Low-density lipoprotein oxidation, antioxidants and risk of atherosclerosis

Lucy P.L. van de Vijver

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Contents

Manuscripts based on studies described in this thesis

Chapter 1
LPL van de Vijver, AFM Kardinaal, DE Grobbee, HMG Princen, G van Poppel. Lipoprotein oxidation, antioxidants and cardiovascular risk (review). *Prostaglandins, Leukotrienes and Essential Fatty Acids* (in press)

HMG Princen, LPL van de Vijver, G van Poppel. AFM Kardinaal. Antioxydanten en coronaire hartziekten. Stipendium Boerhave Symposium 'Hyperlipoproteinemie en atherosclerose; nieuwe ontwikkelingen ten behoeve van diagnostiek en behandeling' 1996:73-88.

Chapter 2
LPL van de Vijver, W van Duyvenvoorde, R Buytenhek, A van der Laarse, AFM Kardinaal, H van den Berg, HMG Princen. Seasonal variation in Low Density Lipoprotein oxidation and antioxidant status. *Free Radical Research* (in press)

Chapter 3
LPL van de Vijver, AFM Kardinaal, W van Duyvenvoorde, HACM Kruijssen, DE Grobbee, G van Poppel, HMG Princen. LDL oxidation and risk of coronary atherosclerosis. (submitted)

LPL van de Vijver, AFM Kardinaal, W van Duyvenvoorde, HACM Kruijssen, G van Poppel, HMG Princen, DE Grobbee. Oxidation of LDL and risk of peripheral atherosclerosis. (submitted)

LPL van de Vijver, R Steyger, G van Poppel, HACM Kruijssen, JC Seidell, HMG Princen. Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls. *Atherosclerosis* 1996;122:245-53.

Chapter 4
LPL van de Vijver, AFM Kardinaal, HACM Kruijssen, DE Grobbee, HMG Princen, G van Poppel. Plasma vitamin C, LDL antioxidants and risk of coronary atherosclerosis. (submitted)

LPL van de Vijver, C Weber, AFM Kardinaal, DE Grobbee, HMG Princen, G van Poppel. Plasma Coenzyme Q_{10} levels are not decreased in patients with coronary atherosclerosis.

Chapter 5
LPL van de Vijver, JCM Witteman, JH den Breeijen, MC de Bruyne, AFM Kardinaal, A Hofman, DE Grobbee. Major dietary antioxidants and risk of silent myocardial infaction: the Rotterdam Study. (submitted)

LPL van de Vijver, AFM Kardinaal, HAM Brants, DE Grobbee, HMG Princen, G van Poppel. Dietary intake of flavonoids is not related to risk of coronary atherosclerosis; a case-control study.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in most industrialized countries. In the Netherlands, it is responsible for 40% of all deaths.[1] Major risk factors for CVD are identified such as smoking, high cholesterol level and hypertension. Other important determinants of cardiovascular risk are nutrition, physical activity and body weight. However, these known risk factors cannot fully explain individual differences in cardiovascular risk. An intriguing hypothesis has been postulated in which the known beneficial effect of a diet high in fruit and vegetables is combined with the known harmful effect of high cholesterol levels or, more specifically, high low-density lipoprotein (LDL) cholesterol levels.[2] This hypothesis describes a high level of LDL cholesterol as a promoting factor in atherosclerosis after LDL has been chemically altered by free radical compounds. The uptake of cholesterol in macrophages is increased and foam cells are formed. These foam cells can cluster just beneath the intima of the vessel wall which is the beginning of the fatty streak and of the atherogenic process. Antioxidants can play a role in protecting LDL from oxidation by scavenging free radicals.

Several lines of evidence have indicated that oxidation occurs *in vivo* and that antioxidants may have a preventive effect. Epitopes of oxidized LDL are found in atherosclerotic lesions.[3] Further, supplementation with vitamin E has been reported to decrease susceptibility of LDL to oxidation.[4,5] Evidence for direct relations between susceptibility to oxidation and risk of cardiovascular diseases, however, is scarce. To investigate susceptibility of LDL to oxidation and the preventive role of antioxidants in relation to atherosclerosis, we performed the studies described in this thesis.

Chapter 1 presents an overview of the current knowledge of the relation between antioxidants and CVD, antioxidants and LDL oxidation and the relation between LDL oxidation and CVD.

Chapter 2 describes a small study on seasonal variation in susceptibility of LDL to oxidation and in determinants of LDL oxidation. This study was performed to determine whether the moment of blood sampling should be taken into account in a study on LDL oxidation.

Chapter 3 comprises three studies on the relation between LDL oxidation and risk of atherosclerosis. In section 3.1 results of a case-control study on susceptibility of LDL to oxidation in relation to risk of coronary atherosclerosis are presented. In section 3.2 the association between susceptibility of LDL to oxidation and risk of

peripheral atherosclerosis is described. The method of measuring LDL oxidation used in these two sections is an *ex vivo* measure, which may not reflect the processes going on *in vivo*. Therefore, we investigated the association between an *in vivo* measure of LDL oxidation, the autoantibody titre against oxidized forms of LDL, and risk of coronary atherosclerosis in a small case-control study. This study is described in section 3.3.

An indirect method for investigating the effect of LDL oxidation on the risk of atherosclerosis is by measuring antioxidant status (Chapter 4), which may reflect the antioxidant defence against oxidation of LDL. The antioxidant status in the LDL particle and plasma vitamin C levels were measured and related to risk of coronary atherosclerosis (section 4.1). Further the association between plasma levels of the antioxidant Coenzyme Q_{10} and coronary atherosclerosis was studied (section 4.2).

Chapter 5 presents two studies on dietary intake of antioxidants in relation to cardiovascular disease. In section 5.1 a study is presented on dietary intake of vitamin C, α-tocopherol and β-carotene in relation to the risk of having a silent myocardial infarction. Data were collected in the Rotterdam Study, a prospective cohort study. Section 5.2 describes results on dietary intake of flavonoids in relation to risk of coronary atherosclerosis.

In the epilogue (Chapter 6) a general discussion of the hypothesis, the strength of the research performed and some indication for further research are given.

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Chapter 1

Lipoprotein oxidation, antioxidants and cardiovascular risk: epidemiologic evidence

1

Lipoprotein oxidation, antioxidants and cardiovascular risk: epidemiologic evidence

ABSTRACT

This review summarizes the scientific evidence for a possible role of antioxidants in the prevention of coronary heart disease (CHD). Dietary antioxidants include vitamin E, vitamin C and β-carotene, whereas selenium is an integral part of the antioxidant enzyme glutathione peroxidase. Experimental studies suggest that the oxidation of low-density lipoproteins (LDL) in the vessel wall plays an important role in the development of atherosclerotic lesions. The resistance of LDL to oxidation is increased by antioxidant supplementation, at least *in vitro*. Epidemiological studies have not demonstrated unequivocally that a high intake of antioxidants leads to a decreased risk of CHD. Studies on dietary intake and serum levels of antioxidants do point into the direction of a preventive effect of antioxidants, whereas the results of intervention studies are less conclusive. β-carotene supplementation is not associated with any decrease in CHD; high doses of vitamin E may be beneficial, but results from large trials are to be awaited. General preventive measures based on antioxidant supplementation are not yet justifiable.

INTRODUCTION

It has long been hypothesized that free-radical reactions mediate in the development of coronary heart disease (CHD) and that antioxidants play a protective role.[1] In recent years interest in the latter compounds has strongly increased, inspired by experimental evidence that the hypothesis regarding the role of blood lipids on the one hand and of antioxidants on the other do not contradict but rather may complement one another.[2] Promising epidemiological findings[3,4] have indicated that a diet adequate in, or supplemented with, antioxidants could help prevent CHD. In this review we will briefly discuss the possible role of antioxidants in the prevention of CHD.

FREE-RADICAL REACTIONS AND LDL OXIDATION

Free radicals are highly reactive because these molecules contain an unpaired electron. They can thus readily oxidize and damage essential biological molecules such as fats, proteins and DNA. Polyunsaturated fatty acids are particularly prone to oxidation (lipid peroxidation).

The formation of free radicals is a biological process taking place, for example, when oxygen is reduced to water in the respiratory chain of live tissues or during enzymatic synthesis of prostaglandins and leukotrienes.[1,5] Under normal physiological conditions, the cell is protected against an overproduction of radicals by the enzymes superoxide dismutase, catalase and the selenium-dependent glutathione peroxidase, and by various antioxidants such as vitamin E, vitamin C and β-carotene.[5] In case of a disturbed balance between formation of free radicals (oxidant stress) and antioxidant defence, free radicals can play a role in the development of various diseases including CHD.

Unsaturated fatty acids in low-density lipoproteins (LDL) are sensitive to oxidation. The three most important cell types in the vessel wall (endothelial cells, smooth muscle cells and macrophages) can release free radicals which affect lipid peroxidation. Oxidized LDL can become atherogenic through a number of mechanisms. Oxidation can elicit a chemotactic response simulating monocytes and T-lymphocytes to be attached to the endothelial monolayer and then migrate into the subendothelial space, the intima.[2,6–8] In the intima the monocytes are transformed to macrophages which efficiently absorb oxidatively modified LDL via the 'scavenger receptor'. The macro-phages subsequently develop into foam cells and thus give rise to 'fatty streak', the first stage of the atherosclerotic process. Further, auto-antibodies against oxidized LDL are formed,[9,10] which form an immune complex with the oxidized LDL and are recognized by the Fc receptor on the macrophages. This process accelerates the uptake of oxidized LDL by macrophages.

In addition to the formation of foam cells, oxidized LDL stimulate the cells in the vessel wall to produce cytokines and growth factors.[6–8,11] This process leads, among other things, to proliferation of smooth muscle cells and production of fibre proteins, thus leading to the next stage of the atherosclerotic process, formation of fibrous plaque. Moreover, oxidized LDL have an inhibitory effect on the production of endothelium-dependent relaxation factor, thus reducing vasodilatation of the atherosclerotic vessel.[7,8,11] Further, oxidized LDL are cytotoxic and can directly damage endothelial

cells.[11] In combination with the stimulation of platelet aggregation and of procoagulant activity on the surface of endothelial cells and macrophages, the damage to endothelial cells will contribute to a further progress of atherosclerosis, leading eventually to complex atherosclerotic lesions.[6-8,11]

In addition to this mechanism via oxidized LDL, free radicals and lipid peroxides in the cell can have a direct toxic effect on endothelial cells by reacting with proteins and lipids in the cell membrane. It has been hypothesized that this process leads to changes in the structure of the membranes and thus to changes in membrane permeability or membrane-bound protein activity.[12] Among other things, it may change the permeability of the endothelial inner lining of the blood vessel.

Various methods are available to investigate whether oxidative processes in the body do occur indeed. Damage to DNA (oxidized DNA bases), proteins (protein carbonyls) and lipids can be measured. To study the relation between oxidative stress and atherosclerosis, lipid peroxidation and its consequences are of particular interest. Two of these methods are the thiobarbituric acid (TBA) test used to measure aldehydes, degradation products of lipid peroxides, and the measurement of conjugated dienes in LDL. Although both methods have their limitations,[13] they have produced indications for oxidative processes to play a role *in vivo* in the development of atherosclerosis. Stringer et al.[14] have used the TBA test to demonstrate the occurrence of elevated levels of lipid peroxides in patients with vascular disorders. Others have found an association between sensitivity of LDL to lipid peroxidation (measurement of conjugated dienes) and coronary sclerosis.[15-17] A new, promising method for the detection of lipid peroxidation is measurement of 8-isoprostanes, oxidation products of arachidonic acid. Plasma levels of 8-isoprostanes have been found to be elevated in smokers.[18]

Subsequent degradation products of lipid peroxidation can form new epitopes in LDL, which can be detected by means of monoclonal antibodies. Immunocytochemical techniques have demonstrated the presence of oxidized LDL in atherosclerotic lesions, but not in unaffected arteries.[2,6] Higher titres of auto-antibodies against modified LDL appear to coincide with an accelerated progression of carotid atherosclerosis,[9,10] although they may not be an indicator of the severity of atherosclerosis.[19,20]

If a causal relationship exists between oxidized LDL and atherosclerosis, then it would follow that antioxidants may protect against atherosclerosis by preventing or retarding the oxidative modification of LDL. In a number of epidemiological and clinical studies this possible preventive effect of antioxidants on CHD has been inves-

tigated. The concentration of antioxidant micronutrients in tissues depends mainly on dietary intake. Antioxidant nutrients for which intake has been studied in relation to CVD include vitamins E and C, β-carotene, and selenium, a co-factor in the enzyme glutathion peroxidase.

OBSERVATIONAL STUDIES

Vitamin E

In the WHO/MONICA study, in which various European countries were compared,[21,22] ischaemic heart disease was found to be inversely associated with average plasma vitamin E levels. This association has also been found in a survey in 19 European and 5 non-European countries,[23] but in Finnish men no relationship was found between vitamin E/cholesterol ratio and prevalence of ischaemic heart disease.[24] Riemersma et al.[25] did find lower plasma vitamin E levels in patients with angina pectoris, whereas the European multi-centre case-controlled EURAMIC study has not revealed an association between α-tocopherol in adipose tissue and the risk for myocardial infarction.[26] Prospective studies on antioxidant intake or blood levels in relation to CVD risk are summarized in Table 1. In three prospective studies,[27-29] men who died of ischaemic heart disease did not have reduced plasma vitamin E levels standardized for cholesterol. However, a higher intake of vitamin E, in particular through supplements, has been found to be associated with a lower incidence of CHD both in the prospective Nurses' Health Study among women[4] and in the Health Professionals Follow-up Study among men.[3] In a large US cohort of post-menopausal women,[30] a more than 60% reduced risk of CHD mortality was found in the group with the highest intake of vitamin E from food, but no association was observed for higher intakes from supplements. In a Finnish cohort with a 14-year follow-up, vitamin E intake tended to be related to a reduced risk for CHD mortality both among women and men, but the risk in the group with the highest vitamin E intake was significantly lower than in the group with the lowest intake for women only.[31] In the ARIC study, too, a significant inverse relation between vitamin E intake and thickness of the carotid artery wall was found only among women.[32] A recent sub-group analysis of a group treated with niacin and colestipol in the Cholesterol Lowering Atherosclerosis Study has demonstrated an association between self-chosen vitamin E supplement use and angiographically confirmed reduction of progression of coronary lesions.[33]

Vitamin C

Vitamin C has also been a subject in the WHO/MONICA study. On a group level, lower plasma vitamin C levels appeared to be associated with a higher cardiovascular mortality.[21,22] In the UK, too, ischaemic heart disease mortality was found to be highest in regions with a relatively low vitamin C intake.[34,35] Patients with angina pectoris, however, had no reduced plasma vitamin C levels after correction for smoking.[25]

In the prospective Basel study, lower plasma vitamin C levels preceded a higher ischaemic heart disease mortality; however,this correlation was not statistically significant.[27] In two large US cohorts,[3,30] no relation could be established between vitamin C intake and CHD incidence. In a 25-year follow-up of middle-aged men, there was no overall association between vitamin C intake and CHD mortality, but men who never smoked had a more than 40% decreased risk when they had an intake of more than 112 mg vitamin C per day.[40] In a prospective study in England, both a high vitamin C intake and high plasma vitamin C levels were found to be associated with a lower risk for cerebrovascular disorders, but not of coronary disease.[36] In contrast, a prospective study in Finland did show a relation with CHD, albeit only for women.[31] A strong inverse relation between vitamin C intake and risk for CHD has been found in NHANES I.[37] Moreover, recent results from the ARIC study reveal a strong inverse relation between vitamin C intake and thickness of the carotid artery wall.[32]

β-carotene

In Israel, a strong inverse relation has been found between dietary intake of vitamin A from fruit and vegetables and cardiovascular mortality.[38] In a case-control study,[39] the incidence of acute myocardial infarction was negatively associated with consumption of carrots, green vegetables and fresh fruit. The European EURAMIC study has reported that patients with acute myocardial infarction had a lower β-carotene content in adipose tissue than had controls[26] which could be indicative of a lower long-term β-carotene intake. However, this relation was found only for smokers and ex-smokers. In the prospective Health Professionals Follow-up Study, a high β-carotene intake was found to be associated with a lower risk of CHD, also for smokers only.[3] In a Finnish cohort study, a protective effect of β-carotene was found neither for women nor for men, but β-carotene did strengthen the favourable effect found for vitamins C and E in this study.[31] In two subsequently reported cohort studies,[30,40] no association of β-carotene with CVD risk was observed.

Table 1. *Prospective studies on antioxidant intake or blood levels in relation to CVD risk*

Name study First author (ref)	Population Gender,age	n	follow-up (yr)	Exposure Contrast in comparison group	Outcome (nr of cases)	Results[1] RR (95% CI)
Dietary intake						
NHANES I, Enstrom[37]	♂ ♀ 25-74 year	11.348	10	SMR group ≥ 50 mg vit C relative to SMR US whites	Fatal CVD (1809)	SMR=0.90 (0.82-0.99) SMR=0.66 (0.53-0.82) (incl suppl)
US health professionals, Rimm[3]	♂ health professionals without CVD; 40-75 yr	39.910	4	Vit E: ≤ 6.9 vs. ≥ 11.0 IU/d (food) 0 vs. ≥ 250 IU/d (suppl) Vit C: median 92 vs. 1162 mg/d Carotene: <5030 vs. ≥ 14.388 IU/d	Fatal CHD, non-fatal MI, bypass, PTCA (667)	RR_{vitE}= 0.79 (0.54-1.15) (food) RR_{vitE}= 0.70 (0.55-0.89) (suppl) RR_{vitC}= 1.25 (0.91-1.71) RR_{caro}= 0.71 (0.53-0.86) RR_{caro}= 0.30 (0.11-0.82) (smokers)
US nurses health study, Stampfer[4]	♀ nurses 34-59 year	87.245	8	Vit E: ≤ 3.5 vs. ≥ 21.6 IU/d median 2.8 vs. 208 IU/d ≤ 3.1 vs. ≥ 6.3 IU/d (food)	Non-fatal MI CHD death (552)	RR_{vitE}= 0.66 (0.50-0.87) (incl suppl) RR_{vitE}= 0.95 (0.72-1.23) (food)
Finland longitudinal population study, Knekt[31]	♂ 30-69 year	2.748	14	Vit E: ≤ 6.8 vs. ≥ 8.9 mg; Vit C: ≤ 60 vs. > 85 mg; carotenoids: ≤ 147 vs. ≥ 258 µg retinol equivalents	Fatal CHD (186)	RR_{vitE}= 0.68 (0.42-1.11)p_{tr}=0.01 RR_{vitC}= 1.00 (0.68-1.45) RR_{caro}= 1.02 (0.70-1.48)
Finland longitudinal population study, Knekt[31]	♀ 30-69 year	2.385	14	Vit E: ≤ 5.3 vs. ≥ 7.1 mg; Vit C: ≤ 61 vs. > 91 mg; carotenoids: ≤ 182 vs. ≥ 383 µg retinol equivalents	Fatal CHD (58)	RR_{vitE}= 0.35 (0.14-0.88) p_{tr}<0.01 RR_{vitC}= 0.49 (0.24-0.98) RR_{caro}= 1.62 (0.30-1.29)
Western Electric study, Pandey[40]	♂ employees 40-55 year	1.556	25	Vit C: ≤ 82 vs. > 112 mg; β-Carotene: ≤ 2.9 vs. > 4.0 mg	Fatal CHD (231)	RR_{vitC}= 0.75 (0.52-1.07) RR_{caro}= 0.84 (N.S.)
Iowa's Womens Health Study, Kushi[30]	♀ postmenopausal 55-69 year	34.486	7	Vit E: ≤ 4.9 vs. ≥ 9.6 IU/d (food) 0 vs. >250 IU/d Vit C: ≤ 112.3 vs. ≥ 391.3 mg/d Carotenoids: ≤ 4421vs. ≥ 13465 IU	Fatal CHD (242)	RR_{vitE}= 0.38 (0.18-0.80) (food) RR_{vitE}= 1.09 (0.67-1.77) (suppl) RR_{vitC}= 1.49 (0.96-2.30) RR_{caro}= 1.03 (0.63-1.70)

Name study First author (ref)	Population Gender,age	n	follow-up (yr)	Exposure Contrast in comparison group	Outcome (nr of cases)	Results[1] RR (95% CI)
Serum levels						
EPOZ-study, Kok[28]	♂ ♀ 37-87 year	10.532	6-9[2]	Vit E: < 645 vs. ≥ 645 Selenium: <105 vs. ≥105 µg/l	Fatal CVD(84)	RR_{VitE}= 0.7 (0.3-1.7) RR_{Se}= 0.6 (0.3-1.25)
Basal study, Gey[27]	♂ working population; mean age 50±9 yr	2.974	12	Vit C: <22.7 vs. ≥22.7 µmol/l Carotene: <0.23 vs. ≥0.23 µmol/l	Fatal IHD (132)	RR_{VitC}= 0.8 (0.5-1.3) RR_{Caro}= 0.7 (0.5-0.9)
MONICA Augsburg study, Hense[29]	♂ ♀ 25-64 year	4.022	5[2]	Vit E: ≤ 27.9 vs. > 27.9 µmol/l	Fatal and non-fatal MI (46)	RR_{VitE}= 1.85 (90%CI: 0.7-4.8)
Street[41]	♂ ♀ 35-65 year	25.802	7-14[2]	Vit E and carotenoids lowest vs. highest quintile	MI(123)	OR_{Caro}= 0.45 (0.22-0.90) OR_{VitE}= 2.4; $OR_{VitE/chol}$= 0.7
Morris[42]	♂ 40-59 years	1.899	13	Carotenoids: <2.3 vs. >3.2 µmol/l	Fatal CVD, non-fatal MI (282)	RR= 0.64 (0.44-0.92) RR= 0.28 (0.11-0.73) (never smokers)
Skin Cancer Prevention trial, Greenberg[43]	♂ ♀ skin cancer patients mean age 63.2 yr	11.881	8.2	β-carotene: <0.21 vs. >0.52 µmol/l	Fatal CVD	RR= 0.57 (0.34-0.95)

[1]RR compared to low intake/level group. [2]nested case-control study. [3]p_{tr} = p for trend. Abbreviations used: CVD=cardiovascular disease, CHD=coronary heart disease, MI=myocardial infarction, PTCA=percutaneous transluminal coronary angioplasty, SMR=standardized mortality ratio, RR=relative risk, OR=odds ratio.

Several studies have looked at blood levels of β-carotene or carotenoids, as marker of dietary intake. In the prospective Basel study, low plasma β-carotene levels were found to be associated with an elevated risk for ischaemic heart disease,[26] whereas such an elevated risk was found only for smokers in a retrospective study.[41] In the cohort of the Lipid Research Clinics Coronary Primary Prevention Trial,[42] CHD risk for men with the highest serum carotenoid levels was lower than for men with the lowest levels (22% and 72% reduction for smokers and non-smokers, respectively). Similarly, patients in the Skin Cancer Prevention Study with initial plasma β-carotene levels in the highest quartile were found to have a 43% decreased risk of CVD mortality, compared to patients in the lowest quartile.[43]

Selenium

Selenium is a co-factor of the antioxidant enzyme glutathione peroxidase and has been studied extensively in relation to CHD. In Dutch patients with acute myocardial infarction, selenium levels measured in erythrocytes, toenails and plasma were lower than in healthy controls.[44] Such an unequivocal relation has not been found, however, in seven prospective studies varying in follow-up period from 5 to 7 years. Three of these studies found low plasma selenium levels to be associated with an elevated risk for CHD and death.[45-47] The other four prospective studies, however, did not find such a relation.[28,48] In the European EURAMIC study, a weak but non-significant overall inverse association between selenium levels in toenails and risk of non-fatal MI was observed.[49] Thus, there is not an unequivocal relation between blood selenium levels and the incidence of CHD. Two studies that did find an association[45,46] were conducted in Finland, where selenium intake has been very low until recently. Likewise, in the EURAMIC study the only country where a significant inverse association was found was Germany (Berlin), the center with the lowest selenium levels. It is conceivable that CHD risk is, indeed, influenced by extremely low blood selenium levels.

In interpreting descriptive epidemiological studies, it should be borne in mind that comparisons among countries simply show associations and provide no proof for causal relations. Moreover, in case-control studies one should always question whether a change in antioxidant status is the cause or the consequence of the disease. This problem does not exist is prospective studies in which antioxidant status is measured some years before the disease is diagnosed. In prospective studies, it is essential that the results are corrected for possible confounding factors. For example, smoking is an established risk factor, and smokers have usually both lower intakes and lower plasma

levels of vitamin C and β-carotene. However, even if the results are adequately corrected for known risk factors, it is certainly conceivable that an elevated plasma concentration of β-carotene, for example, simply reflects a high consumption level for vegetables and that it is not β-carotene, but another component or combination of components of vegetables, that is responsible for the association found. Moreover, a high intake of antioxidants could obviously also be correlated with unknown life-style factors.

Studies described above both investigated dietary intake levels and serum or plasma levels and their relation to the risk of CVD. Serum and plasma levels have been used as a biomarker for dietary intake. However, correlations between dietary and plasma levels of antioxidants are low. Kardinaal et al. reported correlations of 0.05 for α-tocopherol in non-supplemented subjects. However, significant correlations slightly over 0.30 were observed by others (50 and references in 50). For β-carotene slightly higher correlation coefficients have been reported ranging from 0.17 to 0.49.[50] Therefore it seems more reasonable to use plasma and serum levels as a marker for the internal concentration, taking into account the variation in absorbtion and metabolism.

The epidemiological studies discussed above suggest that there might be an inverse association between vitamin E intake and CHD, but that this association may become manifest only at high vitamin E intakes from supplements. For vitamin C, no consistent inverse relation with CHD has been established. As for β-carotene, this antioxidant could play a protective role in particular for smokers, which would confirm the hypothesis that smoking-induced oxidative stress impairs the body's β-carotene status. The results for selenium do not point consistently to a protective effect of high intakes, but an extremely low selenium status could well be a risk factor. The threshold value could be relevant in this context. It should be noted, however, that the studies mentioned cannot demonstrate a cause-effect relation. The best proof is provided in intervention studies exploring the effects of vitamin supplementation on relative risk, morbidity and mortality.

INTERVENTION STUDIES

Animal studies

Convincing evidence that antioxidants can prevent the progression of atherosclerosis has been provided by animal studies. In cholesterol-fed and LDL receptor-deficient rabbits and monkeys, the drug probucol, a potent antioxidant, inhibits the progression

of atherosclerosis. A similar effect has been found for other synthetic antioxidants in some, but not all, other animal studies[51,52] and references therein.Probucol also inhibits the formation of neo-intima after damage to the vessel wall in pigs.[53] This finding suggests that reactive oxygen compounds may play a role in restenosis. Some studies have found that supplementation of the diet with vitamin E slows down the development of atherosclerosis in rabbits. In other studies, however, such an effect was not found.[54] Restenosis is also suppressed by α-tocopherol after balloon dilatation in rabbits.[55]

Wójcicki et al.[56] have reported an additive protective effect of selenium and vitamin E on atherosclerosis. Convincing evidence comes from a study[57] in which primates were supplemented with 70 mg vitamin E daily, resulting in prevention of experimental atherosclerosis as compared with a control group receiving only the atherogenic diet. In another group, which already had developed atherosclerosis, vitamin E supplementation resulted in significant regression.

Some vitamin C supplementation studies in cholesterol-fed rabbits produced a reduction of aortic intimal or more advanced atheromatous lesions, but this effect was not uniformly observed.[58] Supplementation with low doses of vitamin E or β-carotene resulted in preservation of endothelium-dependent vessel relaxation in rabbits after cholesterol feeding,[59] but high doses of vitamin E worsened vasodilative function.[60] A recent study has demonstrated inhibition of atherosclerosis by all-*trans*-β-carotene, but not by 9-*cis*-β-carotene (0.01% in the chow). This finding suggests the involvement of retinoid receptors in the vessel wall in the development of atherosclerosis.[61]

Most of the animal studies report a favourable effect of antioxidant on the development of atherosclerosis, whereas in some studies no clear association was found. This can possibly be explained by the fact that both the level of progression of the atherosclerotic process and the dose of antioxidants in relation to cholesterol levels are important factors. Moreover, some antioxidants have effects other than their antioxidant action.

Human intervention studies

Human intervention studies with antioxidant vitamins have thus far mainly focused on risk factors. None of four well controlled intervention studies conducted in a healthy population has shown any effect of vitamin E supplementation on platelet aggregation.[62] Nor was an effect of vitamin E on symptoms of angina pectoris found in any of six intervention studies.[63] Although five intervention studies have reported a modest

favourable effect of vitamin E on symptoms of intermittent claudication, the sample sizes were small in each of these studies.[63] However, vitamin E supplementation *in vivo* does lead to an increased resistance of LDL to *ex vivo* chemical oxidation,[54,64-68] but the incidence of restenosis after percutaneous transluminal coronary angioplasty was not significantly altered by vitamin E supplementation.[69]

Supplementation with 0.5–1.0 g vitamin C was reported to have favourable effects on plasma lipids, but these effects were only seen in subjects with marginal plasma vitamin C or high serum cholesterol levels.[58,70]

No favourable effects of β-carotene on plasma lipids have been reported,[71,72] and reports on effects of β-carotene on *ex vivo* LDL oxidation are conflicting.[3,64-68] Moreover, these results possibly cannot be extrapolated to *in vivo* conditions because β-carotene is an effective antioxidant in particular under low oxygen tension.[74] This aspect is supported by a study in which β-carotene lowered the concentration of pentane (an indicator of lipid peroxidation) in exhaled air of smokers, but not of non-smokers.[75]

The only intervention study we know (the Probucol Quantitative Regression Swedish Study) in which the effect of probucol on atherosclerosis in the femoral artery was studied, did not find any differences between the experimental and the control group.[76] This could be explained by reduction of high-density lipoprotein (HDL) levels and of plasma levels of fat-soluble dietary antioxidants.

