ARI PALOMÄKI

Multifactorial Prevention, Lovastatin Therapy and Ubiquinone Supplementation in Coronary Heart Disease

Effects on Antioxidative Capacity of Low-density Lipoprotein

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ACADEMIC DISSERTATION

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University of Tampere, Medical School,
Department of Internal Medicine and Department of Pharmacological Sciences
Tampere University Hospital, Centre for Laboratory Medicine
Kanta-Häme Central Hospital, Department of Medicine
UKK Institute for Health Promotion Research
Finland

Supervised by

Docent Kimmo Malminiemi
University of Tampere
Professor Amos Pasternack
University of Tampere

Reviewed by

Docent Petri Kovanen
University of Helsinki
Professor Heikki Vapaatalo
University of Helsinki

Distribution

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Finland

Tel. +358 3 215 6055
Fax +358 3 215 7685
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CONTENTS

ABSTRACT 7

1. LIST OF ORIGINAL PUBLICATIONS 9

2. ABBREVIATIONS AND ACRONYMS 10

3. INTRODUCTION 12

4. REVIEW OF THE LITERATURE 13

4.1. Coronary heart disease 13
4.1.1 Epidemiology 13
4.1.2 Risk factors 13

4.2. Oxidative stress and atherosclerosis 13
4.2.1 Free radicals and the oxidation cascade of lipids 14
4.2.2 Oxidation of the LDL particle 14
4.2.3 Ubiquinol and lipid peroxidation 15
4.2.4 Alpha-tocopherol and lipid peroxidation 15
4.2.5 Tocopherol-mediated oxidation of LDL 16
4.2.6 Oxidatively modified LDL in the vessel wall 17

4.3 Intervention and prognosis of coronary heart disease 17
4.3.1 Lifestyle changes 17
4.3.2 Cholesterol lowering therapy with 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) 19
4.3.3 Antioxidant supplementation 24

4.4 Effects of different treatments on oxidative stress 26
4.4.1 Lifestyle changes 26
4.4.2 Statin treatment 31
4.4.3 Ubiquinone and alpha-tocopherol supplementation 37

5. AIMS OF THE STUDY 43

6. PATIENTS AND METHODS 44
6.1 Patients and study design 44
6.2 Methods 45
6.2.1 Evaluation of dietary habits (I) 45
6.2.2 Evaluation of physical activity (I) 45
6.2.3 Clinical chemistry (I-V) 46
6.2.4 Metal-ion independent oxidation of LDL lipids (I-V) 46
6.2.5 Copper-ion mediated oxidation of LDL (II-V) 48

7. ETHICAL CONSIDERATIONS 49

8. STATISTICAL METHODS 50

8.1 Controlled preventive education study (I) 50
8.2 Double-blind cross-over studies (II-V) 50

9. RESULTS 51

9.1 Effects of comprehensive preventive group education (I) 51
9.1.1 Lipids and other risk factors 51
9.1.2 Total radical antiperoxiding capacity of LDL (LDL TRAP) 51

9.2 Effects of lovastatin therapy (II, IV) 51
9.2.1 Lipids and lipoproteins 51
9.2.2 Antioxidant concentration and total consumption time 53
9.2.3 Copper-ion mediated oxidation 54

9.3 Effects of antioxidant supplementation (III, IV) 55
9.3.1 Lipids and lipoproteins 55
9.3.2 Antioxidant concentration and total consumption time 55
9.3.3 Copper-ion mediated oxidation 56

9.4 Comparison of metal-ion dependent and independent oxidation (V) 57

10. DISCUSSION 59

10.1 Patient allocation and interventions 59
10.2 Effects of group education on diet and physical activity (I) 60
10.3 Effects of group education on LDL antioxidative defence (I) 60
10.4 Comparison of different oxidation methods (V) 61
10.5 Effects of lovastatin therapy on LDL antioxidants and their consumption during oxidation (II, IV) 61
10.6 Effects of lovastatin therapy on the resistance of LDL to copper-mediated oxidation (II, IV) 63
10.7 Effects of ubiquinone and α-tocopherol supplementation on LDL antioxidants and their consumption during oxidation (III, IV) 66
10.8 Effects of ubiquinone and α-tocopherol supplementation on LDL antioxidative defence (III, IV) 67
10.9 Concluding remarks 68

11. CONCLUSIONS 69

12. ACKNOWLEDGEMENTS 70

13. REFERENCES 72

ABSTRACT

Modification of low-density lipoprotein (LDL) particles is an integral part of atherogenesis. It may take place enzymatically or through an oxidation cascade, which leads to oxidative modification of the LDL particle and its accumulation into monocyte-derived macrophages in the arterial intima. Alpha-tocopherol (α–TOH) behaves as antioxidant during an attack with plenty of free radicals but pro-oxidatively during low radical flux. Ubiquinol is the first line antioxidant in the outer surface layer of LDL. It can be regenerated from its oxidised form ubiquinone. It is synthesised via the same pathway as cholesterol. Hence, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) not only decrease plasma cholesterol but also the level of ubiquinol in LDL particles.

Altogether 104 patients with coronary heart disease participated and completed at least one study concentrating on the effects of different treatments on the susceptibility of LDL to oxidation. Effects of preventive group education, lovastatin, and ubiquinone supplementation were analysed.

On the average of three years after coronary bypass grafting, 72 patients participated in a controlled parallel study with a follow-up of one year. The effect of preventive group education was investigated by LDL TRAP (Total Radical Antiperoxiding capacity in LDL), a method using the azo compound 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN) as a radical generator. A significant although transient increase was observed in the resistance of LDL against oxidation. Eight months after preventive education, the difference between the intervention group and the control group was 25% reflecting an almost parallel change in leisure time physical activity. However, LDL TRAP did not differ between the groups after one year. There was a significant difference of 1.2 kg in the body weight in favour of the intervention group after one year. Also circulating white blood cell count decreased in the intervention group, possibly suggesting lower degree of inflammation compared to controls. No clinically significant changes were observed in dietary habits or lipid values.

In a methodological study with 10 patients, LDL TRAP was compared with the lag time to the propagated formation of conjugated dienes, a measurement generally used to study the resistance of LDL against copper-mediated oxidation. Both methods revealed an evident improvement in the antioxidative capacity of LDL during α–TOH supplementation, but the results after lovastatin therapy were incoherent.

In all 32 men participated in two double-blind, placebo-controlled cross-over studies, where effects of lovastatin (20 mg three times daily) therapy were studied with or without α–TOH supplementation. Mean LDL cholesterol level in serum decreased significantly by 44%, apolipoprotein B-100 by 33%, triglycerides by 30%, while HDL cholesterol and apolipoprotein A-1 increased by 16% and 7%, respectively. Lovastatin shortened the consumption time of both ubiquinol and α–TOH during oxidation induced by AMVN in average by 17% and 41%, respectively. According to the copper-mediated oxidation assay, the lag time shortened statisti-

cally significantly by 7%. The maximal amount of conjugated dienes tended to be decreased by lovastatin. Hence, lovastatin had both anti- and pro-oxidative effects on the resistance of LDL against oxidation.

The effect of ubiquinone supplementation during lovastatin therapy was assessed in a double-blind, placebo-controlled cross-over study with 19 male subjects. Dietary ubiquinone (60 mg three times daily) prolonged the depletion time of LDL ubiquinol significantly but not that of α–TOH during oxidation induced by AMVN. In copper-mediated oxidation of LDL, ubiquinone supplementation was followed by a prolongation of the lag time.

In conclusion, preventive education may improve the resistance of LDL against oxidation through life style changes. Because of the scarce effect of education on the lipid values, initiation of statin therapy should not be delayed while waiting for results of repeated preventive counselling. Lovastatin therapy had both anti- and pro-oxidative effects on LDL, and dietary ubiquinone improved the resistance of LDL against the early phase of oxidation during statin therapy. At the moment, routine ubiquinone supplementation has yet no prognostic indication for patients on statin therapy.

1. LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Palomäki AK, Miilunpalo S, Holm P, Mäkinen E, Malminiemi K. Effects of preventive group education on the resistance of LDL against oxidation and risk factors for coronary heart disease in bypass surgery patients. *Ann Med.* 2002;34:272-283.

II Palomäki A, Malminiemi K, Metsä-Ketelä T. Enhanced oxidizability of ubiquinol and alpha-tocopherol during lovastatin treatment. *FEBS Lett.* 1997;410:254-258.

III Palomäki A, Malminiemi K, Solakivi T, Malminiemi O. Ubiquinone supplementation during lovastatin treatment: effect on LDL oxidation *ex vivo*. *J Lipid Res.* 1998;39:1430-1437.

IV Palomäki A, Malminiemi K, Malminiemi O, Solakivi T. Effects of lovastatin therapy on susceptibility of LDL to oxidation during alpha-tocopherol supplementation. *Arterioscler Thromb Vasc Biol.* 1999;19:1541-1548.

V Malminiemi K, Palomäki A, Malminiemi O. Comparison of LDL TRAP assay to other tests of antioxidant capacity; Effect of vitamin E and lovastatin treatment. *Free Radical Res.* 2000;33:581-593.

2. ABBREVIATIONS AND ACRONYMS

Abbreviations

AAPH	2,2´-azobis(2-amidinopropane hydrochloride)
AMI	Acute myocardial infarction
AMVN	2,2-azobis (2,4-dimethylvaleronitrile)
ANOVA	Analysis of variance
apoA-I	Apolipoprotein A-I
apoB-100	Apolipoprotein B-100
α–TOH	Alpha-tocopherol; vitamin E
α–TO$^{\bullet}$	Alpha-tocopheroxyl radical
BMI	Body mass index
CABG	Coronary artery bypass grafting
CHD	Coronary heart disease
CoQ_{10}	Coenzyme Q, also called ubiquinone
EDTA	Ethylene diamine tetra-acetate
HMG CoA	Hydroxymethyl-glutaryl coenzyme A
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
LDL	Low-density lipoprotein
LTPA	Leisure time physical activity
MDA	Malondialdehyde
MET	Multiples of resting metabolic rate
MUFA	Monounsaturated fatty acid
NA	Not available
NS	Non significant
PBS	Sodium phosphate buffer containing NaCl
PnA	Parinaric acid
PTCA	Percutaneous transluminal coronary angioplasty
PUFA	Polyunsaturated fatty acid
RANOVA	Analysis of variance with repeated measurements
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
TRAP	Total radical antiperoxiding capacity *or* Total radical trapping parameter

Acronyms

AF/TexCAPS	Air Force/Texas Coronary Atherosclerosis Prevention Study
ASAP	Antioxidant Supplementation in Atherosclerosis Prevention
ATBC	The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study
CARE	Cholesterol And Recurrent Events study
CARET	The Carotene and Retinol Efficacy Lung Cancer Chemoprevention Trial
CCAIT	The Canadian Coronary Atherosclerosis Intervention Trial
CHAOS	Cambridge Heart Antioxidant Study
CIS	The multicenter Coronary Intervention Study
4S	Scandinavian Simvastatin Survival Study
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico
GISSI-P	GISSI-Prevenzione Trial
HOPE	The Heart Outcomes Prevention Evaluation Study
HPS	Heart Protection Study
KAPS	The Kuopio Atherosclerosis Prevention Study
LIPID	The Long-Term Intervention with Pravastatin in Ischaemic Disease Study
LCAS	Lipoprotein and Coronary Atherosclerosis Study
MAAS	The Multicenter Anti-Atheroma Study
MARS	The Monitored Atherosclerosis Regression Study
PHS	Physicians' Health Study
PLAC I	Pravastatin Limitation on Atherosclerosis in the Coronary Arteries
POSCH	Program on the Surgical Control of the Hyperlipidemias
REGRESS	The Regression Growth Evaluation Statin Study
SCPS	Skin Cancer Prevention Study
STARS	St Thomas' Atherosclerosis Regression Study
WOS	West of Scotland Coronary Prevention Study

3. INTRODUCTION

According to the oxidation hypothesis, oxidative modification of LDL (low-density lipoprotein) particles plays an important role in the formation of the atherosclerotic plaque. Compelling evidence has arisen to support the theory. Biochemical studies have found oxidative changes in active areas of the atherosclerotic plaques. Epidemiological surveys and follow-up studies have discovered an inverse relation between antioxidant supply and the prevalence of coronary heart disease. However, the final evidence for the oxidation hypothesis is still lacking. For antioxidant ethusiasts, the few prognostic trials with antioxidant supplementation have revealed rather disappointing results with no significant advantage from the use of vitamins.

At the same time the old cholesterol hypothesis has been proven. Almost twenty years ago, the structure of the LDL receptor was elucidated by the research group of Nobel-prize winners Brown and Goldstein. Shortly after the synthesis of cholesterol via the mevalonate chain was clarified, new cholesterol-lowering agents *i.e.* hydroxymethyl-glutaryl coenzyme A reductase inhibitors or statins became available. During the last decade, six megatrials showed good prognostic effects of statins not only in patients with coronary heart disease but also in apparently healthy individuals. The medical and mercantile triumphs of statins have shadowed the good results achieved by lifestyle changes of heart patients.

Oxidation of LDL particles may be affected by lifestyle changes, drug therapy or antioxidant supplementation. Understanding of these processes is fundamental in confirming the oxidation hypothesis. Furthermore, stopping of atherosclerotic process is hardly possible without deep understanding of its prerequisites.

4. REVIEW OF THE LITERATURE

4.1 Coronary heart disease

4.1.1 Epidemiology

Coronary heart disease (CHD) is nowadays the most common disease of man in the post-industrial societies. The mortality from CHD arose to its peak 1960-1965 in the United States and a few years later in Finland among other Western European countries (Uemura and Pisa 1988, Braunwald 1997, Reunanen 2000a). Death rates from CHD are still increasing in the former socialistic countries in Europe and in many other countries including the developing part of the world (Uemura and Pisa 1988, Mähönen et al. 2000, Reunanen 2000a).

According to the drug indemnity statistics (National Agency for Medicines and Social Insurance Institution 2002), almost 190,000 patients are suffering from CHD among the five million inhabitants of Finland. During 1991 to 1995, the average annual number of acute myocardial infarction (AMI) was around 20,000. After 1995, the annual numbers of percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) increased to 2,500 and 4,500, respectively (Suomen sydäntautiliitto 1999, Suomen sydänliitto 1999). Notwhitstanding the evident decrease of mortality from CHD in Finland, this disease has remained the most important killer because of the ageing of population (Reunanen 2000a).

4.1.2 Risk factors

Male gender, age, and a positive family history of CHD are unchangeable risk factors for CHD. The classical acquired risk factors for CHD are an elevated level of blood (LDL) cholesterol, smoking, and arterial hypertension. Besides them, low high density lipoprotein (HDL) cholesterol level, central obesity, physical inactivity and diabetes mellitus increase the risk of CHD (Reunanen 2000b). During the last decades also many other factors have been found to be associated with CHD or AMI. Nevertheless, the evidence of their independent causal roles in the pathogenesis of CHD or AMI is still incomplete. Examples of such factors are oxidative stress (Steinberg et al. 1989), infections like *Chlamydia pneumoniae* (Saikku et al. 1988), elevated serum levels of triglycerides (Tenkanen et al. 1994, Stampfer et al. 1996), homocysteine together with a low level of folate (Duell and Malinow 1997), fibrinogen (Meade 1995), urate (Brand et al. 1985), and lipoprotein(a) (Bostom et al. 1996), possibly partly via its pro-oxidative effect (Riis Hansen et al. 1994).

4.2 Oxidative stress and atherosclerosis

A high content of lipid peroxides in plasma is associated with obvious occlusive arterial diseases (CHD and peripheral arterial disease) (Stringer et al. 1989). Also in numerous studies, the susceptibility of LDL to oxidation is associated with coronary atherosclerosis or angina pectoris (Liu et al. 1992, Regnström et al. 1992, de Rijke et al. 1992, Chiu et al. 1994, Duthie et al. 1994, Kovacs et al. 1997, Ehara et al. 2001). Elevated levels of antibodies against oxidised LDL seem to predict subsequent myocardial infarction (Puurunen et al. 1994). In a Finnish epidemiological study, intake of the pro-oxidative transition metal mercury was associated with an excess risk of AMI as well as coronary, cerebrovascular, and all cause mortality (Salonen et al. 1995). High levels of biomarkers of oxidative stress as well as low levels of antioxidants in the circulation are associated with an increased cardiovascular morbidity and mortality (Gey et al. 1991, Gey et al. 1993, Street et al. 1994, Kontush et al. 1999, Gackowski et al. 2000).

A comprehensive theory claiming a significant role of LDL oxidation in atherogenesis was presented already more than ten years ago (Palinski W et al. 1989, Steinberg et al. 1989, Ylä-Herttuala et al. 1989). In spite of increasing evidence supporting it, the oxidation hypothesis is still today by no means fully confirmed (Steinberg 2000).

4.2.1 Free radicals and the oxidation cascade of lipids

A free radical is a chemical species with an unpaired electron. Formation of superoxide radical ($O_2^{\bullet}$) in the respiratory chain may lead to the formation of other reactive oxygen species (ROS) like the non-radical hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$) (Frei 1994). Hydroxyl radical is the most reactive and aggressive of the various ROS. It can damage cellular membranes and lipids by a process called lipid peroxidation (Halliwell 1993). Hydroxyl radical is capable of attacking polyunsaturated fatty acids (PUFAs) and abstract hydrogen atoms from their side chains (Halliwell 1993). The hydroxyperoxyl radical ($HO_2^{\bullet}$), the protonated form of the superoxide radical generated e.g. by smooth muscle cells, endothelial cells or leukocytes, may also react with PUFAs. A carbon-centered lipid radical is formed in both initial reactions, which may lead to a cascade of lipid oxidation (Halliwell 1993, Frei 1994). If not stopped by antioxidants, initiation may be followed by peroxidative modification of the various components of the LDL particle. Free radicals may penetrate the LDL particle via different mediators such as a tryptophan residue of apolipoprotein B-100 (apoB-100) or α–tocopherol (α–TOH) (Stocker 1994, Giessauf et al. 1995, Witting et al. 1995). Except by ROS, this cascade may be initiated auto-oxidatively, when a hydrogen atom ($H^{\bullet}$) is abstracted from a methylene group ($-CH_2-$) between two double-bonds of a PUFA and a lipid radical is formed (Frei 1994). When the lipid radical rapidly reacts with O_2, a peroxyl radical is formed. Lipid peroxyl radical is able to react with a nearby PUFA forming lipid hydroperoxide and another carbon-centered lipid radical, thus propagating the radical chain reaction. Large amounts of lipid hydroperoxides can be formed during an auto-oxidation cascade of PUFAs triggered by a single initiating reaction (Frei 1994). Copper ions are widely used *in vitro* to stimulate peroxidation of LDL (Esterbauer et al. 1989). If metal ions or haem proteins are present close to the lipids, they will decompose into peroxides thus stimulating the peroxidation cascade. Copper ions are not able to cause peroxidation in completely peroxide-free LDL (Halliwell 1993).

4.2.2 Oxidation of the LDL particle

The surface of the human LDL particle is formed by a lipid monolayer, composed of phospholipids, free cholesterol, and one single molecule of apoB-100 (Hevonoja et al. 2000). Cholesteryl esters together with small amounts of triglycerides and unesterified (free) cholesterol are located in the core of the LDL particle. ApoB-100 has some parts in the surface region (outer surface layer and interfacial layer) as well as in the core (Hevonoja et al. 2000). The LDL particle contains lipid soluble antioxidants like α–tocopherol and coenzyme Q_{10} (CoQ_{10}) in its reduced form ubiquinol. Quantitatively the most important antioxidant is α–TOH: the LDL particle contains about 6 to 12 molecules of α–TOH, while the amount of ubiquinol is only one molecule in every 1 to 2 particles of LDL (Esterbauer et al. 1990, Stocker 1994).

Radical attack may very fast damage some 8 to 9 out of the 37 tryptophan residues of apoB-100, which may play a role in initiating the lipid peroxidation process in LDL (Giessauf et al. 1995). However, the importance of this finding is still unclear (Okada et al. 1997). The surface lipid peroxidation leads to formation of phospholipid hydroperoxides (Frei 1994). More long-lasting radical attack is shifted from the surface region of the LDL particle into its core with peroxidation of PUFAs of cholesteryl esters or of the few triglyceride molecules and formation of lipid hydroperoxides (Frei 1994, Stocker 1994, Tribble 1995). In cholesteryl esters, linoleate is

the most important substrate for peroxidation, but cholesterol may also be oxidised (Stocker 1994, Steinberg et al. 1989). Fatty acid hydroperoxides subsequently break down to form malondialdehyde and other reactive compounds followed by the oxidation of the lipophilic part of apoB-100. Modification of the LDL particle is completed by the degradation of apoB-100 (Steinberg et al. 1989, Frei 1994).

Structural changes in LDL during oxidation can be detected by studying lipid peroxidation products, surface charge, and spectrophotometric patterns (Okada et al. 1997). Dense LDL is more susceptible to oxidative modification than buoyant LDL (de Graaf et al. 1991, Tribble et al. 1995), maybe because of smaller antioxidant (α–tocopherol and ubiquinol) activity in the dense LDL subfractions (Tribble et al. 1995, de Rijke et al. 1997). Macrophages recognise oxidatively modified LDL particles by scavenger receptors, and LDL is taken up in an unregulated manner (Frei 1994). Lack of the normal down-regulation mediated by intracellular cholesterol content leads macrophages to accumulate lipids from oxidised LDL. With time macrophages are converted into foam cells, which are the hallmark of the atherosclerotic fatty streak (Frei 1994).

4.2.3 Ubiquinol and lipid peroxidation

Except in brain and lung, coenzyme Q appears mostly in its reduced form, ubiquinol (Åberg et al. 1992). It is the only lipid soluble antioxidant, which can be synthesised *de novo* by animal cells and regenerated by enzymatic mechanisms from its oxidised form ubiquinone (Ernster and Forsmark-Andrée 1993, Dallner and Sindelar 2000).

Ubiquinol acts in the initiation of lipid peroxidation independently of vitamin E by scavenging one radical (Frei et al. 1990, Forsmark et al. 1991). Doing so, ubiquinol is converted to ubisemiquinone, which may scavenge another radical (Frei et al. 1990). Alternatively, ubisemiquinone radical is disproportionated to reduced ubiquinol and oxidised ubiquinone (Q_{10}) (Frei et al. 1990). Ubiquinol, as well as vitamin C, can sustain the effect of α–TOH by regenerating vitamin E from the α–tocopheroxyl radical (Frei et al. 1990, Maguire et al. 1992, Ernster and Forsmark-Andrée 1993, Ingold et al. 1993).

In LDL peroxidation experiments, ubiquinol acts markedly in the start of the initiation period (Stocker et al. 1991, Kontush et al. 1994). It might be relevant in the prevention of formation of minimally modified LDL (Lynch et al. 1994). In ascorbate-free LDL, formation of lipid hydroperoxides is slow as long as ubiquinol is present, but it increases rapidly after the consumption of ubiquinol in spite of the presence of α–TOH (Stocker et al. 1991).

