mg fat. Any random error in vitamin assessment (which will be larger in small samples) is thus multiplied by the random error in the fatty acid assessment. However, it is necessary to perform this correction, because the biopsies show a large variation in proportion of fat tissue, cases having on average higher proportions of fat in their biopsies than controls. This measurement error, which results in a certain amount of random "misclassification" of exposure status and therefore may weaken the association with disease, may be limited by obtaining larger samples, washing the samples before weighing and standardizing the biopsy procedure as much as possible.

EURAMIC Study results in the light of recent studies

Recent publications on antioxidants and cardiovascular disease can be divided into four mainstreams: animal experiments on the prevention of atherosclerosis by antioxidants; observational studies on antioxidants and cardiovascular disease end-points in human subjects; ex vivo studies on the susceptibility of LDL to oxidation; investigations into the relationship between LDL oxidation and coronary heart disease (CHD). It is not the intention of this chapter to provide a complete review of all these studies, but to highlight some important studies that are relevant to the interpretation of our own results. A review of the literature available until 1991 is given in Chapter 2.

Animal studies

Until recently, almost no data were available on the effectiveness of dietary antioxidants in the prevention of experimental atherosclerosis in animal models[11]. Studies had only been performed with antioxidant drugs, such as probucol[12]. Now, two studies in rabbits have been reported in which vitamin E suppletion resulted in less atherosclerotic plaque formation, either with[13] or without[14] lowered blood cholesterol levels. Another study also reports lowered plasma total and LDL cholesterol in vitamin E-fed rabbits, although no inhibition of plaque formation was observed[15]. Wojcicki et al.[16] report an additive protective effect of selenium and vitamin E on atherosclerosis. Convincing evidence comes from a study[17] in which primates were supplemented with 79 mg vitamin E daily, resulting in prevention of experimental atherosclerosis compared with a control group receiving only the atherogenic diet; in another group, which already had developed atherosclerosis, vitamin E suppletion caused a significant regression. To our knowledge, no such experiments have been reported for β-carotene.

Epidemiological studies

Vitamin E has also received most attention in epidemiological studies. The preliminary results of the first long-term, randomized primary-prevention trial were reported only recently[18]. Among male smokers receiving 50 mg α-tocopherol daily for 5 to 8 years, there were fewer, but not significantly less, deaths caused by ischaemic heart disease than in the control group. Overall mortality did not differ significantly between the α-tocopherol and control groups, although more deaths from haemorrhagic stroke were observed among the men who received the supplement. Rimm et al.[19] have reported an inverse association of vitamin E intake with risk of CHD in a prospective cohort study among US health professionals. The association was restricted to supplement users and was not observed at normal dietary intake levels; a similar result was reported for women[20]. Of course, dietary intake assessment has major limitations and the use of supplements is probably much easier to recall than the habitual use of specific foods. Moreover, exact information on the vitamin E content of supplements is readily available, whereas the vitamin E content of foods may vary and the calculations of intake by means of a food composition table can only provide a best estimate at a particular moment. Together with the smaller contrast in the normal range, this may partly explain the lack of association with dietary intake[21]. Limitations of the study design include the impossibility to adjust for blood cholesterol, the potential residual confounding by general health-related

behaviour and the possible confounding of the association between vitamin E and CHD by other vitamins. However, the study by Rimm et al. is certainly suggestive of a beneficial role for vitamin E in the prevention of CHD. Analysis of the effect of the number of years of supplement use showed hardly an association in subjects using vitamin E for less than 2 years, but a significant inverse association in those using supplements for 2-4 years. Rimm et al.[19] argue that this indicates a role in the atherogenic process, and not primarily in the acute events leading to sudden death or infarction; Byers[21] uses the same data to arrive at the opposite conclusion. Two recent prospective studies using serum vitamin E showed no association with MI[22] and CHD[23], supposedly because vitamin E status was above a presumed critical threshold level. In a German study[22], the relative risk estimate in the lowest tertile of serum vitamin E, when subjects with CHD at baseline were excluded, was 0.54 (90% CI 0.21-1.38). The association was not dependent on fatality of MI. So far the only observational study (apart from ecological comparisons) that has shown an inverse association of plasma vitamin E with cardiovascular disease was reported from Scotland[24], in patients with previously undiagnosed angina. (In Scotland, vitamin E status is relatively low. In the EURAMIC Study, mean adipose tissue α-tocopherol among cases in Edinburgh was 25% lower than among controls ($p < 0.01$).)