Results of several intervention studies with disease incidence as end-point have recently been reported[43,77-80] and are summarized in Table 2. The first was a randomized primary prevention trial with β-carotene and/or vitamin E among 29,133 male Finnish smokers.[77] Among men receiving 50 mg α-tocopherol daily for 5–8 years, there were not significantly fewer deaths caused by ischaemic heart disease than in the control group. Overall mortality did not differ significantly between the α-tocopherol and the control group, although more deaths from cerebral haemorrhages were seen among the tocopherol-supplemented men. For β-carotene-supplemented subjects (20 mg/day), more deaths from ischaemic heart disease were reported after 5–8 years. In this same study, the incidence of angina pectoris in the vitamin E and β-carotene intervention groups was not significantly different from the groups using placebo.[81]

Table 2. *Human intervention studies on antioxidant supplementation in relation to CVD risk*

Name study (reference)	Population Gender,age	n	Intervention	Duration mean (range)	Outcome (number of cases)	Results [1] RR (95% CI)
ATBC study-group[76]	♂ smokers; 50-69 year	29.000	β-carotene (20 mg) and/or vitamin E (50 mg) / d	6.1 (5-8) yr	Death from IHD (1239)	RR_{vitE}= 0.98 (0.89-1.08) RR_{caro}= 1.11 (0.99-1.23)
ATBC, Rapola[80]					Incidence of Angina Pectoris (1983)	RR_{vitE}= 0.91 (0.83-0.99) RR_{caro}= 1.06 (0.97-1.16)
CARET, Omenn[78]	♂♀ smokers + workers exposed to asbestos; 45-69 year	18.314	30 mg β-carotene + 25.000 IU retinol / d	4.0 yr	Death from CVD	RR= 1.26 (0.99-1.61)
PHS, Hennekens[79]	♂ physicians; 40-84 year	22.000	50 mg β-carotene / 2 d	12.0 yr	CVD events (1939)	RR = 1.00 (0.91 -1.09)
Linxian Blot[87]	♂♀ rural subjects 40-69 year	29.584	β-carotene (15 mg) + vitamin E (30 mg) + selenium (50 µg) / d	5.25 yr	Cerebrovascular death (523)	RR= 0.90 (0.76-1.07))
SCPS Greenberg[43]	♂♀ skin cancer patients; 27-84 year	1.730	50 mg β-carotene / d	8.2[2] yr	CVD (127)	RR= 1.16 (0.82-1.64)
CHAOS, Stephens[77]	♂♀ patients with coronary disease	2.002	800 or 400 IU α-tocopherol	510 days (3-981 days)	CVD, non-fatal MI (105)	RR[3]= 0.53 (0.34-0.83)

[1]Intervention compared to placebo. [2]4.3 years of supplementation. [3]α-tocopherol supplement users (both 400 and 800 IU) vs. placebo. Abbreviations used: CVD=cardiovascular disease, IHD=ischemic heart disease, MI=myocardial infarction, RR=relative risk.

Thus, the results of this Finnish trial provide no evidence for a beneficial effect of β-carotene and vitamin E. Major criticism on this study was based on the sample of heavy smokers, many of whom had smoked for more than 30 years so that the atherosclerotic progress might have progressed too far for a successful intervention, and the choice of low doses of vitamin E. However, the lack of a beneficial effect of β-carotene was reproduced in other large randomized trials.[43,79,80] The relative risk of having a major cardiovascular event in the Physicians' Health Study, after a follow-up of 12 years, was 1.00 for the group receiving 50 mg β-carotene on alternate days.[80] In a randomized trial with a high dose of alpha-tocopherol (25-50 times the usual dietary intake) a significant reduction (77%) of the risk of non-fatal MI was observed.[78]

DISCUSSION

Experimental data based on *in vitro* studies suggest that free-radical reactions play a major role in the development of CHD. The results of *in vitro* studies and of animal models examining the role of antioxidants and oxidative changes are less consistent. For example, some antioxidants may exert prooxidant action under specific experimental conditions. The protective (sparing) effect of vitamin C on vitamin E can be easily demonstrated *in vitro*, but evidence for a prominent role *in vivo* is scarce. *In vitro* studies seem to indicate that vitamin C is very important as an antioxidant, but large prospective studies exploring the relation between antioxidant and CHD have revealed a prominent role for vitamin E, but not for vitamin C. Although some epidemiological studies have produced intriguing results, they have not established unequivocally that high intake of antioxidants reduces the risk for CHD. This lack of unanimity may be attributed in part to differences in levels of intake, study population or disease outcome measure. Also, vitamin E, vitamin C and β-carotene are not the only antioxidants worthy of being considered. For example, flavonoids contained in red wine have been proposed as an explanation of the 'French paradox', i.e. the low cardiovascular mortality in France in spite of a high prevalence of known risk factors.[82] Flavonoids are found in various products of vegetable origin (grapes, tea, onions, apples). The prospective Zutphen study has shown that a higher intake of flavonoids is associated with a lower CHD risk.[83] Similarly, it has been suggested that other carotenoids than β-carotene, such as alpha-carotene or lycopene may exert a protective effect. Further, the intake of pro-oxidants such as iron, zinc and copper could be relevant[84] as well as the intake of polyunsaturated fatty acids, the most important substrate for oxidative

reactions. It has been suggested that substitution of monounsaturated fatty acids for polyunsaturated fatty acids may reduce the oxidizability of LDL.[85,86] The epidemiological studies assess intake of antioxidants, through food and supplements, which is probably indicative of a long-term exposure. Therefore results from these studies may indicate an effect at earlier stages of atherosclerosis. On the other hand, it cannot be excluded that persons with a high intake also differ with respect to intake of other dietary factors or life-style characteristics related to CVD risk, which may explain the inverse associations found.

The preventive trials do not support a beneficial effect of β-carotene on CVD risk. For vitamin E, we have too little information yet. The dose used in the Finnish trial was relatively low and their high risk study population may not have been the most appropriate, if an effect on early stage atherosclerosis is assumed. However, high doses have been shown to prevent myocardial infarction in a high risk group.[78] Several ongoing trials can hopefully give us some answers in the coming years.[87]

In conclusion, general preventive measures based on antioxidant supplementation are not justifiable on the basis of current knowledge, although there is certainly support for a preventive effect of high doses of vitamin E on cardiovascular disease risk. The effect of other compounds from fruit and vegetables may be explored. Fundamental research into the pathogenesis of atherosclerosis and the role of lipid peroxidation may help to select the most appropriate study designs in terms of type of antioxidant, dose, population and duration of the intervention.

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Chapter 2

Seasonal Variation in Low Density Lipoprotein Oxidation and Antioxidant Status

2

Seasonal Variation in Low Density Lipoprotein Oxidation and Antioxidant Status

ABSTRACT

Accumulating evidence indicates that oxidative modification of low-density lipoproteins is atherogenic and that antioxidants may play a role in protection of LDL against oxidation. Several studies have reported a seasonal fluctuation in antioxidant levels, but to date nothing is known about seasonal fluctuations in parameters of oxidizability.

We collected blood from 10 volunteers at four different periods over one year (February, May, September and December), and measured levels of plasma lipids, plasma antioxidants, lipid and fatty acid composition of the LDL particle, LDL antioxidant content, LDL particle size and oxidation parameters (resistance time and maximum rate of oxidation).

No seasonal fluctuation for resistance time and maximum rate of oxidation of copper ion-induced LDL oxidation was found. Small seasonal fluctuations were observed for some determinants of LDL oxidation, e.g. plasma and LDL vitamin E and LDL particle size, and for plasma lipids, plasma and LDL lutein and LDL β-carotene. Fatty acid composition of LDL did not change during the year. The main determinant of oxidation susceptibility was the fatty acid composition of LDL.

We conclude that LDL oxidation parameters do not change over the year.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) by free radicals has been implicated as an important determinant in the atherogenic process. By oxidative modification the uptake of LDL by macrophages is accelerated which is the beginning of the fatty streak.[1] LDL is protected against the free radical attack by antioxidants in plasma and in the particle itself. High plasma levels of vitamin E,[2-4] vitamin C[2,3,5] and β-carotene[5,6] have been suggested to be associated with a decreased risk of cardiovas-

cular diseases.

In the literature seasonal variation in plasma lipid and vitamin levels has been reported. Seasonal variation in β-carotene,[7-9] ascorbic acid[10,11] and α-tocopherol[7,8] may result either directly or indirectly in seasonal variation in LDL oxidizability through variation in plasma or LDL antioxidant activity. Other well-known parameters influencing the oxidizability of LDL are the size and composition of the LDL particle[12,13] and its fatty acid composition.[14,15] To our knowledge, no data are available on seasonal variation of these parameters. We therefore studied the influence of the time of the year on the antioxidant status and parameters of oxidizability of LDL cholesterol in healthy subjects.

METHODS

Study design

We collected blood of 10 volunteers at four different periods over one year (February, May, September and December). The six men and four women were apparently healthy and had a mean age 39.6 ± 7.4 years. Two of them were current smokers. Two persons had only three blood collections. All participants in this study gave their informed consent.

Blood was collected in EDTA-containing Vacutainer tubes (1 mg/ml) between 8.30 and 9.00 am at all four time points. The subjects had consumed a light breakfast. Blood was immediately placed on ice and cooled to 4°C. Plasma was prepared, frozen in liquid nitrogen in small portions, leaving as little empty space as possible in the tubes, and stored at -80°C. This procedure was completed within 1 hour from venapuncture. Laboratory analyses were performed at the end of the year of study.

Preparation and oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from the method described by Esterbauer and colleagues[16] with some major modifications as described previously in detail.[17,18] Briefly, from each subject 2 ml of frozen plasma, stored at -80°C was rapidly thawed and used for isolation of LDL by ultracentrifugation at 4°C in the presence of 10 μM EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants,[19] the LDL was not dialysed.[16] By omitting dialysis a more stable LDL preparation is obtained, which can be stored in the dark at 4°C under nitrogen for several days without affecting resistan-

ce time and maximum rate of oxidation.[17,18,20] This improves the precision of the method, since each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, resistance time was 90 ± 2 min one hour after LDL isolation in a LDL preparation which had not been dialyzed; 24 hours after LDL isolation resistance time was 91 ± 3 min (n = 3). Dialysis under nitrogen for 4 hours (2 changes) at 4°C against 1000 volumes of an oxygen-free buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4 resulted in resistance times of 52 ± 5 min, directly after dialysis and 23 ± 4 min after storage of this LDL under nitrogen for 24 hours (n = 3). In agreement with these observations, recently, a loss of lipophilic antioxidants during dialysis was reported.[19]

LDL oxidation is performed under hypersaline conditions (1.18 mol/L NaCl) and in the presence of 10 µmol/L EDTA. Oxidation of LDL under hypersaline conditions results in a higher resistance time compared with oxidation in physiological saline (0.15 mol/L).[17] In a test we found a resistance time of 90 ± 2 min in 1.18 mol/L NaCl and 58 ± 5 min in 0.15 mol/L NaCl both with 10 µmol/L EDTA and 40 µmol/L $CuSO_4$ (n = 3 independent oxidations with a reference LDL on different days). Ten µmol/L EDTA is added during ultracentrifugation to protect the LDL against oxidation and in the oxidation assay to have equal EDTA concentrations in all assays. To overcome the 10 µmol/L and because of the hypersaline conditions, 40 µmol/L $CuSO_4$ is added to initiate lipid peroxidation.[17,18] Under these conditions (high salt and 40 µmol/L $CuSO_4$) the presence of EDTA does not affect kinetics of LDL oxidation.

The kinetics of the LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm.[16-18] All samples from one person were analysed in parallel in the same oxidation run. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. Means were calculated based upon these three observations. The intra-assay coefficients of variation for resistance time and maximum rate of oxidation calculated from measurements obtained at one day were 2.6% and 3.1%, respectively. The inter-assay coefficients of measurements performed on different days were 4.9% and 7.4%, respectively.[18] In every oxidation run one reference LDL, prepared from a reference plasma stored at -80°C, was used as a control. Oxidation runs with a higher than 10% deviation from the mean values of former reference measurements were omitted.[17,18] By using this highly standardized method, resistance time and maximum rate of oxidation do not differ between LDL prepared from plasma frozen in liquid nitrogen and that from freshly collected plasma from the same subject. In addition, no differences in these parameters were found upon

storage of plasma at -80°C up to 18 months.[17,18]

Analytical measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit nr. 236.691 and Triglyceride kit nr. 701.904, Boehringer-Mannheim, Mannheim, Germany). Phospholipid concentrations were determined using a commercially available colour reagent (Wako Chemicals GmbH, Neuss, Germany). 100 µl of LDL (0.25 mg protein/ml) sample and 750 µl colour reagent were mixed for 10 minutes at 37°C and the concentration was measured at a wavelength of 500 nm.

High density lipoprotein (HDL)-cholesterol was measured after precipitation of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) using the precipitation method with sodium phosphotungstate-Mg^{2+}.[21] LDL-cholesterol concentrations were calculated by the Friedewald formula.[22] LDL size was determined by analysis of 7.5-10 µl plasma using 2-16% nondenaturing polyacrylamide gradient gel electrophoresis (Pharmacia LKB, Uppsala, Sweden).[23] High molecular weight standards (Pharmacia, Piscataway, N.J., U.S.A.) were used, together with a reference serum obtained from a pool of normolipidemic sera. In our institution the reference value for LDL particle size is 25.8 nm. After staining with Sudan Black B gels were scanned with an LKB 2202 Ultrascan laser densitometer (LKB, Paramus, N.J., U.S.A.).

Fatty acid composition of LDL was determined by gas-liquid chromatography using a Chrompack gas chromatograph (model 438S) equipped with a CP-Sil88 column (50 m x 0.25 mm i.d.) and a flame ionisation detector, as described previously.[17] We calculated the amount of poly-unsaturated fatty acids (PUFAs = C18:2 + C20:3 + C20:4 + C22:6), mono-unsaturated fatty acids (MUFAs = C16:1 + C18:1), and saturated fatty acids (SFAs = C14:0 + C16:0 + C18:0).

Alpha-tocopherol and carotenoids in plasma and LDL (stored at -80°C) were quantified by reverse-phase HPLC with spectrophotometric detection.[24]

Statistical analysis

Data analysis was conducted using the statistical package BMDP.[25] First, means and standard deviations for the four time periods were calculated for plasma and LDL lipid levels, antioxidant levels in plasma and in LDL, LDL particle size and fatty acid composition and oxidation parameters. Within-person seasonal fluctuation for these

parameters was assessed by analysis of variance (5V module). For those parameters which showed a significant seasonal fluctuation paired t-tests were performed to detect which periods differed signicantly from each other ($p<0.05$). Further, partial correlations were calculated between the oxidation parameters and the determinants of oxidation, e.g. antioxidant vitamins, fatty acid composition and particle size. Adjustment was made for person number, to adjust for dependency of the measures of one subject over the four moments.

RESULTS

Table 1. *Seasonal variation in lipids and vitamins (mean ± SD)*

	February (n = 10)	May (n = 9)	September (n = 10)	December (n = 9)
Plasma:				
Cholesterol, mmol/L*	5.65 ± 0.95	5.36 ± 0.94[3]	5.63 ± 1.04[2]	5.38 ± 0.94
Triglycerides, mmol/L*	1.49 ± 0.63[2]	1.12 ± 0.45[1,3]	1.44 ± 0.64[2]	1.31 ± 0.55
HDL, mmol/L *	1.19 ± 0.22	1.32 ± 0.16	1.16 ± 0.27	1.32 ± 0.45
LDL, mmol/L *	3.78 ± 0.78	3.53 ± 0.83[3]	3.82 ± 0.78[2]	3.46 ± 0.90
Vitamin E, µmol/L *	33.08 ± 6.54	33.53 ± 6.80	33.62 ± 5.39	29.55 ± 5.60
Lycopene, µmol/L	0.31 ± 0.20	0.30 ± 0.16	0.41 ± 0.30	0.25 ± 0.11
β-Carotene, µmol/L	0.38 ± 0.16	0.34 ± 0.11	0.38 ± 0.15	0.32 ± 0.08
Lutein, µmol/L *	0.25 ± 0.07[3]	0.28 ± 0.08[3]	0.20 ± 0.05[1,2]	0.23 ± 0.09
α-Carotene, µmol/L	0.07 ± 0.03	0.06 ± 0.03	0.07 ± 0.03	0.05 ± 0.03
LDL:				
Vitamin E, nmol/mg protein *	22.18 ± 2.37[4]	24.10 ± 2.66[3,4]	19.73 ± 3.64[2]	18.91 ± 1.83[1,2]
Lycopene, nmol/mg protein	0.25 ± 0.14	0.25 ± 0.16	0.23 ± 0.16	0.21 ± 0.08
β-Carotene, nmol/mg protein *	0.22 ± 0.12	0.23 ± 0.12	0.29 ± 0.16	0.23 ± 0.11
Lutein, nmol/mg protein *	0.06 ± 0.02	0.08 ± 0.03[3,4]	0.05 ± 0.02[2]	0.05 ± 0.02[2]

*significant overall seasonal fluctuation ($p<0.05$). [1]significantly different from levels in February ($p<0.05$). [2]significantly different from levels in May ($p<0.05$). [3]significantly different from levels in September ($p<0.05$). [4]significantly different from levels in December ($p<0.05$).

Table 1 shows the mean and standard deviation for the plasma lipids, the plasma antioxidant vitamins, and the vitamin status of the LDL particle for the four time periods. Seasonal fluctuation was found for plasma lipids, plasma vitamin E and

plasma lutein. Plasma total-cholesterol and LDL-cholesterol levels were significantly lower in May compared to September and triglyceride levels were lower in May compared to both September and February. Plasma lutein was significantly lower in September compared to February and May. Further seasonal fluctuation was found for the vitamin E, β-carotene and lutein content of the LDL particle. Both LDL vitamin E and lutein had higher values in May compared to September and December, whereas for vitamin E the values in December were also lower than those in February.

Table 2. *Seasonal variation in parameters of LDL oxidation, LDL size and LDL fatty acid composition*

	February (n = 10)	May (n = 9)	September (n = 10)	December (n = 9)
Resistance time, min	100 ± 6	99 ± 5	101 ± 8	97 ± 7
Maximum rate of oxidation, nmol dienes/min/mg protein	9.0 ± 0.8	9.0 ± 0.7	9.1 ± 0.6	9.2 ± 0.9
LDL particle size, nm*	26.6 ± 0.5[3,4]	27.1 ± 0.4[4]	27.0 ± 0.5[1,4]	26.0 ± 0.3[1,2,3]
LDL fatty acid composition: Total fatty acids, µmol/ mg protein	4.2 ± 0.4	4.7 ± 0.5	4.5 ± 0.5	4.5 ± 0.4
saturated fatty acids, %	28.2 ± 3.2	28.3 ± 4.0	28.0 ± 2.8	29.5 ± 3.2
mono-unsaturated fatty acids, %	20.0 ± 2.6	20.9 ± 3.7	21.4 ± 3.1	20.6 ± 2.5
poly-unsaturated fatty acids, %	51.8 ± 4.1	50.8 ± 3.6	50.6 ± 4.5	50.0 ± 3.0

*significant overall seasonal fluctuation ($p<0.05$). [1]significantly different from levels in February ($p<0.05$). [2]significantly different from levels in May ($p<0.05$). [3]significantly different from levels in September ($p<0.05$). [4]significantly different from levels in December ($p<0.05$).

Seasonal variation in oxidation parameters, LDL particle size and LDL fatty acid composition are presented in Table 2. No significant seasonal variation for resistance time and maximum rate of oxidation was found. LDL particle size did show seasonal fluctuation, with the lowest particle size in December. There were no differences in fatty acid composition of the LDL over the four periods.

The oxidation parameters were not significantly correlated to LDL particle size as assessed by partial correlation ($r = -0.08$ and $r = 0.30$ for maximum rate of oxidation and resistance time, respectively). A positive correlation was found between the amount of poly-unsaturated fatty acids in LDL and maximum rate of oxidation (Pearson's $r = 0.65$, $p<0.01$) (Figure 1). The partial correlation for the association between

poly-unsaturated fatty acids and maximum rate of oxidation was slightly higher ($r = 0.73$, $p<0.01$). Further, maximum rate of oxidation was negatively correlated with LDL mono-unsaturated fatty acids ($r = -0.56$, $p<0.05$) and saturated fatty acids ($r = -0.51$, $p<0.05$). No significant correlation was found between fatty acid composition and resistance time.

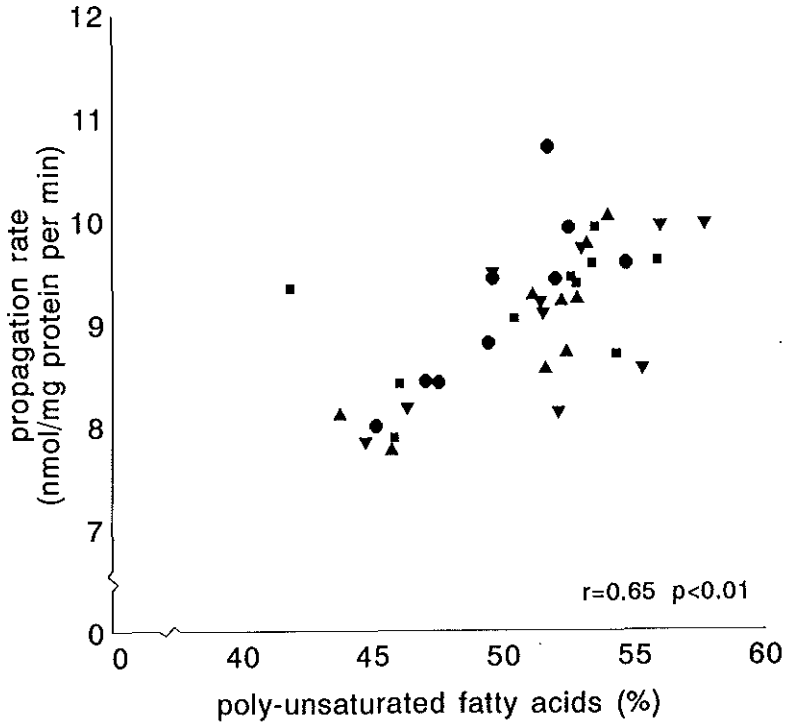

Figure 1. Plot showing the correlation between maximum rate of oxidation of LDL oxidation and the percentage LDL poly-unsaturated fatty acids per moment; February (▾), May (▴), September (■) and December (●). The Pearson correlation is calculated over the total of 38 measures.

DISCUSSION

The purpose of this study was to assess the influence of the time of the year on the antioxidant status and parameters of LDL oxidizability. Parameters of oxidation remained stable throughout the year. However, small fluctuations in plasma lipid levels, plasma and LDL vitamin E and lutein levels, LDL β-carotene levels and particle size over the seasons were found.

Our study sample comprised only a small number of subjects. Of the 10 participants two subjects had no blood sample taking at one moment. To optimize the use of all available data we used the repeated measures module, which adequately handles missing data. To ensure that the difference in means in the periods could not be ascribed to the different number of subjects, we calculated the means in the subset of subjects with complete data. Only marginal differences were detected and resulted in similar patterns as those presented in the tables (results not shown).

We are aware that the differences over the seasons found in some parameters are only small. Though statistically significant, this does not imply that these differences are also of physiological relevance.

In our study significant differences in plasma lipids over the seasons were found. The fluctuation pattern for total cholesterol, LDL and triglycerides were comparable, high in February and September, low in May and December. The HDL levels showed the opposite pattern. Several groups described seasonal fluctuation in cholesterol levels,[26-29] but from these and our studies it is still inconclusive in which months the peaks and throughs occur.

For vitamins we found a seasonal fluctuation in plasma vitamin E and lutein levels and LDL vitamin E, β-carotene and lutein levels. A seasonal variation in serum vitamin E levels, however, was not shown in other studies.[7,8] Several studies considering carotenoids found seasonal variations for β-carotene,[7-9] α-carotene, β-cryptoxanthin and lutein.[8] However, Cantilena and colleagues did not find a seasonal within-person fluctuation for α- and β-carotene and cryptoxanthin[30] and no seasonal fluctuations have been found for lycopene and zeaxanthin.[8,30] To our knowledge no data are available on fluctuations of vitamin concentrations in LDL.

Seasonal fluctuations in vitamin levels in plasma and LDL may be ascribed to seasonal fluctuations in intake of these vitamins. Dietary variation of antioxidant intake over the year has been described by several groups.[9,31] As a result of better preservation techniques and marketing facilities seasonal variation in food intake gets less pronounced, but may still be important. In present study no dietary data were assessed, so we can only speculate on influences of intake on plasma and LDL levels measured over different seasons. There is no variation in LDL fatty acid composition over the year, which suggests that qualitative intake of oils and fats (with respect to composition) remained stable over the year.

To our knowledge this is the first time that seasonal influence on oxidation parameters has been assessed. We did not find a difference in parameters of oxidation, e.g.

resistance time and maximum rate of oxidation, over the four time periods. Oxidizability of LDL is influenced by fatty acid composition,[14,15,20,32] LDL triglycerides,[32] LDL particle size,[12,13] LDL vitamin E[17,18,33] and plasma vitamin E levels,[17,18] but not by carotenoids.[18,34] From the above mentioned parameters a significant seasonal fluctuation was found for LDL particle size and LDL vitamin E. For vitamin E correlations with parameters of oxidation have been reported only after vitamin E suppletion.[17,18,33] The same holds for fatty acids in most studies.[14,15,20,32] Therefore an intervention seems necessary to find a sufficiently large contrast. Nevertheless, we found a possitive correlation between the total amount of poly-unsaturated fatty acids in LDL and maximum rate of oxidation in this study. Similar associations have been reported in other studies in unsupplemented normal and hypertriglyceridemic subjects,[20,35] indicating that fatty acid composition of the LDL particle is an important parameter determining LDL oxidizability in an unsupplemented (group of the) population. The LDL particle size in our study was not correlated to resistance time or maximum rate of LDL oxidation. This could be due to the small sample size of this study, or to the small diversity in particle sizes. Following the classification of LDL particles by Musliner and Krauss,[36] the predominant LDL type measured in our study is the large LDL type I. Other studies in which a relationship between particle size and oxidation parameters is reported, showed a greater diversity in particle sizes with separation of the various density classes by ultracentrifugation.[12,13] In our study one LDL preparation has been isolated and particle size of the most abundant LDL particles has been determined.

In spite of small fluctuations in determinants of LDL oxidtion, no seasonal fluctuation in LDL oxidation was found. Therefore it appears unnecessary to consider seasonality in a study on LDL oxidation.

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Chapter 3

Susceptibility of LDL to oxidation and atherosclerosis

3.1

LDL oxidation and risk of coronary atherosclerosis

ABSTRACT

Evidence has accumulated for oxidative modification of low-density lipoproteins (LDL) to play an important role in the atherogenic process. Therefore, we investigated the relation between coronary atherosclerosis and susceptibility of LDL to oxidation in a case-control study in which men between 45 and 80 years of age participated. Cases and hospital controls were selected from subjects undergoing a first coronary angiography. Subjects with severe coronary stenosis (≥ 85 % stenosis in one and ≥ 50% stenosis in a second major coronary vessel) were classified as cases (n = 91). Hospital controls with no or minor stenosis (≤ 50% stenosis in no more than 2 of the three major coronary vessels (n = 94)) and population controls free of plaques in the carotid artery (n = 85) were pooled for the statistical analysis into one control category. Enrolment procedures allowed for similar distributions in age and smoking habits.

Cases had higher levels of total and LDL cholesterol and triglycerides and lower levels of HDL cholesterol. Resistance time and maximum rate of oxidation were measured *ex vivo* using copper-induced LDL oxidation. A borderline significant inverse trend was observed for coronary atherosclerosis risk at increasing resistance time. Odds ratios (95% confidence interval) for the successive quartiles were 1.0 (reference), 0.77 (0.39-1.53), 0.67 (0.33-1.34) and 0.55 (0.27-1.15) (p_{trend} = 0.07). No relationship with maximum rate of oxidation was found. The main determinant of oxidation was the fatty acid composition of LDL.

We conclude that although LDL resistance to oxidation may be a factor in atherogenesis, the *ex vivo* measure is not a strong predictor of severity of coronary atherosclerosis.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) by free radicals has been implicated as an important determinant in the development of atherosclerosis. Oxida-

tive modification accelerates the uptake of LDL by macrophages which is the beginning of formation of a fatty streak.[1,2] LDL is thought to be protected against attacks of free radicals by antioxidants in plasma and in the particle itself.[3]

Circumstantial evidence indicates that oxidation occurs *in vivo* in humans. Epitopes of oxidized LDL have been found in plasma[4,5] and atherosclerotic lesions of experimental animals and human[6] and autoantibodies against these epitopes have been detected in human plasma.[5,7-10] Furthermore, an increased susceptibility of LDL to oxidation has been described in patients with coronary heart disease.[11-13] The susceptibility of LDL to oxidation is decreased by vitamin E supplementation[14-17] and increased by adding unsaturated fatty acids to the diet.[18-20] A reduction in risk of cardiovascular disease (CVD) at higher plasma antioxidant levels[21] has been reported and higher dietary antioxidant levels have been proposed to be associatied with a reduced risk of CVD.[22-24]

To determine the relation between oxidative stress and atherosclerosis, lipid peroxidation and its consequences is of particular interest. *Ex vivo*, the peroxidation process can be mimicked by incubating isolated LDL with the pro-oxidant Cu^{2+} and by following the production of conjugated dienes from polyunsaturated fatty acids. The time elapsing until the onset of diene production, the resistance time, depends on the strength of the antioxidant defence in the LDL particle,[25] and may, therefore, reflect the resistance to oxidation *in vivo*.[1] By using the copper-induced oxidation method, several studies have described individual variation in susceptibility to LDL oxidation.[11-20,26] In previous studies we have detected subtle changes in susceptibility of LDL to oxidation after supplementation of only 25 mg/day vitamin E[17] and by adding 5 g fish oil to the diet,[20] using this oxidation method.