4.2.4 Alpha-tocopherol and lipid peroxidation

In native human LDL, α–TOH is quantitatively the most abundant antioxidant (Esterbauer et al. 1990). One LDL particle contains approximately 1600 molecules of cholesteryl esters, 600 molecules of free cholesterol, and 100 molecules of triglycerides. Among the 1300 molecules of polyunsaturated fatty acids, linoleic acid accounts for the majority, *i.e.* 1100 molecules (Esterbauer et al. 1990, Thomas et al. 1994). The concentration of vitamin E in LDL is correlated with that of PUFAs. During oxidation of PUFAs, the 20:4 fatty acids are degradated much faster than 18:2 fatty acids, both in the presence or absence of copper ions (Esterbauer et al. 1990). Alpha-tocopherol concentration alone and α–TOH to cholesterol ratio in LDL correlate significantly with total antioxidant activity of LDL in supplemented individuals, while in the non-supplemented group this correlation is not evident (Miller et al. 1995). According to a study of non-human primates with different diets and two initiation methods of oxidation, the ability of α–TOH to inhibit oxidation depends upon the lipid environment and the mode of initiation of oxidation (Thomas et al. 1994).

In a homogenous solution α–TOH and α–tocotrienol are consumed at an equal rate but faster than γ–tocotrienol during oxidation. Ubiquinol added to the solution has a sparing effect on α–TOH as well as on α– and γ–tocotrienol (Suarna et al. 1993). Parinaric acid (PnA) has been used as a probe to detect the very early changes in copper-mediated oxidation (Tribble et al. 1994). Increased PnA oxidation occurs immediately after the consumption of antioxidants and before the propagation of conjugated diene formation. The initiation of LDL oxidation is related to the ubiquinol concentration and to a lesser extent to the α–TOH concentration of LDL. In this setting, susceptibility of small dense LDL to oxidation is associated to reduced levels of ubiquinol and α–TOH, which are about half of those in the buoyant LDL fraction (Tribble et al. 1994).

The small dense LDL is often seen in patients with combined hyperlipidaemia, especially in its familial form. Reduced resistance of the small dense LDL against lipid peroxidation seems to be related to a reduced ubiquinol/ubiquinone ratio compared to normal subjects with buoyant LDL (de Rijke et al. 1997). The plasma or tissue levels of coenzyme Q do not change according to age, at least in animals (Lönnrot et al. 1998). Both the level of CoQ_{10} in plasma and CoQ_{10}/LDL ratio have been found to be significantly lower in CHD patients than in normal subjects (Hanaki et al. 1993), although this finding was not confirmed in another study (Kontush et al. 1999).

4.2.5 Tocopherol-mediated oxidation of LDL

An aqueous peroxyl radical on the surface of LDL particle may be scavenged by α–TOH (Stocker 1994). The formed tocopheroxyl radical (α–TO$^{\bullet}$) transfers the radical attack from the aqueous phase into the inner part of the LDL particle (Stocker 1994, Kalyanaraman et al. 1995, Witting et al. 1995). In ubiquinol-free isolated human LDL, α–TOH acts as a pro-oxidant during low radical flux, when Cu^{2+}/LDL particle ratio is 2.5 or less (Witting et al. 1995). Inside the LDL particle, propagation of a chain reaction may occur, when bisallylic hydrogen group is oxidised by α–TO$^{\bullet}$ and the formed carbon-centred lipid radical is then transformed to lipid peroxyl radical. Alpha-tocopherol may then scavenge the lipid peroxyl radical, but the formed α–TO$^{\bullet}$ continues the peroxidation cascade (Witting et al. 1995).

Except in the reaction of a radical with ubiquinol, non-radical products may be formed inside the LDL particle by the tocopheroxyl radical when reacting with a peroxyl radical or with another α–TO$^{\bullet}$ (Frei 1994). On the surface of the particle, α–TO$^{\bullet}$ may react with an aqueous antioxidant (like vitamin C) or radical, thus terminating the peroxidation cascade (Stocker 1994, Bowry et al. 1995, Witting et al. 1995, Thomas et al. 1996).

In more intense oxidation conditions with a higher concentration of copper (or other pro-oxidant), vitamin E switches from pro- to antioxidant. Then, α–TOH is a potent antioxidant being capable of rapidly scavenging the chain-propagating lipid peroxyl radical (Witting et al. 1995).

Except formation of tocopheroxyl radicals during oxidation of α–TOH, also apoB-100-derived radicals are formed under both aerobic and anaerobic conditions in peroxidase-induced oxidation of LDL (Kalyanaraman et al. 1995). The question whether α–TOH acts as a pro-oxidant or an anti-oxidant during copper-mediated oxidation of LDL is still controversial. Also other factors besides pro- and antioxidant contents, such as the amount of polyunsaturated fatty acids in LDL, seem to be involved in the resistance of LDL against oxidative stress (Esterbauer et al. 1990, Maiorino et al. 1995).

4.2.6 Oxidatively modified LDL in the vessel wall

An essential phenomenon for the development of an atherosclerotic plaque is the accumulation of cholesterol-rich LDL particles in the subendothelial space of the arterial wall. LDL particles need to be modified before they can be taken up in an uncontrolled manner by the cells in the arterial intima (Steinberg et al. 1989, Thorne et al. 1996). According to the oxidation hypothesis, this modification may be a consequence of a peroxidative insult against the LDL particle. Blood contains a substantial amount of different antioxidants, e.g. albumin (Deigner et al. 1992), ascorbate (Retsky et al. 1993, Ma et al. 1994, Lehr et al. 1995), bilirubin (Neuzil and Stocker 1994, Wu et al. 1994), urate (Ma et al. 1994), and thiols (Ferguson et al. 1997), which already may stop the radical attack in the aqueous phase. Hence, peroxidation of the LDL particle happens mostly outside the circulation, apparently in the subendothelial space of the arterial wall.

Once oxidatively modified, LDL induces synthesis of chemokines, such as monocyte chemoattractant protein 1 (MCP-1), thus attracting circulating monocytes into the arterial intima, where they are converted into macrophages (Ylä-Herttuala et al. 1991, Chait and Heinecke 1994). Oxidatively modified LDL also inhibits the return of the cells to the circulation (Steinberg et al. 1989, Greenspan et al. 1997). At the same time oxidised LDL may damage the endothelium exposing it to recurrent microthrombosis (Steinberg et al. 1989). The extent of oxidation of LDL determines its cytotoxicity to coronary artery cells (Thorne et al. 1996). However, already minimally oxidised LDL enhances macrophage survival and DNA synthesis (Hamilton et al. 2001). Minimally oxidised LDL particles also have a more evident proliferative effect on vascular smooth muscle cells than totally oxidised LDL particles (Lähteenmäki et al. 1998). Macrophages continue to endocytose oxidised LDL and ultimately they transform into foam cells, which contain plenty of lipids. Unable to move back to the circulation, foam cells die in the intima leaving there accumulations of lipids and thus promoting the development of an atheroma (Steinberg et al. 1989). Oxidised LDL also regulates expression/secretion of matrix metalloproteinase-1 and its inhibitor by vascular endothelial cells resulting in a net increase in collagen-degradating activity (Huang et al. 2001). Partial breakdown of the matrix may promote the rupture of the atherosclerotic plaque.

4.3 Intervention and prognosis of coronary heart disease

4.3.1 Lifestyle changes

Favourable effects of a multifactorial intervention program on CHD risk factors and sudden death after AMI have been demonstrated already more than 20 years ago (Kallio et al. 1979). Drug therapy for CHD has since become more efficient. Later on, angiographic studies (Ornish et al. 1990, Schuler et al. 1992, Watts et al. 1992) as well as prognostic trials (Singh et al. 1992, de Lorgeril et al. 1994 and 1999) have demonstrated, that lifestyle modification by nutritional advice or a multidimensional prevention program can still have positive effects on patients with CHD (Table 1). Multidimensional programs have included intensive physical exercise in group training sessions combined with a low-fat, low-cholesterol diet (Schuler et al. 1992) or a lifestyle programme including a low-fat vegetarian diet, moderate aerobic exercise, stress management training, cessation of smoking, and group support (Ornish et al. 1990).

Table 1. Effect of lifestyle modification on the prognosis of coronary heart disease.

Angiographic trials

Trial	Follow-up	Patients	Means	Mean lumen diameter *	Minimal lumen diameter *	p-value
Lifestyle	1 year	48 CHD	MP	- 2.2%	-	0.001 (two-tailed)
			C	+ 3.4%	-	
Heidelberg	1 year	113 men with CHD	MP (RPE and D)	-	+ 0.01 mm	< 0.05
			C	-	+ 0.13 mm	
STARS	39 months	74 men with CHD	DC	- 0.103 mm (1	- 0.117 mm (2	0.012 (1 and 0.003 (2 (p for trend)
			D	- 0.003 mm (1	- 0.030 mm (2	
			C	+ 0.201 mm (1	+ 0.232 mm (2	

Prognostic trials

Trial	Follow-up	Patients	Means	Non-fatal MI	Cardiac Mortality	Overall Mortality
Indian	1 year	406 CHD	Diet rich in fruits and vegetables	30 *vs.* 48 (p<0. 01)	20 *vs.* 34 (p<0.01)	21 *vs.* 38 (p<0.01)
Lyon	4 years	605 post 1. AMI	Mediterranean diet	8 *vs.* 25 (p=0.0001)	6 *vs.* 19 (p=0.01)	14 *vs.* 24 (p=0.03)

* Progression (+), regression (-)
CHD = Coronary heart disease, AMI = Acute myocardial infarction, MP = Multifactorial prevention, C = Controls with usual care, DC = Diet + cholestyramine, D = Diet, RPE = Regular physical exercise

Trials:
Lifestyle (Ornish et al. 1990)
Heidelberg (Schuler et al. 1992)
STARS St Thomas' Atherosclerosis Regression Study (Watts et al. 1992)
Indian (Singh et al. 1992)
Lyon (de Lorgeril et al. 1994 and 1999)

The insidence of cardiac events and deaths may even be reduced with Mediterranean diet rich in α–linolenic acid without significant quantitative changes in common fasting serum lipids (de Lorgeril et al. 1994). Nevertheless, according to a few smaller studies postprandial hypertriglyceridaemia can be reduced by rehabilitation programs for cardiac patients, i.e. by augmented intake of dietary n-3 fatty acids or regular exercise (Harris et al. 1988, Yanes et al.1989). GISSI-Prevenzione trial is a well-known intervention study carried out with a 2 x 2 factorial design. Altogether 11324 patients surviving recent myocardial infarction were randomised into four arms. They were given supplements of n-3 polyunsaturated fatty acids (PUFAs), vitamin E, both, or none (GISSI-Prevenzione Investigators 1999). According to the four-way analysis, n-3 PUFAs decrease cardiac deaths by 35% ($p<0.05$) and all-cause mortality by 20% ($p<0.05$) against the placebo arm (GISSI-Prevenzione Investigators 1999). The results - although diluted - remained statistically significant also in the two-way

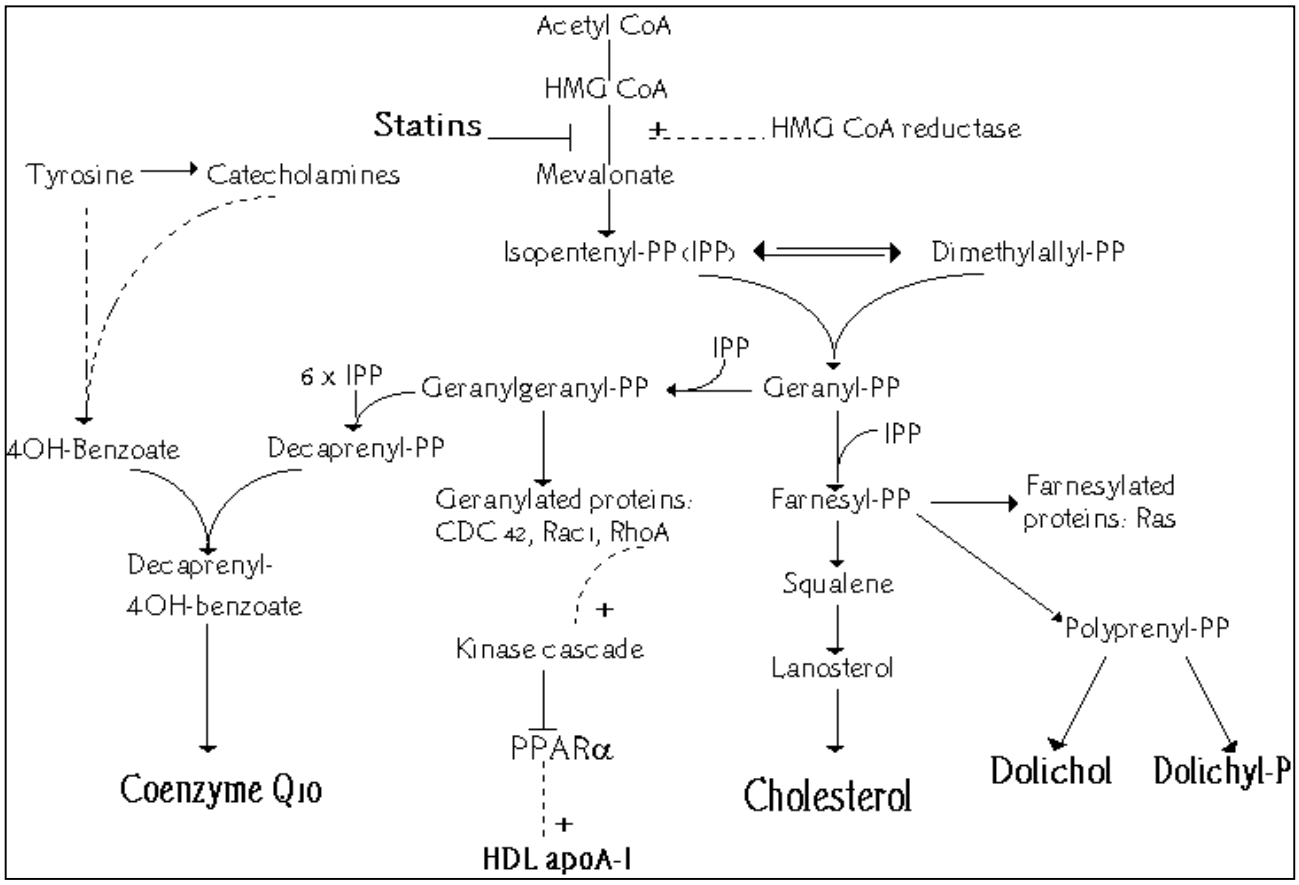
analysis of the whole study population. In another, exercise-based prevention trial, regression of coronary lesions were only observed in patients expending on the average 2,200 kcal/week in leisure time physical activity (Hambrecht et al. 1993). In practice, it means regular physical exercise for 5 to 6 hours weekly. Because comprehensive and continuous individual education is time consuming an requires special skills, its utilisation in routine hospital practice is limited.

4.3.2 Cholesterol-lowering therapy with 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins)

In the circulation, hydrophobic cholesterol is transported to extrahepatic cells in LDL particles (Brown et al. 1981). In time, LDL is removed from plasma, when it is taken up by LDL receptors in the liver. It secretes sterol in the bile. People in Western societies often have higher LDL levels than appears to be appropriate for the receptor system, which predisposes them to atherosclerosis (Brown et al. 1981).

Although cholesterol has been recognised as a risk factor for CHD already in the first Framingham data, evidence of its role was not convincing enough for all sceptics before the statin era (Ravnskov 1992). Reduction of cholesterol synthesis by the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors or statins takes place by competitive inhibition of this early key enzyme of the mevalonate pathway (Fig 1) (Goldstein and Brown 1990, Dallner and Sindelar 2000, Martin et al. 2001). The first inhibitor of HMG CoA reductase, mevastatin (formally called compactin or ML-236B), was discovered in the early 1970's and published in 1976 (Endo 2000). According to Endo (2000), it was isolated independently from the species of *Penicillium* by Endo and by Brown with their co-workers. Studies with mevastatin were discontinued after lovastatin (mevinolin) was isolated and marketed as the first statin available for clinical use. It was isolated from *Aspergillus terreus* by the investigators at Merck, and from *Monascus ruber* by those at Sankyo. Lovastatin was slightly more effective than mevastatin, and it was soon followed by other HMG CoA reductase inhibitors like pravastatin, simvastatin, fluvastatin, and atorvastatin (Jones et al. 1998, Endo 2000).

Figure 1. A schematic representation of the mevalonate pathway and biosynthesis of cholesterol and non-steroid derivatives, like coenzyme Q_{10} (Goldstein and Brown 1990, Dallner and Sindelar 2000, Martin et al. 2001).



4-Hydroxybenzoate may be synthesised from tyrosine or catecholamines. In lower organisms the synthesis goes through 4OH-phenylpyruvate, 4OH-phenyllactate, and 4OH-cinnamate, but the biosynthetic pathway in mammals and especially in humans is not yet explained. The rate-limiting enzyme of the mevalonate chain is HMG-CoA reductase, which is inhibited by statins. Inhibition of the mevalonate chain does not only affect the biosynthesis of cholesterol but also other products. This effect may be both beneficial and detrimental.

CoA = Coenzyme A, HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A, P = Phosphate, PP = Pyrophosphate, PPARα = Peroxisome proliferator-activated receptor α

Istvan and Deisenhofer (2001) have recently determined the crystal structure of the catalytic portion of HMG CoA reductase. They found, that the binding of different statins blocks the access of HMG CoA to the catalytic center. Conformational change in HMG CoA reductase is followed by enzyme dysfunction.

Mechanisms of action

Istvan and Deisenhofer (2001) have divided HMG CoA reductase inhibitors in two groups. Type 1 statins (lovastatin, pravastatin, and simvastatin) are smaller and they have less interaction with the enzyme than type 2 statins (atorvastatin, cerivastatin, fluvastatin, and rosuvastatin). The type 2 drugs are better inhibitors of the receptor, and with the exception of fluvastatin they are also clinically more effective in lowering the plasma level of LDL cholesterol than the others (Jones et al. 1998, Istvan and Deisenhofer 2001, Tanaka et al. 2001).

Inhibition of mevalonate synthesis leads to depletion of cholesterol in liver cells, thus forcing them to rely on lipoproteins to get

cholesterol for the synthesis of bile acids (Brown et al. 1981). Therefore, the cholesterol-lowering effect of statins is markedly a result of enhanced clearance of LDL from plasma mediated by an increased amount of hepatic LDL receptors (Brown et al. 1981, Kovanen et al. 1981). In clinical work, it is possible to decrease the mean LDL cholesterol level in plasma at least 50% by therapy with an effective HMG CoA reductase inhibitor (Jones et al. 1998).

In addition to the synthesis of steroids and cholesterol, HMG CoA reductase is needed in the formation of many other non-sterol products like dolichol, ubiquinol, heme, as well as prenylated or farnesylated proteins (Goldstein and Brown 1990, Martin et al. 2001). Except their cholesterol-lowering effect, HMG CoA reductase inhibitors possess many other functions. They have been shown to decrease migration and proliferation of smooth muscle cells, to reduce the expression of matrix metalloproteinases, to inhibit the production of pro-inflammatory cytokines, C-reactive protein, and cellular adhesion molecules, and to decrease the adhesion of monocytes to endothelial cells or to prevent their activation into macrophages (Koh 2000).

Endothelial dysfunction, characterised by deterioration of endothelial vasodilator function, may be an intermediate stage in the process, where a normal artery becomes an atherosclerotic one (Vapaatalo and Mervaala 2001). Cholesterol lowering with statin therapy improves entohelium-dependent vasomotion in CHD patients, and this effect may even be boosted by antioxidant therapy (Anderson et al. 1995, Treasure et al. 1995, Tsunekawa et al. 2001). The beneficial prognostic effects of statins may be contributed by non-lipid mechanisms that affect endothelial function, inflammatory responses, and plaque stability (Koh 2000).

Clinical evidence

The milestone angiographic studies in the treatment of hyperlipidaemia are the Program on the Surgical Control of the Hyperlipidemias (POSCH) and the Monitored Atherosclerosis Regression Study (MARS), where cholesterol was lowered *either* by partial ileal bypass surgery *or* by lovastatin with or without resin therapy, respectively (Buchvald et al. 1990, Blankenhorn et al. 1993, Buchwald et al. 1995). Since then, several angiographic studies and six prospective mega trials have been published with mere statin medication as lipid-lowering drug treatment (Table 2).

These studies show unequivocally, that therapy by inhibitors of HMG CoA reductase not only retards or prevents the development of coronary artery stenosis, but also diminishes the risk of complications such as myocardial infarction, sudden death, and the need of PTCA or CABG (Table 2) [Scandinavian Simvastatin Survival Study Group 1994, Shepherd et al. 1995, Sacks et al. 1996, Downs et al. 1998, The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group 1998)]

Very aggressive statin therapy may have additional positive effects on the prognosis of CHD patients. Lowering of blood LDL cholesterol to the mean level of 2.4 to 2.5 mmol/l is beneficial when compared to modest hypocholesterolaemic treatment in CABG patients or to PTCA in low-risk patients with stable CHD (The Post-CABG Trial Investigators 1997, Pitt B 1999). In a massive MRC/BHF Heart Protection Study (HPS) with more than 20,000 participants, simvastatin therapy has reduced cardiovascular events proportionally likewise in patients already having the baseline LDL cholesterol level under 2.6 mmol/l as in those with higher levels (Heart Protection Study Collaborative Group 2002a). Even after the release of the results of the HPS, it still remains open how much blood cholesterol should be lowered in CHD patients (Callister et al. 1998, Heart Protection Study Collaborative Group 2002a).

Even in the range of normal values, minimally elevated C-reactive protein as an inflammatory marker is associated with impaired prognosis of

CHD (Ridker et al. 2001a). Therapy with cerivastatin has been shown to be able to reduce the level of C-reactive protein already in eight weeks (Ridker et al. 2001b). According to the analysis of the blood samples from the great LIPID and AF/TexCAPS trials, also therapy with pravastatin and lovastatin significantly reduces circulating C-reactive protein levels. This effect of statin therapy is associated with improved prognosis and it is independent of the lipid-lowering effect of the drug (Ridker et al. 1999, Ridker et al. 2001a). However, according to data from the Treat to Target study comparing atorvastatin and simvastatin, the change in C-reactive protein is inversely associated with the change of HDL-cholesterol (Strandberg et al. 2000).

A recent finding in the West of Scotland Coronary Prevention Study shows, that the assignment of pravastatin is followed by a 30% reduction in the hazard of becoming diabetic (Freeman et al. 2001). The reason of this favourable but surprising finding is unclear. The authors speculate that three known effects of statin therapy might play a role in the development of diabetes, *i.e.* favourable effects on plasma triglyceride level, inflammation, and endothelial function. Taking into account the limited length of these trials and the lack of effect of other triglyceride-lowering drugs on the rise of diabetes, two latter effects seem to be relevant in further speculations (Freeman et al. 2001).