For β-carotene, epidemiological studies have provided more support for a protective role in cardiovascular disease. In the US Health Professionals Follow-up Study[19], a significant inverse association between β-carotene and risk of CHD was observed among former and current smokers, but not among men who had never smoked. Another prospective study, among a cohort of elderly men and women, also showed a significant inverse association between dietary intake of β-carotene and cardiovascular death[25]. Preliminary results have been reported of an inverse association between serum and plasma carotenoids and CHD events[26] or progression of carotid atherosclerosis[27]. In contrast with these findings are the preliminary results of the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study[18], in which more deaths due to ischaemic heart disease were found among participants who had received 20 mg β-carotene a day for 5 to 8 years (77.1 per 10,000 person-years) than in the control group (68.9 per 10,000 person-years). These results provide certainly no evidence for a beneficial effect of β-carotene, at least at the dosis used. Therefore, it cannot be excluded that the inverse associations found in observational studies only indicate that β-carotene is marker for other dietary factors or for a life-style that is preventive of CHD.

In comparison with vitamin E and β-carotene, the role of selenium in atherogenesis has received little attention in the past few years, in contrast with the earlier burst of studies. In Denmark, a 3-year follow-up of a cohort of over 3000 men showed a 55% increased risk of ischaemic heart disease among subjects with serum selenium levels below 1 μmol/l, approximately the lowest tertile, after adjustment for cholesterol, social class, smoking and age[28].

Biological mechanism

The current major hypothesis linking antioxidants to coronary heart disease focuses on the lipid peroxidation of the LDL cholesterol particle, enhancing its uptake by macrophages in the arterial wall and the formation of fatty streaks, an early stage of atherosclerosis. Moreover, oxidized LDL may be a key component in endothelial injury[29], playing a role in later stages. Another hypothesis supposes an effect of antioxidants, in particular vitamin E, on platelet aggregation[30] or adhesion[31]. Observational and experimental studies have accumulated evidence for the role of oxidative modification of LDL in human atherosclerosis. Salonen et al.[32] have found the titre of autoantibodies against malondialdehyde-modified LDL to be an independent predictor of the progression of carotid atherosclerosis in men. Regnström et al.[33] found the susceptibility of LDL to oxidation to be associated with severity of coronary atherosclerosis in survivors of myocardial infarction. The in vitro oxidizability of LDL was shown to be dependent on vitamin E content[34-37], and on fatty acid composition: replacement of linoleic acid with oleic acid reduces the oxidation rate and inhibits the uptake by macrophages[38-45]. Supplementation with β-carotene does not result in protection of LDL against oxidation in vitro[35,37]. In how far the method of copper- or cell-mediated oxidation of LDL in vitro reflects any of the actual processes going on in the atherosclerotic vessel remains unclear.

EURAMIC Study

The a priori hypothesis was that antioxidant status would be lower in MI cases than in controls. Furthermore, we postulated that an inverse association between β-carotene and MI would be most pronounced in smokers; selenium would interact with vitamin E in the association with MI.