To address the question whether LDL oxidation is related to the severity of coronary heart disease we compared the susceptibility of isolated LDL to copper-induced oxidation in a large group of patients with angiographically determined coronary atherosclerosis and a control group.

METHODS

Study population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, The Netherlands, in the period 1993-1995. The study was approved by an ethical committee on human research and all participants gave their informed consent.

We selected a group of coronary atherosclerosis patients, a group of hospital controls and a group of population controls. The groups consisted of men between 45 and 80 years of age. Enrolment procedures allowed for similar distributions of age (in 5-year categories) and smoking habits (smoking, non-smoking).

Selection of the two hospital groups was based on angiographic reports. To reduce the impact of the disease on dietary and life-style patterns we selected only those patients who underwent their first angiography and who had not experienced a myocardial infarction (MI) in the year prior to the study. For the same reason, blood was collected within 2 months after angiography. Subjects using HMG-CoA reductase inhibitors were excluded, because of a possible influence of this medicine on LDL oxidation.

In the study period 2830 patients underwent coronary angiography for suspected coronary atherosclerosis, which included 1966 male subjects. Subjects were ineligible if they met one of the following exclusion criteria: under 45 or over 80 years of age (n = 144), not the first coronary angiography (n = 289), MI in the 12 months prior to the study (n = 180), diabetes mellitus (n = 84), liver, kidney or thyroid disease (n = 15), alcohol or drug abuse (n = 4), use of HMG-CoA reductase inhibitors (n = 82), vegetarian diet (n = 3), psychiatric complaints (n = 2). For 88 subjects more than 2 months had elapsed between angiography and case selection and 12 patients had died in the meantime, leaving a population of 1063 eligible subjects. Of this group 124 refused to participate and 50 could not be contacted or were otherwise indisposed. From the remaining 889 men, 92 cases were selected who had at least 85% stenosis in one and at least 50% stenosis in a second of the three major coronary vessels. Ninety-five hospital controls were selected with less than 50% stenosis in no more than two of the three major coronary vessels. The percentage of stenosis was scored by the cardiologist performing the angiography.

Population controls were selected from participants in the Rotterdam Study. The rationale and design of this population-based prospective cohort study have been described previously.[27] No angiography data were available, but subjects were selected without any plaques in the carotid artery as assessed by ultrasound echography. Also these subjects had no history of cardiac disease or treatment, had no diabetes mellitus or liver, kidney or thyroid disease, did not use HMG-CoA reductase inhibitors nor were vegetarian. As the participants in the Rotterdam Study were all 55 years and over at baseline we additionally recruited men between 45 and 55 years of age through an advertisement in a local newspaper. Recruitment took place in the area the other

population controls originated from. A questionnaire was used to obtain information on medical history; when candidates fulfilled the inclusion criteria they were invited to the research centre. Enrolment in the study took place after it had been echographically ascertained that the carotid artery was free of plaques.

No oxidation parameters were measured in one case, and two control subjects (one population and one hospital control) had invalid resistance time measurements, and were hence excluded from the statistical analysis. The final study population consisted of 91 cases with severe coronary atherosclerosis, 94 hospital based controls with no or minor coronary atherosclerosis and 85 population based controls.

Data Collection

For the hospital groups information on medical history was obtained from the medical file and through a questionnaire within 2 months after angiography. Information on dietary, smoking, and drinking patterns, drug use, use of vitamin supplements, occupation and family history of CVD was obtained. Weight, height and blood pressure were measured. A fasting venous blood sample was collected in EDTA Vacutainer tubes and immediately placed on ice and cooled to 4°C. Plasma was prepared within 1 h after blood collection by centrifugation at 1750 x g for 15 min, frozen in methanol of -80°C or liquid nitrogen, and stored at -80°C.

Preparation and oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from Esterbauer et al.[25] with some major modifications as described previously in detail.[15,17] Briefly, for each subject, 2 ml of frozen plasma stored at -80°C was rapidly thawed and used for isolation of LDL by ultracentrifugation at 4°C in the presence of 10 μM EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants,[28] the LDL was not dialysed.[15,17,20] By omitting dialysis a more stable LDL preparation is obtained, which can be stored in the dark at 4°C under nitrogen for several days without affecting resistance time and maximum rate of oxidation.[15,17,20] This improves the precision of the method since each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, resistance time was 90 ± 2 min one hour after LDL isolation in a LDL preparation which had not been dialysed; 24 h after LDL isolation resistance time was 91 ± 3 min (n = 3). Dialysis under nitrogen for 4 h (2 changes) at 4°C against 1000 volumes of an oxygen-free buffer containing 150 mmol/l NaCl and 10 mmol/l sodium phosphate, pH

7.4, resulted in resistance times of 52 ± 5 min directly after dialysis and 23 ± 4 min after storage of LDL under nitrogen for 24 h (n = 3). In agreement with these observations a loss of lipophilic antioxidants during dialysis was recently reported.[28]

The kinetics of LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm.[15,17,25] Absorbance curves of LDL preparations obtained from an equal number (3) of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. Means were calculated on the basis of these three observations. The intra-assay coefficients of variation for resistance time and maximum rate of oxidation calculated from measurements obtained at one day were 2.6% and 3.1%, respectively. The inter-assay coefficients of measurements performed on different days were 4.9% and 7.4%, respectively.[15,17] In every oxidation run one reference LDL, prepared from a reference plasma stored at -80°C, was used as a control. Oxidation runs with a deviation higher than 10% from the mean values of former reference measurements were omitted.[15,17] By using this highly standardized method, resistance time and maximum rate of oxidation do not differ between LDL prepared from plasma frozen in liquid nitrogen and that from freshly collected plasma from the same subject. In addition, no differences in these parameters were found upon storage of plasma at -80°C up to 18 months.

Analytical measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit 236.691 and Triglyceride kit 701.904, Boehringer-Mannheim, Mannheim, Germany). Phospholipid concentrations in LDL were determined using a commercially available colour reagent (Wako Chemicals, Neuss, Germany). 100 µl of LDL (0.25 mg protein/ml) sample and 750 µl colour reagent were mixed for 10 min at 37°C and the concentration was measured at a wavelength of 500 nm. The protein content of the LDL preparations was measured according to Lowry et al.[29]

High-density lipoprotein (HDL)-cholesterol was measured after precipitation of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL using the precipitation method with sodium phosphotungstate-Mg^{2+}.[30] LDL-cholesterol concentrations were calculated by the formula of Friedewald et al.[31]

Fatty acid composition of LDL was determined in duplicate by gas liquid chromatography as previously described.[17] Heptadonoic acid (C17:0) was added as an internal

standard. We calculated the amount of polyunsaturated fatty acids (C18:2 + C18:3 + C20:2 + C20:3 + C20:4 + C20:5 + C22:6), monounsaturated fatty acids (C14:1 + C16:1 + C18:1 + C20:1 + C22:1), and saturated fatty acids (C12:0 + C14:0 + C15:0 + C16:0 + C18:0 + C20:0 + C22:0).

Concentrations of LDL antioxidants were determined by reversed-phase HPLC and spectrophotometric detection.[32] LDL antioxidant concentrations were standardized by calculating antioxidant concentrations per mg of LDL protein.

Statistical analysis

Characteristics of the case group and the control groups were compared with Student's t-test for unpaired samples. Because the two control groups were comparable regarding the lipid levels, age and smoking status and to increase statistical power data analyses was performed with the two control groups pooled. Age-adjusted means were compared by analysis of covariance. Odds ratios (ORs) were calculated to quantify the association between parameters of oxidation and coronary stenosis. Quartile distributions for calculation of ORs were based on distributions of oxidation parameters in the control group. The trend analysis was performed over the oxidation parameters as a continuous variable in the logistic model. To determine which variables are important in predicting resistance time and maximum rate of oxidation we used univariate analysis. These analyses were performed in the control group. Variables found to be significant contributors in univariate analysis were examined in multiple linear regression to assess which variable was the most important predictor. Data analysis was conducted using the statistical package BMDP.[33]

RESULTS

Table 1 lists characteristics of cases and controls. Groups were comparable regarding the prestratification factors age and smoking status. Total cholesterol, LDL cholesterol and triglyceride levels were significantly lower in the control group, whereas HDL cholesterol was higher in controls. No differences were seen in blood pressure, body mass index, diet use and family history of CVD. Cases reported more often a history of MI, use of antihypertensive and lipid-lowering medication other than HMG-CoA reductase inhibitors and use of aspirins and coumarin derivatives.

Table 1. *Characteristics of the study population (Mean ± SD)*

	Cases (n = 91)	Controls (n = 179)
Age (years)	61.6 ± 9.2	60.0 ± 8.4
Smokers (%)[1]	31.9	31.3
Ex-smokers (%)[1]	54.9	50.8
Number of cigarettes (smokers only)[2]	11.1 ± 8.4	12.4 ± 6.7
Body mass index (kg/m^2)	26.4 ± 2.5	26.1 ± 3.2
Total cholesterol (mmol/l)	6.1 ± 1.2	5.6 ± 1.0[5]
Triglycerides (mmol/l)	2.1 ± 0.9	1.7 ± 0.9[5]
HDL cholesterol (mmol/l)	0.9 ± 0.2	1.0 ± 0.3[5]
LDL cholesterol (mmol/l)	4.3 ± 1.1	3.8 ± 0.9[5]
Systolic blood pressure (mmHg)	133.3 ± 17.6	135.2 ± 17.2
Diastolic blood pressure (mmHg)	81.6 ± 8.1	83.7 ± 8.8
Under treatment by a cardiologist (years)	3.5 ± 5.0	2.4 ± 4.7
Diet use (%)	13.6	8.4
-Cholesterol-restricted (% in diet users)	58.3	20.0
-Fat-restricted (% in diet users)	36.4	46.7
Antioxidant supplementation (%)[3]	5.5	3.4
Medication (%)		
-Antihypertensive medication (%)	94.5	43.6[5]
-Lipid-lowering medication (%)[4]	6.6	0.6[5]
-Aspirin + coumarin derivatives	93.4	20.7[5]
Family history of CVD	27.5	20.6
History of MI	37.8	4.3[5]

[1]ex-smokers stopped smoking more than one year ago; otherwise they are categorized as current smokers. [2]number of smokers among cases (n = 14) and controls (n = 37). [3]use of vitamin A, vitamin C or vitamin E supplements. [4]lipid-lowering medication other than HMG CoA reductase inhibitors. [5]significant difference ($p<0.05$) in age-adjusted differences (analysis of covariance).

LDL composition is given in Table 2. No differences in α-tocopherol level and fatty acid composition were seen. Comparison of the LDL composition revealed higher percentages of total cholesterol, esterified cholesterol and phospholipids in the controls, whereas the percentage of triglycerides was lower in the control group. When expressed as absolute amounts of lipids per mg LDL protein only triglycerides were significantly lower in the control group (results not shown).

Table 2. *LDL vitamin E, LDL fatty acids content and LDL composition (mean ± SE)*

	Cases (n = 91)	Controls (n = 179)
α-Tocopherol (ng/mg protein)	5156.0 ± 118.1	4908.0 ± 88.3
Total fatty acids in LDL (μg per mg protein)	1372.3 ± 18.6	1406.1 ± 14.8
Polyunsaturated fatty acids(%)	58.0 ± 0.8	57.6 ± 0.4
Monounsaturated fatty acids (%)	18.9 ± 0.6	19.8 ± 0.4
Saturated fatty acids (%)	22.5 ± 0.2	22.2 ± 0.1
Total cholesterol (%)	40.7 ± 0.2	41.2 ± 0.1[1]
Free cholesterol (%)	8.5 ± 0.2	8.0 ± 0.2
Cholesterol ester (%)	32.2 ± 0.3	33.3 ± 0.2[1]
Triglycerides (%)	6.6 ± 0.2	5.8 ± 0.1[2]
Phospholipids (%)	25.1 ± 0.2	25.6 ± 0.1[1]
Protein (%)	27.5 ± 0.2	27.3 ± 0.2

[1]$p<0.05$. [2]$p<0.01$.

Parameters of LDL oxidation

Table 3 lists parameters of LDL oxidation with age-adjusted differences. Resistance time and maximum rate of oxidation were not significantly different. However, a borderline significant lower resistance time was seen in the cases ($p = 0.07$). Surprisingly, a significant difference in oxidation maximum, i.e. maximum diene production, was found with a higher maximum production being found in the control group.

Table 3. *Parameters of LDL oxidation (mean ± SE) and age-adjusted differences*

	Cases (n = 91)	Controls (n = 179)	Adjusted difference[1] ± SE
Resistance time (min)	87 ± 1	89 ± 1	-2 ± 1
Maximum rate of oxidation (nmol diene/min per mg protein)	10.3 ± 0.1	10.1 ± 0.1	0.1 ± 0.15
Maximum diene production (nmol/mg)	406 ± 2	420 ± 3	-14 ± 4[2]

[1]case minus control. [2]$p<0.01$.

Odds ratios (ORs) and 95% confidence intervals were calculated for the risk of coronary atherosclerosis per quartile of resistance time and maximum rate of oxidation. For resistance time a slightly (non-significant) decreased risk for coronary atherosclerosis was found. For maximum rate of oxidation no associations were found (Table 4).

Table 4. *Odds ratios for the risk of coronary atherosclerosis per quartile of resistance time and maximum rate of oxidation*

Quartile	I (reference)	II	III	IV	Trend[1]
Resistance time	<85	85-89	89-94	>94	
number of cases	30	23	21	17	
OR (95% CI)	1.0	0.77 (0.39-1.53)	0.67 (0.33-1.34)	0.55 (0.27-1.15)	p=0.07
Max. rate of oxidation	<9.4	9.4-10.3	10.3-11.0	>11.0	
number of cases	20	26	19	26	
OR (95% CI)	1.0	1.27 (0.62-2.61)	0.91 (0.43-1.93)	1.30 (0.63-2.67)	p=0.43

[1]the trend is calculated over the oxidation parameters as a continuous variable.

The ORs calculated for the oxidation parameters as continuous variables in the model resulted in an OR of 0.97 (0.94-1.00) per minute increase of resistance time and 1.09 (0.87-1.37) per unit of maximum rate of oxidation. The difference between the lowest 10% point of distribution and the 90% point produced an OR of 0.58 (0.32-1.03) for resistance time and 1.31 (0.67-2.57) for maximum rate of oxidation. The same ORs as found for the pooled control group were observed after calculation of ORs for the control groups separately.

As more subjects in the case group had a history of MI and a history of MI could have had impact on dietary patterns, we repeated the analyses with MI survivors excluded. No relevant differences in ORs were detected.

Stratified analyses for smokers (n = 85) and non- and ex-smokers (n = 185) were performed. ORs were 0.97 (0.91-1.03) and 0.97 (0.93-1.01) per unit of resistance time and 1.11 (0.78-1.59) and 1.08 (0.81-1.45) per unit of maximum rate of oxidation in smokers and non-smokers plus ex-smokers, respectively.

Determinants of oxidation parameters

We investigated which variables were determinants of the oxidation parameters in the control group. For this analysis age, BMI, smoking status, plasma lipids, α-tocopherol and fatty acid content of the LDL particle and LDL composition were considered. By univariate analysis determinants which were significantly correlated to the oxidation parameters were detected and included in multiple linear regression analysis to identify those determinants which give the highest contribution in explaining the outcome. The only significant association with resistance time was found in the percentage of saturated fatty acids in the LDL particle (r = 0.18). Maximum rate of oxidation was positively related to percentage of polyunsaturated fatty acids (r = 0.55) and inversely to the

percentage of monounsaturated (r = -0.36) and saturated fatty acids (r = -0.53). In addition, an association was found between maximum rate of oxidation and body mass index (r = -0.18) and percentage total cholesterol (r = 0.24) and triglycerides (r = -0.23) in the LDL particle. Multiple linear regression ascribed the most relevant contributions to the maximum rate of oxidation to the percentages saturated and monounsaturated fatty acids in the LDL particle (R^2 = 0.53).

DISCUSSION

We investigated the relationship between parameters of LDL oxidation and severity of coronary atherosclerosis in a case-control study. Resistance time, as a reflection of resistance to oxidation *in vivo*, was expected to be lowest in subjects with coronary atherosclerosis. We indeed found a slight, but not significant, decreased risk of coronary atherosclerosis with increasing resistance time. Propensity to oxidation may also be reflected in a higher maximum rate of oxidation. However, this could not be confirmed in our study.

In this study selection of both case and hospital control groups was based on angiography. The mean percentage of stenosis in the case group was 75% and 55% of the cases had narrowing of at least 50% in all three coronary vessels, whereas the hospital controls had a mean of 4% stenosis and 76% of these controls had no substantial narrowing in the 3 major coronary vessels. The contrast between cases and controls was thus sufficient with virtual exclusion of misclassification of disease. Moreover, we included a group of population controls of whom we had a measure of CVD, i.e, echography of the carotid arteries.

In this study a positive correlation between the percentage of polyunsaturated fatty acids and maximum rate of oxidation was found. The percentages of monounsaturated and saturated fatty acids were inversely related to the maximum rate of oxidation, whereas resistance time was positively correlated with percentage of saturated fatty acids. Assessment of determinants important in predicting susceptibility to oxidation demonstrated that fatty acid composition of LDL may be most important. These results coincide with other studies reporting increased susceptibility to oxidation with the degree of unsaturation of fatty acids, which leads to a decreased resistance time and an increased maximum rate of oxidation and maximum diene production.[18-20] In our study the mean level of fatty acids, however, did not differ between the groups. Despite equal amounts of polyunsatured fatty acids in the LDL particle, we did find a signifi-

cant higher maximum diene production in controls, most pronounced in the population control group. We do not know yet how to interprete this result, but diene production seems to be an unsuitable parameter to study LDL oxidation as risk factor for coronary atherosclerosis.

In our study we found small but significant differences in LDL lipid composition between the case group and the control group, with higher total cholesterol and phospholipid levels in the LDL particle and lower LDL triglyceride levels in the control group. These differences, however, were not reflected in a significant difference in resistance time and maximum rate of oxidation between the groups.[26] The reason for this may be the small diversity of LDL particles, despite significant differences between cases and controls.

No difference in fatty acid composition of the LDL particle between cases and controls was observed, indicating that dietary intake of fatty acids was similar in the groups. The use of a prescribed diet was not different between groups, and to further exclude dietary changes as a result of angiography blood samples were taken within two months after catheterization. ORs for coronary atherosclerosis risk for the two separate control groups did not differ. Therefore, it seems unlikely that the hospital groups have been more prone to dietary changes. Another reason for changed dietary patterns could have been the experience of MI, which was more common in the case group. Analyses with MI survivors excluded, however, yielded essentially similar results.

Supplementation with vitamin E has been reported to increase resistance time and decrease maximum rate of oxidation.[14-17] About 5% of our study population reported the use of antioxidant supplements. Most common was the use of vitamin C which does not have an effect on LDL oxidizability.[16] Only one control subject reported the use of vitamin E and one case used vitamin A. One case and eight controls reported the use of multivitamins. As in multivitamins the concentration of antioxidants are usually low in the Netherlands, subjects who used multivitamins were not categorized as being supplement user. Analyses with supplement users excluded did not change the results.

Only three previous studies reported on the relation between oxidation parameters and coronary heart disease. De Rijke et al. found a higher susceptibility of LDL to oxidation in coronary bypass patients who had shown progression compared to those without progression after seven years of follow up.[13] Regnström et al. described an inverse association between resistance phase and severity of coronary stenosis in young

MI survivors.[11] Cominacini et al.[12] observed a lower lag phase in coronary artery patients than in hyperlipidaemic patients or valvular heart disease patients. In our study we also found a lower (though not significantly lower) risk of coronary atherosclerosis with increasing resistance time. As in the study of de Rijke et al., no significant difference in maximum rate of oxidation was found. The association of resistance time and risk of coronary heart disease was weaker in our study than in the other studies, even though the sample sizes in the other studies were relatively small and the difference between coronary atherosclerosis patients and controls is less discriminative in Cominacini's study compared to ours. Similar to Regnström et al.[11] and Cominacini et al.[12], we had only one measure of CVD and we had no data on progression of atherosclerosis. The progression of atherosclerosis may differ between persons and those in the most active stage of atherogenesis may be most susceptible to oxidation.

Intervention studies have shown a clear relationship between vitamin E[14-17] and unsaturated fatty acid supplementation[18-20] and LDL susceptibility to oxidation. From this it has been concluded that the *ex vivo* oxidation can mimick the oxidative process *in vivo*. In this study we found borderline significant associations between risk of coronary atherosclerosis and reduced resistance time, indicating that coronary heart disease does not lead to a significant contrast in oxidation parameters as was found after vitamin E intake or intake of specific fatty acids.[14-20] In addition it is likely that this method of assessing oxidizability will not really reflect the oxidative process active in development of atherosclerosis *in vivo*. The oxidative process is influenced not only by antioxidants and fatty acids in the LDL particles themselves, but is part of a larger mechanism, in which also plasma antioxidants and cell constituents play a role. Direct methods for measuring oxidation *in vivo* may therefore be more successfull as predictive parameters. Measurement of autoantibodies against oxidized LDL[7-10] or epitopes of oxidized LDL[4,5] appears to be promising, but so far results are contradicting. Possibly autoantibodies are not an indicator of severity of atherosclerosis, i.e. the extent of thickening of the vessel wall, but can be used as an indicator of the active atherogenic process.[10] The same may hold true for LDL oxidation and thickening of the vessel wall.

In conclusion, our data do not support the presence of an association between risk of coronary atherosclerosis and LDL oxidation in patients with severe coronary heart disease. This may be due to the phase of the atherosclerotic process, or failure of the method used to measure LDL oxidation to sufficiently reflect *in vivo* oxidation.

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3.2

Oxidation of LDL and risk of peripheral atherosclerosis

ABSTRACT

Evidence has accumulated for oxidative modification of low-density lipoproteins (LDL) to play an important role in the atherogenic process. Therefore, we investigated the relation between susceptibility of LDL to oxidation and risk of peripheral atherosclerosis among 249 men between 45 and 80 years of age. Participants initially selected to study the relation between coronary atherosclerosis and LDL oxidation were recategorized according to their ankle-arm blood pressure index. The ankle-arm index was calculated for both legs as the ratio of systolic blood pressure in the leg divided by the arm systolic blood pressure. The lowest of both ankle-arm indices was used to categorize subjects into groups with and without peripheral atherosclerosis. Thirty-nine men had an ankle-arm index <1.00 (16% of the study population).

Subjects with peripheral atherosclerosis reported more often the use of a special diet and the use of antihypertensive medication, aspirin and coumarin derivatives. No significant differences in total, LDL and HDL cholesterol and triglycerides were present between groups. Resistance time and maximum rate of oxidation were measured *ex vivo* using copper-induced LDL oxidation. Subjects with peripheral atherosclerosis had a significantly lower resistance time, whereas the maximum rate of oxidation tended to be increased in subjects with Peripheral atherosclerosis. Odds ratios (ORs, and 95% confidence interval) for the successive tertiles of resistance time were 1.00 (reference), 0.37 (0.15-0.89) and 0.37 (0.16-0.86) (p_{trend}<0.01). ORs for the successive tertiles of maximum rate of oxidation were 1.00 (reference), 1.34 (0.47-3.82) and 1.50 (0.55-4.15). This inverse association was borderline significant (p_{trend}=0.07).

These results support a role for LDL oxidation in the development of peripheral atherosclerosis.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) by free radicals has been

implicated as an important determinant in the development of atherosclerosis. By oxidative modification the uptake of LDL by macrophages is accelerated which is the beginning of formation of fatty streak.[1,2] Antioxidants in plasma and in the LDL particle itself are thought to protect LDL against attacks of free radicals.[3]

Circumstantial evidence indicates that oxidation occurs *in vivo* in humans. Epitopes of oxidized LDL have been found in plasma[4,5] and atherosclerotic lesions of experimental animals and humans[6] and autoantibodies against these epitopes have been detected in human plasma.[7,8] The susceptibility of LDL to oxidation is decreased by vitamin E supplementation[9-11] and increased by adding unsaturated fatty acids to the diet.[12-14] A reduction in risk of cardiovascular disease (CVD) at higher plasma antioxidant levels[15] has been reported and higher antioxidant levels have been proposed to be associated with a reduced risk of CVD.[16-18]

Susceptibility of LDL to oxidative stress is measured *ex vivo* by determining the production of conjugated dienes from polyunsaturated fatty acids after incubating isolated LDL with the pro-oxidant Cu^{2+}. The time elapsing until onset of diene production, the resistance time, depends on the strength of antioxidant defence in the LDL particle[19] and may, therefore, reflect the resistance to oxidation *in vivo*.[1]

A few studies have investigated the relationship between oxidation of LDL and risk of CHD.[20-22] Thus far, no studies investigated whether LDL oxidation plays a role in development of peripheral atherosclerosis. Higher levels of lipid peroxides[23,24] and autoantibodies against oxidized LDL[25] were found in patients with peripheral vascular disease. In the present study, initially designed to study the relationship between coronary atherosclerosis and susceptibility to oxidation, we investigated the association between susceptibility to oxidation and peripheral atherosclerosis by measuring resistance time, maximum oxidation rate and determinants of oxidation in subjects with and without peripheral atherosclerosis.

METHODS

Study population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, the Netherlands (1993-1995), initially to study the relationship between angiographically documented coronary atherosclerosis and LDL oxidation. The study was approved by an ethical committee on human research and all participants gave their informed consent. Subjects with a history of cardiovascular disease (CVD) were

selected out of patients undergoing their first coronary angiography. Excluded were subjects who had experienced a myocardial infarction in the year prior to the study. Further, subjects without a history of CVD were selected from participants in the Rotterdam Study, a population-based prospective cohort study[26] and through advertisement in a local newspaper.** Of this latter group, only those subjects without any plaques in the carotid artery as assessed by echography participated.

All subjects were men between 45 and 80 years of age. Exclusion criteria were; use of HMG-CoA reductase inhibitors because of a possible influence of this medicine on LDL oxidation; diabetes mellitus; liver, kidney or thyroid disease; alcohol- or drug abuse; vegetarian diet and psychiatric complaints. Of the 273 participants in the initial study, ankle-arm index as a measure of peripheral artery disease was measured in 252 subjects. We classified patients with peripheral atherosclerosis as those subjects with an ankle-arm ratio lower than the 20% cut-off point of distribution. This cut-off point was situated at 1.00. As an ankle-arm ratio of 1.00 was found in 33 subjects, we decided to categorize as patients with peripheral atherosclerosis only those with an ankle-arm ratio lower than 1.00 (16% of the subjects). No oxidation parameters were measured in one subject without peripheral atherosclerosis and two subjects without peripheral atherosclerosis were excluded from the statistical analysis because of invalid resistance time values. This resulted in a study population of 39 patients with and 210 subjects without peripheral atherosclerosis.

Data Collection

For the participants with a CVD history information on medical history was obtained from the medical file and through a questionnaire within 2 months after angiography. For the others information on medical history was obtained from baseline data of the Rotterdam Study and via questionnaires. For all subjects information on dietary, smoking, and drinking habits, drug use, use of vitamin supplements, occupation and family history of CVD was obtained and weight and height were measured.

Systolic and diastolic blood pressure in the right upper arm were measured in duplicate with the subject in sitting position. To evaluate the presence of atherosclerosis in the lower arteries systolic blood pressure of the posterior tibial artery at both left and right ankle was determined using an 8 MHz continuous wave doppler probe (Imex Pocketdop-II) and a random-zero sphygmomanometer with the subject in supine

**for detailed information see chapter 3.1

position. Ankle-arm index was calculated as the ratio of the systolic blood pressure in the ankle to the systolic blood pressure in the arm. The lowest ankle-arm index in either leg was used in the analysis.

Finally, a fasting venous blood sample was collected in EDTA Vacutainer tubes and immediately placed on ice and cooled to 4°C. Plasma was prepared within 1 h after blood collection by centrifugation at 3000 rpm for 15 min, frozen in methanol of -80°C or liquid nitrogen, and stored at -80°C.