As seen in HPS, a wide range of patients may benefit from statin therapy (Heart Protection Study Collaborative Group 2002a). However, according to the analysis of the Finnish subgroup of 4S participants, a minority of CHD patients have low synthesis of cholesterol in the liver. For those subjects other means than statins may be a better choice or at least good supplementation to lower cholesterol level and thus improve their prognosis (Miettinen et al. 1998).

Table 2. Effects of statin therapy on CHD.

Selected randomised, controlled angiographic trials

Trial	Follow-up	Patients	Medication, mean daily dose	Mean lumen diameter *	Minimal lumen diameter *	p-value
CCAIT	2 years	331	Lovastatin, 36 mg Placebo		0.05 mm 0.09 mm	0.01
MAAS	4 years	341	Simvastatin, 20 mg Placebo	0.02 mm 0.08 mm	0.04 mm 0.13 mm	Both <0.05; combined p=0.006
PLAC I	3 years	320	Pravastatin, 40 mg Placebo	0.02 mm 0.04 mm	0.03 mm 0.05 mm	Mean: NS Minimum: 0.04
REGRESS	2 years	778	Pravastatin, 40 mg Placebo	0.06 mm 0.10 mm	0.03 mm 0.09 mm	Mean: NS Minimum: 0.001
LCAS	2.5 years	429	Fluvastatin, 40 mg Placebo		0.028 mm 0.100 mm	0.005
CIS	2.3 years	205 men	Simvastatin, 34.5 mg Placebo		0.02 mm 0.10 mm	0.002

The great prognostic trials

Trial	Follow-up	Patients	Medication, mean daily dose	Non-fatal MI	Cardiac mortality	Overall mortality
4S	5.4 years	4444	Simvastatin, 27 mg	- 33 % (p<0.0000001)	- 35 % (p=0.0003)	- 30 % (p=0.0003)
WOS	5 years	6595	Pravastatin, 40 mg	- 31 % (p<0.001)	- 32 % (p=0.033)	- 22 % (p=0.051)
CARE	5 years	4159	Pravastatin, 40 mg	- 23 % (p=0.02)	-	- 9 % (p=0.37)
LIPID	6 years	9014	Pravastatin, 40 mg	-	- 25 % (p=0.0001)	- 23 % (p=0.00002)
AF/Tex-CAPS	5 years	6605	Lovastatin, 30 mg	-	- 25 % (p<0.003)	+ 1 % (NS)
HPS	5 years	20536	Simvastatin, 40 mg	- 38 % (p<0.0001)	- 18 % (p=0.0005)	- 13 % (p=0.0003)

* Progression of the diameter

Acronyms in Table 2

MAAS = The Multicenter Anti-Atheroma Study (MAAS Investigators 1994)
CCAIT = The Canadian Coronary Atherosclerosis Intervention Trial (Waters et al. 1994)
PLAC I = Pravastatin Limitation on Atherosclerosis in the Coronary Arteries (Pitt et al. 1995)
REGRESS = The Regression Growth Evaluation Statin Study (Jukema et al. 1995)
CIS = The multicenter Coronary Intervention Study (Bestehorn et al. 1997)
LCAS = Lipoprotein and Coronary Atherosclerosis Study (Herd et al. 1997)
4S = Scandinavian Simvastatin Survival Study (Scandinavian Simvastatin Survival Study Group 1994)
WOS = West of Scotland Coronary Prevention Study (Shepherd et al. 1995)
CARE = Cholesterol and recurrent events study (Sacks et al. 1996)
LIPID = The Long-Term Intervention with Pravastatin in Ischaemic Disease Study [The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group 1998]
AFCAPS/TexCAPS = Air Force/Texas Coronary Atherosclerosis Prevention Study (Downs et al. 1998)
HPS = MRC/BHF Heart Protection Study (Heart Protection Study Collaborative Group 2002a)

4.3.3 Antioxidant supplementation

A few cross-sectional (Gey et al. 1991, Riemersma et al. 1991, Hertog et al. 1993, Kardinaal et al. 1993) and follow-up studies (Gey et al. 1993, Rimm et al. 1993, Stampfer et al. 1993, Kushi et al. 1996) have produced promising evidence on the preventive role of antioxidants in CHD. However, in prospective studies with vitamin E supplementation alone or together with vitamin C, antioxidants did not attenuate the progression of atherosclerosis in rabbits with high cholesterol level (Morel et al. 1994, Kleinveld et al. 1995). So far, results of prospective controlled human trials using antioxidant supplementation have not been encouraging as presented in Table 3. In two out of the four great trials carried out in industrialised countries, the overall outcome in the group with vitamin A supplementation was significantly inferior compared to that of the placebo group, while the increase in cardiovascular mortality did not reach statistical significance in any of the studies (Table 3) (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994, Greenberg et al. 1996, Hennekens et al. 1996, Omenn et al. 1996).

CHAOS was the first prognostic intervention trial using α−TOH with a dose great enough to cause measurable effects on the resistance of LDL against oxidation (Jialal et al. 1995, Stephens et al. 1996, Devaraj et al. 1997). The number of non-fatal AMIs (14/1035 *vs.* 41/967, p=0.0001) was much smaller in the vitamin group than in the placebo group (Stephens et al. 1996). However, the cardiovascular or total mortality did not differ statistically significantly between the treatment groups (Table 3). When only patients with angina pectoris at baseline were taken into account in the ATBC study, neither α−TOH nor β−carotene supplementation was associated with any beneficial effects on cardiac morbidity or mortality (Rapola et al. 1997). The results of vitamin supplementation in the GISSI-prevenzione trial are presented in Table 3. Four-way analysis (vitamin E only *vs.* placebo only), suggests that vitamin E had a beneficial effect on coronary, cardiac, and sudden death, which were decreased by 25%, 23%, and 34%, respectively ($p<0.05$ in all). Nevertheless, the positive effect on cardiovascular mortality was diluted in the two-way analysis of the total population, where the patients on n-3 PUFA were included (Table 3) (GISSI-Prevenzione Investigators 1999)

The latest big trial with α−TOH as monotherapy was the randomised, placebo-controlled 2 x 2 factorial HOPE study (The Heart Outcomes Prevention Evaluation Study Investigators 2000). In this study, α−TOH did not demonstrate any benefit in coronary heart disease patients compared to placebo treatment.

According to the studies already published, monotherapy with either β−carotene or α−TOH is not associated with better prognosis in CHD than placebo. Hence, the recent or ongoing trials have concentrated on finding a proper combination of antioxidants. The results of Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (α−TOH together with vitamin C) with the change of the carotic intima-media thickness as an endpoint were promising (Salonen et al. 2000). According to ASAP, combined antioxidant supplementation retards the progression of common carotid atherosclerosis in men. The HPS had a 2 x 2 factorial design, with comparisons of simvastatin *vs.* placebo (Table 2) and vitamin combination *vs.* placebo (Heart Protection Study Collaborative Group 2002b). Combination of vitamins E and C with β−carotene had eventually no effect on the prognosis of patients with a diagnosis of or an evident risk for CHD. Reasons for the use of β−carotene in this study were not quite clear.

In heart transplant patients, oxidised LDL is associated with the extent of posttransplant coronary artery disease (Holvoet et al. 1998). Effect of combined treatment with vitamins C and E was studied in 40 heart transplant patients with one-year follow-up (Fang et al. 2002). In this recent study, vitamin supplementation retarded the early progression of coronary atherosclerosis.

Table 3. Effects of randomised, controlled prognostic trials with antioxidant supplementation.

Trial	Follow-up	Patients	Means	Cardiovascular mortality	Overall mortality
ATBC	6.1 years	29133	Vitamin E 50 mg/d β–carotene 20 mg/d	-2% (NA) +11% (NA)	+2% (NS) +8% (p=0.01)
PHS	12 years	22071 men	β–carotene 50 mg/2d	+ 9 % (NS)	+ 2 % (NS)
CARET	4 years	18314 smokers	β–carotene 15 mg/d and retinyl palmitate	+26% (NS)	+ 17 % p=0.02
SCPS	8.2 years	1720	β–carotene 50 mg/d	+16% (NS)	+ 3 % (NS)
CHAOS	510 days	2002	Vitamin E 400 mg/d or 800 mg/d	+10% (NS)	+ 29 % (NS)
GISSI-P. 3.5 years	3.5 years	11324	Vitamin E 300 mg/d	-20% (p < 0.05; 4-way), -6% (NS; 2-way)	-14 % (NS; 4-way) -8% (NS; 2-way)
HOPE	4.5 years	9541	Vitamin E 400 mg/d	+5% (NS)	-0% (NS)

Abbreviations and acronyms in Table 3.

NS = non significant, NA = Not applicable; 4-way and 2-way = four-way and two-way analysis in the 2 x 2 factorial trial

ATBC	The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994)
CARET	The Carotene and Retinol Efficacy Lung Cancer Chemoprevention Trial (Omenn et al. 1996)
PHS	Physicians' Health Study (Hennekens et al. 1996)
SCPS	Skin Cancer Prevention Study (Greenberg et al. 1996)
CHAOS	Cambridge Heart Antioxidant Study (Stephens et al. 1996)
GISSI-Prevenzione	GISSI-Prevenzione Trial (GISSI-Prevenzione Investigators 1999)
HOPE	The Heart Outcomes Prevention Evaluation Study (The Heart Outcomes Prevention Evaluation Study Investigators 2000)

4.4 Effects of different treatments on oxidative stress

4.4.1 Lifestyle changes

Dietary counselling

A study consisting of 309 subjects with the mean initial value of total cholesterol just over 7 mmol/l resulted in an equal but only modest effect of dietary advise when given in general practice either by a dietician, a practice nurse, or a diet leaflet alone (Neil et al. 1995). When data from the groups were pooled together, the decrease in total cholesterol during a 6-month period was 0.13 ($p<0.001$) and in LDL cholesterol 0.15 mmol/l ($p<0.001$). No significant change was noted in plasma α–TOH or β–carotene concentrations, but total carotenoids in plasma showed a statistically significant increase of 2.2% (Neil et al. 1995).

Cessation of smoking

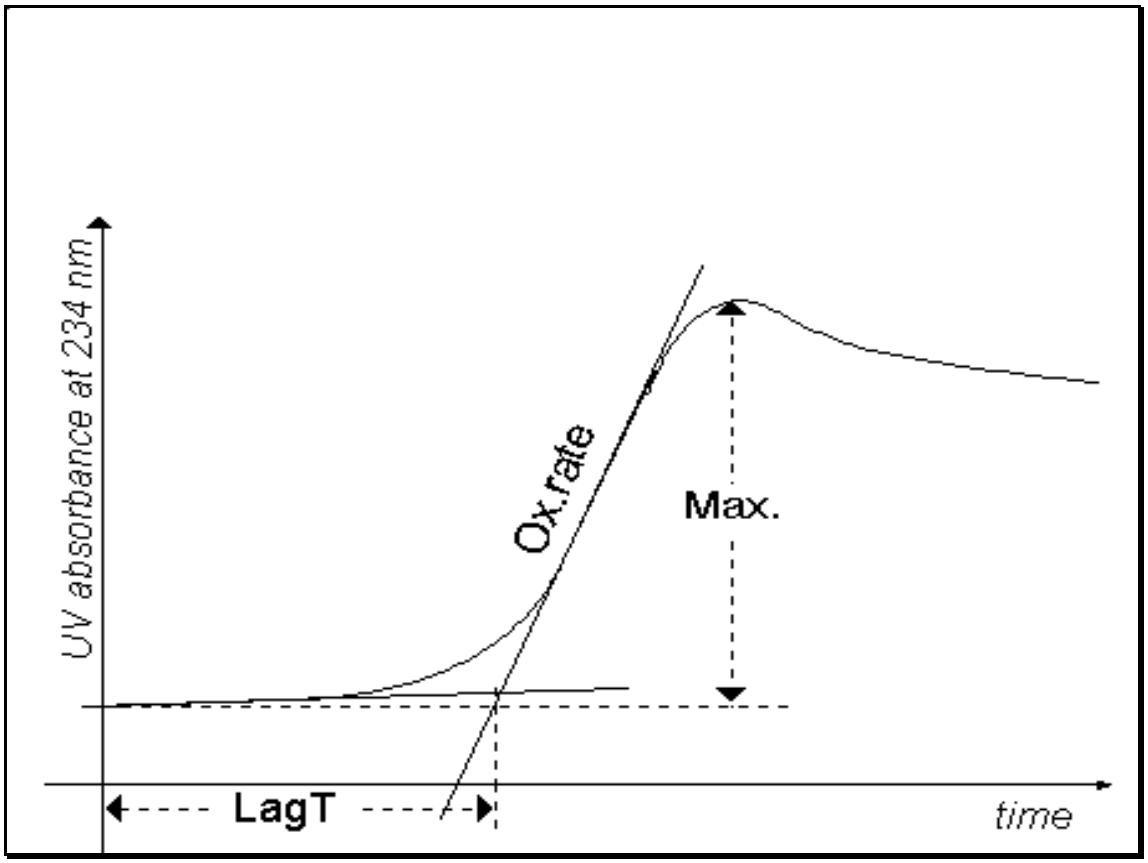
Smoking is a classical risk factor for CHD. Together with hypercholesterolaemia it synergistically impairs vascular endothelial function, which may predispose to accelerated development of atherosclerosis (Heitzer et al. 1996). The combined presence of smoking and hypercholesterolaemia is associated with increased plasma levels of autoantibodies against oxidised LDL (Heitzer et al. 1996).

Different methods have been used to elucidate the possible effects of smoking and its cessation on oxidative stress. Except non-selective thiobarbituric acid reactive substances, *i.e.* TBARS in plasma (van den Berkmortel et al. 2000), the *lag time* of lipoproteins during metal-ion dependent (Priemé et al. 1998, van den Berkmortel et al. 2000) or independent oxidation (Sasaki et al. 1997) have been measured.

Parameters describing copper-mediated oxidation are presented in Fig 2. The lag time, the maximal rate of oxidation and the maximal amount of conjugated dienes can be determined according to the method of Esterbauer et al. (1989). In addition to *in vitro* or *ex vivo* methods used, urine or plasma levels of bioactive prostaglandin F_2-like compounds, F_2-isoprostanes, have been analysed as a possible method for evaluating oxidation injury *in vivo* (Morrow et al. 1995, Reilly et al. 1996, Oguogho et al. 2000).

When smokers and non-smokers were compared with each other, parameters measuring the resistance of LDL against copper-mediated oxidation (*i.e.* the lag time, oxidation rate, and the maximal amount of conjugated dienes) did not reveal any difference (van den Berkmortel et al. 2000). According to Morrow et al. (1995), the mean plasma levels of antioxidants do not differ between smokers and non-smokers, except for vitamin C. In smokers, it was only half of that found in non-smokers. Similarly, an ATBC sub-study with 336 participants did not reveal any correlation between serum α–TOH or β–carotene and smoking variables (Karhumäki 1993). F_2-isoprostanes measured in plasma or urine were significantly higher in smokers than in non-smokers (Morrow et al. 1995, Reilly et al. 1996, Oguogho et al. 2000). Furthermore, urinary excretion of F_2-isoprostanes correlated significantly with the concentration of circulating free F_2-isoprostanes both in smokers and in non-smokers ($r=0.97$, $p<0.001$) (Morrow et al. 1995).

Figure 2. Parameters describing copper-mediated oxidation.



Formation of conjugated dienes is indicated by an increase in absorbance at 234 nm. Lag time is the delay from the beginning of oxidation to the start of the propagation phase in diene formation. It is defined by the intersection of lines drawn through the linear portions (first 5 minutes) of the lag phase and the maximal propagation. The maximal rate of oxidation (expressed as μmol/l dienes formed per min) is obtained from the slope of the absorbance curve during the propagation phase (Esterbauer et al. 1989). Maximum concentration of dienes formed is calculated from the difference in absorbance at zero time and at diene peak and it is presented normalised by the protein content in LDL fraction. Oxidation is often mediated by 1.67 μmol/l $CuCl_2$ or $CuSO_4$ (Esterbauer et al. 1989, Ramos et al. 1995).

LagT = Lag time; Ox.rate = Maximal rate of oxidation; Max. = Maximal amount of conjugated dienes.

Table 4. Effects of smoking cessation on oxidative stress.

First author	Year	Subjects	Time of sampling*	Oxidation method	Change	p-value
Morrow JD.	1995	8/10 Quitters	2 weeks	P-F_2-isoprostanes, free P-F_2-isoprostanes, esterified	-38% -25%	0.03 0.02
Reilly M.	1996	6/6 Quitters	2 weeks 3 weeks	U-8-epi-PG-$F_{2\alpha}$	-21% -23%	<0.05 <0.05
Sasaki A.	1997	14/14 Quitters 9/9 Smokers	3 months	Lag time in 4-methoxy-2,4-dimethylvalerinitrile induced oxidation	+90% ~0 %	<0.005 NS
Priemé H.	1998	62/100 Quitters 72/82 Smokers 29/100 Quitters	4 weeks 4 weeks 26 weeks	Lag time ($CuSO_4$)	+11% +13% -7%	NS ** NS ** NS
Van den Berkmortel FW.	2000	17/47 Quitters	3 months 12 months	TBARS Lag time Maximal amount of dienes Lag time Maximal amount of dienes	-14% +4% -3% +2% -4%	0.016 NS NS NS 0.025
Oguogho A.	2000	7/7 Quitters 7 Controls	4 weeks	P-8-epi-PG-$F_{2\alpha}$	~ -43% ~0%	<0.01 ***

* First samples were taken before quitting and those during follow-up as indicated
** Change in 62 quitters *versus* 72 smokers also NS.
*** Change in 7 quitters vs. 7 non-smoking controls
P-8-epi-PG-$F_{2\alpha}$ = 8-*epi*-prostaglandin $F_{2\alpha}$; $CuSO_4$ = Copper sulphate

The main results of smoking cessation on oxidative stress are presented in Table 4. In general, oxidisability of VLDL+LDL (Priemé et al. 1998) or LDL (van den Berkmortel et al. 2000) measured during copper-mediated oxidation did not change significantly. However, when a lipid-soluble diazo compound was used as a radical generator, the lag time of LDL was prolonged already at two weeks after smoking cessation (Sasaki et al. 1997). The reason for the discrepancy between the results of different methods is not clear.

Measurements of oxidative stress in a hydrous environment (*i.e.* plasma) demonstrated uniform and significant results after smoking cessation (Table 4). Plasma TBARS decreased significantly in quitters, when compared to baseline (van den Berkmortel et al. 2000). Three *in vivo* studies gave consistent results in terms of the decrease of F_2-isoprostanes (Morrow et al. 1995, Reilly et al. 1996, Oguogho et al. 2000). Interestingly, supplementation with the lipid-soluble vitamin E had no effect on urinary excretion of F_2-isoprostanes, while the water-soluble vitamin C significantly suppressed it (Reilly et al. 1996). Taking the results together, smoking cessation diminishes oxidative stress in plasma, while the effects on lipoproteins are controversial.

Physical activity

According to Vasankari et al. (2000) who have studied three groups of teenage girls, gymnasts are physically the most active during leisure time with their mean METs (multiples of resting metabolic rate) of 77, while runners consume 45 METs and controls only 11 METs per week ($p<0.0001$). In oxidation measurements, the groups differed significantly in formation of conjugated dienes in LDL (Table 5). However, LDL antioxidative capacity did not differ significantly between the groups (Vasankari et al. 2000).

Table 5 consists of results concerning mostly strenuous physical activity. Effects of exercise on oxidation seem to be related to the method used. Methods determining oxidation in hydrous phase showed an increased resistance against oxidation after exercise (Vasankari et al. 1997, Liu et al. 1999). However, those methods studying changes in lipid phase produced conflicting results concerning the resistance of LDL against oxidation related to physical activity (Ginsburg et al. 1996, Liu et al. 1999, Vasankari et al. 1997 and 2000). These results are confusing and certainly at least partly affected by de- and rehydration during the activity. For example, the drinks taken during the long-lasting performance may have contained anti- or pro-oxidative ingredients. Ginsburg et al. (1996) have found a significant decrease of 45% in the plasma iron level, while that of vitamin C has stayed unchanged during exercise.

Table 5. Effects of physical activity on oxidative stress.

First author	Year	Subjects	Comparison of samples	Oxidation method	Change	p-value
Ginsburg GS.	1996	39 Men completing the Triathlon World Championship	Before and after	Vitamin E Lipid peroxides	+3 % -47%	NS <0.001
Vasankari TJ.	1997	8 Trained runners completing a 31-km run	Before and after	S-α–TOH S- Conjugated dienes S-TRAP LDL-Conjugated dienes LDL-TRAP	+29 % +9 % +22% +9 % -9 %	<0.05 <0.01 <0.001 NS NS
		22 Keep-fit runners completing the Paavo Nurmi Marathon	Before and after	S-α–TOH S- Conjugated dienes S-TRAP LDL-Conjugated dienes LDL-TRAP	+7 % +7 % +16 % +3 % -14 %	<0.05 NS <0.01 NS NS
Liu M-L	1999	11 Men completing the Helsinki City Marathon	Before and after the race Before and four days after the race	Lag T of LDL ($CuSO_4$) P-TRAP Lag T of LDL ($CuSO_4$) P-TRAP	-16% +25% -14% +12%	<0.001 <0.001 <0.001 <0.001
Vasankari TJ.	2000	Teenage girls: 64 Gymnasts 61 Runners 58 Controls	Cross-sectional comparison	LDL-Conjugated dienes	9.8 10.2 11.5	0.013
		Teenage girls: 64 Gymnasts 61 Runners 58 Controls	Cross-sectional comparison	LDL-TRAP	21.2 21.4 22.3	NS

Special diets

Results of selected dietary studies are compiled in Table 6. The duration of studies varied from those with a single meal (Staprãns et al. 1994) to those analysing long-standing dietary habits (Korpela et al. 1999).

In general, LDL was more resistant to oxidation after a diet rich in monounsaturated fatty acids (MUFAs) than before (Aviram and Eias 1993, Corboy et al. 1993) or even after a diet rich in PUFAs (Reaven et al. 1991, Reaven et al. 1993). Although a diet with high fish intake seemed to be protective, its positive effect was not explained by any association to the resistance of LDL against oxidation *in vitro*, which was even weaker than during a diet rich in saturated fat (Korpela et al. 1999).

Staprãns et al. (1994) have found, that prooxidative attack may take place through the intestine. They studied effects of three meals differing in their content of conjugated dienes and separated from each other by 14 days. After ingestion of a single meal with a high amount of conjugated dienes, serum chylomicrons became more oxidisable than after a control meal. The content of conjugated dienes in chylomicrons increased almost 5-fold and their lag time shortened significantly by 25%.