α-Tocopherol

The findings of the EURAMIC Study do not suggest an important role of α-tocopherol, in the physiological range, in the protection of myocardial

infarction. We found no overall significant association of α-tocopherol with MI. At the lowest selenium quintile, there may be a trend towards an inverse association, but at all other levels of selenium status the direction of the association is even positive. There is no effect modification by level of PUFA in the adipose tissue. This lack of association is in agreement with most studies using plasma or serum vitamin E levels to examine the association with CHD. Moreover, normal dietary intake of vitamin E in other studies was also not associated with CHD risk. However, intake of vitamin E supplements was found to be inversely associated with risk of CHD, in line with supplementation studies in animals. This apparent inconsistency may be related to the different ranges of intake. The recent suggestion that plasma α-tocopherol levels may be regulated within a certain range under normal circumstances supports the notion that normal intake levels are not related to CHD risk. Only at a very high or low vitamin E status[24] a linear inverse association with CHD risk could become apparent. The biological mechanism need not be the same in such extreme situations; the pharmacological effect can be different from an antioxidant effect. Under specific circumstances, α-tocopherol may even act as a pro-oxidant[46]. Thus, in contrast to in vitro and animal experiments, the EURAMIC Study has not confirmed the hypothesis that α-tocopherol in particular may provide protection against CHD.

β-Carotene

The EURAMIC results support the hypothesis that β-carotene may play a role in the protection of polyunsaturated fatty acids against oxidation and thereby prevent myocardial infarction. The inverse association of β-carotene with risk of MI might be attributed to a lower intake in patients. The association is strongest in smokers and in subjects with a high PUFA status. These form the groups with relatively high oxidative stress and high susceptibility to lipid peroxidation. These findings are in line with those from dietary intake studies[19,47], although a possible interaction with PUFA intake has not yet been examined to our knowledge. The results of the Finnish prevention study[18], however, do not support a causal role of β-carotene suppletion in the prevention of CHD. Although further reports from this study are to be awaited, this may indicate that β-carotene is merely a marker of a protective dietary pattern or life-style. On the other hand, as for α-tocopherol, different biological mechanisms may prevail at different ranges of exposure.

Selenium

The observed association of toenail selenium with MI was only weak

and not statistically significant. In the lowest selenium quintile the risk of MI was 55% higher than in the highest quintile. The finding that a significant association between selenium and MI existed at low vitamin E levels (although the interaction term was not significant), suggests that selenium may act as an antioxidant somewhere in the process of atherogenesis or thrombogenesis. However, the observed interaction with PUFA was not in line with the antioxidant hypothesis. At low PUFA, we observed an inverse association, but not at medium or high PUFA levels. Of course, as this modifying effect of PUFA status on the risk estimates for selenium was not significant, it may well be explained by chance. It was not due to a low vitamin E status in the low-PUFA group, because there was no overall association between vitamin E and PUFA in our population. It is possible that the interaction between selenium and PUFA may be explained by some residual confounding by smoking: the largest proportion of smokers was found at the lowest PUFA level. The biological role of selenium in the body has been shown to be primarily its function as an antioxidant[48] and a nutritional interaction with vitamin E was established in studies on animal diseases, decades ago. Our results are in line with animal experiments showing a combined effect of vitamin E and selenium on atherosclerosis[16] and platelet function[49]. To our knowledge, no human (experimental or observational) studies have been performed to investigate a possible interaction of selenium and vitamin E in atherogenesis.

Public health consequences

Our conclusions about the possible role of selenium, α-tocopherol and β-carotene in the prevention of myocardial infarction are based on biomarker status. Comparison of biomarker status with dietary intake indicates that extrapolation to conclusions about dietary intake can only be made very cautiously.

General recommendations of antioxidant supplements are not warranted before large, long-term prevention trials have established a clear benefit[50] - not only because the effectiveness of a preventive or therapeutic measure should be known, but also because it is not completely sure that the long-term intake of very large doses of antioxidant nutrients will be harmless. The preliminary results of the first large primary-prevention trial justify such reservations[18]. More data from metabolic studies in healthy subjects as well as CHD patients are needed to clarify the biological mechanism, the impact

of dosage and the relationship with diet, including the role of different food sources and dietary patterns, before dietary antioxidants may be optimally used in primary and secondary prevention of CHD. However, there are certainly no objections to a generous intake of antioxidants through a normal dietary pattern.