Preparation and oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from Esterbauer et al.[27] with some major modifications as described previously in detail.[9,11] Briefly, for each subject, 2 ml of frozen plasma stored at -80°C was rapidly thawed and used for isolation of LDL by ultracentrifugation at 4°C in the presence of 10 μM EDTA. To minimize the time between isolation and oxidation and to prevent loss of lipophilic antioxidants,[28] the LDL was not dialysed.[29] By omitting dialysis a more stable LDL preparation is obtained, which can be stored in the dark at 4°C under nitrogen for several days without affecting resistance time and maximum rate of oxidation.[9,11,14] This improves the precision of the method, since each LDL preparation can be oxidized consecutively in triplicate. In a representative experiment, resistance time was 90 ± 2 min one hour after LDL isolation in a LDL preparation which had not been dialysed; 24 h after LDL isolation resistance time was 91 ± 3 min (n = 3). Dialysis under nitrogen for 4 h (2 changes) at 4°C against 1000 volumes of an oxygen-free buffer containing 150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4, resulted in resistance times of 52 ± 5 min directly after dialysis and 23 ± 4 min after storage of this LDL under nitrogen for 24 h (n = 3). In agreement with these observations, recently, a loss of lipophilic antioxidants during dialysis was reported.[28] The kinetics of the LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm.[9,11,27] Absorbance curves of LDL preparations obtained from an equal number of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. Means were calculated on the basis of these three observations. The intra-assay coefficients of variation for resistance time and maximum rate of oxidation calculated from measurements obtained at one day were 2.6% and 3.1%, respectively. The inter-assay coefficients of measurements performed on different days were 4.9% and 7.4%, respectively.[9] In every oxidation run one reference LDL, prepared from a reference

plasma stored at -80°C, was used as a control. Oxidation runs with a higher than 10% deviation from the mean values of former reference measurements were omitted.[9,11] By using this highly standardized method, resistance time and maximum rate of oxidation do not differ between LDL prepared from plasma frozen in liquid nitrogen and that from freshly collected plasma from the same subject. In addition, no differences in these parameters were found upon storage of plasma at -80°C up to 18 months.[29]

Analytical measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP) kit and Triglyceride kit, Boehringer-Mannheim, Mannheim, Germany). Phospholipid concentrations were determined using a commercially available colour reagent (Wako Chemicals, Neuss, Germany). 100 µl of LDL (0.25 mg/ml protein) sample and 750 µl colour reagent were mixed for 10 min at 37°C and the concentration was measured at a wavelength of 500 nm. The protein content of the LDL preparations was measured according to Lowry et al.[30]

High-density lipoprotein (HDL)-cholesterol was measured after precipitation of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) using the precipitation method with sodium phosphotungstate-Mg^{2+}.[31] LDL-cholesterol concentrations were calculated by the formula of Friedewald et al.[32]

Fatty acid composition of LDL was determined in duplicate by gas-liquid chromatography as previously described.[11] Heptadonoic acid (C17:0) was added as internal standard. We calculated the amount of polyunsaturated fatty acids (C18:2 + C18:3 + C20:2 + C20:3 + C20:4 + C20:5 + C22:6), monounsaturated fatty acids (C14:1 + C16:1 + C18:1 + C20:1 + C22:1), and saturated fatty acids (C12:0 + C14:0 + C15:0 + C16:0 + C18:0 + C20:0 + C22:0).

Concentrations of LDL antioxidants were determined by reversed-phase HPLC[33] and spectrophotometric detection. LDL-antioxidant concentrations were standardized by calculating antioxidant concentrations per mg LDL protein.

Statistical analysis

Characteristics of the subjects with and without peripheral atherosclerosis were compared with Student's t-test for unpaired samples. Age-adjusted means were compared by covariance analysis. For comparison of LDL antioxidant concentrations log-transformed data were used. Odds ratios (ORs) were calculated to quantify the association

between parameters of oxidation and peripheral atherosclerosis. Classification in tertiles for calculation of ORs was based on distributions of oxidation parameters in the group without peripheral atherosclerosis. The p for trend was assessed by calculating the ORs per unit of resistance time or maximum rate of oxidation. Data analysis was conducted using the statistical package BMDP.[34]

RESULTS

Table 1 lists characteristics of subjects with and without peripheral atherosclerosis. Patients with peripheral atherosclerosis were significantly older. No differences were seen in body mass index (BMI), plasma lipid levels, smoking status, systolic and diastolic blood pressure and family history of CVD. Patients with peripheral atherosclerosis reported more frequently use of a special diet (20.5% in patients with vs. 8.2% in subjects without peripheral atherosclerosis), use of antihypertensive medication (76.9% vs. 55.2%) and use of aspirins and coumarin derivatives (61.5% vs. 40.0%). No significant differences in family history of CVD (23.1% vs. 23.8%) and history of MI (33.3% vs. 18.0%) were reported.

Table 1. *Characteristics of the study population (Mean ± SD)*

	Subjects with peripheral atherosclerosis (n=39)	Subjects without peripheral atherosclerosis (n=210)
Age (years)	64.1 ± 8.1	59.9 ± 8.7 *
Smokers (%)[1]	33.3	28.6
Ex-smokers (%)[1]	53.8	52.9
Body mass index (kg/m^2)	25.6 ± 3.1	26.1 ± 3.0
Total cholesterol (mmol/l)	5.7 ± 0.9	5.7 ± 1.0
Triglycerides (mmol/l)	1.8 ± 0.6	1.8 ± 0.9
HDL (mmol/l)	0.9 ± 0.2	1.0 ± 0.3
LDL (mmol/l)	4.0 ± 0.9	3.9 ± 1.0
Systolic blood pressure (mmHg)	137.7 ± 20.7	133.6 ± 17.0
Diastolic blood pressure (mmHg)	82.6 ± 9.4	83.0 ± 8.8
Under treatment by a cardiologist (%)	79.5	63.3
Years under treatment (years)	2.6 ± 4.1	3.1 ± 5.1

[1]ex-smokers quit smoking more than one year ago, otherwise current smoker. *significant difference $p<0.05$.

Table 2 lists potential determinants of LDL oxidation, i.e. LDL antioxidant concentrations, fatty acid content and LDL composition. Subjects with peripheral atherosclerosis had significantly lower lutein/zeaxanthin levels, while the other antioxidants did not

differ. No significant differences were seen in fatty acid composition and LDL composition. However, differences in proportions of poly-unsaturated and mono-unsaturated fatty acids were borderline significant.

Table 2. *Concentrations of LDL antioxidants, LDL fatty acid content and LDL composition in subjects with and without peripheral atherosclerosis (mean ± SE)*

	Subjects with peripheral atherosclerosis (n=39)	Subjects without peripheral atherosclerosis (n=210)	p-value[2]
γ-Tocopherol (ng/mg protein)[1]	572.2 ± 97.4	640.1 ± 105.6	0.65
α-Tocopherol (ng/mg protein)[1]	5149.6 ± 191.8	4994.1 ± 80.0	0.43
Lutein/Zeaxanthin (ng/mg protein)[1]	50.0 ± 3.7	60.2 ± 2.1	0.03
β-Cryptoxanthin (ng/mg protein)[1]	58.8 ± 8.0	54.4 ± 3.0	0.67
Lycopene (ng/mg protein)[1]	86.2 ± 10.3	117.6 ± 7.1	0.46
α-Carotene (ng/mg protein)[1]	17.4 ± 2.8	21.4 ± 1.3	0.12
β-Carotene (ng/mg protein)[1]	124.6 ± 9.9	136.0 ± 5.9	0.66
Total fatty acids in LDL (mg per mg protein)	1364.8 ± 24.0	1406.7 ± 13.8	0.37
Polyunsaturated fatty acids (%)	60.1 ± 0.8	58.2 ± 0.3	0.05
Monounsaturated fatty acids (%)	17.6 ± 0.8	19.4 ± 0.4	0.07
Saturated fatty acids (%)	22.1 ± 0.2	22.1 ± 0.1	0.76
Total cholesterol (%)[3]	41.3 ± 0.3	41.1 ± 0.1	0.42
Free cholesterol (%)[4]	8.4 ± 0.4	8.0 ± 0.2	0.49
Cholesterol ester (%)[4]	32.9 ± 0.5	33.1 ± 0.2	0.93
Triglycerides (%)[3]	6.3 ± 0.3	6.0 ± 0.1	0.73
Phospholipids (%)[3]	25.1 ± 0.3	25.6 ± 0.1	0.24
Protein (%)[3]	27.4 ± 0.3	27.3 ± 0.1	0.96

[1]test of significance on log transformed data. [2]p-value for age-adjusted difference. [3]number of cases (n=38) and controls (n=205). [4]number of cases (n=31) and controls (n=174).

Parameters of LDL oxidation are listed in Table 3. Resistance time was lower in the group with peripheral atherosclerosis, whereas a borderline significant difference was seen for the maximum rate of oxidation.

Table 3. *Oxidation characteristics of subjects with and without peripheral atherosclerosis (mean ± SE)*

	Subjects with peripheral atherosclerosis (n=39)	Subjects without peripheral atherosclerosis (n=210)	p-value[3]
Resistance time (min)	85 ± 2	89 ± 1	<0.01
Maximum rate of oxidation (nmol diene/min per mg protein)	10.5 ± 0.2	10.1 ± 0.1	0.07

ORs were calculated per tertile of resistance time and maximum rate of oxidation (Table 4). The risk of peripheral atherosclerosis was significantly decreased in the two highest tertiles compared to the lowest tertile of resistance time. In the higher tertiles of maximum rate of oxidation risk of peripheral atherosclerosis was increased, though not significantly. The ORs (95% confidence interval) calculated for the oxidation parameters as a continuous variable in the model resulted in an OR of 0.94 (0.90-0.98) per minute increase in resistance time and 1.34 (0.97-1.85) per unit increase in maximum rate of oxidation. The difference between the lowest 10% point of distribution and the 90% point produced an OR of 0.34 (0.15-0.75) for resistance time and 2.21 (0.93-5.23) for maximum rate of oxidation.

Table 4. *Odds ratios per tertile of resistance time and maximum rate of oxidation*

Tertiles	I (reference)	II	III	p-value
Resistance time (min)	**<86**	**86-92**	**>92**	0.008
Number of cases	22	8	9	
OR	1.0	0.37 (0.15-0.89)	0.37 (0.16-0.86)	
Max. rate of oxidation	**<9.7**	**9.7-10.6**	**>10.6**	0.07
Number of cases	8	12	19	
OR	1.0	1.34 (0.47-3.82)	1.50 (0.55-4.15)	

More subjects in the case group reported the use of a prescribed diet or had a history of myocardial infarction (MI). This latter also could have had impact on dietary patterns. ORs calculated with diet users or MI survivors excluded, yielded no relevant differences in ORs (results not shown).

We performed analysis with exclusion of subjects (10 subjects without peripheral atherosclerosis) who had an ankle-arm index larger than 1.5, as these unusually high values may reflect a high degree of arterial calcification.[35] Exclusion, however, resulted in similar ORs (results not shown).

We performed stratified analysis for smoking habits. ORs for smokers (n = 73) and non- and ex-smokers (n = 176) were 0.90 (0.82-0.98) and 0.95 (0.90-1.00) per minute increase in resistance time and 1.42 (0.85-2.36) and 1.27 (0.84-1.91) per unit increase of maximum rate of oxidation, respectively.

DISCUSSION

We investigated the relationship between parameters of LDL oxidation and peripheral

atherosclerosis. Resistance time was used as a measure of resistance to oxidation *in vivo*, which was expected to be lower in subjects with peripheral atherosclerosis. Further, a decline in resistance to oxidation may be reflected in an increased maximum rate of oxidation. In this study resistance time was reduced in subjects with peripheral atherosclerosis, whereas the maximum rate of oxidation was borderline significantly increased.

This study was initially performed to study the association between oxidation parameters and risk of coronary atherosclerosis. For the initial study three groups were selected: subjects with angiographically assessed severe coronary atherosclerosis, subjects with angiographically assessed no or minor coronary atherosclerosis and healthy population controls with no history of CVD. In the present study, we recategorized subjects according to their lowest ankle-arm index. In both groups patients with known CVD were included. As adjustment for severity of coronary atherosclerosis yielded similar ORs (results not shown), we feel that the association between oxidation parameters and peripheral atherosclerosis is independent of coronary atherosclerosis. Because two-thirds of our study population underwent angiography for suspected CVD, changes in dietary and life-style habits as a result of their disease status may have occurred. To minimize dietary changes as a result of the angiography blood samples were taken within 2 months after catheterisation. Subjects who had undergone angiography did not differ in fatty acid composition of the LDL from those subjects without a CVD history, which indicates that dietary intake of fatty acids did not differ between these groups. Another reason for changing dietary patterns could be the experience of MI, which was more commonly reported in the case group. Exclusion of MI survivors did not change the results.

This study did not intend to produce a representative sample of men aged 45-80. One third of all participants was free of plaques in the carotid artery. As peripheral atherosclerosis is related to presence of carotid artery disease,[36] the chance of finding peripheral atherosclerosis patients in this group was reduced. The same holds for those patients who were selected because of no or minor stenosis in the coronary arteries. Therefore, the arbitrary cut-off point 0.90 for ankle-arm index used commonly in studies on peripheral atherosclerosis[37,38] was not appropriate in this study as only 19 subjects had an index lower than 0.90. As the objective of our study was to disclose the presence of susceptibility to LDL oxidation in peripheral artery disease rather than to describe those phenomena in the population at large, we feel that the cut-off point chosen does not import our findings.

Categorization of subjects was based on a blood pressure measurement at one point in time. As blood pressure can fluctuate over time, misclassification could have occurred. The ankle-arm index, however, is a ratio between two systolic blood pressures at one specific time point, which is more stable than blood pressure itself.

The difference in susceptibility of LDL to oxidation between subjects with and without peripheral atherosclerosis could not be explained by differences in known determinants of LDL oxidation. Other studies have described an increased susceptibility to oxidation with increasing degree of unsaturation of fatty acids,[12-14] with decreasing LDL vitamin E concentration[9-11] and with decreasing LDL particle size.[39,40] No correlations were found between fatty acid composition and resistance time, but the fatty acid composition was correlated with maximum rate of oxidation. The same holds for antioxidant concentrations. No correlations were found between antioxidants and resistance time and only γ-tocopherol was inversely correlated with maximum rate of oxidation. In this study LDL particle size was not measured but the LDL composition can reflect this; small LDL particles are lower in cholesteryl esters and phospholipids and relatively higher in triglycerides and protein than large particles. However, we did not find differences in LDL composition between the groups. In short, none of the known determinants could explain the difference in resistance time between the groups.

Few previous studies have investigated the relation between oxidation parameters and CHD. Coronary bypass patients with progression after 7 years showed a higher susceptibility to oxidation than those without progression,[22] an inverse association between resistance time and severity of coronary stenosis was described in young MI survivors,[20] and resistance time was found to be lower in coronary artery patients than in hyperlipidaemic patients or valvular heart disease patients.[21] Further, higher TBARS-MDA concentrations were described in coronary artery patients[41] and in peripheral vascular disease patients[23,24] than in controls. Also elevated levels of autoantibodies against oxidized LDL have been reported in young patients with peripheral vascular disease.[25] Although our results agree with these findings, it should be noted that in the initial study only a borderline significant association between the oxidation parameters and risk of coronary atherosclerosis was found. LDL oxidation seems to play a more prominent role in the pathogenesis of peripheral atherosclerosis than in coronary atherosclerosis. Other studies have indicated that peripheral atherosclerosis is a strong independent predictor of mortality even in patients with known CHD.[42] Further, the susceptibility to atherogenic risk factors appears to vary across vascular

areas,[42] which indicates that the pathogenesis of peripheral atherosclerosis may be different from that of coronary atherosclerosis and that oxidative modification of LDL is a more important risk factor in peripheral atherosclerosis.

To our knowledge, this is the first study in which oxidation of LDL has been investigated as a potential risk factor of peripheral atherosclerosis. We conclude that the susceptibility of LDL to oxidation measured as resistance time is associated with peripheral atherosclerosis, with a decreased resistance time seen in cases with peripheral atherosclerosis.

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3.3

Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls

ABSTRACT

Autoantibodies against oxidized low-density lipoprotein (LDL) have been reported to be associated with atherosclerosis. However, data are not consistent.

We compared the titres of autoantibodies to malondialdehyde-modified LDL in three groups, a case group with angiographically documented severe coronary stenosis (>80% stenosis in at least 1 vessel, n = 47), a hospital control group with minor stenosis on the coronary angiography (<50% stenosis in all three major vessels, n = 47) and a healthy population control group with no history of coronary heart disease (n = 49). Age ranged from 26 to 68 years. Subjects were frequency-matched for gender distribution and storage time of the blood samples. No relevant differences in autoantibody titre between case and control groups were found. The mean autoantibody titres (± SD) were 1.44 ± 1.82, 1.46 ± 1.40 and 1.62 ± 1.95 for cases, hospital controls and population controls respectively. No correlations were found between autoantibody titre and age, number of cigarettes smoked and LDL or total cholesterol. Autoantibody titres were correlated with body mass index (r = 0.2) and high-density lipoprotein (HDL) (r = -0.2). Odds ratios (OR) were calculated by tertiles of autoantibody titres for the hospital control group and the population control group, respectively. Age-adjusted OR (95% confidence interval) for medium and high compared to low autoantibody titre were 0.76 (0.27-2.14) and 1.09 (0.39-2.95) for the comparison between cases and hospital controls and 1.09 (0.39-3.07), 0.90 (0.32-2.56) for the comparison between cases and population controls. Adjustment for gender, body mass index, smoking habits and HDL yielded essentially the same results.

This study does not support an association between autoantibody titres to oxidized LDL and the extent of coronary stenosis.

INTRODUCTION

Studies implying that oxidative modification of low-density lipoprotein (LDL) takes place in vivo and may play an important role in atherogenesis have accumulated over recent years.[1-4] Oxidized LDL can activate endothelial cells and induce endothelial damage, thereby allowing blood elements and monocytes to enter the sub-endothelial space, which is the beginning of the atherogenic process. As a consequence of oxidation, the uptake of LDL by macrophages is accelerated and foam cells are formed. Furthermore, modified LDL is immunogenic and has the ability to induce the formation of autoantibodies.[4,5] Autoantibodies against epitopes of oxidized LDL have been found in several studies in both human[6-15] and rabbit[6,15-16] plasma and atherosclerotic lesions. However, data on the relation between autoantibody titres and coronary atherosclerosis are not consistent.[6-10,12,13,17-19] Though the LDL-oxidation hypothesis is attractive in explaining the mechanism of development of atherosclerosis, direct evidence from human studies is still scarce.

To investigate the association between autoantibody titres and atherosclerosis, we studied autoantibody titres of three groups differing in levels of coronary artery disease, namely a group of patients with angiographically documented severe atherosclerosis, a group with angiographically documented minor or non-atherosclerosis and a population based group with no history of cardiovascular disease (CVD).

SUBJECTS AND METHODS

Study population

Groups of patients and hospital controls were selected from participants in a previous hospital-based case-control study on angiographically documented CVD and plasma levels of cholesterol oxidation products. A group of population controls consisted of participants in the Dutch National Cardiovascular Disease Risk Factor Monitoring Project.[20] The hospital groups consisted of patients who had undergone a coronary angiography for suspected CVD in the period 1991 and 1992 in Rotterdam. Ineligible were those patients: over 68 years of age; with a previous bypass surgery; with a myocardial infarction (MI) in the 12 months prior to the study period; under cardiac care for more then 2.5 years; in whom more then 2 months elapsed between angiography and case selection; who had diabetes mellitus, liver, kidney or thyroid disease, or showed evidence of alcohol or drug abuse. Of the 387 patients eligible for this

study, 51 refused to participate, 22 could not be contacted or were otherwise indisposed and 7 had died. From the remaining 307 patients, cases were selected on the basis of having more then 80% stenosis in at least one of the three major coronary vessels, and controls having less then 50% stenosis in all three major coronary vessels. This left 159 patients (80 cases and 79 controls) for the original study. For the study reported here, 50 cases and 48 controls were randomly selected for determination of plasma autoantibodies.

The population control group is a sample out of 36.000 participants in the Cardiovascular Disease Risk Factor Monitoring Project (1987-1991).[20] For the original study a random sample of men and women aged 20-59 were selected from the civil registry of three cities in the Netherlands. The study was performed at the basic health service in each city. For the study reported here, the following selection criteria were used: domiciled in Amsterdam, the Netherlands, without use of hypercholesterolaemic drugs, not under cardiac care, no heart surgery (such as bypass surgery), without a myocardial infarction. The 51 persons selected were frequency-matched for gender distribution and storage time of the blood samples to subjects of the hospital study.

Data collection

For all three groups, information on medical history, use of medication, dietary, smoking and drinking habits, occupation and family history of CVD was obtained through a questionnaire. Further, data on height, weight and blood pressure were gathered. The hospital cases and controls were seen within 2 months after angiography. Fasting venous blood samples were collected into a 10 ml EDTA vacutainer tube and the isolated plasma was stored at -80°C. For the population control group non-fasting venous blood samples were collected in 10 ml EDTA vacutainer tubes and after centrifugation plasma was stored at -20°C. The mean storage period of blood samples was 31 ± 3 months (mean ± SD). Storage periods for cases and control groups were similar.

Measurement of anti-MDA-modified LDL autoantibodies

Autoantibody titres were measured by bi-site sandwich ELISA using polystyrene microtitre plates (Greiner, number 655001, Alphen a/d Rijn, the Netherlands). The microtitre plates were coated with MDA-LDL (malondialdehyde-modified LDL) as antigen (10 µg/ml, 100 µl/well) in phosphate-buffered saline (PBS) for 16 h at 4°C. Plates were washed 4 times with PBS and the residual binding sites were blocked with

1% ($^{w}/_{v}$) casein (Merck) in PBS (200 µl/well) for 2 h at room temperature. Plates were washed 4 times with PBS, 100 µl/well of diluted samples for autoantibody determination was added, and the plates were incubated for 16 h at 4°C. Five dilutions, 1:8, 1:16, 1:24, 1:32, 1:64 ($^{v}/_{v}$), in blocking buffer containing 0.05% ($^{w}/_{v}$) Tween 20 (Merck) were applied. After washing 5 times with PBS containing 0.05% ($^{w}/_{v}$) Tween 20, 100 µl/well goat-anti-human IgG-Fc fragment conjugated to horseradish peroxidase (Nordic, Tilburg, the Netherlands), diluted 1:11000 in blocking buffer containing 0.1% ($^{w}/_{v}$) casein and 0.05% ($^{w}/_{v}$) Tween 20, was added and the plates were incubated for 2 h at 37°C. Plates were washed 4 times and the peroxidase-labelled conjugate was visualized using 3,3',5,5'-tetramethylbenzidine and H_2O_2 as substrate mixture[21] (Organon Technika BV, Boxtel, the Netherlands). Each microtitre plate contained equal numbers of samples from the three study groups.

Extensively modified MDA-LDL was used and prepared essentially as described by Palinski et al.[6] by incubating 1 mg/ml LDL (prepared from pooled plasma from 5 male and 7 female healthy volunteers aged 21-35) in PBS, pH 7.4 for 4 hours at 37°C with 0.1 M MDA, freshly prepared from malonaldehyde-bis-dimethylacetal (Kodak Eastman Co.). After conjugation, MDA-LDL was extensively dialysed against PBS and stored in the presence of 10% sucrose at -80°C. As additional antigen, native LDL (prepared from the above mentioned pool) was used in the assay. This LDL was protected from oxidation by addition of 10 µM EDTA and 20 µM butylated hydroxytoluene (Sigma) to PBS and stored in the presence of 10% sucrose at -80°C.

Each microtitre plate contained a dilution series (1:8, 1:16, 1:24, 1:32, 1:64 $^{v}/_{v}$) of the above-mentioned pool plasma in triplicate, which was used as a reference standard. From the response of these dilution series, a reference line for autoantibody response was constructed. Intra- en inter-assay coefficients of variation were 6.0% and 8.3%, respectively, for the reference curves. The autoantibody titre is defined as the ratio between the dilution on the reference line belonging to the autoantibody response in the sample and the original dilution of the sample and is in general the mean of the four autoantibody titres obtained at dilutions 1:8, 1:16, 1:24 and 1:32 ($^{v}/_{v}$). In most cases the optical density (OD) at the 1:64 ($^{v}/_{v}$) dilution was near the background and deviated from the linear curve. This dilution was then omitted from the calculation. The slopes of the calibration curve constructed with reference standard and of the curves of the individual samples from the patient group and the control groups were not identical. About 15% of the sample curves deviated from the reference curve, indicating that the values calculated for the human samples can only be considered as

indicators of autoantibody concentrations. This allows comparisons between different samples but cannot be considered as an accurate measure of the absolute autoantibody mass in each sample.

Alternatively, data are expressed as the absolute value for the ratio of autoantibody binding to MDA-LDL/native LDL (both as OD), as applied by Salonen et al.[9] The presented value is the mean of the ratios obtained at dilutions 1:8 and 1:32, as the response to native LDL was only measured at these dilutions. Binding to native LDL, considered as a non-specific control, is defined as the ratio between the autoantibody response in the sample to native LDL (as OD) and the autoantibody response in the reference standard to native LDL (as OD).

Analytical measurements

HDL-cholesterol and triglycerides were determined as described by Sullivan et al.[22] and Warnick et al.[23] Total-cholesterol was determined with a spectrum analyser (Abbott Laboratories, USA) with CHOD-PAP reagent (cat.no. 236691, Boehringer Mannheim). LDL-cholesterol was calculated with the Friedewald formula.[24] With this formula triglyceride concentrations must not exceed 4.52 mmol/l.[25] Subjects who had a triglyceride level above 4.52 were excluded from further analysis (3 cases, 1 hospital control and 2 population controls).

Data analysis

Data analysis was conducted using the BMDP statistical package.[26] Basic characteristics for the three groups were compared by Student's t-test for unpaired samples ($p < 0.05$) and by the Mann-Whitney test for non-normal distributions. Pearson χ^2 analysis was applied for class variables. By means of the Pearson correlation coefficient the associations between autoantibody titres and continuous variables in the total group were quantified.

To adjust differences between cases and controls for possible confounders, multiple linear regression was used. Stratified analysis was performed to identify confounders or effect modifiers. Odds ratios were calculated to quantify the association between autoantibody titre and coronary stenosis, the patients were divided into tertiles based on autoantibody titres in the hospital or population control group. Multiple logistic regression adjusted the odds ratio for potential confounders.

RESULTS

In Table 1 the baseline characteristics of the three study groups are presented. The population control group was slightly, though significantly, younger than the two hospital groups. No differences were seen for body mass index (BMI). The hospital control group had higher HDL (high-density lipoprotein) and lower LDL and triglycerides than the cases. The population controls had lower total cholesterol, HDL, LDL and systolic and diastolic blood pressure than the cases. After adjustment for age and smoking habits the significant difference in HDL disappeared.

Table 1. *Baseline characteristics (mean ±SD)*

	Cases (n = 47)	Hospital controls (n = 47)	Population controls (n = 49)
Age (years)	54.2 ± 8.9	53.5 ± 9.2	49.6 ± 7.8[1,2]
Body mass index (kg/m^2)	26.2 ± 3.3	25.4 ± 2.8	25.9 ± 3.4
Plasma cholesterol (mmol/l)	6.3 ± 0.9	5.9 ± 1.1	5.6 ± 1.0[1]
HDL-cholesterol (mmol/l)	1.1 ± 0.3	1.3 ± 0.4[1]	1.0 ± 0.3[1,2]
LDL-cholesterol (mmol/l)	4.4 ± 0.8	4.0 ± 1.0[1]	3.9 ± 0.9[1]
Plasma cholesterol/HDL	6.0 ± 1.6	4.9 ± 1.7[1]	6.3 ± 1.9[2]
Plasma triglycerides (mmol/l)	1.8 ± 0.8	1.5 ± 0.8[1]	1.7 ± 0.9
Systolic pressure (mmHg)	135.0 ± 9.4	134.0 ± 15.1	127.0 ± 16.5[1,2]
Diastolic pressure (mmHg)	84.8 ± 0.6	83.4 ± 10.9	79.3 ± 10.3[1]
% Male	68.1	68.1	67.3
% Smokers	25.5	27.7	38.8

[1]significant difference ($p<0.05$) with cases. [2]significant difference ($p<0.05$) with hospital controls.

Autoantibody titres between cases (1.44 ± 1.82 (mean ± SD)), hospital controls (1.46 ± 1.40) and population controls (1.62 ± 1.95) were not significantly different. Figure 1 shows frequency histograms of the autoantibody titres of the three study groups. At the 1:8 and 1:32 dilutions the autoantibody response to native LDL was measured. In 42 out of 143 persons a response higher than 0.2 OD in both dilutions was found. These higher responses to native LDL were equally distributed over the three groups as determined with frequency calculations. Titres to native LDL (mean (± SD)) were similar for the three groups. The OD assessed at dilution 1:8 were 0.34 ± 0.35, 0.37 ± 0.36 and 0.32 ± 0.35 and at the 1:32 dilution 0.23 ± 0.31, 0.22 ± 0.22 and 0.21 ± 0.20 for cases, hospital controls and population controls, respectively. None of the differences reached significancy.

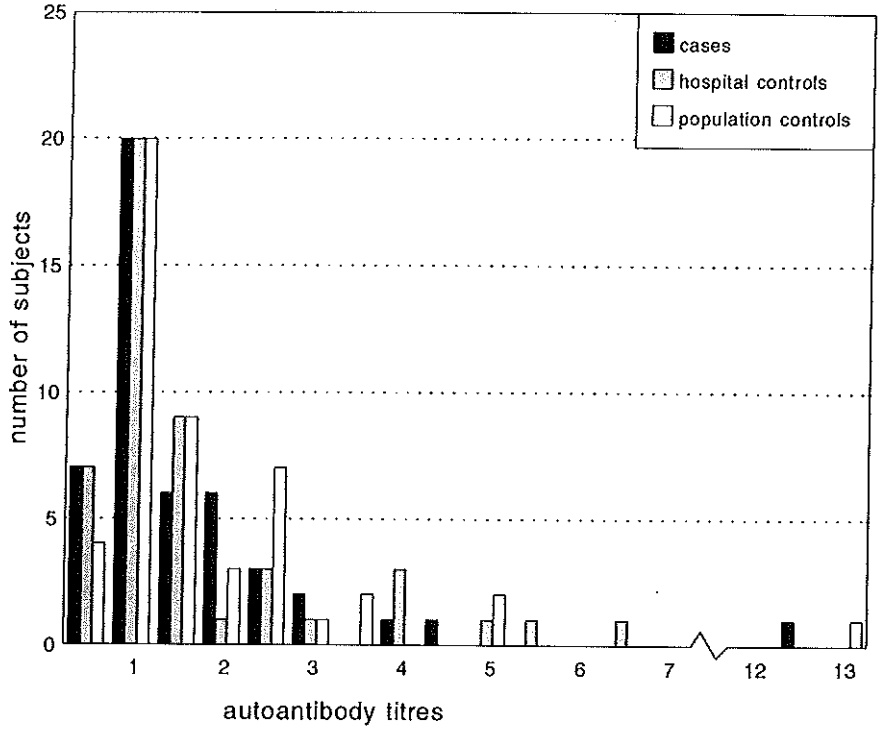

Figure 1: Distribution of autoantibody levels against MDA-LDL among cases, hospital controls and population controls.