Table 6, page 31

[a] $p<0.05$ and [b] NS, both against baseline.
[c] Separate comparisons of conjugated dienes in chylomicrons: diets with high and medium *vs.* low quantity of oxidised lipids.
[d] Comparisons with diet with high fish intake are presented. The difference between high fat and vegetarian diet was not statistically significant.
[e] Comparisons with baseline NCEP-1 diet: Olive oil diet NS; Sunflower oil diet $p<0.001$.
NA= Not applicable, NS= Not significant.

Table 6. Effects of special diets on antioxidative capacity of LDL

First author	Year	n	Samples	Setting	Diet	Oxidation parameter	Change/ value	p-value
Reaven	1991	4 5	4 weeks	Parallel	PUFA-rich *vs.* MUFA-rich	Conjugated dienes Lag time	1.6-fold half	<0.0001 NA
Bonanome	1992	11	3 weeks	Cross-over	MUFA-rich *vs.* baseline PUFA-rich *vs.* baseline	Inhibition time (AAPH)	+13 % +17 %	NS NS
Sarkkinen	1993	18 19	28 weeks	Parallel	AHA Low-fat	TBARS	-16 % [a] +7 % [b]	0.026
Aviram & Eias	1993	10	2 weeks	Before and after	Olive oil –rich	Conjugated dienes LDL peroxides LDL uptake	-32 % -73 % -61 %	<0.01 <0.01 <0.01
Corboy	1993	5	4 weeks	Before and after	Rapeseed oil & muesli	Lag time	+68 %	<0.001
Reaven	1993	7 6	8 weeks	Parallel	PUFA-rich *vs.* MUFA-rich	Conjugated dienes	+50 %	NA
Staprãns	1994	6	3 x single meal	Cross-over	Ox. Lipids: low Ox. Lipids: medium Ox. Lipids: high	Conjugated dienes	+126 % [c] +376 % [c]	<0.005 <0.005
Korpela	1999	9 11 7	Long-lasting diet	Cross-sectional	High fish intake Vegetarian Saturated fat	Lag time	116 min. 165 min. 129 min.	<0.05 [d] NS [d]
Castro	2000	22	4 weeks	Cross-over	MUFA-rich olive oil MUFA-rich sunflower oil	Lag time	+5 % +24 %	<0.01 [e]

To summarise, 1) smoking cessation in general is related to decreased oxidative injury, especially in studies analysing oxidative stress in plasma *in vivo*. 2) Continuous physical training may be related to a slightly increased resistance of LDL against oxidation. The antioxidative capacity of the water phase may be improved during prolonged strenuous exercise, while in the lipid phase it remains unchanged or is even decreased. 3) The resistance of LDL against oxidation can be improved by low-fat diet rich in vegetables and MUFAs.

4.4.2 Statin treatment

Effect on antioxidant concentrations

As presented in Fig 1, cholesterol is synthesised via the same pathway as dolichol and coenzyme Q_{10}. Hence, it is conceivable, that statins might inhibit not only the synthesis of cholesterol but also that of non-sterol end-products. Since ubiquinol (the reduced form of 2,3-dimethoxy-5-methyl-6-decaprenyl benzoquinone) is an effective antioxidant, antioxidant studies on the possible secondary effects of statins have primarily concentrated on the synthesis of coenzyme Q_{10}.

Animal studies

The main results of selected animal studies are presented in Table 7a. According to Löw et al. (1992), the decrease in ubiquinone level in liver and heart tissue varies from 10 to 30 % depending on the dose of mevinolin (a lovastatin analogue).

Table 7a. Effects of statins on antioxidant levels in animal studies

First author	*Year*	n	Animals	Treatment	*Duration*	Measured antioxidant	*Change*	p-value
Willis	1990	15 vs. 15	Rats	Lovastatin *vs.* Controls	4 weeks	Serum CoQ_9 Heart CoQ_9 Liver CoQ_9 Serum CoQ_{10} Heart CoQ_{10} Liver CoQ_{10}	-33 % -8 % -30 % -28 % -14 % -17 %	<0.01 NS <0.01 NS <0.01 NS
Löw	1992	7 to 8	Rats	Mevinolin intra-peritoneally *vs.* Controls	15 days	Blood CoQ_{10} Muscle CoQ_{10} Liver CoQ_{10} Heart CoQ_{10} Brain CoQ_{10}	Increased +150 % Decreased Decreased ~0 %	<0.05 <0.05 <0.05 <0.05 NS
		7 to 8		Mevinolin perorally *vs.* Controls	30 days	Blood CoQ_{10} Muscle CoQ_{10} Liver CoQ_{10} Heart CoQ_{10} Brain CoQ_{10}	Increased -20 % Decreased Decreased ~0 %	NS <0.05 <0.05 <0.05 NS
Diebold	1994	12 vs. 12	Young Guinea pigs	Lovastatin *vs.* Controls	4 weeks	Cardiac whole muscle CoQ_{10} Cardiac mitochondrial CoQ_{10}	-5 % -16 %	NS NS
		12 vs. 12	Older Guinea pigs	Lovastatin *vs.* Controls	4 weeks	Cardiac whole muscle CoQ_{10} Cardiac mitochondrial CoQ_{10}	-31 % -37 %	<0.05 <0.05
Chen	1997	4 vs. 6	Rats	Lovastatin *vs.* Controls	12 weeks	Serum vitamin E *	-43 %	NS
Singh	1997	10 10	Rabbits	Lovastatin Placebo	12 weeks	Plasma vitamin E	+5 % -10 %	<0.05 <0.05

* Vitamin E in this study was determined as alpha-, beta- and gamma-tocopherol
CoQ_9 = coenzyme Q_9 , CoQ_{10} = coenzyme CoQ_{10}

In general, the levels of both Coenzyme Q with nine (CoQ_9) and ten (CoQ_{10}) isoprene units are decreased almost in all tissues during statin treatment (Willis et al. 1990, Löw et al. 1992, Diebold et al. 1994). An exception was the steady concentration of CoQ_{10} in the brain (Löw et al. 1992). Also, some discrepancy existed in the effect of statins on blood or serum levels of ubiquinone in the first two studies.

The two studies with vitamin E determinations have also given non-coherent results. The hypocholesterolemic effect of lovastatin was accompanied by a clear decrease of serum total vitamin E level in one study, while plasma vitamin E was increased slightly in the other (Chen et al. 1997, Singh et al. 1997).

Human studies

The results of human studies on the concentration of CoQ_{10} are more coherent (Table 7 b). During treatment with HMG CoA reductase inhibitors, the level of CoQ_{10} was lower both in plasma or serum and low density lipoproteins (Elmberger et al. 1991, Ghirlanda et al. 1993, Watts et al. 1993, Bargossi et al. 1994, Laaksonen et al. 1994, Davidson et al. 1997). Exceptionally, a recently published small study with 12 healthy students and an open cross-over design did not reveal statistical significance in the change of the whole blood CoQ_{10} concentration during pravastatin or atorvastatin treatment (Bleske et al. 2001).

Few studies have been carried out with a comparison of two statins (Ghirlanda et al. 1993, Davidson et al. 1997, Bleske et al. 2001). The effects of different statins on ubiquinone

concentration were quite comparable with each other (Table 7 b). However, no comparative studies on CoQ_{10} determinations have yet been published with equipotent dosages (in terms of reduction of LDL cholesterol level) of various statins.

The effect of statin treatment on CoQ_{10} levels has been studied in patients with hypercholesterolaemia of familial (Elmberger et al. 1991) or non-familial origin (Ghirlanda et al. 1993, Watts et al. 1993, Bargossi et al. 1994, Laaksonen et al. 1994, Davidson et al. 1997) as well as in healthy subjects (Ghirlanda et al. 1993, Bleske et al. 2001). After cessation of simvastatin therapy, an evident increase of serum CoQ_{10} concentration was seen already after four weeks (Laaksonen et al. 1994).

Concentration of vitamin E or α–tocopherol has been monitored in two small studies (Table 7 b). According to them, statins do not have any systematic effect on vitamin E concentration in LDL (Hoffman et al. 1992, Leonhardt et al. 1997).

Table 7b. Effects of statins on antioxidant levels in human studies

First author	Year	n	Statin, daily dose (mg)	Duration	Antioxidant measured	Change	p-value
Elmberger	1991	6 FH	Pravastatin, 40	12 weeks	LDL-CoQ_{10}	-29 %	<0.05
Hoffman	1992	6	Pravastatin, 20	6 weeks	LDL-vitamin E	-36 %	NA
Ghirlanda	1993	10 10 10	Pravastatin, 20 Simvastatin, 20 Placebo	12 weeks	P-CoQ_{10}	-50 % -54 % -17 %	<0.001 <0.001 NS
		5 5	Pravastatin, 20 Simvastatin, 20	4 weeks		-33 % -26 %	<0.05 <0.05
Watts	1993	20 20 20	Simvastatin, 10-80 (A) Diet alone (B) Healthy controls (C)	15 months	CoQ_{10}/LDL-chol (A vs. B) CoQ_{10}/LDL-chol (A vs. C)	-29 % -30 %	<0.01 <0.05
Bargossi	1994	15	Simvastatin, 20	90 days	P- CoQ_{10}	-22 %	<0.03
Laaksonen	1994	17 15	Simvastatin, 35, stopped Then Lovastatin, 20	4 weeks 6 weeks	S-CoQ_{10}	+34 % -26 %	<0.001 <0.001
Davidson	1997	789 260	Atorvastatin, 12.5 (mean) Lovastatin, 29.5 (mean)	52 weeks	(NA)-CoQ_{10}	-38 % -27 %	<0.05
Leonhardt	1997	10 10	Fluvastatin, 80 Placebo	8 weeks	LDL-α–TOH	+9 %	NS
Bleske	2001	12 12	Pravastatin, 20 Atorvastatin, 10	4 weeks	B-CoQ_{10}	-8 % -8 %	NS NS
Oranje	2001	7 7	Atorvastatin, 10 Placebo	3 months	P-CoQ_{10}	-37 % -3 %	0.009

FH = Patients with familial hypercholesterolemia; CoQ_{10} = Coenzyme CoQ_{10} in plasma (P-) or serum (S-);
LDL-α–TOH = Alpha-tocopherol in LDL
NS = Not significant, NA = Not applicable

Effect on antioxidant capacity of LDL

In vitro measurements

It is possible to analyse the *in vitro* change in free radical formation by macrophages, direct effect of statin on radicals, oxidation products of LDL particles, physicochemical changes in LDL particles, and interaction between LDL particles and macrophages. So, at least eight

different methods with modifications have been used to assess the effects of statins on the resistance of LDL against oxidation and its possible consequences *in vitro*, *i.e.* 1) superoxide production of macrophages, 2) free radical scavenging capacity of statin, formation of 3) TBARS or 4) conjugated dienes during different systems of LDL oxidation, 5) electrophoretic mobility of LDL, 6) cellular binding of LDL and 7) LDL cholesterol esterification by macrophages, together with 8) LDL degradation by macrophages (Aviram et al. 1991, 1992, and 1998, Hoffman et al. 1992, Giroux et al. 1993, Chen et al. 1997, Hussein et al. 1997, Tanaka et al. 2001). With few exceptions, these studies have revealed antioxidative effects of statins on LDL *in vitro*.

Giroux et al. (1993) have incubated monocyte-derived macrophages with increasing concentrations of simvastatin. Macrophages were then activated by phorbol myristate acetate. During incubation, simvastatin inhibited superoxide production dose-dependently by 26% to 54%. This inhibition was completely reversed by addition of mevalonic acid to the medium before incubation (Giroux et al. 1993).

Free radical scavenging capacity of atorvastatin and its metabolites has been analysed by the 1,1 diphenyl-2 picryl-hydrazyl (DPPH) assay, where the drug was mixed with DPPH in ethanol and the time course of the change in optical density at 517 nm was monitored. Atorvastatin metabolites caused a time-dependent reduction in absorbance suggesting that the metabolites have substantial free radical scavenging abilities (Aviram et al. 1998). Neither atorvastatin itself nor fluvastatin had any radical scavenging effect (Hussein et al. 1997, Aviram et al. 1998).

Three oxidation mediators, *i.e.* J-774A.1 macrophages, $CuSO_4$, and 2,2´-azobis(2-amidinopropane hydrochloride) (AAPH), have been used to study the effect of atorvastatin on LDL as measured by the TBARS assay (Aviram et al. 1998). AAPH is a water-soluble azo-compound used to generate a steady flux of radicals at 37^{o}C (Niki et al. 1991). According to this experiment, atorvastatin itself does not inhibit LDL oxidation. However, the formation of TBARS was substantially inhibited by ortho- and para-hydroxy metabolites of the parent drug in all these oxidative systems studied (Aviram et al. 1998). The same oxidation systems were used selectively in other studies. Simvastatin, lovastatin, fluvastatin and especially its metabolites M2 and M4 inhibited the formation of TBARS during oxidation of LDL (Aviram et al. 1992, Giroux et al. 1993, Hussein et al. 1997, Tanaka et al. 2001), while pravastatin had no significant effect on the formation of TBARS during oxidation of LDL (Hoffman et al. 1992, Tanaka et al. 2001).

The formation of conjugated dienes has been continuously monitored by Chen et al. (1997) for six hours during leukocyte-induced and copper-mediated oxidation of LDL. Lovastatin delayed the start of the propagation phase during incubation with activated leukocytes, whereas the greater concentration of lovastatin totally blocked the oxidation. During copper-mediated oxidation of LDL, lovastatin had no effect on the formation of conjugated dienes (Chen et al. 1997). In contrast, fluvastatin has been found to prolong the lag time to the propagated formation of dienes (Hussein et al. 1997). Furthermore, the lag time was prolonged from 58 to 190 minutes by atorvastatin metabolites when compared to either control or atorvastatin-treated LDL (Aviram et al. 1998).

A rise in the electrophoretic mobility of LDL reflects an increase in the negative charge of LDL during an oxidative process. Simvastatin, lovastatin, and fluvastatin with its metabolites prevented the increase in the electrophoretic mobility of LDL (Aviram et al. 1991, Giroux et al. 1993, Hussein et al. 1997, Tanaka et al. 2001). Pravastatin had no significant effect on the mobility of LDL in electrophoresis (Aviram et al. 1991, Tanaka et al. 2001).

Cellular binding together with LDL uptake by macrophages as assessed by the cholesterol

esterification rate have been studied by Aviram et al. (1991). Lovastatin-treated ^{125}Iodide-labelled LDL was bound to macrophages to a lesser extent than control LDL. However, both lovastatin and simvastatin increased cholesterol esterification by macrophages (Aviram et al.1991), though in another study lovastatin decreased esterification of LDL cholesterol (Aviram et al. 1992). Also fluvastatin and its metabolites decreased the esterification rate of LDL cholesterol (Tanaka et al. 2001). Pravastatin, again, had no significant effect on esterification of LDL cholesterol by macrophages.

The results on LDL degradation have been rather inconsistent. Again, in the study of Aviram et al. (1991) lovastatin and simvastatin increased whereas in other studies they decreased LDL degradation by macrophages (Aviram et al. 1992, Giroux et al. 1993). Pravastatin had no effect on LDL degradation (Hoffman et al. 1992).

In most of the above-mentioned *in vitro* settings, LDL is protected against oxidation when incubated together with an HMG CoA reductase inhibitor or its metabolite. Pravastatin seems to behave somewhat differently compared to other statins. It does not prevent the production of TBARS or the increase in negative charge of LDL (electrophoretic mobility). Furthermore, it neither inhibits esterification of LDL cholesterol nor degradation of LDL by macrophages (Aviram et al. 1991, Hoffman et al. 1992, Tanaka et al. 2001).

Animal experiments

Animal studies have been carried out at least with lovastatin, pravastatin, and simvastatin (Chen et al. 1997, Singh et al. 1997, Ribeiro Jorge et al. 1997). Malondialdehyde is one of the end products of lipid peroxidation in tissues. It is often determined as TBARS. According to Chen et al. (1997), lovastatin treatment for 24 weeks (rabbits) significantly decreases the formation of TBARS in serum when the reaction mixture was heated at 95^{o}C for 75 minutes. Lovastatin treatment for 12 weeks also decreased TBARS production in rat plasma with similar heating for 30 minutes (Singh et al. 1997). Furthermore, a time- and dose-dependent decline of aortic cholesterol and TBARS was induced by 4- to 6-day treatment with simvastatin and especially with pravastatin reflecting decreased lipid peroxidation of vessel wall (Ribeiro Jorge et al. 1997). In none of these studies the actual oxidation products of LDL were analysed.

Human studies

During copper-mediated oxidation, the lag time reflects the initial phase of LDL oxidation (see Fig. 2). In human studies, it has been measured to elucidate the effect of statin treatment on the susceptibility of LDL to oxidation *ex vivo* (Table 8).

Three statin studies have been published with parallel (Salonen R et al. 1995, Oranje et al. 2001) or cross-over (Zhang et al. 1995) protocol and comparison against placebo. One parallel study was carried out with a placebo arm but without any statistical comparisons between the groups (Leonhardt et al. 1997). Two studies were head-to-head comparisons with two active drugs (Kleinveld et al. 1993, Bredie et al. 1995). One study was a non-controlled one (Hussein et al. 1997). According to the six controlled human studies, the lag time appears not to change significantly during statin therapy (Table 8). According to results in Table 7b, statin treatment lowers CoQ_{10} concentration in LDL. It has been suggested, that the lag time may be too crude as a parameter in analysing the effect of ubiquinol during the initiation of lipid peroxidation (Frei and Gaziano 1993).

The maximal rate of oxidation during the propagation phase has been calculated in six studies (Kleinveld et al. 1993, Bredie et al. 1995, Salonen R et al. 1995, Zhang et al. 1995, Leonhardt et al. 1997, Oranje et al. 2001). Fluvastatin decreased the rate significantly by 15% (Leonhardt et al. 1997), whereas pravastatin had no effect on it (Kleinveld et al. 1993, Zhang et al. 1995) or only decreased it

slightly (Salonen R et al. 1995). Simvastatin had also a minor decreasing effect on the maximal rate of oxidation (Kleinveld et al. 1993, Bredie et al. 1995), while atrovastatin had no significant effect (Oranje et al. 2001).

The maximal amount of conjugated dienes has only been determined in four studies (Kleinveld et al. 1993, Salonen R et al. 1995, Leonhardt et al. 1997, Oranje et al. 2001). Atorvastatin, fluvastatin, pravastatin, and simvastatin diminished the amount of diene formation statistically significantly but only by 3 to 16%.

Table 8. Effect of statin treatment on the lag time to conjugated-diene formation during LDL oxidation

First author	Year	Subjects	Design	Placebo controlled?	Therapy, mg/day	Duration	Change of the lag time	p-value
Kleinveld	1993	12 11	Parallel	No	Simvastatin, 20→40 Pravastatin, 20→40	18 weeks	~0 % +4 %	NS NS
Bredie	1995	18 17	Parallel	No	Simvastatin, 20 Gemfibrozil, 1200	12 weeks	NA	NS
Salonen R	1995	212 212	Parallel	Yes	Pravastatin, 40 Placebo	3 years	+1 % vs. placebo	NS
Zhang	1995	20	Cross-over	Yes	Pravastatin, 20 Placebo	12 weeks	-1 % vs. placebo	NS
Hussein	1997	10	Before and after	No	Fluvastatin, 40	24 weeks	+150 %	NA
Leonhardt	1997	10 10	Parallel	Yes	Fluvastatin, 80 Placebo	8 weeks	+1 % -1 %	NS NS
Oranje	2001	9 10	Parallel	Yes	Atorvastatin, 10 Placebo	3 months	-5 % -13 %	NS

Abbreviations: NS = Not significant, NA = Not applicable

In the Kuopio Atherosclerosis Prevention Study (KAPS) consisting of 424 patients, LDL TRAP was analysed with a method originally introduced by Timo Metsä-Ketelä (Salonen R et al. 1995). According to KAPS, pravastatin treatment increases the resistance of LDL against oxidation systematically but only by 9% ($p=0.007$). In the same study, LDL was also studied with oxidation by haemin in the presence of hydrogen peroxide (H_2O_2). During continuous monitoring the lag time was significantly longer by 42% in the pravastatin group than in the placebo group. The rate of maximal oxidation was slightly decreased (Salonen R et al. 1995).

In a recent study on elderly diabetic patients, the concentration of circulating F_2 isoprostanes as a marker of oxidant stress decreased already three days after cerivastatin administration (Tsunekawa et al. 2001). In a few other *ex vivo* studies the antioxidative capacity of LDL was assessed in more or less non-standard settings. After 24-hour incubation of LDL, the amount of conjugated dienes decreased after pravastatin and non-specific TBARS decreased after pravastatin or lovastatin (Aviram et al. 1992, Hoffman et al. 1992). The same group noted, that cholesterol esterification rate, reflecting uptake of oxidised LDL by macrophages, decreased significantly after lovastatin (Aviram et al. 1992) but not after pravastatin treatment (Hoffman et al. 1992).

To summarise, HMG CoA reductase inhibitors in general diminish the tissue levels of CoQ with nine or ten isoprene units in animals.

CoQ_{10} in human LDL is also decreased by statin treatment. Statins also may decrease the level of circulating α–TOH via their lipid-lowering effect. Nevertheless, the effect of statins on vitamin E level in serum or in LDL is not consistent.

Effects of statins on the susceptibility of LDL to oxidation *in vitro* and in animal studies have been studied with several different methods. According to them, statins or their metabolites seem to have some antioxidative activity in LDL. According to human studies the lag time *i.e.* initiation of oxidation of LDL isolated from human subjects treated with statins does not change significantly during copper-mediated oxidation, but it is prolonged during oxidation induced by haemin+H_2O_2. In the later phases, oxidation of LDL seems to diminish slightly. It is conceivable, that here the decrease in formation of conjugated dienes may, at least partly, be related to the cholesterol lowering effect of HMG CoA reductase inhibitors.

4.4.3 Ubiquinone and alpha-tocopherol supplementation

Effect on antioxidant concentrations

In vitro and animal studies

Both *in vitro* and animal studies have given consistent results on the effect of antioxidant supplementation on their concentration in LDL. Incubation of plasma with α–TOH has a dose- and time-dependent effect on the concentration of LDL α–TOH (Esterbauer et al. 1991). Alpha-tocopherol has three chiral centres, which could result in a total of eight different stereoisomers (Kaneko et al. 2000). Naturally occurring α–TOH consists of a single stereoisomer, RRR-α–TOH. Although synthetic all-racemic α–TOH contains equimolar isomers with lower biopotencies, a comparable dose-dependence of *in vitro* supplementation exists without significant difference in LDL α–TOH concentrations between the use of all-racemic α–TOH and RRR-α–TOH (Devaraj et al. 1997, Kaneko et al. 2000).