A recent evaluation of vitamin E intake in the Netherlands, based on the first National Food Consumption Survey, indicated an adequate level of intake for the population[51]: median intake in all age-categories was above the 0.67 α-tocopherol equivalents (α-TE) per gram PUFA, defined as the intake that covers minimum requirements for 97.5% of the population. Indeed, only 1-2% of the adult population had an intake of less than that minimum requirement (0.4 α-TE/gram PUFA). Considering the adequate provision with vitamin E, our results indicate that selenium status is not likely to be an important factor in the prevention of CHD in the Netherlands. However, it is evident from the comparison among the EURAMIC centres that the selenium status in the Netherlands is among the lowest in Europe. In the lowest range of selenium status, it may be important to avoid a simultaneous low vitamin E status. As yet, on the basis of our study and other available evidence in human populations, there is no reason to raise the recommendations for dietary vitamin E intake, neither absolutely nor relative to polyunsaturated fatty acids.

Fruits and vegetables, the main sources of β-carotene, and partly good sources of vitamin E as well, also contain other health-promoting compounds[52]. Moreover, there is accumulating evidence that fruit and vegetables may protect against specific forms of cancer[53,54]. In the light of this notion, it is worrying that the consumption of fruits and vegetables has decreased between 1987 and 1992 in the Netherlands, by almost 10%[55]. The average consumption of fruit is limited to one portion, and the mean consumption of vegetables is only 128 g per day, which is 50-100 g less than what is desirable according to the Netherlands Bureau for Nutrition Education[56], and certainly less than the recommendation of 5 daily servings issued by the American National Research Council[57]. An active policy to increase the consumption of fruit and vegetables in the Netherlands, especially among younger age groups, may be warranted.

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Summary

The epidemiologic study presented in this thesis aimed to find evidence in support of the hypothesis that antioxidant nutrients may protect against coronary heart disease.

Coronary heart disease is the major cause of death among men in most European countries. The underlying process, atherosclerosis, involves the accumulation of cholesterol and other lipids in the arterial wall. In an advanced stage of atherosclerosis, the lumen of the vessel may become completely obstructed by the resulting fibrous plaque, or by the formation of a blood clot. If that happens in the coronary arteries, the blood flow to the heart is interrupted, causing a myocardial infarction. One of the main risk factors for atherosclerosis is a high serum cholesterol level, in particular cholesterol transported in low-density lipoproteins (LDL). Recent experimental studies suggest that LDL in itself is not atherogenic, but becomes so when modified by oxidation processes naturally occurring in the body. Especially the polyunsaturated fatty acids in the LDL particle are susceptible to oxidation. Oxidation processes in the body are restrained by an antioxidant defense mechanism. This defense consists of several enzymes, including the selenium-containing glutathione peroxidase, and diet-derived compounds such as vitamin E and β-carotene (pro-vitamin A). It is conceivable that the beginning and progress of atherosclerosis is dependent on the balance between oxidative stress by internal and external factors, the susceptibility to oxidation of fatty acids and other biomolecules, and the availability of antioxidants acting to prevent oxidation (Chapter 2).

The EURopean study on Antioxidants, Myocardial Infarction and Cancer of the breast, in short the EURAMIC Study, was designed to examine the association between antioxidants and the risk of coronary heart disease. The levels of α-tocopherol (vitamin E) and β-carotene in adipose tissue and of selenium in toenails were compared between patients with a first acute myocardial infarction (MI) and control subjects without a history of MI. We hypothesized that these levels would be lower in patients than in controls. Moreover, the relative intake of polyunsaturated fatty acids was estimated by assessing the fatty acid composition of the adipose tissue. Patients were recruited from coronary care units of hospitals in 8 European countries and Israel. Control subjects were, in most countries, population controls, but in some countries hospital controls were a more feasible comparison group. Patients as well as controls were below 70 years of age, had had stable

dietary habits (including supplement use) and a stable body weight in the previous 12 months. The antioxidant levels in the samples were analyzed in a central laboratory, to ensure comparability among centers. The data from all centers were pooled to calculate the risk of MI at low versus high antioxidant levels (Chapter 3).