Table 2 presents correlation coefficients for the total population between risk factors for CVD and autoantibody titres. Only BMI and HDL showed a significant relationship with autoantibodies. When LDL is being oxidized, a series of oxidative products of cholesterol are produced.[27,28] We, therefore, also assessed the relationship between cholesterol oxidation products (total oxysterols, 7α-OH cholesterol (the main oxysterol, but also an important intermediate in the bile acid synthetic pathway) and total oxysterols without 7α-OH cholesterol) and autoantibody titres in the case and the hospital control group. Correlations were -0.05, -0.02 and -0.07 for total oxysterols, 7α-OH cholesterol and total minus 7α-OH cholesterol, respectively. None of these correlations reached significance.

Table 2. *Correlations for all data between autoantibody titres and risk factors for CVD*

Total (n = 143)	Autoantibody titre	Ratio titre[1]
Age	-0.01	-0.14
Number of cigarettes/day	0.06	-0.08
Body mass index	0.20[2]	0.09
HDL	-0.19[2]	-0.15
Total cholesterol	-0.08	-0.13
Triglycerides	0.03	0.02
LDL	-0.03	-0.10
Systolic pressure	0.05	0.04
Diastolic pressure	0.00	0.02

[1]autoantibody titres were calculated acording to the method proposed by Salonen et al.[9]. [2]$p<0.05$.

Odds ratios for the risk of CVD and autoantibody titre were calculated with logistic regression analyses for tertiles of autoantibody titre for cases and hospital controls and for cases and population controls. Results are shown in Table 3a. No significant associations were observed for autoantibody titres and CVD.

Table 3. *Odds ratios and 95% confidence intervals (CI) for the risk of CVD in tertiles of autoantibody titres in the controlgroup, for case and hospital control groups and cases and population controls*

3a.

Autoantibody titre	Age-adjusted	Multivariate adjusted[1]
cases/hospital controls		
<0.76	1.0[2]	1.0[2]
0.76-1.17	0.76 (0.27-2.14)	0.63 (0.21-1.93)
>1.17	1.09 (0.40-2.95)	0.77 (0.26-2.27)
cases/population controls		
<0.75	1.0[2]	1.0[2]
0.75-1.42	1.09 (0.39-3.07)	1.32 (0.45-3.89)
>1.42	0.90 (0.32-2.56)	1.08 (0.36-3.25)

3b.

Ratio titre[3]	Age-adjusted	Multivariate adjusted[1]
cases/hospital controls		
<2.2	1.0[2]	1.0[2]
2.2-4.9	2.22 (0.75-6.58)	1.51 (0.46-4.95)
>4.9	2.04 (0.67-6.21)	1.60 (0.49-5.23)
cases/population controls		
<3.0	1.0[2]	1.0[2]
3.0-5.9	0.98 (0.36-2.68)	0.93 (0.32-2.76)
>5.9	0.71 (0.25-2.06)	0.74 (0.25-2.22)

[1]adjusted for gender, age, smoking habits, HDL, body mass index. [2]reference. [3]autoantibody titres were calculated according to the method proposed by Salonen et al.[9]

Additionally, we performed the analysis on data obtained by using the calculation method proposed by Salonen et al.,[9] in which autoantibody titres are expressed as the ratio titre (binding to MDA-LDL divided by binding to native LDL, both as OD). The mean (± SD) ratio titres for cases, hospital controls and population controls were 4.54 ± 2.96, 4.26 ± 2.72 and 4.90 ± 3.06, respectively. Comparable to the above mentioned approach, no relevant differences among the groups were found.

When assessing the correlation coefficients between the ratio titre and risk factors for CVD, none of the variables age, number of cigarettes, BMI, systolic and diastolic blood pressure, total cholesterol, HDL, LDL and triglycerides showed a significant association (Table 2).

Odds ratios calculated for tertiles of the ratio titre in the control group are presented in Table 3b. No significant association was found between cases and hospital controls and between cases and population controls. Adjustment for gender, age, smoking habits, HDL and BMI had no impact on the results.

DISCUSSION

In this study no association between coronary heart disease and autoantibody titres was found.

It is unlikely that the absence of differences in autoantibody titres is due to flaws in the study design. Blood samples were stored at -80°C or -20°C degrees. There is no reason to assume that differences in storage temperature have affected the association, as autoantibody titres in plasma at the different storage temperatures were within the same range. Storage time for the three groups was comparable.

As it is conceivable that dietary or life-style changes may affect LDL oxidation in vivo[29-32] and thus may alter autoantibody production, the cases and hospital controls were examined within 2 months after the angiography. Thus, dietary and life-style changes as a response to the cardiology report are restricted.

The two hospital groups were slightly older than the population group (mean age of 54 and 50 for hospital and population groups, respectively). This could have introduced bias, but no association between autoantibody titres and age was found. Further, the result could have been biased by smoking habits, since there were more smokers in the population control group. Smoking might be related to oxidative stress[33-36] and thus could increase oxidation of LDL. Yet, no difference in autoantibody titres between smokers and non-smokers was found and calculation of correlations between autoanti-

body titres and number of cigarettes smoked did not produce a relevant association. In addition, in a previous report we did not observe an effect of smoking on susceptibility of LDL to oxidation.[36]

We compared the autoantibody titres of the cases to both a hospital and a population control group. An objective discrimination between cases and hospital controls is possibly based on the angiographical data, implying as a control group a group with documented minor stenosis. However, in the control group 64% were scored as having no stenosis, 23% had less than 10% stenosis in the 3 vessels and the mean percentage stenosis over the 3 vessels did not exceed 30%. The population control group was a selection of healthy men and women without self-reported history of CVD. However, we cannot rule out the possibility of people with unknown, clinically non- manifested CVD entering the control group.

In this study, we defined autoantibody titres in two different ways: one related to a reference line constructed from the response to a reference plasma and the other being the ratio between the response to MDA-LDL and that to native LDL. The advantage of the first method is the use of several dilutions. The autoantibody titres presented are generally the mean of the autoantibody titres obtained at four dilutions. We believe to have thus reduced the approximation error. When plotting the OD against the concentration, individual curves do not parallel each other. As pointed out by Virella et al.,[19] we therefore should not use these autoantibody titres as an absolute measure, but rather as an indicator of autoantibody concentrations. The second method for expressing autoantibody titres was calculated at the 1:8 and 1:32 dilutions. Both methods were significantly correlated ($r = 0.34$, $p<0.001$). As both ways of expressing the data generate the same result, it is justified to conclude that there is no reason to assume that our result can be ascribed to our definition of autoantibody titres.

The results of our study are consistent with reports from several groups, in which no difference in levels of antibodies were found,[6,17-19] but are inconsistent with others.[7,9,10,12-14] In most studies, elevated levels of autoantibodies against epitopes of oxidized LDL are reported both in subjects with coronary heart disease and in healthy controls. Findings of Maggi et al.,[10] Salonen et al.,[9] Puurunen et al.,[13] as well as the results of Virella et al.[18] who described higher (though not significantly higher) autoantibody titres in hyperlipidaemic persons and in subjects with minor atherosclerosis in comparison to persons with known CVD, support the idea that antibodies are not an indicator of the severity of atherosclerosis, i.e. the extent of thickening of the vessel wall, but can be used as an indicator for an active atherogenic process. In our study no

data on the change in extent of stenosis in recent years were available. We, therefore, can not exclude the possibility that subjects were in a stable state of atherogenesis. This may provide an explanation for the lack of difference in our study. Another explanation for not finding a relation with the extent of CVD is pointed out by Virella et al.,[19] who suggest that different persons may have different populations of antibodies with a different affinity to antigens.

We conclude that this study does not support an association between autoantibody titres to oxidized LDL and thickening of the vessel wall. We suggest that clinical studies focus on assessment of the relationship between autoantibody levels and development of new lesions e.g. by measurement of intima-media thickening by ultrasound.

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Chapter 4

LDL and Plasma antioxidant levels and atherosclerosis

4.1

Plasma vitamin C, LDL antioxidants and risk of coronary atherosclerosis

ABSTRACT

Antioxidants have been implicated to play a protective role in the atherosclerotic process, possibly through inhibition of low-density lipoprotein (LDL) oxidation. We studied plasma vitamin C, LDL tocopherols and LDL carotenoids and risk of coronary atherosclerosis in a case-control study among men aged 45-80 year. Subjects with severe coronary atherosclerosis were classified as cases (n = 92). Hospital controls with no or minor atherosclerosis (n = 95) and population controls free of plaques in the carotid artery (n = 86) were combined into one control category.

No difference in mean plasma vitamin C levels between cases and controls was found. LDL γ-tocopherol levels were higher and LDL lutein/zeaxanthin and LDL α-carotene levels were significantly lower in cases. Unexpectedly, a positive association between LDL tocopherol levels and risk of coronary atherosclerosis was seen. Odds ratios calculated for the successive quartiles of LDL α-tocopherol were 1.00 (reference), 1.34 (0.55-3.30), 2.61 (1.15-5.90) and 2.26 (0.97-5.26) (p_{trend} = 0.02). LDL carotenoids, especially α-carotene and lutein/zeaxanthin, showed slightly decreased risks of coronary atherosclerosis at higher levels, which however disappeared after correction for blood cholesterol level. No intermediate effect of LDL oxidation in the association between antioxidants and the risk of coronary atherosclerosis could be detected.

We conclude that in this study only high circulating levels of α-carotene showed a slight preventive effect against coronary atherosclerosis. For the antioxidants in general no protective effect was seen on coronary atherosclerosis as measured by LDL antioxidants. The association between antioxidants and coronary atherosclerosis can not be ascribed to their inhibition of LDL oxidation.

INTRODUCTION

Antioxidants have been implicated to play a protective role in the atherogenic process. In several epidemiological and clinical studies the preventive potential of antioxidants on coronary heart disease (CHD) has been investigated. A preventive effect of dietary and supplementary vitamin E against CHD has been described in prospective studies,[1-3] whereas prospective studies on plasma vitamin E did not reveal clear associations with CHD.[4-6] Further, the risk of angina pectoris was significantly decreased at high plasma vitamin E levels,[7] but no reduction in risk of myocardial infarction was found at high vitamin E levels in adipose tissue.[8] Dietary vitamin C was strongly inversely related to risk of CHD[9] and thickness of the carotid artery wall,[10] but such a reduced risk of CHD at higher levels of vitamin C intake was not observed in the studies by Rimm and colleagues[1] and Stampfer and colleagues.[2] For dietary intake of β-carotene lower risk of CHD at high intake levels has been observed in a prospective study,[1] but no association was found in a Finnish cohort study.[11] Low plasma levels of β-carotene, however, were associated with an increased risk of CHD[4] and myocardial infarction (MI),[12] and high levels of adipose β-carotene were related to decreased risk of MI.[8] Results from trials on antioxidant supplementation do not support a beneficial effect of β-carotene.[13-15] For vitamin E data is scanty, no effect has been observed for CHD mortality,[13,16] whereas it may have a preventive effect on CHD morbidity, e.g. high doses were found to prevent non-fatal MI in coronary atherosclerosis patients[16] and the incidence of angina pectoris.[17]

One mechanism by which antioxidants are thought to have a protective effect is through inhibition of low-density lipoprotein (LDL) oxidation. Oxidative modification of LDL by free radicals has been implicated as an important step in the development of atherosclerosis. Oxidative modification accelerates the uptake of LDL by macrophages which is the beginning of the fatty streak.[18,19] LDL is thought to be protected against free radicals by antioxidants in plasma and in the particle itself. Antioxidants incorporated in the LDL particle may therefore be relevant. From supplementation studies evidence arose that increased LDL vitamin E, the most prominent lipid soluble antioxidant, lowers the susceptibility of LDL to oxidation,[20-23] and an inverse association has been reported recently between LDL vitamin E concentrations and coronary artery disease.[24] Not much is known about other LDL antioxidants such as carotenoids and their relation to atherosclerosis.

Many studies have examined the role of antioxidants in the development of CHD

in subjects with MI. The pathogenesis of MI comprises both atherogenic and thrombotic processes. To gain more insight into the mechanism by which antioxidants can play a role in the atherogenic process we performed a case-control study in which the relationship between plasma vitamin C and LDL concentrations of various lipid soluble antioxidants and risk of angiographically determined coronary atherosclerosis was investigated. Also the susceptibility to LDL oxidation was measured in order to address the question whether antioxidant concentrations are related to LDL oxidation and whether LDL oxidation plays an intermediate role in the relation between antioxidants and atherosclerosis.

METHODS

Study population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, the Netherlands, in the period 1993-1995. The study was approved by an ethical committee on human research and all participants gave their informed consent. We selected a group of CHD patients, a group of hospital controls and a group of population controls. The groups consisted of men between 45 and 80 years of age. Enrolment procedures allowed for similar distributions of age (in 5-year categories) and smoking status (smoking, non-smoking).

Selection of the two hospital groups was based on angiographic reports. To reduce the impact of disease on dietary and life-style patterns we selected only those patients who underwent their first angiography and who had not experienced a MI in the year prior to the study. For the same reason, blood was collected within 2 months after angiography. Subjects using HMG-CoA reductase inhibitors were excluded because of a possible influence of this medicine on LDL oxidation.

In the study period 2830 patients underwent coronary angiography for suspected CHD, which included 1966 male subjects. Subjects were ineligible if they met one of the following exclusion criteria: under 45 or over 80 years of age (n = 144), not the first coronary angiography (n = 289), MI in the 12 months prior to the study (n = 180), diabetes mellitus (n = 84), liver, kidney or thyroid disease (n = 15), alcohol or drug abuse (n = 4), use of HMG-CoA reductase inhibitors (n = 82), vegetarian diet (n = 3), psychiatric complaints (n = 2). For 88 subjects more than 2 months elapsed between angiography and case selection and 12 patients had died in the meantime, leaving a population of 1063 eligible subjects. Of this group 124 refused to participate

and 50 could not be contacted or were otherwise indisposed. From the remaining 889 men, 92 cases were selected who had at least 85% stenosis in one and at least 50% stenosis in a second of the three major coronary vessels. Further, 95 hospital controls were selected with less then 50% stenosis in no more than two of the three major coronary vessels. The percentage of stenosis was scored by the cardiologist performing the angiography.

Population controls were selected from participants in the Rotterdam Study. The rationale and design of this population-based prospective cohort study have been described previously.[25] We selected subjects without any plaques in the carotid artery as assessed by ultrasound. Further, these subjects reported not to have a history of cardiac treatment, not to have diabetes mellitus, or liver, kidney or thyroid disease, not to use HMG-CoA reductase inhibitors nor to be vegetarian. As the participants in the Rotterdam Study were all 55 years and over at baseline, we additionally recruited men between 45 and 55 years of age through an advertisement in a local newspaper. Recruitment took place in the area the other population controls originated from. A questionnaire was used to obtain information on medical history; when candidates fulfilled the inclusion criteria they were invited to the research centre. Enrolment in the study took place after it had been echographically ascertained that their carotid artery was free of plaques. Eighty six population-based male controls were included, which led to a study population consisting of 92 patients with severe coronary atherosclerosis, 95 hospital-based controls with no or minor coronary atherosclerosis and 86 population-based controls.

Data Collection

For the hospital groups information on medical history was obtained from medical status records and through a questionnaire within 2 months after angiography. Information on dietary, smoking and drinking patterns, medicine use, use of vitamin supplements, occupation and family history of CVD was obtained. Weight, height and blood pressure on ankle and arm were measured. A fasting venous blood sample was collected in EDTA Vacutainer tubes and immediately placed on ice and cooled to 4°C. Plasma was prepared within 1 h after blood collection by centrifugation at 1750 x g for 15 min, frozen in methanol of -80°C or liquid nitrogen, and stored at -80°C.

Preparation and oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from Ester-

bauer et al.[26] with some major modifications as described previously in detail.[21,23] The kinetics of LDL oxidation was followed by continuously monitoring the change of absorbance at 234 nm.[21,23,26] Absorbance curves of LDL preparations obtained from an equal number (3) of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. Means were calculated on the basis of these three observations.

Analytical measurements

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit 236.691 and Triglyceride kit 701.904, Boehringer-Mannheim, Mannheim, Germany).

High-density lipoprotein (HDL)-cholesterol was measured after precipitation of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL using the precipitation method with sodium phosphotungstate-Mg^{2+}.[27] LDL-cholesterol concentrations were calculated by the formula of Friedewald et al.[28]

Plasma vitamin C concentrations were assessed within 1 month after blood sampling with HPLC and fluorometric determination.[29] Concentrations of LDL antioxidants were determined by reversed-phase HPLC and spectrophotometric detection.[30] Lutein and zeaxanthin were combined. LDL antioxidant concentrations were standardized by calculating antioxidant concentrations per mg LDL protein.

Statistical analysis

Characteristics of the case group and the control groups were compared with Student's t-test for unpaired samples. Because the two control groups were comparable regarding lipid levels, age and smoking status, further data analysis was performed with the two control groups pooled. Age-adjusted means were compared by analysis of covariance. Log transformations were performed to compare non-normal distributed variables. Odds ratios (OR) were calculated to quantify the association between antioxidant concentrations and coronary atherosclerosis. Quartile distributions for calculation of ORs were based on distributions in the control group. Trend analysis was performed over the antioxidants as a continuous variable in the logistic model. Confounding factors taken into account were; body mass index (BMI), total, HDL- and LDL-cholesterol and diastolic and systolic blood pressure. Data were analyzed with the statistical package BMDP.[31]

RESULTS

Characteristics of the study groups are described in Table 1. No differences were present in age, smoking habits, BMI and blood pressure. Total cholesterol, LDL-cholesterol and triglycerides were significantly higher in the case group, whereas HDL-cholesterol was significantly lower in the cases. Cases more frequently reported the use of antihypertensive medication, lipid-lowering medication other than HMG-CoA reductase inhibitors and aspirin or coumarin derivatives. A history of MI was more common in the case group.

Table 1. *Characteristics of the study population (Mean ± SD)*

	Cases (n = 92)	Controls (n = 181)
Age (years)	61.8 ± 9.2	60.1 ± 8.4
Smokers (%)[1]	31.5	31.5
Ex-smokers (%)[1]	54.3	50.3
Body mass index (kg/m^2)	26.4 ± 2.5	26.1 ± 3.2
Total cholesterol (mmol/l)	6.0 ± 1.2	5.6 ± 1.0 *
Triglycerides (mmol/l)	2.1 ± 0.9	1.7 ± 0.9 *
HDL (mmol/l)	0.9 ± 0.2	1.0 ± 0.3 *
LDL (mmol/l)	4.3 ± 1.1	3.8 ± 0.9 *
Systolic blood pressure (mmHg)	133.4 ± 17.6	135.3 ± 17.1
Diastolic blood pressure (mmHg)	81.7 ± 8.1	83.7 ± 8.8
Under treatment by a cardiologist (years)	3.4 ± 5.0	2.4 ± 4.7
Diet use (%)	13.5	8.3
-Cholesterol-restricted (% in diet users)	58.3	20.0
-Fat-restricted (% in diet users)	36.4	46.7
Antioxidant supplementation use (%)[2]	5.4	3.3
Medication use		
-Antihypertensive medication (%)	93.5	43.1 *
-Lipid-lowering medication (%)[3]	6.5	0.6 *
-Aspirin + coumarin derivatives (%)	93.5	20.4 *
Family history of CVD	27.2	21.0
History of MI	37.4	4.2 *

[1]ex-smokers stopped smoking more then one year ago, otherwise current smoker. [2]use of vitamin A, vitamin C or vitamin E supplements. [3]other than HMG CoA reductase inhibitors. *significant age-adjusted difference (analysis of covariance), $p<0.01$.

Table 2 lists plasma vitamin C and LDL antioxidant levels. Cases had a higher γ-tocopherol level, but lower levels of α-carotene and lutein/zeaxanthin. One case had an extremely high γ-tocopherol value (21,099 ng/mg protein), but analysis after exclusion of this subject still resulted in a significantly higher γ-tocopherol level in the

cases (adjusted mean ± SE 777.7 ± 61.2). Expressing tocopherol as tocopherol to LDL-cholesterol ratio instead of tocopherol to LDL-protein ratio resulted in significant higher γ- and α-tocopherol levels in the cases. Mean α-tocopherol levels (±SE) were 1338.1 ± 29.8 vs. 1250.7 ± 21.9 (p = 0.02) and mean γ-tocopherol levels were 257.43 ± 57.9 vs. 123.1 ± 11.7 for cases vs. controls.

Table 2. *Antioxidant concentrations (mean ± SEM) and age-adjusted differences between cases and controls*

	Cases (n = 92)	Controls (n = 181)	p-value[1]
plasma vitamin C (μmol/l)[2]	47.1 ± 2.1	50.6 ± 1.7	0.21
LDL antioxidants:[3]			
γ-Tocopherol (ng/mg protein)	997.3 ± 231.1	474.9 ± 40.2	<0.01
α-Tocopherol (ng/mg protein)	5157.8 ± 116.8	4906.3 ± 87.4	0.08
Lutein/Zeaxanthin (ng/mg protein)[4]	52.8 ± 2.4	60.1 ± 2.3	0.04
β-Cryptoxanthin (ng/mg protein)[4]	54.8 ± 5.2	53.1 ± 3.1	0.92
Lycopene (ng/mg protein)[4]	99.5 ± 11.0	113.7 ± 6.8	0.08
α-Carotene (ng/mg protein)[4]	16.3 ± 1.4	21.9 ± 1.5	0.01
β-Carotene (ng/mg protein)[4]	117.9 ± 8.1	136.1 ± 6.0	0.06

[1]test of significance for age-adjusted difference. [2] no data for 2 cases and 6 controls. [3]test of significance on log transformed data. [4]no data for 1 case.

Antioxidants and risk of coronary atherosclerosis

We calculated the ORs for risk of coronary atherosclerosis per quartile of antioxidant level. Crude ORs and ORs adjusted for age and smoking status were only marginally different; therefore only the ORs adjusted for age and smoking status are presented in Table 3. A significantly elevated risk of coronary atherosclerosis was seen in the highest compared to the lowest quartile of γ-tocopherol. Significant trends were found for γ-tocopherol, lutein/zeaxanthin and α-carotene. We expanded the logistic model, which resulted in a model with further adjustment for total cholesterol, HDL-cholesterol and diastolic blood pressure. The positive association for γ-tocopherol remained, whereas for α-tocopherol the positive association with risk of coronary atherosclerosis now reached significance. The inverse association of lutein/zeaxanthin and α-carotene no longer reached significance. For γ-tocopherol the continuous OR (95% CI-interval) per ng/mg LDL protein increase was calculated which resulted in an OR for a 100 ng increase of 1.08 (1.02-1.14) and an OR for the contrast between the lowest 10% point of distribution compared to the 90% point of distribution of 1.70 (1.16-2.48). For α-tocopherol the OR calculated per ng/mg LDL protein increase resulted in an 1.03

(1.01-1.06) for a 100 ng/mg increase with the 10-90% point contrast of 2.26 (1.16-4.46).

Cases more frequently reported a history of MI, which could have influenced dietary patterns and consequently could have resulted in a changed antioxidant pattern in the LDL. Exclusion of MI survivors, however, had only marginal effects on the ORs (results not shown).

Stratified analyses for smoking status were performed. The mean levels of vitamin C and the LDL antioxidants were lower in smokers compared to never/ex-smokers. For smokers vs. never/ex-smokers mean levels ± SE were 41.1 ± 2.5 vs. 53.3 ± 1.5 µmol/l ($p < 0.01$) for vitamin C, 4779.5 ± 130.4 vs. 5093.6 ± 82.4 ng/mg protein ($p = 0.04$) for α-tocopherol and 112.2 ± 8.6 vs. 138.4 ± 5.8 ng/mg protein ($p = 0.01$) for β-carotene. ORs calculated for smokers and never/ex-smokers separately showed an inverse association between plasma vitamin C and LDL β-carotene and risk of coronary atherosclerosis for never/ex-smokers only. We, however, could not disclose a significant interaction between smoking status and antioxidant levels, and no significant correlations were found between antioxidant levels and number of cigarettes smoked by current smokers. This may be due to the small number of smokers and the inaccuracy of measuring number of cigarettes.

In the results in Table 1-3, the two control groups were pooled for the statistical analyses, as the groups were comparable according to the plasma lipid levels, BMI and blood pressure. We also calculated the ORs for the control groups separately. The same associations were found as in the pooled analysis, with exception of γ-tocopherol. The positive trend for γ-tocopherol was seen only when comparing cases with population controls ($p<0.01$).

Associations with oxidation parameters

A possible mechanism by which antioxidants may play a role in the atherogenic process is by inhibition of LDL oxidation. Resistance time, as a measure of LDL resistance to oxidation, however, was not related to antioxidant levels, and only γ-tocopherol was weakly inversely correlated to maximum rate of oxidation ($r = -0.15$, $p<0.04$). Adjustment of the ORs for risk of coronary atherosclerosis per quartile of antioxidant concentration for the parameters of LDL oxidation, such as resistance time and maximum rate of oxidation, resulted in marginal differences (results not shown).

Table 3. *Odds ratios for the risk of coronary atherosclerosis per quartile of antioxidant concentration in LDL*

Quartile	II[1]	III[1]	IV[1]	trend
plasma vitamin C (μmol/l)	**34.1-50.2**	**50.2-69.0**	**≥69.0**	
number of cases	23	29	13	
OR model 1[2]	0.88 (0.43-1.80)	1.03 (0.51-2.08)	0.48 (0.21-1.08)	p=0.17
OR model 2[3]	1.17 (0.52-2.63)	1.19 (0.55-2.59)	0.57 (0.23-1.38)	p=0.37
LDL antioxidants:[4]				
γ-Tocopherol (ng/mg)	**207.7-380.3**	**380.3-558.0**	**≥558.0**	
number of cases	20	14	46	
OR model 1	1.57 (0.67-3.63)	1.21 (0.49-2.95)	3.82 (1.77-8.24)	p<0.01
OR model 2	1.52 (0.62-3.76)	1.23 (0.47-3.17)	3.65 (1.58-8.43)	p<0.01
α-Tocopherol (ng/mg)	**403.8-493.1**	**493.1-568.7**	**≥568.7**	
number of cases	16	33	26	
OR model 1	0.95 (0.42-2.15)	1.83 (0.89-3.79)	1.50 (0.70-3.20)	p=0.08
OR model 2	1.34 (0.55-3.30)	2.61 (1.15-5.90)	2.26 (0.97-5.26)	p=0.02
Lutein/Zeaxanthin (ng/mg)	**38.4-55.3**	**55.3-74.8**	**≥74.8**	
number of cases	29	24	15	
OR model 1	1.18 (0.59-2.36)	0.96 (0.47-1.97)	0.58 (0.26-1.28)	p=0.04
OR model 2	1.21 (0.56-2.60)	1.41 (0.63-3.15)	0.70 (0.29-1.66)	p=0.23
β-Cryptoxanthin (ng/mg)	**24.0-41.1**	**41.1-69.1**	**≥69.1**	
number of cases	20	30	19	
OR model 1	0.74 (0.35-1.60)	1.29 (0.64-2.62)	0.75 (0.35-1.60)	p=0.74
OR model 2	0.94 (0.40-2.18)	1.50 (0.68-3.33)	1.01 (0.43-2.37)	p=0.47
Lycopene (ng/mg)	**49.1-84.5**	**84.5-167.5**	**≥167.5**	
number of cases	16	23	16	
OR model 1	0.45 (0.22-0.93)	0.64 (0.32-1.27)	0.49 (0.23-1.08)	p=0.50
OR model 2	0.50 (0.23-1.09)	0.71 (0.33-1.53)	0.74 (0.31-1.78)	p=0.28
α-Carotene (ng/mg)	**8.6-15.6**	**15.6-27.6**	**≥27.6**	
number of cases	26	18	17	
OR model 1	0.78 (0.39-1.55)	0.47 (0.22-1.00)	0.49 (0.23-1.05)	p=0.02
OR model 2	0.88 (0.41-1.89)	0.73 (0.32-1.67)	0.61 (0.27-1.41)	p=0.06
β-Carotene (ng/mg)	**77.4-115.3**	**115.3-172.3**	**≥172.3**	
number of cases	26	21	15	
OR model 1	0.47 (0.22-1.03)	0.63 (0.30-1.30)	0.47 (0.22-1.03)	p=0.10
OR model 2	0.76 (0.36-1.63)	0.71 (0.32-1.60)	0.63 (0.27-1.48)	p=0.77

[1]compared to the first quartile. [2]adjusted for age and smoking status. [3]model 1 + adjustment for total cholesterol, HDL cholesterol and diastolic blood pressure. [4]calculated per mg protein.

DISCUSSION

We investigated the relationship between LDL antioxidant and plasma vitamin C

concentrations and risk of coronary atherosclerosis. High antioxidant concentrations have been hypothesized to play a protective role against atherogenesis. Lutein/zeaxanthin and α-carotene levels were indeed inversely related to the risk of coronary atherosclerosis, however, these associations disappeared after adjustment for blood cholesterol and blood pressure. On the other hand, γ-and α-tocopherol levels were positively related to risk of coronary atherosclerosis. We did not find indications that these relations may be ascribed to an intermediate role of LDL oxidation.