Lönnrot et al. have repeatedly studied the effects of ubiquinone supplementation in different settings. In a study consisting of 150 rats, the effect of CoQ_{10} supplementation was compared to that of control chow at four different ages (Lönnrot et al. 1998). Typically for non-supplemented rats, CoQ_9 concentration in plasma and tissues was higher than that of CoQ_{10}. Concentration of CoQ_9 and CoQ_{10} increased in plasma and liver but not in kidney, heart or brain during supplementation. In both groups, there was a decline in CoQ_{10} concentration in plasma and liver of the oldest animals (Lönnrot et al. 1998). According to Suarna et al. (1993), supplementation of rats for three weeks with varying amounts of tocotrienol-rich fraction of palm oil results in a proportional increase in plasma tocopherols and tocotrienols. The increase in α–TOH concentration was more evident than that of gamma-tocopherol or tocotrienols.

Human studies

Human studies with vitamin E or ubiquinone supplementation have been carried out in different settings. The daily dose of ubiquinone has varied between 90 mg (Kaikkonen et al. 1997) and 300 mg (Mohr et al. 1992) and that of α–TOH from 100 IU (Devaraj et al. 1997) to 1600 IU (Reaven et al. 1995). The length of the studies has ranged from five days (Thomas et al. 1996) to three years (Porkkala-Sarataho et al. 2000). Not only healthy volunteers (Lilja et al. 1996, Devaraj et al. 1997) and especially smokers have been studied (Kaikkonen et al. 1997, Fuller et al. 2000), but also patients with diabetes mellitus (Upritchard et al. 2000), chronic renal insufficiency (Islam et al. 2000) or hypercholesterolaemia (Kaikkonen et al. 2000).

Treatment with ubiquinone or α–TOH has systematically increased the concentration of the supplemented antioxidant in LDL. Concentration of α–TOH normally increased 2- to 3-fold during α–TOH supplementation (Reaven et al. 1995, Devaraj et al. 1997, Fuller et al. 2000, Islam et al. 2000). CoQ_{10} concentration increased 2- to 4-fold in LDL

(Mohr et al. 1992, Lilja et al. 1996, Thomas et al. 1996, Kaikkonen et al. 1997) and 6-fold in plasma during ubiquinone supplementation (Kaikkonen et al. 2000). The concentrations of antioxidants in plasma and in LDL increased already 6 to 8 hours after a single oral dose (Mohr et al. 1992, Suarna et al. 1993, Thomas et al. 1996). Coenzyme Q_{10} appears in LDL mostly (~80% to 85%) in its reduced form, ubiquinol. The proportion of reduced ubiquinol and oxidised ubiquinone remains constant, although the supplementation has been carried out with the oxidised form, ubiquinone (Mohr et al. 1992).

In general, vitamin E supplementation has no effect on CoQ_{10} concentration in LDL. However, the proportion of reduced ubiquinol to total coenzyme Q_{10} in plasma decreased during vitamin E supplementation (Kaikkonen et al. 2000). Ubiquinone supplementation has no systematic effect on α–TOH concentration (Mohr et al. 1992, Thomas et al. 1996, Kaikkonen et al. 1997, Kaikkonen et al. 2000).

Effect on the resistance of LDL against oxidation

In vitro and animal studies

Coenzyme Q_{10} efficiently inhibits lipid peroxidation in submitochondrial particles in the absence of vitamin E (Forsmark et al. 1991). Hence, the radical scavenging effect of CoQ_{10} is direct, and it does not need the mediation of α–TOH. Kontush et al. (1995) have later studied oxidation of LDL and found, that incorporation of CoQ_{10} in LDL decreases the formation of lipid peroxidation products significantly and dose-dependently. According to the latter study, the *in vitro* effect of CoQ_{10} is more evident than that of vitamin E at the same concentration.

As described earlier, the time of incubation of plasma with **α–tocopherol** influences the α–TOH content of LDL in plasma (Esterbauer et al. 1991). According to the same study, both increased concentration and longer incubation time of α–TOH with plasma before LDL isolation prolong the lag time of LDL during copper-mediated oxidation. In other studies using copper as oxidation mediator, the increase in the lag time of LDL by α–TOH was comparable and dose-dependent (Devaraj et al. 1997, Seccia et al. 1997).

Three oxidation methods have been evaluated by Seccia et al. (1997). During oxidation of LDL in the presence of copper sulfate, AAPH, or horse radish peroxidase (HRP) with H_2O_2, the lag time was significantly prolonged. Interestingly, the α–TOH content and the resistance of native LDL against oxidation were not correlated with each other when using any of those methods. After supplementation, the difference in α–TOH concentration correlated well with that in the lag time. Comparison of the oxidation protocols revealed that only the lag times measured after AAPH and HRP+H_2O_2 correlated with each other ($r=0.77$, $p=0.002$) (Seccia et al. 1997).

Vitamin E added in LDL solution before incubation shortened the time delay to propagated oxidation during radical flux initiated by HRP+H_2O_2 (Santanam and Parthasarathy 1995). The finding is opposite to that in copper-mediated oxidation. Because HRP is a plant peroxidase, the observations were extended to a mammalian peroxidase system using bovine milk lactoperoxidase as initiator with similar results (Santanam and Parthasarathy 1995). Also oxidation of α–TOH enriched LDL in the iron-containing Ham's F-10 medium (without macrophages) resulted in increased peroxidation of LDL when compared to native LDL (Thomas et al. 1996).

Plasma obtained from tocotrienol supplemented rats has been exposed to peroxyl radicals generated by AAPH (Suarna et al. 1993). The concentration of vitamin E derivatives showed a clear plateau before tocopherols and tocotrienols were consumed suggesting, that in the rat plasma there is prime exhaustion of water-soluble antioxidants such as vitamin C or urate (Suarna et al. 1993). Guinea pigs on high cholesterol diet had about 2-fold increase in serum TBARS, compared to animals with

either normal chow or with high cholesterol diet supplemented by vitamin E (Qiao et al. 1993).

Human studies

Coenzyme Q_{10} supplementation. Dietary ubiquinone supplementation has been given with daily doses of 180 mg (Lilja et al. 1996) and 90 mg (Kaikkonen et al. 1997) for four weeks and two months, respectively. The slight changes in the lag time during copper-mediated oxidation of LDL were not statistically significant (Lilja et al. 1996, Kaikkonen et al. 1997, Kaikkonen et al. 2000). Three other methods for studying the susceptibility of LDL to oxidation, *i.e.* haemin+H_2O_2 -induced oxidation, LDL TRAP, and TBARS, did not either show any significant differences between the supplementation and placebo groups (Kaikkonen et al. 1997).

Mohr et al. (1992) have studied antioxidant consumption and formation of hydroperoxides during AAPH-induced oxidation in one healthy man after supplementation with 300 mg/d ubiquinone. In the samples taken before the supplementation, ubiquinol was oxidised rapidly to ubiquinone after initiation of oxidation. When most of the ubiquinol had been exhausted, cholesteryl ester hydroperoxides and then also phospholipid hydroperoxides were formed. The finding was confirmed in a later study, in which ubiquinol was consumed before vitamin E derivatives, which again was followed by the formation of cholesteryl ester hydroperoxides (Suarna et al. 1993). After dietary ubiquinone supplementation, formation of lipid peroxides was inhibited for a time about 2-fold longer than before supplementation (Mohr et al. 1992). Thomas et al. (1996) compared the effect of dietary ubiquinone supplementation to that of α−TOH on the oxidation of LDL in low radical flux induced by Ham's F-10 medium containing iron ions and a very small amount of copper ions. Dietary ubiquinone supplementation increased LDL resistance to oxidation, but α−TOH supplementation was associated with increased susceptibility to oxidation. Supplementation of α−TOH with ubiquinone efficiently protected LDL against the pro-oxidative effect of α−TOH.

Alpha-tocopherol supplementation. To assess the resistance of LDL against oxidation in high radical flux with plenty of copper-ions, the method of Esterbauer et al. (1989) has been widely used (see Fig 2) (Table 9). In these circumstances, α−TOH behaves as a powerful antioxidant in contrast to studies carried out with low radical flux, in which Cu^{2+}/LDL particle ratio is 2.5 or less (Witting et al. 1995). During copper-mediated oxidation the lag time is prolonged, when the daily dose of α−TOH supplementation is 400 IU or more (Jialal et al. 1995, Devaraj et al.1997, Mabile et al. 1999).

Table 9. Effect of dietary vitamin E supplementation on the lag time to conjugated diene formation monitored continuously during LDL oxidation.

First author	Year	Subjects	Supplement-ation	Dose (IU/d)	Duration	Comparison	Change	p-value
Belcher	1993	9	α–TOH acetate	800	2 w	Before and after	+79 % *	<0.001
Jialal	1995	8 8 8 8 8 8	α–TOH	0 60 200 400 800 1200	8 w	Before and after	-7 % +4 % +6 % +27 % +80 % +69 %	NS NS NS <0.01 <0.05 <0.001
Devaraj	1997	4 38 37	α–TOH	0 100-200 400-800	8 w	Before and after	-16 % -3% to +18 % +28% to +65%	NS NS <0.001 to <0.025
Mol	1997	14 Controls 11 DM 12 Smokers	α–TOH acetate	600	4 w	Before and after, Parallel [A]	+47 % +52 % +26 %	<0.001 <0.001 <0.01
Mabile	1999	22	α–TOH	75 200 400	2 w, then 2 w, then 2 w	Before and after	+15 % +26 % +35 %	NS NS <0.05
Marangon	1999	16 15	α–LA α–TOH	600 mg 400	2 mo	Before and after	+11 % +49 %	NS <0.01
Arrol	2000	21 16	α–TOH	200 mg 400 mg	50 d	Before and after	+44 % +90 %	<0.0001 <0.0001
Fuller	2000	8 Smokers 8 Smokers 8 Smokers	Vitamin E Vitamin E (+ C) Placebo	400 IU 400 IU -	8 w	Before and after	+64 % +10 % +4 %	<0.05 NS NS
Islam	2000	15 C 15 HD 15 PD	α–TOH	800	12 w	Before and after, Parallel [B]	+34 % +21 % +54 %	<0.005 <0.05 <0.001
Porkkala-Sarataho	2000	11 10	Placebo α–TOH acetate	182 mg	36 mo	Parallel	-12 % ** +27 % **	<0.001
Upritchard	2000	13 12	Placebo α–TOH	800	4 w	Before and after	-7 % +54 %	NS <0.001

* LDL oxidised with hemin+H_2O_2, ** Lag time of VLDL+LDL fraction

Parallel analyses:
[A] Changes between groups: NS; [B] Change in PD vs. HD: p<0.03

Abbreviations:
C = Healthy controls, DM = Patients with diabetes mellitus, PD = Patients with chronic renal failure on peritoneal dialysis, HD = Patients with chronic renal failure on hemodialysis;
α–TOH = α–Tocopherol, α–LA = α–Lipoic acid; IU = International units; d = Days, w = Weeks, mo = Months;

In this setting, dietary α–TOH is an effective antioxidant in healthy subjects (Arrol et al. 2000, Porkkala-Sarataho et al. 2000), both in non-smokers (Belcher et al. 1993, Jialal et al. 1995, Mol et al. 1997, Mabile et al. 1999, Marangon et al. 1999, Islam et al. 2000) and in smokers (Mol et al. 1997, Fuller et al. 2000). It is also effective in patients with diabetes mellitus (Reaven et al. 1995, Mol et al. 1997, Upritchard et al. 2000) or renal insufficiency (Islam et al. 2000). Unfortunately only few studies with dietary α–TOH supplementation have been placebo controlled (Devaraj et al. 1997, Porkkala-Sarataho et al. 2000, Upritchard et al. 2000). Nevertheless, the results in general seem to be quite similar, *i.e.* they show an

increase in the lag time of LDL oxidation (Table 9).

Jialal and his group have studied effects of dietary α–TOH supplementation in different settings. The lag time was prolonged after α–TOH supplementation (800 IU/d) for 12 weeks and the oxidation rate of conjugated dienes was decreased (Jialal and Grundy 1992). Supplementation with vitamin C and β–carotene, in addition to α–TOH, did not give any extra effect compared to α–TOH alone (Jialal and Grundy 1993). The methods in these three studies were not very precise, since conjugated dienes were monitored every 30 minutes rather than continuously (Jialal et al. 1995). Despite these shortcomings, similar results have been obtained in recent studies, in which continuous monitoring has been applied (Table 9).

Reaven et al. (1995) have compared α–TOH with placebo in patients with type 2 diabetes mellitus. They also used non-continuous monitoring. Vitamin E supplementation resulted in a significant prolongation of the lag time. The results were similar in a recent vitamin E supplementation study with continuous monitoring of dienes and with three control arms, *i.e.* placebo, vitamin C, and tomato juice (Upritchard et al. 2000). Here, the lag time prolonged significantly during supplementation with vitamin E or tomato juice.

As presented in Table 9 (Mol et al. 1997), prolongation of the lag time after dietary α–TOH supplementation was less in smokers than in control subjects or diabetic patients. The maximal rate of oxidation was slightly diminished in controls and in patients with diabetes, but not in smokers (Mol et al.1997). The lag time in young smokers is prolonged considerably more according to a recent study by Fuller et al. (2000).

The group of Jialal has also studied patients with renal insufficiency on dialysis (Islam et al. 2000). According to the non-continuous monitoring used (Jialal et al. 1995), the change in the lag time during dietary α–TOH supplementation seems to be related to the type of dialysis. The prolongation was more pronounced in patients with peritoneal dialysis than those with hemodialysis. They also studied the lag time to the formation of lipid peroxides, where the prolongation was only seen in controls and in patients on peritoneal dialysis. The authors did not explain the greater benefit seen in patients with peritoneal dialysis (Islam et al. 2000).

RRR-α–TOH has been compared to all-racemic α–TOH with different dosages in a randomised placebo-controlled oxidation study by Devaraj et al. (1997). Both preparations resulted in similar antioxidative resistance of LDL, which only was evident at doses of 400 IU/day or more in both forms (Devaraj et al. 1997). In the study of Belcher et al. (1993) with three periods (presupplement, α–TOH alone or with vitamin C, and washout), the cytotoxicity of haemin+H_2O_2 –conditioned LDL to endothelial cells was almost completely prevented by vitamin E (Belcher et al. 1993).

The familial isolated vitamin E deficiency or FIVE syndrome is characterised by a very low level of plasma α–TOH unless supplemented. In an interesting study, Thomas et al. (1996) have studied samples from a 28-year old male patient with the FIVE syndrome after a 5-day discontinuation of supplementation and again after a 3-day further supplementation with vitamin E. After the discontinuation, LDL particles contained on the average only 0.8 molecules of α–TOH and there was practically no formation of cholesteryl ester hydroperoxides during the 12-hour oxidation induced by Ham's F-10 medium. Thus, LDL particles lacking α–TOH were highly resistant to oxidation. During the re-supplementation, α–TOH was increased to 12.5 molecules per LDL particle and the consumption of α–TOH was verified together with the formation of lipid oxidation products (Thomas et al. 1996). The same group analysed the samples further,

and according to Neuzil et al. (1997), addition of any of four different initiators of oxidation failed to induce any marked signs of peroxidation in the α–TOH depleted LDL. In the samples taken during re-supplementation, lipid peroxidation was noted again in relation to α–TOH exhaustion during the water- and lipid-soluble peroxyl radicals AAPH and AMVN [2,2-azobis (2,4-dimethylvaleronitrile)], respectively, during soybean 15-lipoxygenase, and during copper sulfate. The results indicate, that α–TOH generally renders lipoproteins more reactive towards radical oxidants and that α–TOH is required for oxidation of peroxide-free LDL (Thomas et al. 1996, Neuzil et al. 1997).

In summary, both CoQ_{10} and α–TOH are effectively incorporated in LDL by antioxidant supplementation of incubation medium *in vitro*. Their level in plasma and LDL is increased significantly also by dietary supplementation. In spite of different settings and doses, the published results are very consistent.

The effect of dietary antioxidant supplementation on the susceptibility of LDL to oxidation *ex vivo* is more disputed. Ubiquinol is an effective antioxidant during the very initiation of oxidation process, but due to low concentration in LDL its actual importance *in vivo* is still questionable. Alpha-tocopherol on the other hand has a pro-oxidative effect on LDL exposed to low radical flux when Cu^{2+}/LDL particle ratio is 2.5 or less. However, in most of the supplementation studies oxidation has been carried out with high radical flux, where dietary α–TOH with a daily dose of 400 IU or more behaves as an antioxidant. For the time being, no follow-up or intervention studies have been carried out to evaluate the prognostic value of different oxidation methods in CHD. Therefore it is still not possible to conclude, which methods might be of clinical value.

5. AIMS OF THE STUDY

In the present study the resistance of LDL against peroxidation was studied in different clinical settings in CHD patients. The specific aims of the study were to evaluate, whether

1. comprehensive preventive group education given late after coronary artery bypass grafting affects behaviour of the patients and further the resistance of LDL against oxidation or the classical risk factors for coronary heart disease,

2. lovastatin therapy has an effect on ubiquinol or alpha-tocopherol concentration in LDL, their consumption during metal-ion independent oxidation of LDL lipids, or susceptibility of LDL to oxidation during copper-mediated oxidation in patients with or without alpha-tocopherol supplementation,

3. ubiquinone supplementation during lovastatin treatment has an effect on ubiquinol or alpha-tocopherol concentration in LDL, their consumption during oxidation, or susceptibility of LDL to oxidation during Cu^{+2}-ion dependent oxidation,

4. there are substantial differences between metal-ion dependent and independent oxidation methods when measuring oxidisability of α–tocopherol supplemented and non-supplemented LDL *ex vivo*.

6. PATIENTS AND METHODS

6.1 Patients and study design

The overview of Studies I to V is presented in Table 10. The number of patients completing at least one study was altogether 104. Some of them participated in three or four studies.

Table 10. Patients and methods

Study	Number of patients	Age, years, mean ± SD (range)	Protocol	Intervention	Follow-up	Main Outcome Measurements
I	72 CABG patients	59 ± 5 (46-65)	Open, parallel	Comprehensive group education	One year	LDL-TRAP Classical risk factors for CHD
II	27 men with CHD	56 ± 8 (41-69)	Double-blind, randomised, cross-over	Lovastatin vs. placebo	2 x 6 weeks	LDL ubiquinol and α–TOH, Consumption time of antioxidants, Lag time
III	19 men with CHD	55 ± 7 (41-69)	Double-blind, randomised, cross-over	Ubiquinone vs. placebo during lovastatin therapy	2 x 6 weeks separated by 6-week wash-out	
IV	28 men with CHD	56 ± 8 (40-69)	Double-blind, randomised, cross-over	Lovastatin vs. placebo during α–TOH supplementation	2 x 6 weeks	
V	10 men with CHD	56 ± 8 (40-69)	Double-blind, randomised, cross-over	Placebo, lovastatin, α–TOH	Samples after the 6-week periods	Methodological analysis of LDL TRAP, Comparison with antioxidant consumption and copper-mediated oxidation

Study I. Eighty-seven consecutive patients, both men and women, aged 65 years or less had undergone coronary artery bypass grafting (CABG) at least 1½ years before recruitment. They had to be able to perform light daily exercise, such as walking, without symptoms. After analysis of their baseline condition, 77 patients started the study and 72 of them completed the one year follow-up. The mean age of the 41 patients in the intervention group and of 31 controls was 60 ± 4 and 59 ± 6 years, respectively (mean ± SD). There were altogether six smokers (three daily smokers) and six patients with diabetes mellitus.

Preventive councelling was given to the whole intervention group at the same time during six separate sessions. Effects of a feasible education program on total radical antiperoxiding capacity of LDL lipids and on traditional risk factors for coronary heart disease were studied. The intervention group was compared with similar patients allocated as controls according to home municipalities. The patients were followed for 12 months according to an open, controlled and parallel study protocol.

Studies II and IV. Twenty-eight hypercholesterolaemic men (fasting serum LDL-cholesterol > 4.0 mmol/l or total to HDL cholesterol ratio >

5.5) with verified coronary heart disease were randomised in both cross-over studies. All patients had fasting serum triglycerides less than 5.0 mmol/l. Of the patients completing Study II, ten had diagnosed hypercholesterolaemia (Fredrickson type II a) and 17 had mixed hyperlipidaemia (type II b). In Study IV the numbers were 10 and 18, respectively. There were three smokers in Study II and two in Study IV. Patients with a severe chronic condition such as malignancy, alcoholism, narcotic use, or a metabolic disease such as diabetes mellitus, thyroid dysfunction or overt hypertriglyceridaemia, were excluded. In Study II, one patient was excluded after randomisation because of concomitant administration of antioxidative agents. In Study IV, all except five patients had participated in Study II.

Lovastatin was compared to placebo in α–tocopherol supplemented (Study IV) or non-supplemented (Study II) patients. The treatments (lovastatin 60 mg daily or placebo) were given in a random order according to a cross-over protocol. The wash-out period without cholesterol lowering medication or antioxidants was at least six weeks before Study II and eight weeks before Study IV. Each treatment period lasted for six weeks.

Study III. Twenty men from Study II participated in Study III with the same inclusion and exclusion criteriae as in Studies II and IV. One patient was excluded from the study because of protocol violation. Five of the men were current smokers. Eight had hypercholesterolaemia (Fredrickson type II a) and 11 had mixed hyperlipidaemia (type II b).

The prerequisites were the same as in Studies II and IV. Here the effects of ubiquinone supplementation were compared to those of placebo during lovastatin therapy. Treatment periods (lovastatin 60 mg daily with placebo or ubiquinone) lasted for six weeks, and they were separated by a 6-week wash-out period with placebos.

Study V. Methodological comparisons were performed on samples obtained during Studies II and IV. Plasma samples were collected from ten hypercholesterolaemic men with coronary heart disease participating in Studies II and IV. Results from copper-mediated oxidation and those from metal ion independent oxidation were compared with each other and with LDL antioxidant contents.

6.2 Methods

6.2.1 Evaluation of dietary habits (I)

Dietary habits were registered using a dietary record which was analysed with a computerised system. After individual guidance, the patients filled in nutritional data on four consecutive days, i.e. from Friday to Monday. All food eaten, including candies and drinks, was further itemised and quantified. Analysis of nutritive substances was carried out by the Nutrica^{R} computer program (Social Insurance Institution 1993, Finland). In statistical analysis, every item was processed as a 24-hour mean of the four days. The following items were measured: total energy, proteins, total fat, saturated fats, monounsaturated fats, polyunsaturated fats, P/S ratio, cholesterol, palmitic acid, stearic acid, linoleic acid, linolenic acid, carbohydrates, starch equivalents, saccarose, ethanol, ash, fibre, equivalents of retinol, thiamine, riboflavin, and niasin, together with the vitamins C, D, and E. The dietary record was filled in before the group education, and after eight months and one year, *i.e.* at the same time points, when the measurements of the total radical antiperoxiding capacity of LDL (LDL-TRAP) were carried out.

6.2.2 Evaluation of physical activity (I)

Following the principles described by the research group of UKK Institute for Health Promotion Research (Haapanen et al. 1996), a leisure time physical activity energy expenditure index (LTPA index, kcal/week) was estimated. The calculation was based on three scaled questions on the weekly frequency of leisure time physical activity sessions (six predetermined choices), the average duration of the session (four choices), and the multiples of resting metabolic rate (MET) values of the reported intensity of exercise (four choices). The LTPA

index is an estimate of extra physical activity to resting metabolic rate. It is 0 kcal/week, when the leisure time physical activity corresponds to only 1 MET. Therefore the range of this index varied from 0 to 2160 kcal per week.