In 683 patients and 727 controls, we examined the association between adipose tissue α-tocopherol, β-carotene and risk of MI. The mean β-carotene concentration was significantly lower in patients than in controls. Persons in the lowest fifth of the β-carotene distribution had a 80% higher risk than persons in the highest fifth. This was particularly due to an increased risk in former and current cigarette smokers. The risk of MI was not associated with the level of α-tocopherol in adipose tissue (Chapter 4).

Mean toenail selenium concentration was 4% lower in cases than in controls. The risk of MI was 55% higher at low than at high selenium levels, but this difference was not statistically significant. However, in subjects with low vitamin E levels, low toenail selenium was associated with a 2.5-fold increased risk of MI compared with high selenium. Among persons with high vitamin E levels, the risk of MI was not dependent on selenium levels. These findings suggest an interaction of selenium and vitamin E in the etiology of MI, possibly through their antioxidant functions (Chapter 5).

As mentioned above, the potential protective effect of antioxidants may also depend on the intake of polyunsaturated fatty acids (PUFA) that are susceptible to lipid peroxidation. The intake of PUFA is reflected in the adipose tissue. The inverse association between β-carotene and MI described in Chapter 4 was indeed dependent on the level of PUFA. At high PUFA levels, low β-carotene status was associated with a more than three-fold increased risk of MI. At lower PUFA levels, there was still an increased risk at low β-carotene status, but less than at high PUFA levels. For α-tocopherol, no interaction with PUFA levels was observed. For selenium, the opposite was found: at low PUFA, but not at high PUFA, there was an inverse association between selenium and MI. This is not in line with our hypothesis; possibly this finding may be explained by the larger number of smokers in the low-PUFA group, because smoking strongly affects selenium levels (Chapter 6).

In the EURAMIC Study, biomarkers of α-tocopherol, β-carotene and selenium were used, assuming that these would reflect dietary intake over a longer period of time. To examine this assumption for α-tocopherol and β-carotene, we compared the levels in plasma and in adipose tissue with dietary intake, as measured by a food frequency questionnaire, in an

additional study. The study was performed among healthy, non-smoking volunteers (38 men and 47 women), aged 50-70. Supplementation with 30 mg β-carotene daily for 6 months resulted in 6-fold increased levels in adipose tissue. However, the association between adipose tissue levels and usual dietary intake was only modest ($r = 0.24$ for α-tocopherol and $r = 0.20$ for β-carotene), but stronger than the association of plasma levels with intake (Chapter 7).

In the Dutch contribution to the EURAMIC Study, in addition to the adipose tissue samples, we collected information on dietary intake of α-tocopherol and β-carotene by means of a food frequency questionnaire. For the biomarkers, the associations with MI were similar to those for the pooled data. For dietary intake, the association between β-carotene and MI was weak and not significant; for α-tocopherol intake, there was a tendency towards an inverse association with MI, which was more or less opposite to the biomarker data. The limited number of subjects does not allow to draw firm conclusions. However, it appears that the biomarkers do not reflect dietary intake very well, as we had seen before; moreover, the relationship between intake and biomarkers differs between MI patients and population controls. This may indicate a different recall of food consumption for patients and controls, but it may also reflect differences in metabolism, either as a cause or a consequence of the disease (Chapter 8).

Chapter 9 gives an account of some of the strengths and weaknesses in the study design and conduct. The results of the EURAMIC Study are discussed in the light of recent findings. We conclude that variations within the normal range of intake of vitamin E are not associated with MI and therefore cannot be considered to protect against coronary heart disease. This does not exclude the possibility that large doses of vitamin E may have a beneficial effect. Dietary selenium may have a modest protective effect on coronary heart disease, in particular in persons with a low vitamin E status. Low β-carotene status is associated with increased risk of myocardial infarction, especially in persons who smoke cigarettes or have done so in the past. Randomized controlled trials with varying doses and combinations of antioxidant nutrients should clarify whether the observed associations are causal. Until then, supplement use is not recommended, but a generous consumption of fruits and vegetables may be encouraged.