In this study both cases and hospital controls were selected on the basis of angiographical reports. The mean percentage of stenosis in the case group was 75% of whom 55% had narrowing of at least 50% in all three coronary vessels, whereas the hospital controls had a mean of 4.3% stenosis of whom 76% had no substantial narrowing in the 3 coronary vessels. The contrast between cases and controls was thus sufficiently high to avoid misclassification of the disease. In addition, we included a group of population controls for whom a measure of CVD was available, namely an ultrasound of the carotid artery.

LDL antioxidant levels and plasma vitamin C levels are a reflection of dietary intake of antioxidants. The use of a biomarker for dietary intake may be more relevant to study the association between antioxidants and diseases, because individual variation in absorption and metabolism of dietary antioxidants is taken into account. Moreover, the chance of under- or overreporting dietary antioxidant intake is excluded. However, in a case-control study the possibility remains that disease status influences dietary and life-style patterns, and thus the antioxidant level in plasma or LDL. Therefore we took blood samples within 2 months after catheterization to minimize dietary changes as a result of the angiography. In addition, we included a control group consisting of both hospital-based and population-based controls. Hospital controls were expected to be equally prone to dietary and life-style changes because of awareness of the disease, whereas population controls without cardiovascular complaints are not likely to have changed their diet. In this study no differences in the use of a prescribed diet between cases and controls were found. Also, no marked differences in associations between antioxidants and risk of coronary atherosclerosis were found between the two control groups. Therefore, dietary changes in this study are not likely to have influenced the results.

Use of antioxidant supplements was reported by 5% of our study population. One control subject reported the use of vitamin E supplements and one case used vitamin A supplements. Most prominent was the use of vitamin C supplements. Analysis with

supplement users excluded did not change the results.

In our study a significant positive association between both γ-tocopherol and α-tocopherol levels and risk of coronary atherosclerosis was found. Based on findings from previous studies this result was not expected.[1-8] Although not significant, some other studies have also suggested higher rather than lower vitamin E levels in serum[6,12,32,33] and atherosclerotic lesions[34] in cases compared to controls. Higher vitamin E levels in cases might be explained if the concentration tocopherol in LDL is increased as a response to atherosclerotic lesions. Especially for γ-tocopherol a difference was present when calculating ORs in the separate control groups. The positive association was most clear in the population control group, suggesting that the physiology of tocopherol uptake and metabolism in cases is different from that in healthy subjects. This has been suggested by Kardinaal et al., who found different correlations between dietary vitamin E intake and adipose tissue markers in MI patients and controls.[35] Another indication that the physiology of vitamin E uptake and metabolism in LDL may be different in healthy subjects and cases is seen in correlations calculated in sub-samples of our study population. In a sub-sample of population controls (n = 67) significant correlations between LDL and plasma levels of γ-tocopherol (r = 0.53) and α-tocopherol (r = 0.42) were found, whereas the correlations in a sub-sample of the case group (n = 69) were virtually absent (-0.01 for γ-tocopherol and 0.13 for α-tocopherol). A difference in correlations between plasma and LDL levels in CHD patients and controls is also described by Regnström et al.[24] Therefore, studies on plasma or serum levels may need to be interpreted differently from studies on LDL levels.

We found higher carotenoid levels in controls compared to the cases, but only for α-carotene and lutein/zeaxanthin this difference was significant. These two carotenoids were inversely related to risk of coronary atherosclerosis in analyses adjusted for age and smoking. However, further adjustment for cholesterol attenuated the result. To date no study has reported on LDL carotenoid levels and risk of CHD in a non-supplemented population. Other studies have described an inverse association between dietary,[1] plasma and serum[4] and adipose tissue[8] β-carotene levels and risk of CHD, but results from recently published intervention studies on β-carotene could not confirm this protective effect.[13-15] In our study no association was found for β-carotene and risk of coronary atherosclerosis.

In our study an inverse association between vitamin C levels and risk of coronary atherosclerosis was seen only in the never/ex-smokers, but after adjustment for choles-

terol this association was no longer significant. In previous studies, intake of vitamin C intake has been strongly and inversely associated with risk of CHD,[9] whereas no relation was found in two large prospective studies.[1,3] Adequate plasma vitamin C levels above a desirable level for protection against CHD (>40-50 µmol/L for non-smokers), could have resulted in lack of association in our study and in the latter prospective studies.[36]

Our second hypothesis concerned a protective effect of antioxidants against atherosclerosis through protection of LDL against oxidation. Vitamin E supplementation in intervention studies resulted in a decreased susceptibility of LDL to *ex vivo* oxidation.[20-23] No clear protective effect has been found after β-carotene supplementation.[21,22,37] We could not find evidence for an association between levels of LDL antioxidants and LDL oxidation. Only marginal differences were found when we included resistance time or maximum rate of oxidation into the logistic model to assess the risk of coronary atherosclerosis at different levels of LDL antioxidants (results not shown). Therefore, we could not confirm an intermediate role for LDL oxidation in the association between antioxidants and CHD. Possibly, this may be ascribed to the small differences in antioxidant levels between case and control group. Large doses may be needed for a sufficiently large contrast in antioxidant levels to be reflected in lower oxidation rates.

In conclusion, no effect of high LDL antioxidant levels and plasma vitamin C on risk of coronary atherosclerosis was found. To the contrary, high LDL-tocopherol levels showed a positive association with risk of coronary atherosclerosis, which may reflect differences in physiology of tocopherol uptake and metabolism in CHD patients. No indications were found that LDL oxidation plays an intermediate role in the relation between antioxidants and risk of coronary atherosclerosis.

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4.2

Plasma Coenzyme Q_{10} levels are not decreased in coronary atherosclerosis patients

ABSTRACT

Coenzyme Q_{10} (CoQ_{10}) is an important mitochondrial electron transfer component and has been postulated to function as a powerful antioxidant protecting LDL from oxidative damage. It could thus reduce the risk of cardiovascular disease. To study the relation between plasma CoQ_{10} levels and coronary atherosclerosis, we performed a case-control study among 71 male cases with angiographically documented severe coronary atherosclerosis and 69 healthy male controls free from symptomatic cardiovascular disease and without atherosclerotic plaques in the carotid artery.

Plasma CoQ_{10} levels (mean ± SE) were 0.86 ± 0.04 vs. 0.83 ± 0.04 µmol/L for cases and controls, respectively. The CoQ_{10} / LDL-cholesterol ratio (µmol/mmol) was slightly lower in cases than in controls (0.22 ± 0.01 vs. 0.26 ± 0.03). Differences in CoQ_{10} levels and CoQ_{10}/LDL-cholesterol ratio did not reach significance. The odds ratios (95% confidence interval) for the risk of coronary atherosclerosis calculated per µmol/L increase of CoQ_{10} was 1.12 (0.28-4.43) after adjustment for age, smoking habits, total cholesterol and diastolic blood pressure.

We conclude that an unsupplemented plasma CoQ_{10} level is not related to risk of coronary atherosclerosis.

INTRODUCTION

Antioxidants have been implicated to play a protective role in the atherogenic process. They are believed to delay atherogenesis by protecting lipid fractions within the low-density lipoprotein (LDL) particle against oxidation by free radicals. Oxidative modification of LDL has been implicated as an important step in the development of atherosclerosis. Oxidative modification accelerates the uptake of LDL by macrophages which is the beginning of formation of fatty streak.[1,2]

Coenzyme Q_{10} (CoQ_{10}) is an important carrier for two-electron transfer within the

mitochondrial membrane and has been shown to function as an endogenous lipid-soluble antioxidant in blood and tissues.[3] Previous studies have shown that reduced CoQ_{10} (ubiquinol) exerts a protective effect on LDL oxidation[4-8] and that reduced CoQ_{10} is depleted before tocopherols during lipid oxidation,[4,9,10] consistent with the notion that reduced CoQ_{10} acts as a scavenger of the tocopheroxyl radical produced during lipid oxidation.[11-13] Ubiquinol is also believed to function as a chain breaking antioxidant in the lipid peroxidation process.[3,9,14]

CoQ_{10} supplementation has been suggested to have beneficial effects in treatment of coronary heart disease (CHD) patients[15,16] and to result in decreased thiobarbituric acid-reactive substances (TBARS) levels[17] and conjugated diene[7] production after oxidative stress. Some authors also have reported decreased levels of CoQ_{10} in cardiomyopathy patients compared to normal controls,[18] and the LDL/ubiquinone ratio has been suggested to be a coronary risk factor.[19] However, little information is available on plasma CoQ_{10} levels in CHD patients and healthy subjects under unsupplemented conditions.

To study the association between plasma CoQ_{10} levels and the risk of coronary atherosclerosis, we performed a case-control study among cases with severe coronary atherosclerosis and healthy controls.

METHODS

Study population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, the Netherlands, in the period 1993-1995. The study was approved by an ethical committee on human research and all participants gave informed consent. We selected a group of patients with coronary atherosclerose and a group of population controls without symptomatic cardiovascular disease. All were men between 45 and 80 years of age. Enrolment procedures allowed for similar distributions of age (in 5-year categories) and smoking status (smoking, non-smoking).

Selection of the cases was based on angiographic reports. To reduce the impact of disease on dietary and life-style patterns, we selected only those patients who underwent their first angiography and who had not experienced a myocardial infarction in the year prior to the study. For the same reason, blood was collected within 2 months after angiography. Subjects using HMG-CoA reductase inhibitors were excluded because of the possible inhibiting effect of this drug on ubiquinone production by

interfering in the mevalonate pathway[20] and its possible influence on LDL oxidation.

In the study period 2830 patients underwent coronary angiography for suspected CHD, including 1966 male subjects. Subjects were not eligible if they met one of the following exclusion criteria: under 45 or over 80 years of age (n = 144), not the first coronary angiography (n = 289), MI in the 12 months prior to the study (n = 180), diabetes mellitus (n = 84), liver, kidney or thyroid disease (n = 15), alcohol or drug abuse (n = 4), use of HMG-CoA reductase inhibitors (n = 82), vegetarian diet (n = 3), psychiatric complaints (n = 2). For 88 subjects more than 2 months had elapsed between angiography and case selection and 12 patients had died in the meantime, leaving a population of 1063 eligible subjects. Of this group 124 refused to participate and 50 could not be contacted or were otherwise indisposed. From the remaining 889 men, 71 cases with at least 85% stenosis in one and at least 50% stenosis in a second of the three major coronary vessels were selected for assessment of plasma CoQ_{10}. Of these subjects, 57% had a narrowing of at least 50% in all three major coronary vessels. The percentage of stenosis was scored by the cardiologist performing the angiography.

Population controls were selected from participants in the Rotterdam Study. The rationale and design of this population-based prospective cohort study have been described previously.[21] We selected subjects without any plaques in the carotid artery as assessed by ultrasound. Further, these subjects reported not to have a history of cardiac treatment, not to have diabetes mellitus, or liver, kidney or thyroid disease, not to use HMG-CoA reductase inhibitors nor to be vegetarian. As the participants in the Rotterdam Study were all 55 years and over at baseline, we additionally recruited men between 45 and 55 years of age through an advertisement in a local newspaper. Recruitment took place in the area the other population controls originated from. A questionnaire was used to obtain information on medical history; when candidates fulfilled the inclusion criteria they were invited to the research centre. Enrolment in the study took place after it had been echographically ascertained that subjects' carotid artery was free of plaques. A total of 69 population controls were included in which plasma CoQ_{10} was assessed.

Data collection

For the cases information on medical history was obtained from hospital records and through a questionnaire within 2 months after angiography. Information on dietary patterns, smoking and drinking patterns, medicine use, use of vitamin supplements,

occupation and family history of CVD was obtained. Weight, height and blood pressure were measured. A fasting venous blood sample was collected in EDTA Vacutainer tubes and immediately placed on ice and cooled to 4°C. Plasma was prepared within 1 h after blood collection by centrifugation at 1750 x g for 15 min, frozen in methanol of -80°C or liquid nitrogen, and stored at -80°C.

Analytical measurements

Procedures for analysis of total CoQ_{10} and tocopherols in plasma were as follows: 100 µl plasma was extracted after addition of 25 µl 1 mg/ml BHT in EtOH, and 900 µl 0.1 M SDS and 2 ml hexane. 1.5 ml of the hexane layer was taken to dryness under nitrogen and redissolved in 200 µl ethanol. 10 µl was used for HPLC analysis (method adapted from Lang et al.).[22] The HPLC analysis was performed on a Waters system with Waters 610 pumps, a Beckman Ultrasphere ODS C-18 column, 4.6 mm i.d., 25 cm, 5 µm particle size, a Waters Wisp 717 autosampler, and Millenium software and using a Coulochem 5100A electrochemical detector (Environmental Sciences Assoc., Bedford, MA., USA), equipped with a Model 5020 Conditioning cell set at -750 mV, and a Model 5011 Analytical cell with 2 electrodes in series, the first set at -750 mV, and the second set at +500 mV. The eluent was ethanol/methanol/isopropanol 715:245:40 containing 0.1% $^{w}/_{v}$ lithium perchlorate at 1.2 ml/min[6] (slightly modified). Measurements were performed in duplicate and quantification was carried out by comparing peak areas to the area of standard curves obtained with authentic compounds.

Cholesterol and triglyceride concentrations were determined enzymatically using commercially available reagents (CHOD-PAP kit 236.691 and Triglyceride kit 701.904, Boehringer-Mannheim, Mannheim, Germany). High-density lipoprotein (HDL)-cholesterol was measured after precipitation of very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL using the precipitation method with sodium phosphotungstate-Mg^{2+}.[23] LDL-cholesterol concentrations were calculated by the formula of Friedewald et al.[24]

Preparation and oxidation of LDL

The procedure for preparation and lipid peroxidation of LDL was adapted from Esterbauer et al.[25] with some major modifications as described previously in detail.[26-28] The kinetics of LDL oxidation were followed by continuously monitoring the change of absorbance at 234 nm.[25-27] Absorbance curves of LDL preparations obtained from an

equal number (3) of subjects from each study group were determined in parallel. Each LDL preparation was oxidized in three consecutive oxidation runs on the same day. Means were calculated on the basis of these three observations.

Statistical analysis

Characteristics of the case group and the control group were compared with Student's t-test for unpaired samples. Age-adjusted means were compared by analysis of covariance. Pearson' correlations were calculated between CoQ_{10} levels and risk factors for CHD. Odds ratios (OR) were calculated to quantify the association between plasma CoQ_{10} concentrations and coronary atherosclerosis. ORs are calculated per µmol/L increase of CoQ_{10}. Confounding factors taken into account were: age, smoking habits, body mass index, total, HDL- and LDL-cholesterol, diastolic and systolic blood pressure. Data were analysed with the statistical package BMDP.[29]

RESULTS

Table 1 lists the characteristics of cases and controls. Groups were comparable regarding the prestratification factors age and smoking status. Total cholesterol, LDL-cholesterol and triglycerides were lower in controls and HDL-cholesterol and diastolic blood pressure were higher in controls.

Table 1. *Characteristics of the study population (Mean ± SD)*

	Cases (n=71)	Controls (n=69)
Age (years)	61.9 ± 9.3	61.4 ± 8.8
Smokers (%)[1]	33.8	21.7
Ex-smokers (%)[1]	9.9	18.8
Body mass index (kg/m^2)	26.3 ± 2.4	26.2 ± 3.3
Total cholesterol (mmol/l)	5.9 ± 1.1	5.4 ± 1.1 *
Triglycerides (mmol/l)	2.1 ± 0.8	1.6 ± 0.9 *
HDL (mmol/l)	0.8 ± 0.2	1.0 ± 0.3 *
LDL (mmol/l)	4.2 ± 1.0	3.6 ± 1.0 *
Systolic blood pressure (mmHg)	132.9 ± 16.6	136.4 ± 18.5
Diastolic blood pressure (mmHg)	81.4 ± 8.0	85.4 ± 9.9 *

[1]ex-smoker stopped smoking more than one year ago, otherwise current smoker. *significant difference $p<0.05$.

The frequency of reported prescribed diet use was similar in both groups (17.6% in cases, 13.0% in controls). Cases more frequently reported use of antihypertensive medication (93.0% vs. 10.1%) and aspirin and coumarin derivatives (93.0% vs. 4.3%), while 37% of the cases reported a history of MI.

The mean levels of plasma antioxidants for the two groups are listed in Table 2. No differences in CoQ_{10} levels between patients and controls were found. A slightly lower CoQ_{10}/LDL ratio was seen in the case group. However, a significant age-adjusted difference was found for α-tocopherol only, with higher tocopherol levels in cases.

Table 2. *Concentrations of plasma antioxidants for patients with coronary atherosclerosis and controls (Mean ± SE)*

	Cases (n=71)	Controls (n=69)	p-value[1]
Coenzyme Q_{10} (μmol/L)	0.86 ± 0.04	0.83 ± 0.04	0.50
CoQ_{10}/LDL cholesterol (μmol/ml)	0.22 ± 0.01	0.26 ± 0.03	0.21
γ-Tocopherol (μmol/L)	2.44 ± 0.14	2.16 ± 0.11	0.13
α-Tocopherol (μmol/L)	29.8 ± 0.6	25.4 ± 0.7	<0.01
β-Carotene (μmol/L)	0.22 ± 0.02	0.26 ± 0.02	0.17

[1]age-adjusted.

In the control group we calculated correlation coefficients for the association between cardiovascular risk factors and the level of CoQ_{10}. Positively correlated ($p<0.05$) to CoQ_{10} levels were: total cholesterol ($r = 0.57$), LDL-cholesterol ($r = 0.49$), diastolic blood pressure ($r = 0.25$), γ-tocopherol ($r = 0.40$) and α-tocopherol ($r = 0.53$). No correlations were found with age, body mass index, systolic blood pressure, HDL-cholesterol and triglyceride levels.

In Table 3 ORs (and 95% confidence interval) for the risk of coronary atherosclerosis per μmol/L increase of plasma CoQ_{10} and per unit increase of CoQ_{10}/LDL-cholesterol ratio are presented. No association was found between plasma levels of CoQ_{10} and risk of coronary atherosclerosis. Because crude and age-adjusted ORs differed only marginally, only the age-adjusted OR is presented. To ensure that other differences between the cases and controls did not confound the risk estimate, we adjusted the OR for the potential confounding factors total cholesterol, diastolic blood pressure and smoking habits (Table 3). The OR was 1.12 which means that every μmol/L increase of CoQ_{10} results in a non-significant 12% increase in risk of coronary atherosclerosis.

Table 3. *Odds ratios (and 95% CI) for risk of coronary atherosclerosis per μmol/L increase of CoQ_{10} and per unit increase in CoQ_{10}/LDL cholesterol.*

	Age-adjusted	Multivariate[1]
CoQ_{10}	1.50 (0.48-4.67)	1.12 (0.28-4.43)
CoQ_{10}/LDL cholesterol	0.18 (0.01-3.55)	1.01 (0.09-11.2)

[1]adjusted for age, smoking, total cholesterol and diastolic blood pressure.

Additional adjustment for α-tocopherol resulted in an OR of 0.37 (0.08-1.78). The interaction between plasma levels of CoQ_{10} and α-tocopherol was not significant ($p = 0.38$). Calculation of the ORs over quartiles of CoQ_{10} levels in the control group yielded essentially similar results. ORs for the successive quartiles were 1.0 (reference), 0.98 (0.34-2.86), 1.00 (0.33-3.05) and 1.31 (0.43-4.02) after adjustment for total cholesterol, smoking habits and diastolic blood pressure.

Stratified analysis in separate strata of total cholesterol (< 5.5 or ≥ 5.5) or smoking status (smokers or never/ex-smokers) did not essentially change the results.

Associations with oxidation parameters

A possible mechanism by which CoQ_{10} and other plasma antioxidants may play a role in the atherogenic process is by inhibition of LDL oxidation. Resistance time, as a measure of LDL resistance to oxidation *ex vivo*, and maximum rate of oxidation did not differ between cases and controls (Table 4) and were not correlated to plasma CoQ_{10} levels. The OR (95% confidence interval) for coronary atherosclerosis per μmol/l increase of CoQ_{10} was 0.99 (0.24-4.06), after further adjustment for resistance time and maximum rate of oxidation.

Table 4. *Oxidation characteristics for patients with coronary atherosclerosis and controls (mean ± SE)*

	Cases (n=71)	Controls (n=68)[1]	p-value[2]
Resistance time (min)	87 ± 1	90 ± 1	0.20
Maximum rate of oxidation (nmol diene/min per mg protein)	10.4 ± 0.1	10.3 ± 0.1	0.54

[1]one control with unreliable data for oxidation parameters was left out for the analyses. [2]age-adjusted.

DISCUSSION

We investigated the relationship between plasma total CoQ_{10} levels and the risk of coronary atherosclerosis in unsupplemented individuals in a case-control study. No association could be detected.

A protective effect against CHD has been ascribed to CoQ_{10}, possibly due to the antioxidant function of the reduced form of CoQ_{10}, ubiquinol. In this study we measured the total CoQ_{10} concentration, but data from other studies indicate that at least 75-80% of the total CoQ_{10} can be found in reduced form,[5,7,17] and therefore differences in total CoQ_{10} levels also reflect differences in the levels of reduced CoQ_{10}.

It seems unlikely that the finding of no difference between the groups is a result of flaws in the study design. As a result of the disease status changes in life-style and nutritional patterns could have occurred in the patient group. CoQ_{10} is present in a wide variety of foods, but is mainly high in organ meats (e.g. heart, liver, kidney), beef, vegetable oils (e.g. soy oil), fish (e.g. sardines, mackerel), and peanuts. A change in dietary patterns towards these products is not very likely, as organ meats are not very popular in the Netherlands, and diets prescribed to CHD patients commonly are energy- and/or fat-restricted. In addition, recent studies have shown that the average CoQ_{10} intake of an average Danish person is 3-5 mg per day, an amount that is not likely to affect the plasma level dramatically.[17]

Another important difference between our two groups is drug use. As many as 93% of our patients used antihypertensive medication, aspirins or coumarin derivatives. From the literature the only drugs which may have an inhibitory effect on ubiquinone production are HMG-CoA reductase inhibitors.[20] In our study subjects who used these drugs were excluded.

The CoQ_{10} level in our study is similar to levels reported by Hanaki et al.[20] and slightly higher than those reported by Weber et al.[17] Several studies have reported on decreased susceptibility to oxidation after supplementation with CoQ_{10}. Weber et al. reported a significant decrease in TBARS production after a 1 μmol/l increase of plasma Q_{10} level.[17] Kontush et al. reported that incorporation of ubiquinol-10 in LDL, which led to an increase in LDL ubiquinol-10 from 0.10-0.20 to 0.55-1.48 mol/mol LDL, resulted in a decreased conjugated diene production.[7] Further, a decrease in hydroperoxide levels was detected after supplementation of LDL with CoQ_{10}.[8] To our knowledge no study has reported on significantly reduced susceptibility to oxidation at physiological plasma levels of CoQ_{10} in unsupplemented subjects. Kontush et al.

described only a small effect of ubiquinone-10 and α-tocopherol incorporated in LDL at physiological levels, whereas at high levels of incorporation (more than eight-fold increase) an antioxidant effect of ubiquinone-10 was seen.[7] Also in a study by Frei et al. no effect of physiological LDL-ubiquinol concentrations on LDL oxidation was found.[30] This is in accordance with the lack of association between CoQ_{10} levels and LDL oxidizability in our study in unsupplemented subjects.

In this study we did not detect differences in plasma levels of CoQ_{10} between cases with coronary atherosclerosis and controls, nor did we find a decreased risk of coronary atherosclerosis at higher CoQ_{10} levels. We conclude that no relation between physiological concentrations of plasma CoQ_{10} levels and the risk of coronary atherosclerosis was detected.

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Chapter 5

Dietary antioxidants and coronary heart disease

5.1

Major dietary antioxidants and risk of silent myocardial infarction: the Rotterdam Study

ABSTRACT

Antioxidants have been implicated to play a protective role in the atherosclerotic process. We studied the relation between dietary intakes of β-carotene, vitamin C and vitamin E and the presence of silent myocardial infarction (MI) in baseline data from the Rotterdam Study (1990-1993), a prospective cohort study among 7983 men and women aged 55 years and over. Silent MI subjects were studied. As they are not aware of their disease status, dietary changes as a result of the disease are unlikely. Dietary intake of major antioxidants was estimated by a semi-quantitative food frequency questionnaire (170 items). The assessment comprised a simple self-reported questionnaire which formed the basis for a detailed interview by a trained dietician. Self-reports of cardiovascular disease (CVD) were validated by medical records. Silent MI was defined as 'ECG evidence of MI without medical information or self-reported history of MI'. Subjects with a silent MI (n = 89) were compared to subjects without any CVD history (n = 2333).

Adjusted odds ratios (95% confidence interval) for risk of MI per quartile of vitamin C were 1.0 (reference), 1.1 (0.7-2.0), 0.6 (0.3-1.1) and 0.8 (0.4-1.4) (p_{trend} = 0.2), for vitamin E 1.0, 1.6 (0.9-2.8), 1.1 (0.6-2.0), 1.0 (0.5-2.0) (p_{trend} = 0.7), and 1.0, 1.4 (0.8-2.3), 0.8 (0.4-1.5), 0.5 (0.2-1.0) (p_{trend} = 0.04) for β-carotene. Associations were more pronounced in women.

In conclusion, β-carotene intake is inversely associated with risk of MI. No association between vitamin C or vitamin E intake and risk of MI was seen. Whether the effect of β-carotene is attributable to β-carotene itself or other components in β-carotene containing foods needs further to be determined.

INTRODUCTION

Antioxidant vitamins have been implicated to play a protective role against coronary

heart disease (CHD). The antioxidant vitamins vitamin E, β-carotene and vitamin C may prevent or slow down the atherogenic process by protecting low-density lipoproteins (LDL) from oxidation. Oxidized LDL may play a role in foam cell formation and in formation of atherosclerotic lesions.[1]

Large prospective studies have shown a lower risk of CHD at high intakes of vitamin C,[2,3] and vitamin E.[3-6] Further, high plasma levels of vitamin E[7] and β-carotene[8] are associated with lower risk of ischaemic heart disease, whereas adipose tissue levels showed a reduction in risk of myocardial infarction (MI) in the highest compared to the lowest quintile of β-carotene but not vitamin E.[9] Results from trials on antioxidant supplementation do not support a benificial effect of β-carotene.[10-12] For vitamin E supplementation no effect on CHD mortality has been observed,[10,13] whereas it may have a preventive effect on CHD morbidity, e.g. high doses were found to prevent non-fatal MI in angiography patients[13] and the incidence of angina pectoris.[14]

We studied the relationship between antioxidant intake and CHD in a large group of subjects who had a silent MI. This group is of particular interest, as they have definitely cardiovascular disease, but are not aware of their disease status. Therefore, change in dietary patterns as a result of the disease and misclassification of subjects due to information bias in reporting dietary intake are unlikely. We compared subjects with silent MI to subjects without any history of cardiovascular diseases in the baseline data from the Rotterdam Study.

SUBJECTS AND METHODS

Study design and subjects

The Rotterdam Study is a single-centre community-based prospective cohort study among 7983 subjects aged 55 years and over, living in Ommoord, an urban district of Rotterdam, the Netherlands. The rationale and design have been described previously.[15] In short, the Rotterdam Study investigates prevalence, incidence, and determinants of cardiovascular, neurological, locomotor, and ophthalmological diseases. Baseline data were collected between 1990-1993. The study comprised an extensive home interview (participation rate 78%) and two visits at a research centre for a clinical examination (participation rate 69%). The study has been approved by the Medical Ethics Committee of the Erasmus University Rotterdam, and written consent was obtained from all participants. Dietary assessment was undertaken to investigate the role of nutrition and nutrition-related factors in the development of chronic diseases.

Dietary assessment

Dietary assessment was performed during the second visit at the research centre. Habitual consumption patterns over the past year were determined by a semi-quantitative food frequency questionnaire. The questionnaire was a modified version of a validated self-administered semi-quantitative food frequency questionnaire previously used in a large-scale prospective cohort study. Measures of the validity and repeatability of the original questionnaire for several nutrients have been reported.[16,17] The questionnaire was adapted to allow an easy and time-efficient dietary assessment in an older population. Furthermore, some additional items were included and more detailed information on vegetable, fruit and meat consumption could be obtained. The modified questionnaire contains 170 food items in 13 food groups and general questions about dietary patterns.

Dietary assessment was undertaken in two consecutive phases. In the first phase a self-administered questionnaire was handed out and explained to each participant during a home visit by a trained research assistant. Participants had to mark the foods they had consumed at least twice a month and whether consumption was season bound (summer or winter). In the second phase a dietary interview was conducted by a trained dietician on the basis of the completed dietary questionnaire. During the dietary interview the dieticians concentrated on obtaining accurate information on quantity and frequency of food items noted by participants as consumed at least twice a month. The conversion from foods to energy and nutrient intake was established with a computerized version of the Dutch Food Composition Table,[18] completed with tables for vitamin E and β-carotene.[19]

No dietary data were available for participants of the pilot study (n = 277), for those living in nursing homes (n = 479), and for persons with reduced cognitive function (n = 122) assessed by a neuropsychological test. For logistic reasons no dietary assessment could be made in an additional 482 persons. On the basis of the judgement of investigators 212 subjects were excluded from analysis due to unreliability of reported dietary intake.

Examination procedures

The participants came to the research center at varying times during the day. At the research center they underwent a brief clinical examination; height and weight were measured and body mass index was calculated (kg/m^2). Blood pressure was calculated as the average of two consecutive measurements with a random zero sphygmomanome-

ter. A 12-lead ECG was recorded with an ESAOTE-ACTA cardiograph with a sampling frequency of 500 Hz and digitally stored. ECG data were evaluated by research physicians using a protocol for standardized clinical ECG evaluation. All ECGs with possible characteristics of MI were checked by an experienced cardiologist who determined the final diagnosis. During the interview questions on history of MI were asked, including the following questions: 'Did you ever have a heart attack?' and, if so, 'At what age?', 'Who made the diagnosis?', and 'Were you admitted to a hospital?'. All ECGs of subjects with self-reported MI without ECG evidence were re-analyzed by the Modular ECG Analysis System (MEANS)[20,21] to detect cases of electrocardiographic MI which had been missed by the research physician. The final diagnosis for these cases was made by another cardiologist with large specialized experience in reading ECGs. From subjects with ECG evidence without self-reported MI, additional clinical information from the GP or cardiologist was obtained to confirm that the MI has occured silent.