In addition to the LTPA index, responses to three questions about daily walking, weekly leisure time activity, and total physical activity during the previous two months were scored and analysed separately. Finally, the patients estimated by a three-part multiple-choice question their ability to climb stairs without rest, to walk two kilometres without rest, and to run about half a kilometre. The whole questionnaire was filled in by both groups before the group education and thereafter on every control visit during the follow-up.

6.2.3 Clinical chemistry (I – V)

Venous blood samples were drawn between 07:30 and 09:00 *a.m.*. The patients were advised to fast, not to take any medication, coffee, or other beverages, and not to smoke for 12 hours before blood sampling. Alcohol was prohibited for 36 hours before sampling. Blood samples were drawn in sitting position after a rest of at least 15 minutes. Plasma was separated by centrifugation immediately after cooling the sample on ice in the dark for 5 minutes. Routine blood tests were performed without delay in Kanta-Häme Central Hospital. After separation, the blood samples for the LDL oxidation measurements were stored at –70 to –85 ^{o}C until analysed. To preserve LDL ubiquinol in the reduced form during the moments between the venipuncture and freezing, the samples were kept cold and in darkness. The analysis of LDL antioxidants before and during metal-ion independent oxidation as well as the total radical antiperoxiding capacity of LDL were carried out in the Research Laboratory of Pharmacology, Medical School of Tampere University. The measurement of Cu^{+2}-mediated oxidation was carried out in Centre for Laboratory Medicine, Department of Clinical Chemistry in Tampere University Hospital.

6.2.4 Metal-ion independent oxidation of LDL lipids (I – V)

Isolation of LDL (I – V)

LDL was precipitated from EDTA plasma by heparin and trisodium citrate in acid-washed Kimax glass tubes (Wieland and Seidel 1983). After careful removal of the supernatant, LDL precipitate was dissolved in chloroform-methanol (1:1) to extract lipids. After short centrifugation the solution was divided for phosphorus and chemiluminescence (LDL TRAP) and/or antioxidant measurements.

Determination of antioxidants (I – V)

Alpha-tocopherol and ubiquinol were determined by high pressure liquid chromatography (HPLC) using a method modified from that published by Lang et al. (1986). Because of variable recovery due to manual extraction of LDL, the results were standardised with LDL phospholipids and expressed as millimoles of α–tocopherol or ubiquinol per mol of LDL phosphorus. LDL phosphorus (Pi) concentration was determined by an inorganic ammonium molybdate reaction with colorimetric detection at 799 nm (Fiske and Subbarow 1925).

Oxidation by AMVN, exhaustion of antioxidants (II – IV)

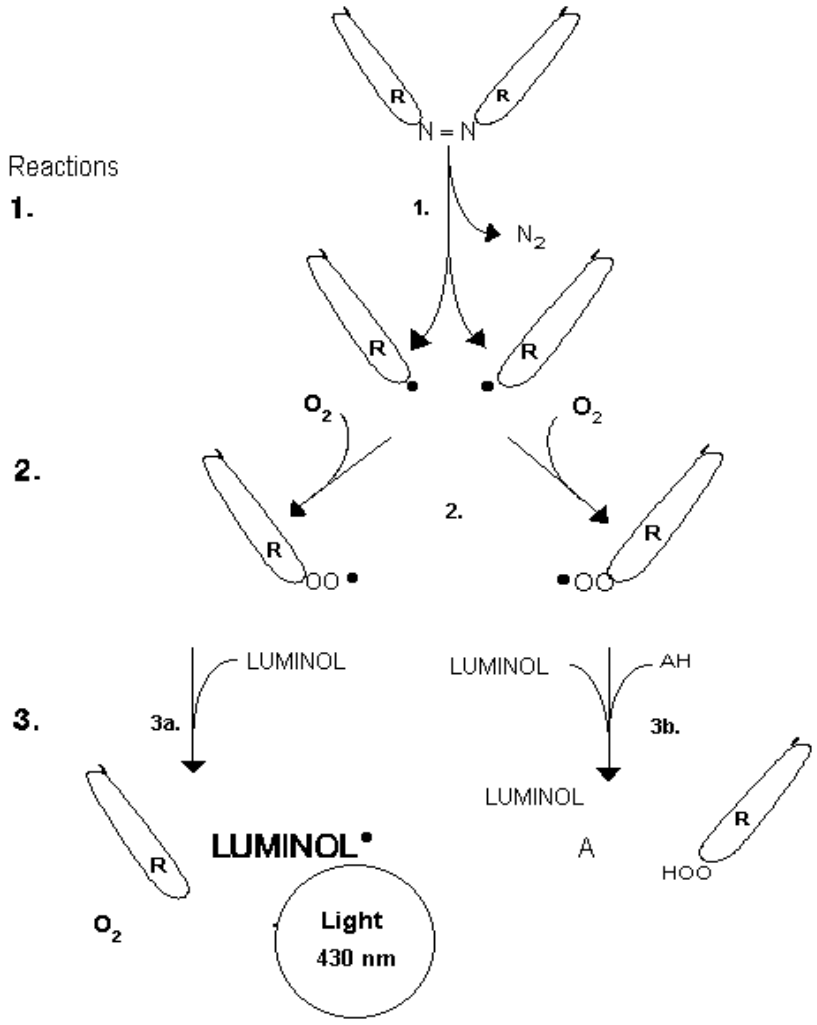
Peroxyl radicals were produced at a constant rate by thermal decomposition of 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN, Polysciences Inc, Warrington, PA, USA) (Niki et al. 1991) dissolved in benzene, which was mixed with chloroform:methanol containing LDL. The final concentration of AMVN was 2.1 mmol/l. For the determination of α–tocopherol and ubiquinol, 100 µl samples were taken every 3 minutes from the test tube placed in a temperature-controlled incubator (37 ^{o}C). Oxidation was stopped by deepfreezing the samples in liquid nitrogen where they were stored until HPLC-analyses.

Oxidation by AMVN, LDL TRAP (I, V)

To measure the overall resistance of LDL against AMVN-induced oxidation, the total radical antiperoxiding capacity of LDL (LDL TRAP) was analysed by the method originally

introduced by the late Timo Metsä-Ketelä (not published) and later adopted by Salonen R et al. (1995) and Kaikkonen et al. (1997).

Figure 3. The principle of radical production and measurement during AMVN induced oxidation and chemiluminescence detection.



Thermal decomposition of 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN) (RN=NR, where R means the rest of the molecule) (reaction 1) produces peroxyl radicals ($ROO^{\bullet}$) at a constant rate (Reaction 2). When luminol is once oxidised by peroxyl radicals (Reaction 3a), it releases nitrogen (N_2) (Popov and Lewin 1994) and produces chemiluminescence at 430 nm. In the presence of an antioxidant (AH), the radical chain is cut off and luminol escapes oxidation (Reaction 3b). Then, no chemiluminescence is detected.

This ex vivo method was modified from the TRAP determination of water soluble compounds, e.g. plasma and buffer solutions (Wayner et al. 1987, Metsä-Ketelä 1991, Alanko et al. 1993, Uotila et al. 1994). The reaction mixture contained AMVN in benzene and luminol dissolved in borate buffer and methanol. The chemiluminescence produced by luminol reacting with the peroxyl radical was measured automatically by a computerised luminometer system (Fig 3). The solution of LDL in chloroform:methanol added to the reaction mixture temporarily extinguishes the chemiluminescence. The duration of extinction corre-

lates linearly to the peroxyl radical scavenging capacity of the sample. The method was calibrated with four known concentrations of α–tocopherol. The concentration of LDL phosphorus was determined as described above. The LDL TRAP results are expressed as tocopherol equivalents: pmol/nmol phosphorus in LDL.

6.2.5 Copper-ion mediated oxidation of LDL (II – V)

Isolation of LDL

To minimise the risk of oxidation during isolation LDL was prepared using a rapid nonequilibrium density-gradient ultracentrifugation method (Chung et al. 1986). After the density of plasma was adjusted with solid anhydrous KBr it was layered beneath saline containing EDTA in a 2-ml quick-seal tube. The tubes were centrifuged for 30 min at 10°C in a bench top ultracentrifuge using a vertical rotor at 338 000 *g*. The distinct LDL band was withdrawn through the side of the tube with a needle and a syringe. Immediately after isolation 0.3 ml of the LDL fraction was applied to a gel filtration column to remove the salt and EDTA. The column was washed with PBS (sodium phosphate buffer containing NaCl) and the LDL eluate was collected.

Oxidation of LDL

Metal-ion dependent oxidation was determined as the Cu^{+2}-mediated production of hydroperoxides with conjugated double bonds (conjugated dienes) by continuously monitoring the change in UV-absorbance at 234 nm as described by Esterbauer et al. (1989). A sample of LDL solution was standardised to contain 0.05 mg protein/ml (~ 0.1 μmol/l LDL) with PBS. The final concentration of $CuSO_4$ in the mixture was 1.67 μmol/l. The temperature was kept at 37°C and UV-absorbance was automatically recorded at 1-min intervals for 5 h. The spectrophotometer was connected to a computer for data collection and analysis of absorbance versus time curves (Ramos et al. 1995). To minimise the effect of variation in the oxidation method on the results of the study, all samples of an individual subject were analysed simultaneously. In addition, one control sample drawn from one healthy subject and kept at –80°C in small portions was always processed with the patient samples. The protein concentration of the eluate was measured with a modification of the Lowry method (Markwell et al. 1981) using bovine serum albumin as standard.

During oxidation, the following measurements were carried out: lag time, maximal rate of oxidation, and the maximum concentration of conjugated dienes (Esterbauer et al. 1989). As presented in Fig 2, lag time (min) to the start of the propagation phase in diene formation is defined by the intersection of lines drawn through the linear portions of the lag phase (first 5 minutes) and the maximal propagation. The maximal rate of oxidation (expressed as μmol/l dienes formed per min) is obtained from the slope of the absorbance curve during the propagation phase (Esterbauer et al. 1989). Maximum concentration of cojugated dienes formed is calculated from the difference in absorbance at zero time and at diene peak and it is presented normalised by the protein content in LDL fraction (Fig 2) (Esterbauer et al. 1989).

7. ETHICAL CONSIDERATIONS

The clinical part of the study was carried out in the cardiology outpatient clinic at Kanta-Häme Central Hospital, Hämeenlinna, Finland. It was approved by the Ethics Committee of the hospital. Studies II to IV with pharmacologic interventions were also approved by the National Agency for Medicines. A written informed consent was given in advance by every patient. Monitoring of the study was carried out according to Good Clinical Trial Practice (EC 1988), and the recommendations of the Declaration of Helsinki were followed in all studies.

8. STATISTICAL METHODS

8.1 Controlled preventive education study (I)

In Study I, the baseline differences were compared using Fischer's exact test, Mann-Whitney U-test and non-paired Students' t-test. Also α–tocopherol concentration of LDL at eight and 12 months was compared with non-paired t-test. Analysis of variance with repeated measurements (RANOVA) was carried out with the follow-up comparisons of continuous variables using Solo Statistical System (BMDP Statistical Software 1991) (Hintze 1991). Interaction p-value between groups and periods was calculated. It describes the probability of similar changes in the variable in the two groups during the trial. Where the interaction p-value was statistically significant, the data of individual time points were compared to the baseline using RANOVA. The changes in graded variables from the baseline during the follow-up were compared with the non-parametric Wilcoxon signed-ranks test. The within-group changes in the leisure time physical activity energy expenditure index and LDL-TRAP at the individual time points *vs.* baseline were analysed with paired t-test. The p-value of < 0.05 was regarded as statistically significant in the two-tailed tests.

8.2 Double-blind cross-over studies (II – V)

The statistical analysis was performed using the above mentioned software (Hintze 1991). In Studies II-V, the rates of individual consumption of ubiquinol and reduced α–tocopherol during AMVN-induced oxidation were calculated using linear regression analysis. The effects of the lovastatin and antioxidant interventions in Studies II-IV were compared with a two-way analysis of variance with repeated measurements (RANOVA), and with the factors treatment and treatment order. The treatment periods were further compared pairwise with a t-test-based contrast analysis (User Contrast, BMDP Solo) and Tukey´s test (Hintze 1991). Two-tailed p-value of 0.05 or less was regarded as statistically significant.

9. RESULTS

9.1 Effects of comprehensive preventive group education (I)

9.1.1 Lipids and other risk factors

After multifactorial preventive group education, there were no significant changes in dietary habits. Leisure time physical activity seemed to increase at the 8-month time point in the intervention group when compared to baseline, but when compared with the control group the difference did not reach statistical significance. The initial number of daily smokers was only three. No significant change was noted in smoking habits. Also blood pressure remained unchanged in both groups. Mean body mass index (BMI) of the intervention group became smaller than in the control group during the whole follow-up period of one year. The difference reached statistical significance at the 12-month time point. The mean weight difference was 1.2 kg at the end of the study. White blood cell count was statistically significantly lower in the intervention group at one year. Fibrinogen was lower in the intervention group at eight and twelve months. However, only the difference at the 8-month time point reached statistical significance.

At baseline, HDL and HDL/LDL ratio were higher in the control group. During the follow-up period, the values in the control group decreased significantly and tended to get closer to those of the intervention group. No other changes in lipids were noted. No clinically significant changes were seen in serum lipid levels in the intervention group.

9.1.2 Total radical antiperoxiding capacity of LDL (LDL TRAP)

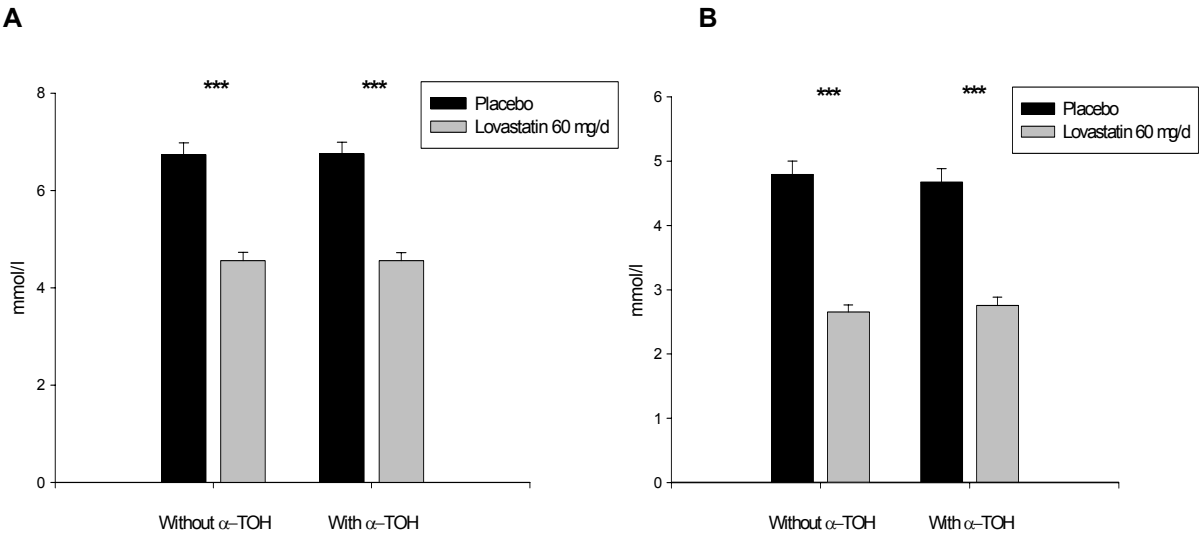
At baseline, LDL TRAP as a measure of total antioxidative capacity in LDL did not differ between the groups. After eight months, LDL TRAP increased significantly in the intervention group while it tended to decrease in the control group. Hence, the 25% net difference between the groups was statistically significant at the time point of eight months. However, at the end of the study, no difference was noted in LDL TRAP. Due to technical reasons, the determination of LDL α−tocopherol concentration was possible only after eight and 12 months. There was no significant difference between the groups at either of these time points.

9.2 Effects of lovastatin therapy (II, IV)

9.2.1 Lipids and lipoproteins

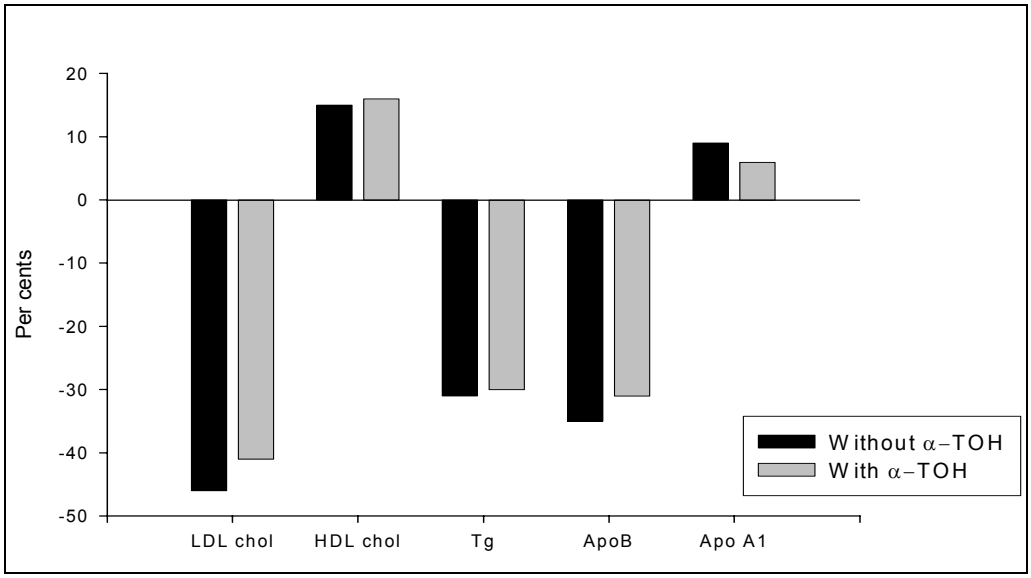
In studies II and IV, lovastatin therapy decreased LDL cholesterol level significantly by 41 to 46% (Fig 4 and 5).

Figure 4. Effect of lovastatin (20 mg three times daily) on serum total cholesterol (A) and LDL cholesterol (B).



The bars represent values obtained after lovastatin and placebo periods with (n=27) or without α–TOH (n=28) supplemention (450 IU/d). Means + SEM are presented. All differences were statistically significant, ***: $p < 0.0001$.

Figure 5. Effect of lovastatin therapy (20 mg three times daily) on serum lipids and lipoproteins compared with the baseline values.



Abbreviations are as follows: LDL chol = LDL cholesterol, HDL chol = HDL cholesterol, Tg = Triglycerides, Apo B = Apolipoprotein B-100, and Apo A1 = Apolipoprotein A1.

HDL cholesterol was increased by 13 to 16% and triglycerides decreased by 17 to 31%. During lovastatin therapy, apoB-100 decreased by 31 to 35%, while apolipoprotein A-I (apoA-I) increased statistically significantly by 6 to 9%. The changes were slightly different depending on whether comparisons were made with baseline or lovastatin-placebo period. Because mere vitamin E treatment increased both HDL-cholesterol and apoA-I concentrations (see 9.3.1), their further increase by lovastatin was only 5% (p=0.02) and 2% (NS), respectively.

9.2.2 Antioxidant concentration and total consumption time

During lovastatin therapy in Studies II and IV, LDL ubiquinol concentration per mol of LDL phosphorus decreased significantly by 13 and 19% (Fig 6). The slight decreases in LDL α–tocopherol were not statistically significant.

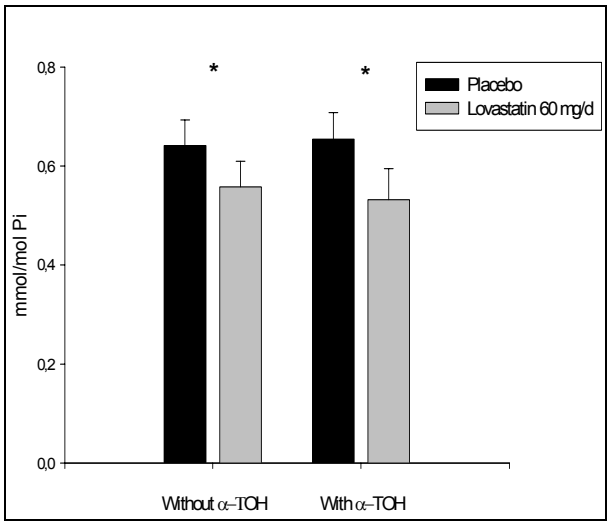
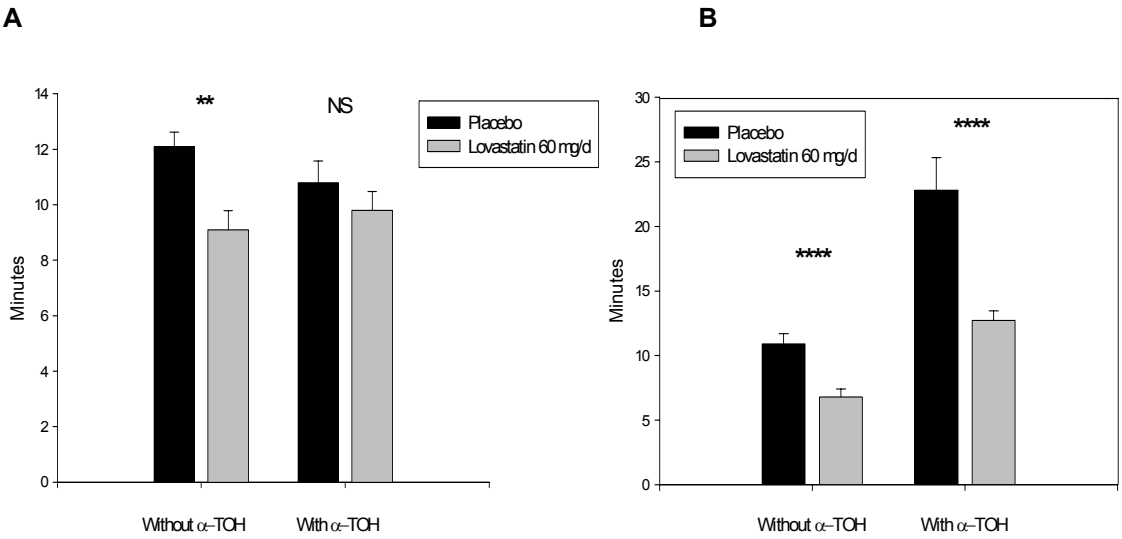


Figure 6. Concentration of reduced ubiquinol in LDL after lovastatin (20 mg three times daily) and placebo periods. Lovastatin and placebo tablets were administrered with (n=28) or without (n=27) α–TOH (450 IU/d). The samples were taken at the end of the 6-week period of each treatments. In both Studies II and IV, the difference was statistically significant. Mean + SEM are presented. Pi = phosphorus in LDL* : $p<0.05$.