Samenvatting

Het doel van het epidemiologisch onderzoek dat in dit proefschrift wordt beschreven, was het onderbouwen van de hypothese dat antioxidanten uit de voeding bescherming kunnen bieden tegen coronaire hartziekten.

Coronaire hartziekten vormen de belangrijkste doodsoorzaak voor mannen in de meeste landen van Europa. Hieraan ten grondslag ligt het proces van atherosclerose, waarbij cholesterol en andere vetten in de vaatwand worden opgeslagen. In een gevorderd stadium van atherosclerose kan de doorgang van het bloedvat worden geblokkeerd door de gevormde fibreuze plaque, of door de vorming van een bloedstolsel. Als dat gebeurt in de kransslagaders van het hart wordt de doorbloeding van het hart belemmerd en ontstaat er een hartinfarct. Een van de voornaamste risicofactoren voor atherosclerose is een hoog serum-cholesterolgehalte, met name het cholesterol dat in het bloed getransporteerd wordt in het lage-dichtheids-lipoproteïne (LDL). Volgens recente experimentele studies zou LDL zelf niet atherogeen zijn, maar dat pas worden wanneer het gemodificeerd wordt door oxidatiereacties, die van nature in het lichaam plaatsvinden. Vooral de meervoudig onverzadigde vetzuren in het LDL-deeltje zijn gevoelig voor oxidatie. Oxidatieprocessen in het lichaam worden binnen de perken gehouden door de beschermende werking van antioxidanten. Deze bescherming wordt gegeven door verschillende enzymen, waaronder het selenium-bevattende glutathion-peroxidase, en door uit de voeding afkomstige stoffen zoals vitamine E en β-caroteen (pro-vitamine A). Het is denkbaar dat het ontstaan en het voortschrijden van atherosclerose afhangt van de balans tussen oxidatieve stress door interne en externe factoren, de gevoeligheid van vetzuren voor oxidatie en de aanwezigheid van antioxidanten (Hoofdstuk 2).

De EURAMIC-studie (EURopean study on Antioxidants, Myocardial Infarction and Cancer of the Breast) is opgezet om het verband tussen antioxidanten en coronaire hartziekten te onderzoeken. De gehalten van α-tocoferol (vitamine E) en β-caroteen in onderhuids vetweefsel en van selenium in teennagels zijn vergeleken tussen patiënten met een eerste acuut hartinfarct en controlepersonen die nooit een hartinfarct hadden gehad. De vooronderstelling was dat deze gehalten lager zouden zijn in patiënten dan in controlepersonen. Verder is gekeken naar de vetzuursamenstelling van het vetweefsel; die geeft een indruk van de relatieve inneming van meervoudig onverzadigde vetzuren via de voeding. Patiënten waren afkomstig van de hartbewakingsafdelingen van ziekenhuizen in 8 landen in Europa en Israel.

De controlegroep werd in de meeste landen gevormd door een steekproef uit de bevolking, maar in sommige landen vormden ziekenhuiscontroles een geschiktere vergelijkingsgroep. Patiënten en controlepersonen waren jonger dan 70, hadden stabiele voedingsgewoonten en een stabiel lichaamsgewicht in de voorafgaande 12 maanden. Het antioxidantgehalte is in de vetweefselmonsters bepaald in een centraal laboratorium, om de resultaten van de verschillende centra goed te kunnen vergelijken. De data van alle centra zijn bij elkaar genomen om het risico op een hartinfarct te kunnen berekenen voor mensen met een laag antioxidantgehalte ten opzichte van mensen met een hoog gehalte (Hoofdstuk 3).