Population for analysis

In this study dietary data and evaluated ECG data were available for 2743 subjects. Excluded were all subjects with self-reported MI with or without matching ECG evidence (n = 228) and subjects with missing information on cardiovascular disease (n = 93). This left 2333 subjects without any history of MI or ECG evidence for MI and 89 subjects with a silent MI (ECG evidence but no self-reported MI and no MI reported in the medical record) for the analysis.

Data analysis

Baseline characteristics were compared by Student's t-test for unpaired samples and Pearson χ^2 analysis ($p<0.05$) and by analysis of covariance. Skewed distributions of vitamin E, vitamin C, β-carotene and energy intake were normalized by natural logarithm transformation. Odds ratios were calculated to quantify the association between antioxidant intake and the risk of a silent MI. Division into quartiles of antioxidant intake was based on antioxidant intake in the baseline population without CVD (n = 2333). Quartile distribution for stratified analysis was based on the intake in the corresponding strata of the baseline. Multiple logistic regression was used to obtain odds ratios adjusted for known risk factors for CVD; i.e. age, gender, smoking habits, systolic blood pressure, total cholesterol. Trend analysis were performed over the medians of the quartiles.

RESULTS

Baseline characteristics of subjects with silent MI and the population without CHD are given in Table 1. Subjects with silent MI were significantly older than those without a history of CHD. Further, subjects with silent MI had higher systolic and diastolic blood pressure and were more likely to be men than in the population without CHD. The use of a prescribed diet was more common, but not significantly so, in the population without CHD.

Table 1. *Baseline characteristics (mean ± sd) of cases with a silent myocardial infaction and controls without cardiovascular disease*

	Silent MI cases (n=89)	Subjects without CHD (n=2333)
Age (years)	70.4 ± 7.4	67.9 ± 7.6[1]
Body mass index (kg/m^2)	26.8 ± 4.7	26.4 ± 3.7
Systolic blood pressure (mmHg)	147.1 ± 22.6	139.3 ± 22.2[1]
Diastolic blood pressure (mmHg)	77.6 ± 13.3	74.3 ± 11.0[1]
Cholesterol (mmol/l)	6.5 ± 1.1	6.7 ± 1.2
HDL (mmol/l)	1.3 ± 0.4	1.4 ± 0.4
Men (%)	51.7	36.8[1]
Smokers (%)	33.7	25.4
Ex-Smokers (%)	39.3	39.4
Diet (%)	7.9	12.8
Vitamin supplement users (%)	9.0	9.3
Mineral supplements users (%)	3.4	2.2
Antidiabetic medication users (%)	3.4	3.8

[1]$p<0.05$.

In Table 2, age- and gender adjusted mean daily intake of antioxidants is shown. Overall, no significant differences between the groups were found.

Table 2. *Age- and gender-adjusted mean daily intake of energy and antioxidants (mean ± SE)*

	Silent MI cases (n=89)	Subjects without CHD (n=2333)	p-value
Energy intake (kJ/d)	7885 ± 201	8264 ± 39	0.07
β-carotene (mg/d)	1.38 ± 0.07	1.51 ± 0.01	0.06
Vitamin E (mg/d)	12.8 ± 0.6	13.6 ± 0.1	0.18
Vitamin C (mg/d)	106.5 ± 5.3	116.5 ± 1.0	0.06

Odds ratios were calculated for quartiles of antioxidant intake, with the lowest quartile as reference (Table 3). No association was found between the risk of MI and vitamin C or vitamin E intake. Only the highest compared to the lowest quartile of β-carotene intake showed a significantly lower risk of MI, when adjusted for age and gender. After adjustment for convential risk factors for CVD the reduced risk in the highest intake group remained (p_{trend} = 0.02). Adjustment for the other antioxidants only resulted in minor differences.

Table 3. *Odds ratio (95% CI) per quartile of antioxidant intake*

Quartile	1 (reference)	2	3	4	p-value for trend
Vitamin C (mg/d)	<83.0	83.0 - 110.3	110.3-141.5	>141.5	
no. of cases	27	28	16	18	
age + gender	1.0	1.1 (0.6-1.8)	0.6 (0.3-1.2)	0.7 (0.4-1.3)	p=0.12
multiple[1]	1.0	1.2 (0.7-2.0)	0.6 (0.3-1.2)	0.7 (0.4-1.4)	p=0.16
multiple+antiox[2]	1.0	1.3(0.7-2.2)	0.8 (0.4-1.5)	1.0 (0.5-1.9)	p=0.30
Vitamin E (mg/d)	<9.3	9.3-12.8	12.8-16.8	>16.8	
no. of cases	20	30	21	18	
age + gender	1.0	1.5 (0.9-2.7)	1.0 (0.5-1.8)	0.8 (0.4-1.5)	p=0.20
multiple[1]	1.0	1.5 (0.8-2.7)	1.0 (0.5-1.8)	0.8 (0.4-1.6)	p=0.24
multiple+antiox[2]	1.0	1.6(0.9-2.9)	1.1 (0.6-2.0)	1.0 (0.5-2.0)	p=0.50
β-Carotene (mg/d)	<1.11	1.11-1.43	1.43-1.80	>1.80	
no. of cases	26	34	19	10	
age +gender	1.0	1.3 (0.8-2.3)	0.7 (0.4-1.4)	0.4 (0.2-0.9)[3]	p=0.01
multiple[1]	1.0	1.4 (0.8-2.3)	0.8 (0.4-1.4)	0.4 (0.2-0.9)[3]	p=0.02
multiple+antiox[2]	1.0	1.4 (0.8-2.3)	0.8 (0.4-1.5)	0.5 (0.2-1.0)[3]	p=0.02

[1]adjusted for age, gender, smoking habits (current, former, never), systolic blood pressure, serum total cholesterol. [2] adjusted for age, gender, smoking habits and other antioxidants. [3]p<0.05.

Use of vitamin supplementation was reported in 9% of all subjects; 4% of the subjects with silent MI and 7% in the subjects without CHD history used vitamin C supplementation, and use of multivitamins was documented in 5% of the silent MI group and in 7% of the population without CHD. Use of vitamin E and carotenoid supplements was absent in the silent MI group and 1.5% and 0.9%, respectively, in the non-CHD group. ORs (95% CI) with supplement users excluded were essentially similar as those presented in Table 3, i.e. 1.0, 1.2(0.7-2.2), 0.7(0.3-1.3), 0.8(0.4-1.6) for the succesive quartiles of vitamin C, 1.0, 1.4(0.8-2.6), 0.8(0.4-1.6), 0.7 (0.3-1.4) for vitamin E and 1.0, 1.3(0.7-2.2), 0.7 (0.4-1.4) and 0.4 (0.2-0.8) for β-carotene.

Stratified analyses for men and women disclosed clear gender differences (Table 4). For men, none of the antioxidants seems to be related to risk of silent MI, whereas in women for all three antioxidants an inverse trend could be detected. The highest quartile of vitamin C and β-carotene showed a 90% reduction in risk of MI. For vitamin E a reduction of 70% was found.

Table 4. *Odds ratio (95% CI) per quartile of antioxidant intake, stratified for gender*

Quartile	1 (reference)	2	3	4	p-value for trend
Men					
Vitamin C (mg/d)	<79.5	79.5-106.8	106.8-137.8	>137.8	
no. of cases	10	14	6	16	
multiple[1]	1.0	1.5 (0.7-3.6)	0.6 (0.2-1.8)	1.8 (0.8-4.2)	p=0.26
Vitamin E (mg/d)	<10.5	10.5-14.2	14.2-18.6	>18.6	
no. of cases	11	11	14	10	
multiple[1]	1.0	1.1 (0.4-2.5)	1.3 (0.6-3.0)	1.0 (0.4-2.5)	p=0.88
β-Carotene(mg/d)	<1.15	1.15-1.49	1.49-1.86	>1.86	
no. of cases	9	19	10	8	
multiple[1]	1.0	2.3 (1.0-5.2)	1.3 (0.5-3.3)	1.1 (0.4-3.0)	p=0.75
Women					
Vitamin C (mg/d)	<85.3	85.3-113.0	113.0-143.1	>143.1	
no. of cases	16	16	9	2	
multiple[1]	1.0	0.9 (0.5-1.9)	0.5 (0.2-1.2)	0.1 (0.0-0.6)	p<0.01
Vitamin E (mg/d)	<8.7	8.7-11.8	11.8-15.9	>15.9	
no. of cases	13	14	12	4	
multiple[1]	1.0	1.2 (0.6-2.6)	0.9 (0.4-1.9)	0.3 (0.1-1.0)	p=0.03
β-Carotene(mg/d)	<1.09	1.09-1.40	1.40-1.77	>1.77	
no. of cases	17	15	9	2	
multiple[1]	1.0	0.9 (0.4-1.8)	0.6 (0.3-1.3)	0.1 (0.0-0.6)	p<0.01

[1]adjusted for age, gender, smoking habits (current, former, never), systolic blood pressure, serum total cholesterol.

In order to test if smoking had any influence on the association between antioxidant levels and risk of MI, we performed stratified analysis for smoking habits. Because of small numbers we calculated ORs in tertiles of antioxidant intake. ORs (95% CI) adjusted for age, gender, serum total cholesterol and systolic blood pressure in smokers (30 silent MI subjects, 593 others) were 1.0, 1.2 (0.5-3.0), 1.2 (0.5-3.0) for vitamin C, 1.0, 0.8 (0.3-2.0), 0.8 (0.3-2.0) for vitamin E and 1.0, 1.9 (0.8-4.5), 0.7(0.2-2.1) for

β-carotene, respectively. In the never and ex-smokers group the ORs were 1.0, 0.7 (0.4-1.2), 0.4 (0.2-0.8) (P_{trend}<0.01) for vitamin C, 1.0, 1.0 (0.5-1.9), 0.8 (0.4-1.5) for vitamin E and 1.0, 1.2 (0.6-2.1), 0.5 (0.3-1.1) for β-carotene.

DISCUSSION

In this study we investigated the relation between the risk of silent myocardial infarction and vitamin C, vitamin E and β-carotene intake in a cross-sectional study. For vitamin C and vitamin E no clear decrease in risk in the higher intake groups could be observed. Beta-carotene intake showed a significant inverse trend with risk of silent MI. Stratified analysis in men and women showed inverse trends for vitamin C, vitamin E and β-carotene in women only.

To assess dietary intake of antioxidants we used a semi-quantitave food frequency questionaire. Food frequency questionaires are commonly used in epidemiological studies to measure diet over a longer period of time.[22] Other dietary methods as recall methods and diet record methods can not give a good indication of usual diet and are less representative for past intake.[22] By means of a food frequency questionaire ranking of subjects into high or low intake categories for specific nutrients is very well possible. In our questionaire 170 food items were included. The calculated daily intake were compatible with other Dutch data. By means of a two-day dietary record method among more than 6000 subjects devided into age and gender categories, slighly lower vitamin C and β-carotene levels were found, whereas vitamin E levels were comparable.[23] In the Dutch part of the EURAMIC study among MI patients vitamin E levels were higher, albeit β-carotene levels were lower as assessed by a written food frequency questionaire.[24]

Assessment of dietary intake is prone to bias. Especially in cross-sectional and case-control studies the influence of changed dietary and lifestyle patterns and differences in recalling dietary intake due to a manifest illness may occur. To overcome this bias we selected people with an ECG documented silent MI who were not aware of their illness and therefore had not yet changed their lifestyle or diet. This assumption seems justifiable, because comparison of the use of prescribed diets between the silent MI group and the group with typical MI (self-reported MI and ECG evidence for MI (n = 110)) in our population showed a significantly higher use of prescribed diets in the clinically diagnosed MI group (24% against 8% in the silent MI group). Of course, the possibility of changed dietary patterns due to physiological changes as a result of

the underlying illness cannot be excluded. Further, the low prevalence of prescribed diets, which is even lower than in the group without history of CVD, could be indicative to a certain indifference towards personal health, which also may help explain why these subjects have missed or neglected symptoms of MI.

Studies on antioxidants and risk of CVD comprise studies on dietary intake, plasma and serum levels and one study used adipose tissue levels to assess long term exposition to antioxidants. Plasma or adipose tissue levels can be used as a biomarker for dietary intake and may be more informative to assess the internal dose, taking into account the variation in absorbtion and metabolism. A preventive effect of dietary and/or supplementary vitamin E against CHD has been described in prospective studies,[3-6] whereas prospective studies on plasma vitamin E did not reveal clear associations with CHD.[8,25-27] In a case-control study an inverse association between plasma levels of vitamin E and risk of angina pectoris was reported.[28] However, MI patients and controls did not differ in vitamin E content of adipose tissue.[9] In our study no relationship between vitamin E intake and risk of silent MI was found. Exclusion of supplement users did not change this result. The vitamin E intake in our study was high compared to levels in the US[4,5] and Finland[3] and may be sufficiently high to achieve desirable plasma levels (≥30 μmol/L) to prevent harmfull effects on CVD risk.[29]

Studies on the relation between MI and β-carotene in adipose tissue,[9] between plasma levels and angina pectoris[28] and between serum levels[8,25,27,30] and dietary intake[4,31] of β-carotene and risk of CVD have shown inverse trends. Results from our study concur with the results from these case-control and cohort studies, but contradict data from recently published intervention studies on β-carotene describing no effect or a slightly increased risk for CVD.[10-12] It seems that the conclusion that a diet rich in β-carotene containing products is protective against CVD still stands, but that this effect may not exclusively be ascribed to β-carotene. Other nutrients in these β-carotene containing foods or a combination of β-carotene with another nutrient may explain this protective effect.

An inverse correlation between dietary vitamin C and risk of CHD has been found in NHANES 1,[2] in a prospective study in Finland[3] and in men, but not in women, in the Scottish Heart Health Study.[31] Patients with angina pectoris, however, had no lower plasma vitamin C levels than controls after correction for smoking[28] and, in two prospective studies, no inverse relation between vitamin C intake and risk of CHD was described.[4,6] The results of our study are compatible with these last findings. For our

study and the latter ones, vitamin C intake could have been sufficiently large to minimize the risk of CVD.

We performed an analysis stratified for gender, which showed a clear inverse association between vitamin C and β-carotene and the risk of a silent MI in women only. We should, however, be careful in interpreting these results because the number of cases was small (46 men, 43 women). Differences in associations between men and women could be due to bias in the measurement of dietary intake. Possibly women are more aware about their diet, especially in this age category (55 years and over), in which it is likely that women do the cooking and hence will better estimate daily intake. However, we reduced this source of bias by means of verification of nutritional questionnaires by a dietician. Further, subjects with unreliable data were excluded from the analyses. Whereas atherosclerosis is a slow-moving process, dietary intake in a period decades before the dietary interview could be of importance. A change in diet after retirement could have taken place, which is most likely to occur in men, as many women in this age category were housewives. Our results describe an association in women only and thus are compatible with findings in the study of Knekt et al.,[3] whereas Kushi et al. did not find any association between antioxidants and CHD in a study among women[6] and in the Scottish Heart Health Study this association was described in men and not in women.[31]

In conclusion, our findings support a reduced risk of MI with higher β-carotene intake, suggesting that a diet rich in β-carotene containing foods may protect against CHD. Whether this is attributable to β-carotene itself or to other nutrients in these foods needs to be further investigated.

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5.2

Dietary intake of flavonoids is not related to risk of coronary atherosclerosis: a case-control study

ABSTRACT

High flavonoid intake has been associated with decreased risk of coronary heart disease (CHD), possible through the antioxidative effect of flavonoids, which protects low-density lipoprotein from oxidation. We investigated this association in a case-control study among men, 45-80 years of age. From subjects undergoing their first coronary angiography 82 cases with severe coronary atherosclerosis and 84 hospital controls with no or minor coronary atherosclerosis were selected. The hospital controls were pooled for statistical analysis with 73 population controls free of plaques in the carotid artery. Dietary intake of the flavonoids, quercetin, kaempferol and myricetin was assessed by means of a self-administered 105 item food frequency questionnaire assessing habitual antioxidant intake.

Total flavonoid intake, or intake of a specific flavonol, did not differ between the case group and the control groups. Mean (± SD) daily flavonoid intake was 20.0 ± 1.8 mg/d for cases and 19.8 ± 1.3 mg/d for controls. Intake of flavonoids was positively associated with intake of vitamin C ($r = 0.33$, $p<0.01$) and β-carotene ($r = 0.19$, $p = 0.02$), whereas no association with vitamin E intake was found. No association was found between dietary flavonoid intake and risk of coronary atherosclerosis. The odds ratio (and 95% confidence interval) for the successive quartiles of total flavonoid intake were 1.0 (reference), 0.88 (0.40-1.93), 0.88 (0.39-1.95) and 1.08 (0.47-2.47) after adjustment for age, smoking and plasma cholesterol level. Further adjustment for other antioxidants had little impact on the results.

We conclude that this study does not support a protective effect of a high intake of flavonoids on risk of coronary atherosclerosis.

INTRODUCTION

High intake of dietary antioxidants can play a protective role against coronary heart

disease (CHD). Large prospective studies have reported preventive effects of vitamin C[1,2] and vitamin E.[2-5] Further, high plasma levels of vitamin E[6] and β-carotene[7] are associated with lower risk of ischaemic heart disease. Other antioxidants worthy of being considered are flavonoids, polyphenolic substances most abundantly present in tea, onions, wine and apples. *In vitro*, flavonoids are scavengers of oxidative agents[8] and can inhibit oxidation of low-density lipoproteins (LDL),[9,10] an important step in the atherogenic process.[11] Further, flavonoids may have an antithrombotic effect through inhibition of platelet aggregation.[12] At present, few studies have investigated this association *in vivo*. The Zutphen Elderly Study, a Dutch cohort study, reported an inverse association between flavonoid intake and risk of CHD mortality.[13] This inverse association was confirmed in a cross-sectional study in 16 cohorts[14] and in a Finnish cohort study,[15] whereas no clear association was found in the prospective Health Professionals Study.[16]

We studied the association between flavonoid intake levels and risk of coronary atherosclerosis in a case-control study of men differing in degree of coronary atherosclerosis.

SUBJECTS AND METHODS

Study population

The study was conducted in several hospitals and clinical centres in Rotterdam and Dordrecht, the Netherlands, in the period 1993-1995. The study was approved by an ethical committee on human research and all participants gave their informed consent. Detailed information on selection procedures have been described elsewhere.[17] In short, cases with severe coronary atherosclerosis and hospital controls with no or minor coronary atherosclerosis were selected from patients undergoing their first coronary angiography. Cases had at least 85% stenosis in one and at least 50% stenosis in a second of the three major coronary vessels. Hospital controls had less than 50% stenosis in no more than two of the three major coronary vessels. The percentage of stenosis was scored by the cardiologist performing the angiography. Further, a group of population controls without symptomatic cardiovascular disease (CVD) was selected from participants in the Rotterdam Study, a population-based prospective cohort study[18] and through an advertisement in a local newspaper. Of this latter group, subjects were included only after echographical confirmation that the carotid artery was free of plaques. Enrolment procedures allowed for similar distributions of age (in 5-year

categories) and smoking habits (smoking, non-smoking).

All subjects were men between 45 and 80 years of age. Exclusion criteria were; use of HMG-CoA reductase inhibitors; diabetes mellitus; liver, kidney or thyroid disease; alcohol or drug abuse; vegetarian diet; and psychiatric complaints. The study population consisted of 92 cases, 95 hospital controls and 85 population controls.

Data collection

For the hospital groups information on medical history was obtained from the medical files and through a questionnaire within 2 months after angiography. Information on dietary, smoking and drinking patterns, medicine use, use of vitamin supplements, occupation and family history of CVD was obtained. Weight, height and blood pressure were measured.

Food frequency questionnaire

Dietary intake of the flavonoids quercetin, myricetin and kaempferol, vitamin E, vitamin C and β-carotene was assessed by means of a self-administered 105 item semi-quantitative food frequency questionnaire, asking the habitual consumption patterns over the past year. The questionnaire was a modified version of the food frequency questionnaire used to assess antioxidant intake in the Dutch part of the Euramic study.[19] To estimate flavonoid intake, extra items were included. Selection of these items was based on flavonoid content of specific food items reported by Hertog et al.[20,21] and the frequency of use of these items in the Dutch population.[22] To the original questionnaire items were added on consumption of onions, tea (including a question on type of tea and use of tea bags or loose tea leaves) and apples (pared or unpared).

Frequency of consumption was reported in 8 categories: never, rarely, once a month, 1 day every 2-3 weeks, 1 day every week, 2-3 days a week, 4-5 days a week, 6-7 days a week. The numbers of servings were quantified by the subject in ordinary household measures (spoons, cups, slices). Items processed in a meal and, therefore, difficult to quantify on an individual level were quantified per meal for the whole family. Individual levels were calculated by dividing the total amount per meal over the number of family members (children under 13 were counted for 0.5). Completeness and reliability of the questionnaire based on reported frequency of consumption, and credibility of reported number of servings was checked. Intake of antioxidants and linoleic acid was calculated with a computerized version of the Dutch Food Composi-

tion Table,[23] completed with a table for flavonoids.[20,21] The questionnaire was not specifically designed to calculate linoleic acid. However, since important sources of vitamin E and linoleic acid are largely the same foods, the questionnaire was considered good enough to obtain an adequate ranking of subjects according to intake.

On the basis of judgement by the investigators 15 subjects were excluded from the analysis due to unreliability of reported dietary data. A missing on one item was handled as follows. Depending on the contribution of an item to a specific antioxidant, the subject was excluded for analyses of this antioxidant. For instance, a missing on mayonnaise resulted in exclusion for vitamin E, but not for the other antioxidants. Depending on the missing item(s) extra subjects were excluded from analysis of flavonoids (n = 6; 3 cases, 2 hospital controls and 1 population control), vitamin C (n = 2; 2 cases), vitamin E (n = 4; 1 case and 3 hospital controls) and β-carotene (n = 2; 1 case and 1 hospital control).

Statistical analysis

Characteristics of the case group and the control groups were compared with Student's t-test for unpaired samples. Because the two control groups were comparable regarding lipid levels, age and smoking status, an increase in statistical power was obtained by pooling the two control groups for data analysis. Age-adjusted means were compared by analysis of covariance. Log transformations were performed to compare non-normally distributed variables. Pearson χ^2 analysis was applied for class variables. Correlations were calculated by Pearson correlation coefficients. Odds ratios (ORs) were calculated to quantify the association between dietary antioxidant intake and coronary atherosclerosis. Quartile distributions for calculation of ORs were based on distribution of intake in the control group. Confounding factors taken into account were; body mass index (BMI), total, high-density lipoprotein (HDL) and LDL cholesterol, diastolic and systolic blood pressure, diet use, medicine use and dietary fibre intake. Data analysis was conducted with the statistical package BMDP.[33]

RESULTS

Characteristics of the study population are presented in Table 1. No differences were seen for age, smoking status, body mass index (BMI), systolic and diastolic blood pressure. Total cholesterol, LDL-cholesterol and triglyceride levels were significantly lower and HDL-cholesterol was significantly higher in controls. Cases reported more

frequently the use of antihypertensive medication (92% in cases vs. 44% in controls), lipid-lowering medication (6% vs. 1%), aspirin + coumarin derivatives (93% vs. 21%) and a history of myocardial infarction (39% vs. 5%). Groups did not differ significantly in frequency of a special diet.

Table 1. *Characteristics of the study population, mean ± SD*

	Cases (n=85)	Controls (n=160)
Age (years)	60.8 ± 9.1	60.2 ± 8.3
Smokers (%)[1]	34.1	29.4
Ex-smokers (%)[1]	50.6	53.1
Body mass index (kg/m^2)	26.3 ± 2.5	26.2 ± 3.3
Total cholesterol (mmol/l)	6.1 ± 1 .1	5.5 ± 1.0 *
Triglycerides (mmol/l)	2.1 ± 0.9	1.7 ± 0.9 *
HDL-cholesterol (mmol/l)	0.9 ± 0.2	1.0 ± 0.3 *
LDL-cholesterol (mmol/l)	4.3 ± 1.1	3.8 ± 1.0 *
Systolic blood pressure (mmHg)	132.4 ± 16.8	135.2 ± 16.9
Diastolic blood pressure (mmHg)	81.4 ± 8.2	83.5 ± 8.5

[1]ex-smokers stopped smoking more than one year ago; otherwise they are categorized as current smokers. *p<0.01.

Table 2 lists mean dietary antioxidant levels in both groups. Mean dietary intake of flavonoids did not differ between the two groups. Intake levels ranged from 0.7 to 96.2 mg/d. With respect to the other antioxidants, the absolute intake of vitamin C was higher in controls, although the difference did not reach significance. No differences were seen for β-carotene and vitamin E, whereas the vitamin E/linoleic acid ratio was significantly higher in cases.

Table 2. *Antioxidant intake levels, mean ± SEM*

	Cases (n=85)	Controls (n=160)	p-value[§]
Flavonoids (mg)[1]	20.0 ± 1.8	19.8 ± 1.3	0.88
Quercetin (mg)[1]	12.0 ± 1.0	12.2 ± 0.8	0.78
Kaempferol (mg)[1]	6.7 ± 0.7	6.4 ± 0.4	0.92
Myricetin (mg)[1]	1.3 ± 0.2	1.2 ± 0.1	0.37
Vitamin C (mg)[2]	115.0 ± 4.9	135.5 ± 6.0	0.12
Beta-carotene (mg)[3]	1.2 ± 0.1	1.3 ± 0.1	0.42
Vitamin E (mg)[4]	16.4 ± 0.8	16.3 ± 0.7	0.88
Vitamin E/linoleic acid[4]	0.93 ± 0.02	1.03 ± 0.02	<0.01

[1]flavonoids= quercetin + keampferol + myricetin (82 cases, 157 controls). [2]83 cases. [3]84 cases, 159 controls. [4] 84 cases, 157 controls. [§]age-adjusted difference on log-transformed data.

Dietary intake of flavonoids was not associated with dietary vitamin E intake (r = -0.06). The correlation coefficients of flavonoid intake with vitamin C, β-carotene and fibre intake were 0.33 ($p < 0.01$), 0.19 ($p = 0.02$) and 0.20 ($p = 0.01$), respectively.

Table 3. *Odds ratios (95% confidence interval) for risk of coronary atherosclerosis per quartile of flavonoid intake*

	I (reference)	II	III	IV
Total flavonoids (mg)[1]	0.7-8.8	8.8-16.5	16.5-27.7	≥27.7
number of cases	22	20	20	20
age-adjusted	1.0	0.91 (0.43-1.93)	0.87 (0.41-1.86)	0.88 (0.41-1.91)
multivariate[2]	1.0	0.88 (0.40-1.93)	0.88 (0.39-1.95)	1.08 (0.47-2.47)
Quercetin (mg)	0.3-5.2	5.2-9.9	9.9-16.5	≥16.5
number of cases	17	26	19	20
age-adjusted	1.0	1.48 (0.70-3.17)	1.11 (0.50-2.47)	1.15 (0.52-2.58)
multivariate	1.0	1.63 (0.73-3.63)	1.18 (0.51-2.71)	1.43 (0.61-3.38)
Kaempferol (mg)	0.1-2.3	2.3-4.5	4.5-9.1	≥9.1
number of cases	17	18	28	19
age-adjusted	1.0	1.05 (0.47-2.35)	1.55 (0.73-3.31)	1.13 (0.50-2.56)
multivariate	1.0	1.03 (0.45-2.40)	1.71 (0.77-3.80)	1.45 (0.60-3.50)
Myricetin (mg)	0-0.2	0.2-0.8	0.8-1.6	≥1.6
number of cases	16	19	31	16
age-adjusted	1.0	1.18 (0.53-2.66)	1.62 (0.76-3.45)	1.04 (0.44-2.44)
multivariate	1.0	1.05 (0.45-2.46)	1.77 (0.80-3.89)	1.28 (0.52-3.16)

[1]flavonoids= quercetin + keampferol + myricetin. [2]adjusted for age, smoking status, plasma total cholesterol.

Table 3 presents ORs (and 95% confidence interval) for the risk of coronary atherosclerosis per quartile of flavonoid intake. No effect of total flavonoids or one of the specific flavonoids was detected. Further adjustment for the prestratification variables age and smoking status and total cholesterol had only a minor influences on the ORs (Table 3). No interaction was observed with smoking status or antioxidant levels. ORs calculated after additional adjustment for vitamin E, vitamin C and β-carotene were 1.0 (reference), 0.84 (0.37-1.93), 0.96 (0.41-2.27) and 1.11 (0.45-2.72) for the successive quartiles of total flavonoid intake.

In the results presented in Tables 1-3, hospital and population controls were pooled for statistical analyses. We also calculated the ORs for the control groups separately. Quartiles were defined on the basis of the distribution in the respective control groups.

The ORs for comparison of the highest to the lowest quartile of total flavonoid intake were 1.68 (0.64-4.44) and 0.97 (0.36-2.58) for comparison with hospital controls and population controls, respectively.

DISCUSSION

The results of this case-control study did not confirm the hypothesis that a high intake of flavonoids reduces the risk of coronary atherosclerosis.