Figure 7. Effect of lovastatin (20 mg three times daily) on the consumption time of LDL antioxidants, reduced ubiquinol (A) and α–TOH (B).



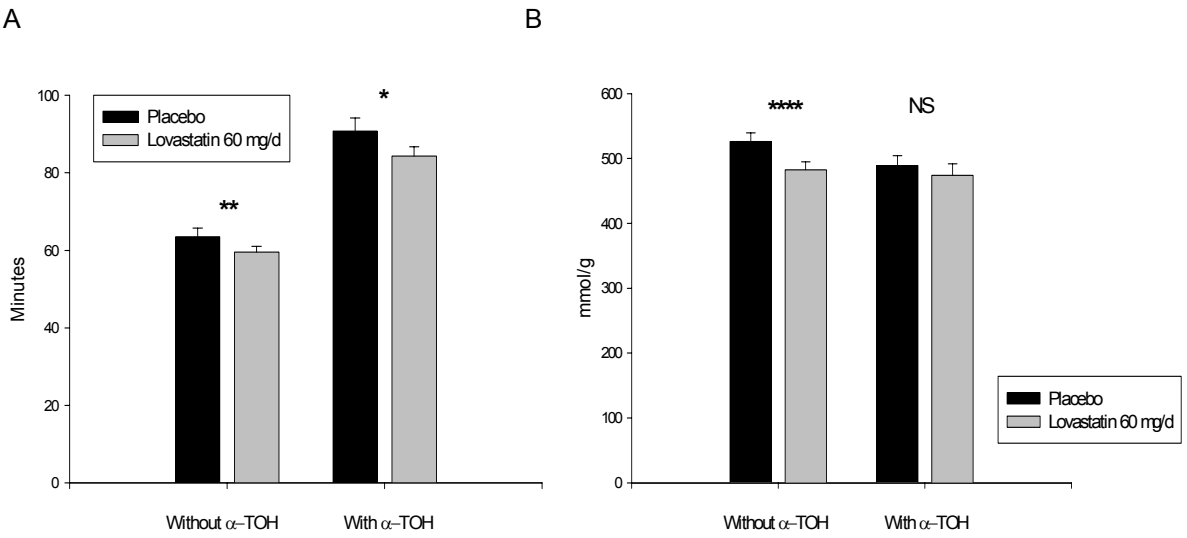
The consumption time of ubiquinol (A) was shortened during lovastatin, although during vitamin E supplementation (α–TOH 450 IU/d) the change did not reach statistical significance.
The prolongation of the consumption times of α–TOH by the supplementation is evident (B). Samples were taken at the end of the 6-week period of each treatment.
Mean + SEM are presented. ** : p<0.01, **** : p<0.0001.

In metal-ion independent oxidation induced by AMVN the total consumption time of LDL ubiquinol was shortened by 25% and that of α–tocopherol by 37%, when lovastatin was compared with placebo (Fig 7 A and B). These changes were statistically highly significant. During α–tocopherol supplementation lovastatin shortened the total consumption time of LDL α–tocopherol by 44% and that of LDL ubiquinol by 9%.

9.2.3 Copper-ion mediated oxidation

Effects of lovastatin therapy on metal-ion dependent oxidation mediated by copper sulfate were small. The lag time shortened slightly but statistically significantly by 6 to 7%, when compared with lovastatin-placebo (Fig 8 A). The rate of maximal oxidation tended to fall slightly, by 3 to 5%, but the differences were statistically non-significant. The maximal amount of conjugated dienes diminished by 3% (NS) to 8% (p=0.0001) (Fig 8 B).

Figure 8. Effect of lovastatin (60 mg/d) on the lag time (A) and maximal amount of conjugated dienes (B) compared to placebo.



The samples were taken at the end of the 6-week period of each treatment. The prolongation of the lag time by α–TOH supplementation is evident (A, right *vs.* left bars). The slight shortening of the lag time by lovastatin was statistically significant in both Studies II (n=27) and IV (n=28). The maximal amount of conjugated dienes (B) was decreased significantly by lovastatin when taken without α–TOH supplementation, whereas during supplementation the maximal amount was not decreased significantly by lovastatin. Mean + SEM are presented.
* : p<0.05; ** : p<0.01; **** : p<0.0001; NS : Not significant.

9.3 Effects of antioxidant supplementation (III, IV)

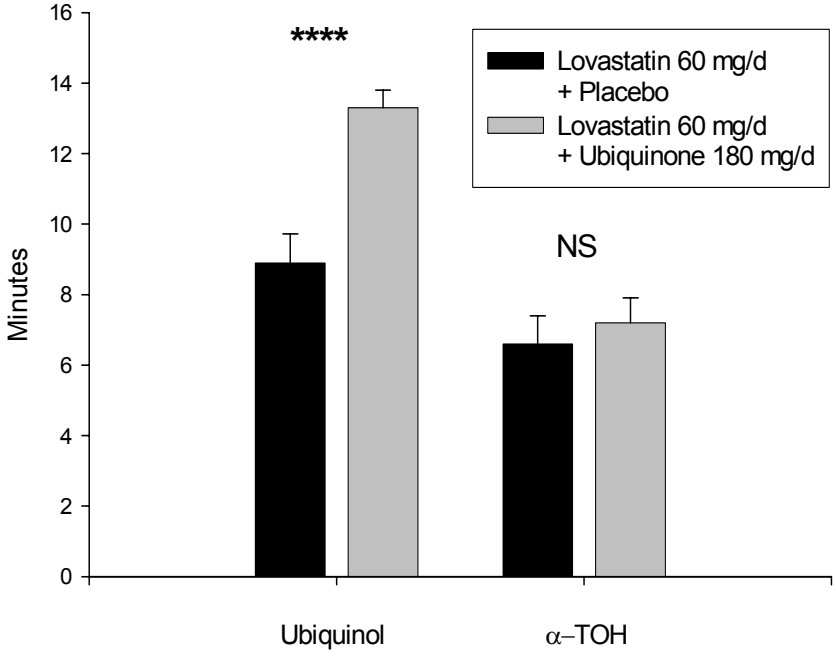
9.3.1 Lipids and lipoproteins

Ubiquinone supplementation during lovastatin therapy did not cause any changes in lipids or lipoproteins. Vitamin E supplementation (α–TOH 450 IU/d) increased HDL cholesterol by 10% and apolipoprotein A-I by 3%. Both changes were statistically significant. The other lipid and lipoprotein levels did not change significantly during α–tocopherol therapy.

9.3.2 Antioxidant concentration and total consumption time

As expected, antioxidant concentrations of LDL per LDL phosphorus changed dramatically during supplementation with ubiquinone or vitamin E. During ubiquinone supplementation, LDL ubiquinol increased by 317% and its consumption time prolonged by 49%. No significant changes were noted in α–tocopherol concentration or total consumption time during ubiquinone supplementation (Fig 9).

Figure 9. Consumption times of reduced ubiquinol and α–TOH in lovastatin-treated patients with or without ubiquinone supplementation.



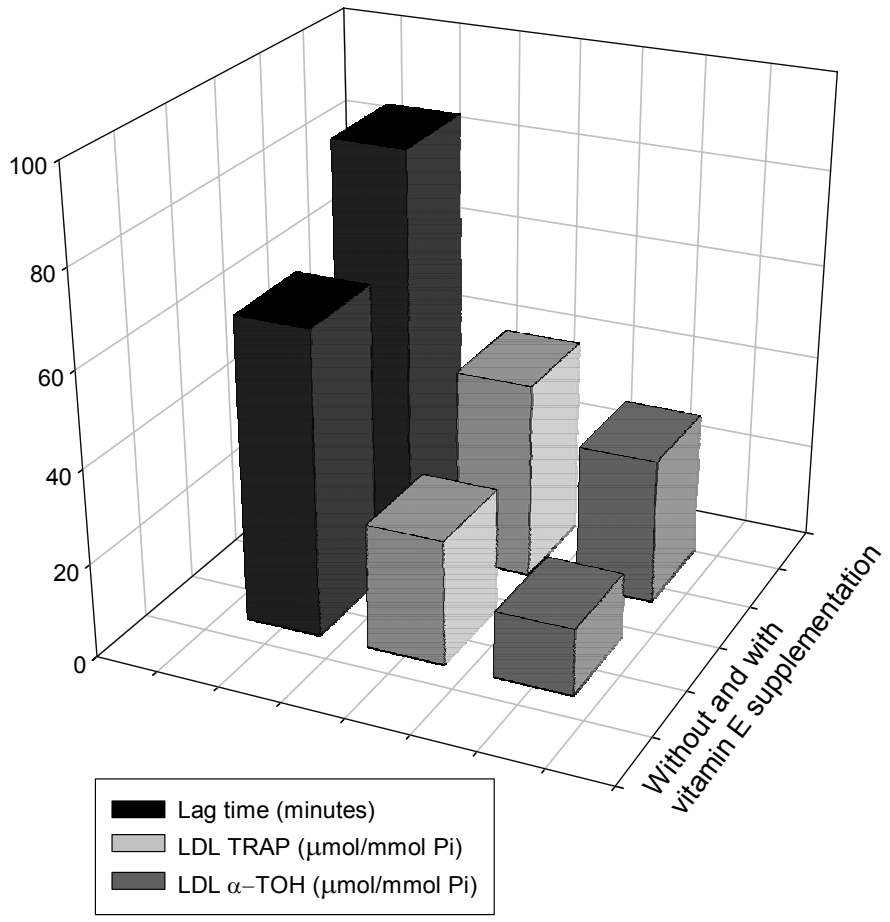
In Study III, the prolongation of ubiquinol exhaustion during oxidation was highly significant, while that of α–TOH was not. **** : $p<0.0001$; NS: not significant. Mean + SEM are presented (n=19).

Vitamin E supplementation increased LDL α–tocopherol concentration by 88% (Fig 10) and prolonged its total consumption time by 102%. Although LDL ubiquinol concentration remained steady during α–tocopherol supplementation, its total consumption time was shortened statistically significantly by 14%.

9.3.3 Copper-ion mediated oxidation

The lag time in copper-mediated oxidation assay was 5% longer ($p=0.02$) after ubiquinol supplementation with lovastatin therapy than after mere lovastatin. The maximal oxidation rate and maximal amount of conjugated dienes remained unchanged.

Figure 10. Effect of vitamin E supplementation on the lag time to propagated diene formation, LDL TRAP, and concentration of α–TOH in LDL.



The bars in the front row represent determinations before oral vitamin E supplementation and the bars in the back row those during the treatment (α–TOH 450 IU/d). During vitamin E supplementation, the lag time prolonged by 43%, LDL TRAP increased by 58%, and concentration of α–TOH in LDL increased by 88%. All changes presented were statistically highly significant ($p<0.0001$).

During vitamin E supplementation, the lag time increased by 43% from baseline (Fig 10), while the rate of maximal oxidation decreased by 16% and the amount of conjugated dienes decreased by 6%. All changes were statistically significant.

9.4 Comparison of metal-ion dependent and independent oxidation (V)

The repeatability of metal-ion dependent oxidation measurements was slightly better than that of AMVN induced oxidation studies. The coefficient of variation within the TRAP assay was 4.4% and between TRAP assays 5.9%. Alpha-tocopherol concentration in LDL did not correlate significantly with the lag time or LDL TRAP at the end of the phases without any antioxidant supplementation. Nevertheless, a statistically significant although not very impressive correlation of LDL TRAP with α–TOH concentration in LDL ($r=0.50$) became evident, when correlation analysis was carried out in-

cluding the periods with lovastatin, vitamin E and vitamin E with lovastatin treatments. During vitamin E supplementation, LDL was more resistant to oxidation according to both metal-ion dependent and independent oxidation method with high radical flux (Fig 10). However, during lovastatin therapy, the individual changes in lag time and LDL TRAP were quite incoherent.

10. DISCUSSION

The present study was focused on the effects of different clinical interventions on the resistance of LDL against oxidation (Table 11). According to Study I, preventive counselling was followed by an evident but transient improvement in LDL antioxidative capacity. In Studies II and IV, lovastatin therapy diminished antioxidant concentrations in LDL and shortened their consumption time. It also slightly shortened the lag time to the start of propagated diene formation. Lovastatin therapy diminished the maximal amount of conjugated dienes, while the maximal rate of oxidation did not change significantly.

Table 11. Effects of different treatments on serum lipids and the resistance of LDL against oxidation.

	Lovastatin	Ubiquinone	α–TOH	Education
LDL cholesterol	▼▼▼	=	=	=
HDL cholesterol	▲	=	▲	=
Triglycerides	▼▼	=	=	=
Lag time	▼	▲	▲▲	ND
LDL TRAP	=	ND	▲▲▲	▲▲ → =

Symbols: Increase ▲, ▲▲, or ▲▲▲; Decrease ▼, ▼▼, or ▼▼▼; No change =; Not determined ND.

Ubiquinone or α–tocopherol treatment in Studies III and IV increased the concentration of the supplemented antioxidant in LDL and prolonged its total consumption time. However, during α–tocopherol supplementation the total consumption time of ubiquinol was shortened. The lag time was prolonged to some extent during ubiquinone supplementation and considerably during α–tocopherol supplementation. The methodological comparisons of copper-mediated and metal-ion independent oxidation in Study V showed, that although the lag time and LDL TRAP behaved similarly during α–tocopherol supplementation, their response to lovastatin therapy was incoherent.

10.1 Patient allocation and interventions

In Study I, the allocation of patients was carried out according to municipalities, not in random. It is possible, that the allocation could have led to some bias between the groups. However, both groups included patients from towns with a hospital and from the countryside. The variables related to the CABG, life style, medications or earlier diseases did not differ between the groups.

The quality of group education was not formally evaluated. So, the immediate impact of education on the intervention group is not known. The patients were followed in blocks randomised in the beginning of the trial. This was done to avoid information change between the groups and possible unconscious biasing of results by the authors. In a trial with open design, it can never be entirely excluded that the investigators might personally have affected the results.

The treatments in Studies II to IV were given randomly, with a double-blind cross-over protocol. Lovastatin tablets and ubiquinone capsules were identical with their placebos in size, shape and colour. The rigorous protocols did not give any possibility to either the patients or the investigators to have systematic subjective influence on the results. No carry-over effects were noted in statistical analysis.

Lovastatin (60 mg daily) was taken in three doses of 20 mg in order to obtain good efficacy with minimal adverse effects. It has been shown more than 10 years ago, that lovastatin, as a relatively short-acting HMG-CoA reductase inhibitor, is somewhat more effective, when the daily dosage is divided than when it is given once daily (Bradford et al. 1991). Ubiquinone and vitamin E dosages were in the range of earlier supplementation studies.

10.2 Effects of group education on diet and physical activity (I)

During the follow-up of Study I, patients in the education group seemed to increase their physical activity from baseline. However, the difference between intervention and control groups was not statistically significant according to ANOVA with repeated measurements. No differences in the dietary behaviour of the two groups were noted. In spite of that, a significant and systematic difference in body mass index was seen during the 12-month follow-up.

Evaluation of physical activity was carried out according to principles studied at UKK Institute for Health Promotion Research, Tampere. The most important questionnaire with three questions described leisure time fitness. According to principles published earlier, it formed the basis on calculation of the physical energy expenditure index (Haapanen et al. 1996). In general, the questionnaire of leisure time physical activity is validated quite well. However, no information is available on its sensitivity to changes in different populations or in different time periods. Hence, it is possible, that after exclusions and a few drop-outs the group size might not have been large enough to reveal definite differences in leisure time physical activity.

Nutritional habits were evaluated with a 4-day dietary record and computerised calculation of daily nutrients. The evaluated weekdays were the days from Friday to Monday, i.e. they included the week-end in which alcohol consumption in Finland is on average higher than during the rest of the week. Using a 4-day dietary record, it is impossible to detect significant changes in nutrients acquired at long intervals, such as β−carotene. However, changes in foodstuffs common in the daily diet are easier to observe.

10.3 Effects of group education on LDL antioxidative defence (I)

In Study I, preventive group education had a positive but transient effect on LDL antioxidative capacity at the 8-month time point. The difference of 25% in LDL TRAP between the groups was greater than that of 9% measured in patients on pravastatin *vs.* placebo in the 3-year Kuopio Atherosclerosis Prevention Study (KAPS) (Salonen R. et al. 1995). Determination of LDL TRAP was carried out in these studies with the same method.

According to the dietary records, no significant difference was observed between the groups, which could explain the variation in the resistance of LDL against oxidation during the follow-up. Instead, the changes in LDL TRAP seemed to reflect those in physical activity, which tended to increase in the intervention group towards the 8-month control visit. A simultaneous increase in LDL TRAP means, that circulating LDL was less prone to be oxidised. However, according to literature the effect of exercise on oxidation is not unambiguous. Firstly, not many studies have been carried out to evaluate the long-term effects of physical activity on the resistance of LDL against oxidation. Secondly, the results may vary depending on the oxidation method used. According to methods determining oxidation in the hydrous phase, exercise may increase capacity of resistance against oxidation (Vasankari et al. 1997,

Liu et al. 1999). On the other hand, those methods comparable to LDL TRAP and studying changes in the lipid phase have indicated both decreased and increased resistance of LDL to oxidation related to physical activity (Ginsburg et al. 1996, Liu et al. 1999, Vasankari et al. 2000). Large, buoyant LDL particles are relatively resistant to oxidation (de Graaf et al. 1991, Tribble et al. 1995). The already known relation between increased physical activity and a favourable LDL profile with a lot of larger particles (Halle et al. 1999) together with the finding, that LDL particle size is associated with increased resistance of LDL to oxidation (de Rijke et al 1997), may explain the possible relation between physical activity and LDL TRAP found in Study I.

It is not likely, that the effect of group education in Study I was too short-lasting, since the systematic changes in the body mass index (BMI) and white blood cell count became statistically significant only after one year. The baseline visit and the 1-year control were scheduled for the coldest winter. First control after the counselling took place after the winter time and the second one in the early autumn reflecting nutritional and physical habits of the late summer. Seasonal variation may at least partly explain low physical activity and reduced LDL antioxidative capacity in winter time (Mänttäri et al. 1993). If the patients are not already physically active, after counselling it is easier to make positive changes in the summer time than during the cold winter. The reason for the decreased trend in LDL TRAP after eight months remains still open, since it can not be explained by the changes in physical activity only. As described in Chapter 10.2, the questionnaire used might have been too crude to evaluate all seasonal changes of physical activity in a small patient group like that in Study I.

10.4 Comparison of different oxidation methods (V)

LDL TRAP is a method analysing the total antioxidative capacity of LDL. The assay was implemented by late docent Timo Metsä-Ketelä. Study V was part of its validation, in which the oxidation methods used in Study I (LDL TRAP) and in Studies II to IV (the lag time) were compared with each other. In Study V, the repeatability of LDL TRAP was quite good although not entirely as predictable as that of copper-mediated oxidation. The comparison of different methods showed, that they do not reflect the antioxidative defence of LDL identically. This finding is congruent with those of earlier studies, in which metal-ion dependent and independent oxidation methods were compared (Frei and Gaziano 1993, Thomas et al. 1994, Porkkala-Sarataho et al. 1998). The correlation of α–tocopherol concentration in LDL with the oxidation parameters of LDL in both metal-ion dependent and independent oxidation became evident only, when measurements during vitamin E supplementation were taken into account. These results are in concordance with earlier studies confirming the findings, that α–TOH concentration in non-supplemented LDL does not account for most of the resistance of LDL against oxidation (Frei and Gaziano 1993, Kontush et al. 1994, Miller et al. 1995, Reaven et al. 1995, Porkkala-Sarataho et al. 1998).

10.5 Effects of lovastatin therapy on LDL antioxidants and their consumption during oxidation (II, IV)

In a hydrous environment like the bloodstream, water-soluble antioxidants are an important means to prevent oxidative damage. Vitamin C (ascorbic acid) and urate are examples of such first line antioxidants (Table 12) (Retsky et al. 1993, Ma et al. 1994, Lehr et al. 1995, Nyyssönen et al. 1997). Besides them, there are plenty of different antioxidants in the blood like albumin, bilirubin or protein-SH groups (Deigner et al. 1992, Neuzil and Stocker 1994, Wu et al. 1994, Ferguson et al. 1997).

Although radical attack may occur for example in the form of oxidised fats through intestine or after smoking through the lungs, and hydroperoxides can to some extent be found in plasma, the most likely site for oxidative modification of LDL is the subendothelial space of the arterial wall (see Chapter 4.2) (Ylä-Herttuala et al. 1989, Chirico et al.1993, Staprãns et al. 1994,

Oguogho et al. 2000). In that environment the importance of lipid-soluble antioxidants, such as ubiquinol or α–tocopherol, is emphasized as the second-line defence (Tribble et al. 1994). Because the therapy with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors is of increasing importance (Shepherd et al. 1995, Sacks et al. 1996, Downs et al. 1998), its effects on antioxidant concentration and the resistance of LDL against oxidation may have clinical implications. Characteristics of some important water- and lipid-soluble antioxidants are presented in Table 12.

Table 12. Properties of selected antioxidants according to Esterbauer et al. (1990), Stocker et al. (1991), Ma et al. (1994), Stocker (1994), Witting et al. (1995), Thomas et al. (1996), Neuzil et al. (1997), and Nyyssönen et al. (1997).

Antioxidant	Water-/lipid-soluble	Advantages	Disadvantages
Ascorbic acid	Water-soluble	Powerful and common antioxidant in nature Turns radical attack from lipid to water phase	Evidence on the effect on the prognosis of CHD is scarce
Urate	Water-soluble	Clinically the most remarkable antioxidant in the blood	Synthesis is associated with the generation of free radicals
Ubiquinol	Lipid-soluble	First and maybe the most Powerful antioxidant in LDL Can be synthesised de novo Can be regenerated from oxidised to reduced form	Not very common (only one molecule in 1-2 LDL particles) No prognostic studies
α–Tocopherol	Lipid-soluble	Quantitatively the most common antioxidant vitamin in LDL Effective antioxidant against high radical flux	Double-edged sword: anti- and pro-oxidant No good results in prognostic trials so far

Lovastatin therapy decreases significantly the ubiquinol concentration in LDL (Elmberger et al. 1991, Ghirlanda et al. 1993, Watts et al. 1993, Bargossi et al. 1994, Laaksonen et al. 1994, Davidson et al. 1997). In Studies II and IV, the concentrations of ubiquinol and α–tocopherol in LDL were decreased during lovastatin treatment (with or without vitamin E supplementation), when compared to those after lovastatin-placebo period, although only the change in LDL ubiquinol was statistically significant. Without vitamin E supplementation, both antioxidants were oxidised faster after lovastatin therapy than without it. With vitamin E supplementation, ubiquinol was exhausted faster than without supplementation, and the further shortening of its exhaustion time by lovastatin was not statistically significant. In the light of earlier *in vitro* studies it does not seem plausible, that a direct pro-oxidative effect of lovastatin itself could explain the findings (Giroux et al. 1993, Chen et al. 1997, Hussein et al. 1997, Aviram et al. 1998, Tanaka et al. 2001).