Bij 683 patiënten en 727 controlepersonen is het verband tussen α-tocoferol en β-caroteen in vetweefsel en het risico op een hartinfarct bestudeerd. De gemiddelde β-caroteenconcentratie was significant lager bij patiënten. Personen in de laagste 20% van de β-caroteen-verdeling hadden een 80% hoger risico op een infarct dan personen in de hoogste 20% van de verdeling. Dit was vooral te wijten aan een verhoogd risico bij rokers en ex-rokers. Het risico op een hartinfarct hield geen verband met het α-tocoferol-gehalte in vetweefsel (Hoofdstuk 4).

De gemiddelde concentratie van selenium in teennagels was 4% lager bij patiënten dan bij controles. Het risico op een infarct bij een laag seleniumgehalte was 55% hoger dan bij een hoog seleniumgehalte; dit verschil was echter niet statistisch significant. Voor personen met een lage vitamine E-status was er echter wel een significant verband tussen een laag seleniumgehalte en het infarctrisico: het risico bij laag seleniumgehalte was dan 2,5 maal zo hoog als bij hoog selenium. Bij personen met een hoge vitamine E status was er geen verband tussen het risico op een infarct en het seleniumgehalte. Deze resultaten wijzen op een mogelijke interactie tussen selenium en vitamine E in de etiologie van het hartinfarct, hetgeen mogelijk verband houdt met de antioxidantfunctie van beide stoffen (Hoofdstuk 5).

Zoals eerder aangegeven wordt de balans tussen oxidatie en antioxidanten mede bepaald door de inneming van meervoudig onverzadigde vetzuren (MOV), die relatief gemakkelijk geoxideerd worden. De inneming van MOV wordt weerspiegeld in de samenstelling van het onderhuids vetweefsel. De sterkte van het inverse verband tussen β-caroteen en het risico op een hartinfarct dat in Hoofdstuk 4 is beschreven, bleek inderdaad af te hangen van het niveau van MOV. Bij een hoge MOV-status was een laag β-caroteen-gehalte geassocieerd met een meer dan drievoudig verhoogd risico op een hartinfarct. Bij een lage MOV-status was er ook een verhoogd risico bij een laag β-caroteengehalte, maar minder hoog dan bij een hoge MOV-

status. Voor α-tocoferol is geen interactie met MOV gevonden. Voor selenium vonden we, in tegenspraak met de vooronderstelling, wel een invers verband tussen selenium en hartinfarct bij een lage MOV-status, maar niet bij een hoge MOV-status. Deze onverwachte bevinding kan mogelijk verklaard worden door het grotere aantal rokers in de groep met een lage PUFA-status, omdat roken het seleniumgehalte sterk beïnvloedt (Hoofdstuk 6).

In de EURAMIC-studie zijn biomerkers voor α-tocoferol, β-caroteen en selenium gebruikt, in de veronderstelling dat deze een weerspiegeling zouden vormen van de inneming via de voeding over een langere periode. Om deze aanname voor α-tocoferol en β-caroteen te onderzoeken, zijn de gehalten in plasma en in vetweefsel vergeleken met de inneming via de voeding, gemeten met een voedselfrequentie-vragenlijst, in een aanvullende studie. Dit onderzoek is uitgevoerd bij gezonde, niet-rokende vrijwilligers (38 mannen en 47 vrouwen) van 50-70 jaar. Suppletie met 30 mg β-caroteen gedurende 6 maanden resulteerde in gemiddeld 6-voudig verhoogde concentraties in vetweefsel. Het verband tussen concentraties in vetweefsel en de gebruikelijke inneming via de voeding was echter vrij zwak ($r = 0.24$ voor α-tocoferol en $r = 0.20$ voor β-caroteen), maar sterker dan het verband tussen concentraties in plasma en de inneming (Hoofdstuk 7).