Flavonoid intake as assessed in our study was lower than the levels in the Zutphen Elderly Study (mean ± SD of 26.6 ± 13.2)[13] and the Netherlands Cohort Study, a prospective study on cancer.[24] In our study quercetin made up 61% of the flavonoids. This percentage is comparable with that reported by Hertog et al. (63%). We calculated dietary intake of the three major flavonoids: the flavonols quercetin, kaempferol and myricetin. Another group of flavonoids, the flavones apigenin and luteolin, were not taken into account. This may have resulted in an underestimation, but, the most important sources of apigenin, celery,[25] parsley and thyme,[16] are no major contributors to flavonoid intake in the Netherlands.[22] Further, differences in assessment of dietary intake may have resulted in differences in intake levels.

Assessment of dietary intake, especially in a case-control study, is prone to bias. To overcome this problem we used two control groups, a group of hospital controls and a group of population controls. Because hospital controls underwent coronary angiography because of suspected CHD, cases and hospital controls will have been equally prone to bias caused by differences in recalling dietary intake. Calculation of the OR for coronary atherosclerosis for the separate control groups yielded similar results as those for the pooled control group, which leads to the conclusion that recall bias has not influenced the results.

The use of a biomarker for flavonoid intake would decrease the chance of potential bias. At present not much is known about the absorption of flavonoids in human beings. Hollman et al. recently reported an uptake of 52% of quercetin in onions.[26] In cooperation with Hollman, flavonoid compounds were measured in fasting plasma samples collected in our study. Fasting flavonoid levels appeared to be very low and barely exceeded the detection level. Because plasma flavonoid levels can give only a short-term impression of intake, it is not an useful tool for assessing usual intake.

To date only four studies have reported on the association between dietary flavonoid intake and risk of CHD. In the Zutphen Elderly Study an inverse association

between intake of flavonoids and risk of coronary death was found after a follow-up of 5 years. The association between flavonoid intake and incidence of myocardial infarction (MI) failed to reach significance.[13] An ecological study in 16 cohorts of the Seven Country Study confirmed the inverse association between flavonoid intake and CHD mortality. The mean intake levels in the cohorts varied from 2.6 mg/d to 68.2 mg/d.[14] Results from ecological studies, however, should be interpreted with caution because cross-cultural differences may confound the association. In a Finnish cohort study among 2748 men and 2385 women, a significant inverse association was seen for coronary mortality in women, whereas in men the association did not reach significance. After further adjustment for other antioxidants and fatty acids associations were no longer significant in women. The mean flavonoid intake reported in this Finnish cohort was 3.4 mg/d, substantially lower than values seen in the Netherlands.[15] Results from the US Health Professionals Study showed no association between flavonoid intake and non-fatal MI after a 6 year follow-up. The average intake in this study was 20.1 mg/d.[16]

Rimm et al. suggested in their discussion that a high flavonoid intake seems to be associated with a decreased risk of coronary mortality only. This may point to a specific effect of flavonoids on thrombosis.[16] Data from our study support this suggestion, as no association was found with prevalent coronary atherosclerosis.

In conclusion, this study among cases with severe coronary atherosclerosis and controls with no or minor atherosclerosis did not reveal an association with flavonoid intake.

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Epilogue

Almost four years have passed since I started this study on the relationship between the susceptibility of low-density lipoprotein (LDL) to oxidation and risk of atherosclerosis. The hypothesis that oxidation of LDL plays a role in the atherogenic process in men was mainly based on *in vitro* studies. Few studies had shown that LDL oxidation occurs *in vivo*,[1-3] and no studies were available to relate LDL oxidation to cardiovascular risk. This motivated the studies presented in this thesis. In summary, our work does not provide strong evidence for the susceptibility of LDL to oxidation to be associated with atherosclerosis, which may indicate that LDL oxidation plays no or a less important role in atherosclerosis. The association between LDL oxidation and risk of coronary atherosclerosis was weak and not statistically significant. However, resistance time to oxidation appears to be positively associated with risk of peripheral atherosclerosis.

That we could not disclose an association between susceptibility of LDL to oxidation and risk of coronary atherosclerosis in our studies is unlikely to be due to the design of the study. The sample size was larger than those in other recently reported studies which did suggest an increased susceptibility of LDL to oxidation,[4-6] and the contrast in atherosclerotic coronary artery disease between our groups should be sufficiently high to reveal an association. Possibly, the measurement of susceptibility of LDL to oxidation by monitoring diene production *ex vivo* after incubating isolated LDL under strong pro-oxidant conditions, i.e. using Cu^{2+} ions, does not reflect the *in vivo* situation. *In vivo* the oxidative process is not only influenced by composition and structure of the LDL particle itself, but is part of a larger mechanism in which plasma antioxidants and cell constituents also play a role. Therefore, as a measure of LDL oxidation *in vivo*, autoantibody titres against oxidized forms of LDL were measured in a smaller case-control study. This study, again, did not reveal an association between autoantibody titres and severity of coronary atherosclerosis. Although the contrast between cases and controls was smaller than in the case-control study on susceptibility of LDL to oxidation, these results indicate that autoantibody titres do not strongly reflect the atherosclerotic process and are not supportive of a marked role for LDL oxidation in the atherogenic process. As we studied atherosclerosis exclusively, a possible role of oxidation of LDL in other processes related to the development of coronary heart disease, such as the process of thrombosis or an influence on the susceptibility to ischemia can not be excluded.

A rather indirect way of measuring the possible role of LDL oxidation in the atherosclerotic process is by measuring antioxidant status. High levels of antioxidants may, through inhibition of LDL oxidation, decrease the risk of coronary atherosclerosis. The results form our study on LDL antioxidants did not support this hypothesis. Unexpectedly, higher LDL tocopherol levels were found in cases which may indicate that tocopherol metabolism changes as a result of the disease status. Therefore, metabolic and genetic differences among individuals, and perhaps between subjects susceptible and those not or less susceptible to cardiovascular disease; should be further elucidated.

The study on LDL and plasma antioxidants made clear that only small differences in antioxidant levels and LDL fatty acid composition occurred between our cases and controls, which may have had an effect on the small differences in the oxidation parameters. However, we did not find a correlation between antioxidant levels and oxidation parameters, whereas fatty acid composition turned out to be an important determinant of the maximum rate of oxidation. Thus far, most data on the influence of antioxidants and fatty acid composition on susceptibility of LDL to oxidation are based on intervention studies.[7,8] Concerning antioxidants, supplementation with α-tocopherol has been shown to decrease susceptibility of LDL to oxidation.[9] This effect is already reached at a low dose (25 IU/d),[7] however, this dose is still twice as high as the daily recommended intake for α-tocopherol. These studies and results from our study indicate that in an unsupplemented population the variation in antioxidant levels is not sufficiently high to be reflected in differences in susceptibility of LDL to oxidation *ex vivo*. A threshold level for tocopherol has been suggested above which tocopherol can actually decrease susceptibility of LDL to oxidation.[8] Below this treshold other factors, such as fatty acid composition, may determine susceptibility of LDL to oxidation. Whether this will also have an effect on oxidation *in vivo* is unknown yet.

In observational studies, a beneficial effect has been described at high levels of antioxidant intake, often through supplementation. This association is most convincing for vitamin E [10,11,chapter 1] In our study on coronary atherosclerosis and susceptibility of LDL to oxidation no differences in dietary antioxidant intake between cases and controls were found. In our analysis in data from the Rotterdam Study we found a reduced risk of silent myocardial infarction at high intake levels of β-carotene. Recall bias is absent or limited in both studies. In the Rotterdam Study patients were not aware of their disease status, and in the LDL oxidation study no differences were found irrespective of whether cases were compared to hospital controls or to popula-

tion controls.

The results from our studies and from other observational studies give no clear indication which antioxidant may be a marker or cause of disease under unsupplemented conditions. With a few exceptions, findings in randomized trials on vitamin E, β-carotene and vitamin C did not support the view that supplementation with a specific antioxidant or a combination of antioxidants decreases the risk of CHD.[10,11,chapter 1]

The discrepancy between results from observational studies on diet and CHD risk and those of intervention studies could imply that the protective effects of antioxidants may not be ascribed to the antioxidant alone, but rather to other compounds or a combination of compounds in foods rich in antioxidants. This seems especially true for β-carotene. Therefore, in future studies emphasis should be placed on foods instead of nutrients, to find out which foods may help to protect against CHD. Another cause of the discrepancy might be that a high consumption of fruit and vegetables is a marker for differences in life-style. A more 'healthy' behaviour in subjects with a high intake of fruits and vegetables may have resulted in an inverse association between a high intake of antioxidants and risk of CHD.

The results from our studies do not indicate that high doses of specific nutrients are necessary to reduce the risk of atherosclerosis. Antioxidant supplementation may have an effect on LDL oxidation, but the importance of LDL oxidation in risk of atherosclerosis is not convincing. Other factors seem to play a more prominent role in determining which subject has an increased risk of developing atherosclerosis.

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Summary

In the studies presented in this thesis we tested the hypothesis that oxidation of low-density lipoproteins (LDL) increases the risk of atherosclerosis and that antioxidants protect against coronary heart disease (CHD), possibly through inhibition of LDL oxidation.

CHD is the major cause of death in most industrialized countries. To reduce mortality and morbidity from CHD, more insight into the process of atherosclerosis is necessary. During the atherogenic process cholesterol and other lipids accumulate in the arterial wall, which may cause partial or complete obstruction of the lumen of the blood vessel. A high level of cholesterol, especially LDL-cholesterol, is a major risk factor for atherosclerosis. A current hypothesis is that the LDL particle becomes even more atherogenic after it has been chemically altered by free radicals and is recognized by the 'scavenger receptor' on the macrophages. This results in accelerated uptake of cholesterol into macrophages in the vessel wall, which is the beginning of the fatty streak. Protection of LDL, in particular protection of polyunsaturated fatty acids in LDL, against oxidation may be achieved by antioxidant defence. Several antioxidant enzymes, including the selenium-containing glutathione peroxidase, superoxide dismutase and catalase, and dietary antioxidants (vitamin E, vitamin C, β-carotene and possibly flavonoids) may have this protective effect. Epidemiological studies have not demonstrated unequivocally that a high intake of antioxidants decreases the risk of CHD. Studies on dietary intake and serum levels of antioxidants do point to a preventive effect, whereas results from intervention studies are less conclusive (Chapter 1).

Several studies have reported seasonal fluctuation in antioxidant levels. Fluctuations in antioxidants may be reflected in differences in susceptibility to oxidation of LDL. We measured resistance time, i.e. the time until onset of LDL oxidation, and maximum rate of oxidation by monitoring *ex vivo* diene production after copper-induced LDL oxidation. In a small experiment blood from 10 healthy free-living subjects was collected at 4 different time points over one year. No seasonal fluctuation in resistance time or maximum rate of oxidation was detected. Small differences were seen in determinants of LDL oxidation, including LDL particle size and vitamin E, lutein and β-carotene levels. No differences were seen in fatty acid composition, although this turned out to be the main determinant of oxidation susceptibility (Chapter 2).

To examine whether the susceptibility of LDL to oxidation plays a role in the

atherogenic process (Chapter 3), we performed a case-control study among men 45-80 years of age. Three groups of subjects participated: a group of cases with angiographically diagnosed severe coronary atherosclerosis (n = 92), a group of hospital controls with no or minor coronary atherosclerosis according to coronary angiography (n = 95) and a group of population controls free of symptomatic cardiovascular disease and with the carotid artery free of plaques (n = 86). For the analyses we pooled the two control groups. To test the hypothesis that susceptibility of LDL to oxidation is elevated in patients with severe coronary atherosclerosis, we measured resistance time and maximum rate of oxidation. Resistance time was inversely associated with risk of coronary atherosclerosis. However, the association was of borderline significance. No association was found with maximum rate of oxidation. Though resistance to oxidation may be a factor in atherogenesis, the *ex vivo* measure of LDL oxidation is no strong predictor of severity of coronary atherosclerosis (section 3.1).

In these participants also the ankle-arm blood pressure index was measured. The ratio of systolic blood pressure in the ankle divided by systolic blood pressure in the arm can be used as a measure of peripheral atherosclerosis. The lowest of the ankle-arm indices in both legs was used to categorize subjects into groups with and without peripheral atherosclerosis. The study population consisted of 39 men with (ankle-arm index < 1.00) and 210 men without (ankle arm index ≥ 1.00) peripheral atherosclerosis. A strong inverse association between resistance time and risk of peripheral atherosclerosis was present, suggesting that patients with peripheral atherosclerosis are more susceptible to LDL oxidation than subjects without peripheral atherosclerosis (section 3.2).

Oxidatively modified LDL has immunogenic properties and has the ability to induce the formation of autoantibodies. We measured autoantibody titres as an *in vivo* measure of LDL oxidation in a sub-study among men and women 26-68 years of age. A group of cases with angiographically detected severe coronary atherosclerosis (n = 47), a group of hospital controls with no or minor atherosclerosis (n=47) and a group of healthy population controls (n = 49) were selected. No association was found between autoantibody titre and atherosclerosis. From our results and results from other studies we conclude that autoantibodies are not related to chronic atheriosclerotic arterial disease although they may play a part at a certain stage of the disease process (section 3.3).

High plasma or LDL antioxidant levels have been hypothesized to decrease the

susceptibility of LDL to oxidation, thereby potentially decreasing the risk of CHD. In the case-control study on LDL oxidation and coronary atherosclerosis, antioxidant levels within the LDL particle and plasma vitamin C levels were measured to assess the association between antioxidant levels and risk of coronary atherosclerosis. In general, no relation of LDL antioxidant levels with risk of coronary atherosclerosis was detected. Marginally decreased risks were seen for high LDL α-carotene and LDL lutein/zeaxanthin levels. Unexpectedly, a positive association between LDL tocopherol levels and risk of coronary atherosclerosis was found. The associations between antioxidants and coronary atherosclerosis could not be ascribed to inhibition of LDL oxidation (section 4.1).

In a sub-sample of participants in this case-control study, consisting of 70 cases and 70 population controls, we assessed plasma levels of the antioxidant Coenzyme Q_{10}. *In vitro* a marked antioxidative potential is ascribed to Coenzyme Q_{10}. No differences were seen in Coenzyme Q_{10} levels between the two groups, making us conclude that the protective effect of Coenzyme Q_{10} is not reached at levels present under normal, unsupplemented conditions (section 4.2).

To study the association between antioxidant intake and risk of CHD we studied baseline dietary data from the Rotterdam Study, a prospective cohort study among men and women aged 55 and over. A group of 89 subjects who had undergone a silent myocardial infarction (MI), defined as 'ECG evidence of MI without medical information or self-reported history of MI', were selected. These subjects with CHD are not aware of their disease status and therefore changes in dietary or life-style patterns can be excluded. Comparison of this group with the baseline population without cardiovascular disease (n = 2333) revealed an inverse association between intake of β-carotene and risk of silent MI. The associations were most pronounced in women (section 5.1).

In addition to LDL antioxidant levels, we estimated dietary intake of antioxidants in the case-control study on LDL oxidation by means of a self-administered food frequency questionnaire in which the habitual antioxidant intake in the previous year was asked. In this study we paid special attention to the intake of flavonoids, which are polyphenolic substances commonly present in fruit and vegetables. No difference in dietary intake of flavonoids or other antioxidants was seen between the cases with severe coronary atherosclerosis and the pooled control groups (section 5.2).

In the studies described in this thesis we were not able to find support for our main hypothesis that susceptibility of LDL to oxidation plays an important role in the

atherogenic process. Possibly, the method of measuring LDL oxidation *ex vivo* is not an adequate parameter. However, LDL oxidation measurement *in vivo* by means of autoantibodies gave the same result. With respect to antioxidants, no clear protective effect of antioxidants in LDL or plasma was detected, whereas dietary antioxidants gave inconclusive results with respect to the question which antioxidant may be important. Further, it is not clear whether the antioxidant intake levels itself, or rather an overall healthy life-style, is responsible for the observed associations. Therefore, the results of our study and from other observational and intervention studies, do not support a general advice in favour of supplementation with antioxidants. A dietary pattern with ample food rich in antioxidants (e.g. fruit and vegetables) is still to be recommended.

Samenvatting

In de studies beschreven in dit proefschrift hebben we de hypothese onderzocht of oxidatie van lage-dichtheidslipoproteïne (transportdeeltjes voor cholesterol in het bloed) het risico op hart- en vaatziekte verhoogt. Antioxidanten zoals vitamine E, vitamine C en β-caroteen kunnen deze chemische omzetting mogelijk voorkomen of vertragen.

Hart- en vaatziekte (HVZ) is de belangrijkste doodsoorzaak in de meeste geïndustrialiseerde landen. Om het risico op ziekte en sterfte als gevolg van HVZ te verlagen is het belangrijk inzicht te krijgen in het proces van atherosclerose ('aderverkalking'). Tijdens het atherosclerotisch proces stapelen cholesterol en andere vetachtige substanties zich op in de wand van de bloedvaten. Hierdoor kan het bloedvat gedeeltelijk of helemaal geblokkeerd worden, waardoor de bloeddoorstroming wordt gehinderd. Hoge cholesterol niveaus in het bloed, vooral hoge hoeveelheden van het 'slechte cholesterol', het lage-dichtheidslipoproteïne (LDL), vormen een belangrijke risico-factor voor atherosclerose. De huidige idee is dat het LDL-deeltje nog een groter risico voor HVZ vormt op het moment dat het deeltje geoxideerd is. Deze oxidatie vindt plaats onder invloed van 'vrije radicalen', reactieve deeltjes die in het lichaam voorkomen. In de vaatwand wordt cholesterol uit het LDL-deeltje afgegeven aan macrofagen. Voor afgifte van cholesterol aan de macrofagen moet een koppeling plaatsvinden tussen het LDL-deeltje en de macrofaag. Daartoe bevinden zich op het ontvangende deeltje receptoren. Dit zijn structuren die herkend worden door het LDL en waar het LDL op past zoals een sleutel op een slot. Na oxidatie wordt het LDL herkend door een andere receptor op de macrofagen. Door de koppeling aan deze zogenaamde 'scavenger receptor' wordt de afgifte van cholesterol aan de macrofaag versneld, resulterend in een versnelde stapeling van cholesterol-geladen macrofagen (schuimcellen) in de vaatwand. Deze opstapeling van schuimcellen is het begin van het atherosclerotisch proces en het begin van de plaques. Antioxidanten kunnen het LDL, of meer in het bijzonder kunnen de meervoudig-onverzadigde vetzuurketens in het LDL, beschermen tegen oxidatie. In ons lichaam komen van nature antioxidant-enzymen voor, zoals superoxide dismutase, het selenium-bevattende glutathion peroxidase en catalase. Andere antioxidanten komen via de voeding binnen. Dit zijn de antioxidant-vitamines vitamine E (tocoferol), vitamine C en β-caroteen (pro-vitamine A), maar mogelijk kunnen ook andere carotenoïden of andere stoffen zoals flavonoïden antioxidatief werken. Epidemiologische studies hebben nog niet eenduidig aangetoond dat een hoge inneming van antioxidanten tot een verlaging van het risico op HVZ

leidt. Studies naar inneming en bloedniveaus van antioxidanten wijzen wel in de richting van een beschermend effect, echter resultaten van interventiestudies waarin mensen voor langere tijd supplementen gebruiken laten een minder duidelijk effect zien (Hoofdstuk 1).

De mate van oxidatie van LDL kan op verschillende manieren worden bepaald. Wij hebben gevoeligheid van LDL voor oxidatie gemeten met behulp van een *ex vivo* (buiten het lichaam) bepaling. Aan een buisje geïsoleerd LDL wordt koper (Cu^{2+}) toegevoegd. Dit Cu^{2+} is een sterke pro-oxidant en zorgt er voor dat LDL gaat oxideren. Afhankelijk van de antioxidantbescherming van het LDL zal de tijd die verstrijkt tussen toevoegen van Cu^{2+} en de daadwerkelijke aanvang van de oxidatie verschillen. Deze periode wordt de resistentie-tijd genoemd. Ook de snelheid waarmee de oxidatie verloopt kan verschillen. De maximale snelheid van oxidatie wordt ook gebruikt als maat voor oxidatie-gevoeligheid.

In hoofdstuk 2 is een studie gepresenteerd waarin gekeken is of oxidatie-gevoeligheid van LDL varieert gedurende het jaar. Uit verschillende studies is gebleken dat er een seizoensvariatie in antioxidant-niveaus bestaat. Een fluctuatie van antioxidant-niveaus zou als gevolg kunnen hebben dat de oxidatie-gevoeligheid van het LDL ook beïnvloed wordt door het moment van bloedafname. In een klein experiment bij 10 gezonde vrijwilligers is de oxidatie-gevoeligheid van het LDL gemeten in 4 periodes over één jaar. Er bleek geen seizoensvariatie in resistentie-tijd of maximale oxidatie-snelheid te zijn. Wel werden kleine verschillen gevonden in determinanten die de oxidatie-gevoeligheid bepalen. De deeltjesgrootte fluctueerde, evenals vitamine E, luteïne en β-caroteen niveaus. De vetzuursamenstelling van het LDL liet geen seizoensvariatie zien. Dit bleek wel een belangrijke factor die de snelheid van oxidatie beïnvloedt.

Om na te gaan of de oxidatie-gevoeligheid van LDL een rol speelt in het proces van atherosclerose (hoofdstuk 3), hebben wij een patiënt-controle onderzoek uitgevoerd bij mannen tussen de 45-80 jaar. Drie groepen deelnemers zijn geselecteerd. Een groep patiënten met ernstige coronaire atherosclerose (aderverkalking rond het hart) (n = 92) en een groep ziekenhuiscontroles met geen of minimale coronaire atherosclerose (n = 95) zijn geselecteerd op basis van catheterisatieverslagen. Verder is een groep populatiecontroles geselecteerd (n = 86) uit deelnemers aan het ERGO-onderzoek, een prospectief vervolgonderzoek uitgevoerd in Rotterdam bij mannen en vrouwen van 55 jaar en ouder, en via een advertentie in een locale krant. Van deze deelnemers was bekend dat ze geen atherosclerose in de halsslagader en geen symptomatische HVZ

hadden. Voor de statistische analyses zijn de twee controlegroepen samengevoegd. Om te testen of de oxidatie-gevoeligheid van patiënten hoger is dan die van controles is de resistentie-tijd en de maximale oxidatiesnelheid tussen patiënten en controles vergeleken. De resistentie-tijd was invers geassocieerd (hoe hoger de resistentie-tijd hoe lager de kans op coronair atherosclerose). Dit verband was echter net niet significant. Geen associatie werd gevonden met de maximale oxidatiesnelheid. Alhoewel uit deze resultaten blijkt dat de resistentie-tijd mogelijk wel een verband houdt met het risico op HVZ, is het verband in ieder geval niet duidelijk. Dit zou erop kunnen wijzen dat de oxidatie-gevoeligheidsmeting door middel van een *ex vivo* maat geen goede weerslag geeft van de processen die zich *in vivo* afspelen (paragraaf 3.1).

Bij de deelnemers van deze studie is ook de enkel-arm bloeddruk index bepaald. De ratio van de systole bloeddruk in de enkel gedeeld door de systole bloeddruk van de arm kan gebruikt worden als een maat voor perifere atherosclerose (aderverkalking in de benen). De deelnemers zijn heringedeeld op basis van de laagste ratio gemeten aan de linker- of rechterzijde van het lichaam. Negenendertig mannen met perifeer atherosclerose (een enkel-arm index lager dan 1.00) zijn vergeleken met 210 mannen zonder perifeer atherosclerose (enkel-arm index groter of gelijk aan 1.00). Een sterk inverse associatie werd gevonden tussen de resistentie-tijd en het risico op perifere atherosclerose, hetgeen een aanwijzing geeft dat mensen met perifere atherosclerose meer gevoelig zijn voor oxidatie (paragraaf 3.2).

Oxidatief veranderde LDL-deeltjes hebben een immunogene werking, wat betekent dat ze de productie van antilichamen kunnen induceren. De concentratie antilichamen in het bloed kan als een *in vivo* maat van LDL-oxidatie worden onderzocht. Wij hebben de concentratie van auto-antilichamen gevormd tegen MDA-gemodificeerd LDL gemeten in een patiënt-controle onderzoek onder mannen en vrouwen tussen de 26 en 68 jaar. Met behulp van catheterisatieverslagen zijn 47 patiënten met ernstige coronair atherosclerose en 47 ziekenhuiscontroles met matige atherosclerose geselecteerd. Verder zijn 47 gezonde populatiecontroles geselecteerd. In bloed van deze deelnemers is de auto-antilichaam titer bepaald. Wij vonden geen associatie met de concentratie auto-antilichamen en het risico op atherosclerose. Uit de resultaten van onze studie en van studies van anderen hebben we geconcludeerd dat de antilichaam-concentratie niet direct gerelateerd is aan chronische atherosclerose, maar dat het meer een maat zou kunnen zijn voor een bepaalde fase in het atherosclerotisch proces (paragraaf 3.3).

Hoge antioxidantniveaus in plasma of in het LDL-deeltje zouden de gevoeligheid

van LDL voor oxidatie kunnen verminderen en op deze manier het risico op HVZ kleiner maken. In ons patiënt-controle onderzoek naar LDL-oxidatie en coronair atherosclerose zijn de antioxidantniveaus in het LDL-deeltje en de plasma vitamine C niveaus gemeten om de associatie tussen antioxidanten en het risico op coronair atherosclerose te meten. Uit dit onderzoek werden geen duidelijke verbanden gevonden. Een matige verlaging van het risico op coronair atherosclerose trad op bij hogere concentraties α-caroteen en luteïne/zeaxanthine concentraties in het LDL. Tegen de verwachting in vonden wij een positief verband tussen de hoeveelheid LDL-tocoferol en het risico op atherosclerose. Het verband tussen de antioxidanten en coronair atherosclerose kon niet toegeschreven worden aan het remmende effect van antioxidanten op de oxidatie van LDL (paragraaf 4.1).

In 70 patiënten en 70 populatie-controles die deelnamen aan het patiënt-controle onderzoek zijn plasmaniveaus van het antioxidant Coenzyme Q_{10} bepaald. *In vitro* studies hebben een sterk antioxidatieve werking aan dit coenzyme toegeschreven. In onze studie hebben we echter geen verschil gevonden in Coenzyme Q_{10} niveaus tussen de twee groepen. Het lijkt erop dat het mogelijk antioxidatieve beschermende effect nog niet wordt bereikt bij van nature voorkomende, niet gesupplementeerde, concentraties in het bloed (paragraaf 4.2).

In hoofdstuk 5 zijn twee studies beschreven waarin we de associatie tussen antioxidantinneming en het risico op HVZ hebben bestudeerd. In data verzameld aan het begin van het ERGO onderzoek is gekeken naar de associatie tussen antioxidantinneming en het risico op een stil myocard infarct. Bij 89 deelnemers werd de diagnose 'stil myocard infarct' gesteld. Deze deelnemers hadden op het ECG (electro cardiogram) duidelijke aanwijzingen van een doorgemaakt infarct, terwijl dit noch bij de deelnemer noch bij zijn/haar arts bekend was. Omdat deze deelnemers zich niet bewust waren van het doorgemaakt infarct, is een verandering van voeding of leefpatroon als gevolg van de ziekte uit te sluiten. We hebben de antioxidant-inneming van deze groep vergeleken met de inneming van de mensen in de beginpopulatie die geen HVZ hadden (n = 2333). Vergelijking van deze twee groepen gaf aan dat een hogere inneming van β-caroteen, of van voedingsmiddelen rijk aan β-caroteen het risico op een stil myocard infarct verlaagde. Deze associatie was het duidelijkst bij vrouwen (paragraaf 5.1).

In het patiënt-controle onderzoek naar LDL-oxidatie en coronair atherosclerose hebben we naast het meten van antioxidantniveaus in plasma en LDL ook de voedingsinneming van antioxidanten gemeten. Door middel van een voedselfrequentie-

vragenlijst, thuis ingevuld door de deelnemers, is de gebruikelijke voeding in het jaar voorafgaande aan de studie bepaald. In deze studie hebben we speciaal gekeken naar de inneming van flavonoïden. Dit zijn polyfenolische deeltjes aanwezig in groente en fruit (belangrijke bronnen: appels, ui, rode wijn en thee). We vonden geen verschil in inneming van flavonoïden of van een van de andere antioxidanten tussen de patiënten en controles (paragraaf 5.2).

In de hier beschreven studies hebben wij geen sterke aanwijzingen kunnen vinden ter ondersteuning van onze belangrijkste hypothese, dat de gevoeligheid van LDL voor oxidatie een belangrijke rol zou spelen in het proces van atherosclerose. Mogelijkerwijs is de methode voor het meten van LDL-oxidatie *ex vivo* niet geschikt als parameter voor het meten van een *in vivo* proces. De meting van auto-antilichamen als *in vivo* maat gaf echter dezelfde resultaten te zien. Met betrekking tot de antioxidanten zijn er geen duidelijk beschermende effecten van antioxidanten in LDL of plasma gevonden. Wat de inneming van antioxidanten via de voeding betreft, is het niet duidelijk welk antioxidant nou speciaal van belang is. Verder is het mogelijk dat bij een gevonden associatie tussen antioxidantinneming en het risico op HVZ niet de antioxidantinneming zelf, maar het totaal gezondere leefpatroon van een persoon met hoge antioxidantinneming een rol speelt in de gevonden associaties. Van de resultaten van onze studie en van andere observationele en interventiestudies kan nog steeds niet geconcludeerd worden dat supplementatie met antioxidanten gunstig zal zijn voor het risico op HVZ. Een voedingspatroon met voedsel rijk aan antioxidanten (zoals groente en fruit) strekt tot de aanbeveling.

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4 jaar zitten erop; het boekje is af. Het wordt tijd om een aantal mensen te bedanken die ieder op zijn of haar manier een bijdrage hebben geleverd aan dit resultaat.

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