The influence of statin treatment on the anti-

oxidative defence of LDL might be mediated through an effect on the synthesis of ubiquinol and thus antioxidant concentration in LDL (Table 8 B) (Laaksonen et al. 1994, Davidson et al. 1997). The already impaired antioxidative defence in the CHD patient group could decrease below some critical level leading to a further decrease in the resistance of LDL to oxidation because of lovastatin treatment. Calculations based on the results of Study III support this possibility. The patients had on average 29 molecules of ubiquinol in 100 LDL particles before lovastatin treatment and only 21 molecules after lovastatin, while in healthy people the numbers vary from 50 to 100 molecules in 100 LDL particles (Stocker 1994). When ubiquinol supplementation was added to lovastatin therapy, ubiquinol concentration increased to a more physiological level, *i.e.* on average 95 molecules in 100 LDL particles. The average numbers of α–TOH molecules in 100 LDL particles were 535, 445, and 504, respectively. These numbers of α–TOH were smaller than in healthy volunteers, which range from 600 to 1200 molecules per 100 LDL particles (Esterbauer et al. 1990, Stocker 1994).

AMVN is an azo-compound, which produces free radicals at a constant rate (Niki et al. 1991). Hence, it is a good pro-oxidant for *in vitro* and *ex vivo* studies. As a very toxic compound it has no value in *in vivo* oxidation. Studies carried out with azo-compounds may still have clinical relevance for it is thought, that *in vivo* LDL oxidation is not an inheritent event, but rather initiated when the particle is attacked by radicals generated by cellular or non-cellular systems in the body (Tribble 1995). Once the cascade of lipid peroxidation has begun, the process is no more dependent on the initial radical-producing agent. For example, initiation of oxidation mediated by copper-ions is dependent on the presence of hydroperoxides, but the susceptibility of LDL to oxidation through radical chain reaction is also related to its antioxidant content (Frei and Gaziano 1993, Tribble et al. 1994).

Because the recovery of LDL in the isolation procedure is variable, antioxidant concentrations in LDL were standardised with LDL phosphorus. Moreover, because there is only one apoB-100 molecule in one LDL particle, division of antioxidant concentrations by apoB-100 should reflect the amount of LDL-bound antioxidants. However, in correlation calculations the results remained the same, even if antioxidant concentrations were proportioned to apoB-100.

Results may be partly explained by variable recovery of LDL in the isolation, while radical production during the oxidation of lipids remained constant. When studying correlations of different oxidation methods in Study V, the calculated consumption time of α–tocopherol and ubiquinol was divided by LDL phosphorus. In this analysis, the consumption time of α–TOH correlated well with LDL TRAP and the lag time. Antioxidant depletion in Studies II to V was carried out in conditions with high radical flux. It can be argued, that such an intense oxidative stress may not be biologically relevant. Because of the lack of prospective long-term clinical follow-up studies comparing clinical value of different oxidation methods, this argument remains to be answered in the future. Moreover, antioxidant concentration in LDL and its consumption time are only mediatory variables between lovastatin treatment and peroxidation of LDL. Therefore these results should be interpreted cautiously and only together with other markers of LDL oxidation.

10.6 Effects of lovastatin therapy on the resistance of LDL to copper-mediated oxidation (II, IV)

In Studies II and IV, the initial phase of the antioxidative defence in LDL (*i.e.* the lag time) was impaired by lovastatin treatment. These results are different from earlier findings. On the other hand, the indifferent or favourable results on oxidation rate and maximal amount of conjugated dienes were similar to those in earlier studies (Kleinveld et al. 1993, Bredie et al. 1995, Salonen R. et al. 1995, Zhang et al. 1995, Leonhardt et al. 1997).

Differences in the protocols of Studies II and IV compared to those published earlier

Vitamin E supplementation prolongs the lag time of conjugated diene formation (Esterbauer et al. 1991, Jialal and Grundy 1992 and 1993, Jialal et al. 1995, Devaraj et al. 1997). Whether this could be affected by lovastatin therapy, had not yet been studied. One reason for differences in results of Studies II (*i.e.* lovastatin *vs.* placebo) and IV (lovastatin with vitamin E *vs.* lovastatin-placebo with vitamin E) compared to those from earlier studies may be the fact, that methods used for oxidation measurements in *ex vivo* trials have been diverse. Unvalidated methods, slow isolation of LDL, long-lasting high-flux oxidation for up to 24 hours, and uncontrolled settings in some of the previous studies are other possible reasons which may explain some of the discrepancies (See e.g. 4.4.2. Statin treatment, Effect on antioxidant capacity of LDL, Animal experiments). In an uncontrolled human study with 10 patients taking fluvastatin for 24 weeks, the lag time was exceptionally prolonged from 80 to 200 minutes (Hussein et al. 1997). This result may be due to many confounding factors, like seasonal change in the diet or physical activity. In other, controlled studies, statin therapy has not had any significant effect on the lag time (Kleinveld et al. 1993, Bredie et al. 1995, Salonen R. et al. 1995, Zhang et al. 1995, Leonhardt et al. 1997). Dissimilarities of the protocols in Studies II and IV compared to the earlier controlled human studies, which might explain why some of the results were different, are outlined herein.

Earlier controlled human studies used other medication than lovastatin as the drug therapy. The studies were carried out with simvastatin (Kleinveld et al. 1993, Bredie et al. 1995), pravastatin (Kleinveld et al. 1993, Salonen R. et al. 1995, Zhang et al. 1995), and fluvastatin (Leonhardt et al. 1997). The effects on LDL oxidation might vary from statin to statin, but no larger comparative studies have been published yet. Kleinved et al. (1993) gave 40 mg of simvastatin (n=12) or pravastatin (n=11) and found no evident difference in the lag time. However, the study was too small even to show the different efficacies of simvastatin and pravastatin in reducing the level of LDL cholesterol.

Effect on LDL oxidation may be related to the efficacy of statin therapy, which has not been uniform in different studies. In the study of Kleinveld et al. (1993) using efficient medication, LDL cholesterol was even after treatment as high as 4.96 mmol/l due to its high initial level. Except that study, the absolute and relative cholesterol-lowering effect of statin therapy was most impressive in Studies II and IV, where the mean LDL cholesterol level decreased to 2.65 and 2.75 mmol/l, respectively.

The copper-mediated oxidation method is based on that described originally by Esterbauer et al. (1989). It is widely used and technically well validated, although no prospective trials have been carried out to study its relevance as a prognostic indicator of CHD. Possible differences in the analytical practices hardly explains the differences of the results in the studies. Instead of that, the isolation procedure may give one explanation for the differences. In Studies II to V, LDL isolation was carried out using rapid ultracentrifugation for 30 minutes to avoid consumption of antioxidants (Chung et al. 1986). In some of the earlier studies, centrifugation has lasted for many hours predisposing LDL to oxidation before analysis (Kleinveld et al. 1993, Bredie et al. 1995).

After venipunctures, the samples were processed without delay and stored at –70 to –85 ^{o}C. The oxidation measurements were carried out several months after all treatment phases. In spite of careful handling, it is possible that concentrations of reduced antioxidants in LDL were diminished by so called auto-oxidation during the storage before analyses were carried out. Studies II to IV were double-blinded, randomised cross-over trials, where lovastatin treatment was partially associated with more unfavourable results than placebo. If auto-oxidation factually was a part of the finding, that LDL samples after lovastatin therapy were more susceptible to the early phase of oxidation than those after placebo, the unfavourable ef-

fects of lovastatin *ex vivo* have relevance *in vivo*.

Possible reasons for defective effect of lovastatin

As described above, effective statin therapy for CHD patients, potentially already impaired antioxidative defence of patients, fast isolation of LDL, possible auto-oxidation of the samples during the delay before analysis, and placebo-controlled, critical, cross-over study design with proper statistical analysis are possible reasons for differing results in Studies II and IV compared to earlier human studies. In the following, possible reasons for increased susceptibility of LDL to the initial phase of oxidation during lovastatin therapy will be discussed.

The influence of statin therapy on LDL oxidation *in vivo* extends beyond the decrease of cholesterol concentration. Lovastatin therapy is followed by an impressive decrease in the "by-products" of cholesterol synthesis (*i.e.* coenzyme Q_{10}), when the therapy is vigorous enough (Ghirlanda et al. 1993, Davidson et al. 1997). A decrease of ubiquinol concentration in LDL has been suspected to lead to a decreased resistance of LDL against oxidation (Willis et al. 1990, Elmberger et al. 1991). According to the results of Studies II and IV, where ubiquinol concentration was decreased and its consumption time and also the lag time shortened, this opinion is credible.

In principle, other changes might also explain the findings. Statin treatment could influence the subfraction profile or other biochemical composition of LDL. In theory, there might be a shift in the age distribution of LDL particles during the first weeks of statin treatment; or medication itself could have some direct pro-oxidative effects on LDL. These alternatives are outlined below.

It has been shown earlier, that small dense LDL is more susceptible to oxidation than buoyant LDL (de Graaf et al. 1991, Tribble et al. 1995, de Rijke et al. 1997). Because lovastatin does not affect all components of an LDL particle in the same manner, one could presume, that a change in the subclass pattern of LDL explains differences in LDL oxidation. LDL subclasses were studied in the Angiographic Monitored Atherosclerosis Regression Study (MARS) (n=220) (Mack et al. 1996). Although lovastatin (80 mg daily) decreased least the densest LDL subfraction, there was as a whole a shift to larger, more buoyant LDL particles during the medication (Mack et al. 1996). In an earlier study, a change in plasma triglycerides reflected the qualitative changes of LDL during lovastatin treatment (40 to 80 mg daily). If plasma triglycerides decreased, there was a shift to a more buoyant profile and *vice versa* (Tilly-Kiesi 1991). In Studies II and IV, lovastatin decreased the mean triglyceride level by 17% and 28% compared to placebo. Hence, a shift to a more buoyant LDL subfraction profile is possible, although no qualitative measurements of LDL subfractions were carried out. According to the earlier findings (Tilly-Kiesi 1991, Mack et al. 1996), the decrease of the initial antioxidative defence of LDL during lovastatin therapy could hardly be a consequence of unfavourable qualitative changes in LDL subfraction profile.

In the study mentioned above (Tilly-Kiesi 1991), the ratio of free cholesterol to apoB-100 in LDL decreased by 12%, while the phospholipids to apoB-100 ratio fell less than 10% during lovastatin treatment. Unesterified cholesterol is mainly located in the outer surface layer of an LDL particle (Hevonoja et al. 2000). It has been hypothesised, that cholesterol itself could act as an antioxidant (Smith 1991). Free cholesterol was not quantifed in the present studies. However, the decrease in LDL cholesterol level by 41% and 45% and the smaller decrease in apoB-100 by 31% and 33% (lovastatin *vs.* lovastatin-placebo) might mirror a decrease in the content of free cholesterol in LDL particles. In the study of Tilly-Kiesi (1991), the decrease in the cholesteryl ester to apoB-100 ratio was the very same 12% as the decrease in the free cholesterol to apoB-100 ratio. If the hypothesis of Smith is correct, a decrease in the amount of free cholesterol could weaken the initial protection of LDL against a peroxidative

insult. On the other hand, the decrease of cholesteryl esters in the core of LDL might diminish the oxidisability of LDL during the later phases of oxidation.

In hyperlipidaemia, LDL particles remain in the circulation longer than in normolipidaemia (Arca et al. 1994). Furthermore, older lipoproteins *per se* are more susceptible to oxidation than younger ones, and hyperlipidaemic patients also have lipid peroxidation products in plasma as measured *ex vivo* (Chirico et al. 1993, Walzem et al. 1995). As a potential artefact related to treatment periods of a limited duration, the change in the age of LDL particles could be one explanation for the results in Studies II and IV. The treatment phases of six weeks were possibly long enough to allow changes in the age distribution of LDL. During the first weeks of statin treatment, LDL pool might shift towards older ones, because of depletion of younger particles due to inhibition of cholesterol synthesis. However, it has been shown quite convincingly, that the cholesterol-lowering effect of statins is mediated by enhanced uptake of LDL by hepatic LDL-receptors (Brown et al. 1981, Kovanen et al. 1981), which in turn shifts the age distribution towards younger particles. Results from clinical studies determining LDL kinetics have been in favour of the latter alternative, since increased clearance of LDL particles was primarily attributable to the cholesterol-lowering effect of lovastatin and pravastatin treatments (Kovanen et al. 1981, Arca et al. 1994, Toto et al. 2000).

In vitro studies using incubation of LDL with statins have shown, that statins themselves or their metabolites have some direct chemical antioxidative effect on LDL (Aviram et al. 1991 and 1992, Hoffman et al. 1992, Giroux et al. 1993, Chen et al. 1997, Hussein et al. 1997, Aviram et al. 1998, Tanaka et al. 2001), although the results have been somewhat controversial (Hoffman et al. 1992, Diebold et al. 1994). Mevalonate is also the precursor of non-steroidal derivatives, whose synthesis preceeds the superoxide generation or radical formation in neutrophils and macrophages mediated by rho-related proteins like rac1 and rac 2 (Glomset and Farnsworth 1994) (see also Fig 1). Therefore, HMG CoA reductase inhibitors should have an effect on this metabolic process. According to *in vitro* studies, statins or their metabolites have direct effects on the production of free radicals by macrophages together with the ability to scavenge free radicals (Giroux et al. 1993, Aviram et al. 1998). However, published results concerning this relation still seem to differ between studies: some observed pro-oxidative (Böger et al. 1997) and some antioxidative effects (Giroux et al. 1993, Singh et al. 1997) of statins.

To get back to the question, whether lovastatin therapy acts antioxidatively or pro-oxidatively, maybe it is most important to keep in mind, that the interpretation of measurements differ from each other. The lag time as well as antioxidant depletion mirror the initial phase of LDL oxidation. Therefore the effect of intense lovastatin therapy may at the same time be *unfavourable,* when measured by the lag time or antioxidant depletion, and *indifferent* or *favourable,* when measured by oxidation rate and maximal amount of dienes reflecting later phases of peroxidation. Furthermore, all the time periods used in *in vitro* and *ex vivo* studies are short, when compared to the potentially long residence times of LDL in the arterial intima, where oxidation of LDL takes place during atherogenesis.

10.7 Effects of ubiquinone and α–tocopherol supplementation on LDL antioxidants and their consumption during oxidation (III, IV)

As in earlier studies, ubiquinone supplementation in Study III had no significant effect on α–TOH concentration in LDL (Mohr et al. 1992, Thomas et al. 1996, Kaikkonen et al. 1997, Kaikkonen et al. 2000). However, administration of vitamin E influenced the kinetics of ubiquinol during oxidation. The depletion time of ubiquinol shortened by 14%. This result supports earlier findings on tocopherol-mediated peroxidation (Stocker 1994, Witting et al. 1995, Kaikkonen et al. 2000).

In Study III, high LDL ubiquinol concentration after supplementation was associated with faster, although longer-lasting consumption of the antioxidant. This unexpected phenomenon was very consistent both within-subjects and between-subjects. Every subject in Study III had a faster ubiquinol consumption rate after oral CoQ_{10} loading than without it. The reason is highly speculative. Ubiquinol is consumed fast as the first antioxidant in LDL, whenever it is available (Stocker et al. 1991). It may form a reserve to protect LDL by sparing other antioxidants like α–tocopherol (Suarna et al. 1993). The consequence of the principle "what you have, you consume" would be a non-linear consumption of the antioxidants. However, in Studies II to V with high radical flux, the 10% sparing effect of α–TOH during ubiquinone supplementation was not statistically significant.

Ubiquinone supplementation might make the ratio of reduced ubiquinol to oxidised ubiquinone lower in the LDL particle and especially in the solution of LDL in chloroform:methanol used in the laboratory experiments. Reduced ubiquinol works as an antioxidant in the LDL particle partly by carrying the radical attack from the lipid core to the surface and the water phase (Stocker 1994). By doing so, it can be reduced again for instance by vitamin C or urate. Lipid peroxidation by AMVN in Studies I to V was carried out in conditions without such interface between the lipid and water phase *i.e.* in the absence of vitamin C and urate, which could be a reason for the fast exhaustion of ubiquinol in the experiment. If LDL solution can not upregulate a refreshing system of ubiquinol from the semiquinone radical or from ubiquinone in one way or another, the net result would be a lower ubiquinol:ubiquinone ratio and faster consumption of ubiquinol during CoQ_{10} supplementation.

10.8 Effect of ubiquinone and α–tocopherol supplementation on LDL antioxidative defence (III, IV)

In Study IV, supplementation of vitamin E (300 mg = 450 IU daily) was associated with a significant prolongation of the lag time and an increase in LDL TRAP (analysed in Study V) indicating improved resistance of LDL against high-radical flux. These findings are congruent with those from earlier studies (Esterbauer et al. 1991, Jialal and Grundy 1992 and 1993, Jialal et al. 1995, Devaraj et al. 1997, Porkkala-Sarataho et al. 2000, Upritchard et al. 2000). Interestingly, vitamin E supplementation was also associated with an exceptional but distinct and statistically significant increase in both apoA-I and HDL-cholesterol (Jialal and Grundy 1992, Brown et al. 2001). While HDL inhibits LDL oxidation (Reaven et al. 1993, Navab et al. 2000a and 2000b), this finding is probably related to the impaired antioxidative defence of CHD patients. Hence, the net effect of vitamin E supplementation on this special patient population might have been beneficial *in vivo*.

The possible positive effect of ubiquinone supplementation on LDL antioxidative defence *in vivo* has been questionable. In earlier studies carried out in Finland, ubiquinone supplementation for healthy individuals did not result in any positive effects on the lag time of conjugated diene formation during copper-mediated oxidation (Lilja et al. 1996, Kaikkonen et al. 1997). However, delicate effects are still possible as established in studies carried out *in vitro* and *in vivo* (Mohr et al. 1992, Thomas et al. 1996). Study III was the first published trial concerning ubiquinone supplementation during effective lovastatin treatment. The results suggest, that ubiquinone supplementation may have beneficial effects during intense statin therapy. Although consumption of ubiquinol during oxidation took place at a faster rate than without supplementation, it lasted longer and was associated with a prolongation of the lag time. The significance of ubiquinone supplementation on LDL oxidisability especially during the treatments was supported by the results of multiple linear regression analysis. When ubiquinol concentration in LDL and the rate of ubiquinol consumption were included in the analysis, they explained (r^2) only 5% (NS) of the lag time during copper-mediated oxidation after the wash-out period. However, after lovastatin therapy they explained 35% (p=0.034) and

after lovastatin with ubiquinone 49% (p=0.004) of the lag time.

In Study III, the net effect of ubiquinone supplementation (180 mg daily) on LDL oxidation was positive. In theory, it might have important clinical consequenses in the future, if the goals of LDL-cholesterol level for CHD patients on statins will be set very low. However, since the validity of the oxidation hypothesis in atherogenesis still remains open and the findings after supplementation are based on a single study with a limited number of CHD patients, recommendation of supplementation can not be given at present.

10.9 Concluding remarks

According to earlier human studies, lovastatin therapy significantly decreases ubiquinol concentration in LDL (Elmberger et al. 1991, Ghirlanda et al. 1993, Watts et al. 1993, Bargossi et al. 1994, Laaksonen et al. 1994, Davidson et al. 1997). However, its effect on the oxidisability of LDL has remained controversial (Kleinveld et al. 1993, Bredie et al. 1995, Salonen et al. 1995, Zhang et al. 1995, Leonhardt et al. 1997). In Studies II and IV, lovastatin therapy decreased ubiquinol concentration in LDL, shortened its consumption time during AMVN-induced oxidation and also impaired the lag time during copper-mediated oxidation. Resistance of LDL against oxidation was, at least partially, restored by ubiquinone supplementation.

Results of LDL oxidation experiments depend on the radical flux used. In Studies I and V, high radical flux generated by AMVN was used to oxidise LDL lipids. In Studies II to V, the final concentration of copper sulfate was 1.67 μmol/l. In these conditions, α–tocopherol acts as an antioxidant and the significance of ubiquinol is not prominent (Esterbauer et al. 1990, Witting et al. 1995, Kaikkonen et al. 1997). However, when radical production is slower, α–tocopherol behaves as a pro-oxidant and the importance of ubiquinol as an antioxidant becomes more evident (Witting et al. 1995). From the clinical point of view, the oxidative status of individual patients vary e.g. because of infections, physical activity, dietary pattern or smoking habits. Long-term prospective studies have not yet revealed possible favourable effects of antioxidants on cardiovascular morbidity and mortality. Therefore, it is not justified to transfer the antioxidant supplementation regimens of separate oxidation studies, although with apparently favourable results, into general clinical practice.

11. CONCLUSIONS

The aim of this study was to evaluate whether resistance of LDL against oxidation in CHD patients is affected by comprehensive group education, lovastatin treatment, or antioxidant supplementation during effective statin therapy. Also, methodological comparisons of metal-ion dependent and independent oxidation assays were of special interest. The main results of the study were:

1. Multifactorial preventive group education given late after CABG was followed by a transient improvement in the antioxidative capacity of LDL. It may be related to the changes in physical activity. The changes in diet and plasma lipids were scarce, but the favourable effect on weight and circulating white blood cell count was more consistent.

2. Lovastatin therapy decreased ubiquinol and α–tocopherol concentration in LDL, and shortened their consumption time during metal-ion independent oxidation. During copper-mediated oxidation, the lag time was shortened by lovastatin in patients with and without α–tocopherol supplementation. The maximal amount of conjugated dienes was smaller after lovastatin than after placebo treatment. Thus, efficient lovastatin therapy had both pro- and antioxidative effects on LDL.

3. Ubiquinone supplementation during lovastatin treatment increased both ubiquinol and α–tocopherol concentration in LDL, lengthened their consumption time in oxidation, and also improved LDL antioxidative capacity slightly during metal-ion dependent oxidation.

4. LDL TRAP and copper-mediated oxidation methods were different in analysing samples of non-supplemented LDL and the changes in LDL after lovastatin treatment. However, changes in LDL after α–tocopherol supplementation were comparable when studied by either method.

In conclusion, multifactorial preventive group education is feasible as a part of routine hospital work. However, for CHD patients statin therapy should be started without delay not waiting for results of repeated education. Effective statin treatment behaves antioxidatively, possibly by a direct effect or by decreasing the amount of lipids in the core of LDL. It may also have pro-oxidative effects by reducing the amount of free cholesterol in the outer surface layer of LDL and by inhibition of the ubiquinol synthesis through the mevalonate chain. Ubiquinol concentration, kinetics and resistance of LDL against oxidation may, at least partially, be reverted by ubiquinone supplementation. The findings should be reassessed using effective new statins (like atorva-, rosuva-, or pitavastatin). Because the oxidation hypothesis of atherosclerosis and its clinical complications have not been proven yet, the antioxidant supplementation results can not at present be transferred into clinical practice.

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