In de Nederlandse bijdrage aan de EURAMIC-studie is, naast vetweefsel, ook informatie verzameld over de inneming van α-tocoferol en β-caroteen via de voeding, met behulp van dezelfde voedselfrequentie-vragenlijst. Voor de antioxidanten in vetweefsel vonden we vergelijkbare resultaten als in de internationale dataset. De resultaten voor antioxidanten uit de voeding waren min of meer tegenovergesteld: voor β-caroteen was er geen verband met het risico op een hartinfarct en voor α-tocoferol was er een tendens naar een invers verband. Dit is onder andere het gevolg van het feit dat de gebruikte biomerkers de inneming via de voeding niet bijzonder goed weer lijken te geven, zoals we in de aanvullende studie al hadden gezien. Verder is de relatie tussen inneming en biomerkers verschillend voor infarctpatiënten en de controlepersonen. Door de beperkte omvang van de populatie in deze deelstudie is het niet mogelijk hier harde conclusies aan te verbinden. Het kan een aanwijzing zijn dat de vragen over de voeding verschillend worden beantwoord door patiënten en controles, maar het kan ook betekenen dat er verschillen in metabolisme zijn tussen patiënten en controles, hetzij als oorzaak hetzij als gevolg van de ziekte (Hoofdstuk 8).

In Hoofdstuk 9 worden enkele aspecten van de onderzoeksopzet en -uitvoering uitvoeriger besproken. De resultaten van de EURAMIC-studie worden belicht in het kader van recente resultaten van andere studies. De

conclusie is dat normale inneming van vitamine E geen bescherming lijkt te bieden tegen coronaire hartziekten, hoewel dit niet uitsluit dat grote hoeveelheden vitamine E mogelijk wel een gunstig effect hebben. Selenium beschermt misschien enigszins tegen coronaire hartziekten, vooral bij personen met een lage vitamine E-status. Een lage β-caroteen-status is geassocieerd met een verhoogd risico op een hartinfarct, vooral bij personen die roken of hebben gerookt. Gecontroleerde interventiestudies met verschillende doses antioxidanten moeten in de toekomst opheldering geven over de mogelijke causaliteit van de verbanden die we hebben waargenomen. Tot die tijd is er geen goede reden om suppletie met antioxidanten aan te bevelen; de consumptie van ruime hoeveelheden groenten en fruit kan echter zeker geen kwaad.

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Verschillende collega's hebben een duidelijke bijdrage aan dit boekje geleverd. Soms heel herkenbaar: Geert van Poppel, Henny Brants en Henk van den Berg als co-auteur, Arthur van Aken voor de figuren, Hanny Leezer de perfecte vormgeving en Monica Stam de prachtige omslag. Of wat meer impliciet: Willy van Dijk, door alle voedingsvragenlijsten in te voeren, Marco Bouman, voor de conversie van de uiteenlopende data naar hetzelfde format, anderen door belangstelling, adviezen en zo nu en dan een schouderklopje als dat nodig was. Ellen, het was op het laatst wel eens lastig (vooral voor de (co)promotoren) dat we zo synchroon liepen, maar ik vond het erg opbeurend om iemand in de buurt te hebben die ook in paniek kon raken bij het idee dat de nullen en enen misschien verwisseld waren.

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About the author

Alwine Kardinaal was born on 17 May 1961 in Den Ham (O), the Netherlands. After finishing secondary school (VWO, Pius X College, Almelo) in 1978, she studied Human Nutrition at Wageningen Agricultural University. She majored in human nutrition, family sociology and public health and graduated in March 1984. From 1984 to 1991 she was involved in the management of the Dutch Food Intolerance Databank, first at the Wageningen Agricultural University, and from 1988 as project leader at the TNO Nutrition and Food Research Institute, department of Human Nutrition. For three years (1988-1990) she was also coordinator of the bureau of the NEVO Nutrient Databank. In 1990, she became project leader of a study on the prevalence of food intolerance among adults, which was finished in 1992, and of the Dutch part of the EURAMIC study on antioxidants and myocardial infarction. During the conduct of this multi-centre study, she was part of the TNO coordinating team. At present, she is involved in several studies at the Epidemiology Department of the TNO Nutrition and Food Research Institute, including new collaborative actions within Europe.