EFFECTS OF DIETARY FAT OXIDATION PRODUCTS AND FLAVONOLS ON LIPOPROTEIN OXIDATION

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ABSTRACT

Various studies suggest that oxidative modifications of low density lipoprotein (LDL), and also other lipoproteins, have an important role in the development of atherosclerosis. In addition to the oxidation products formed endogenously, oxidised triacylglycerols (TAG) and oxysterols in the diet contribute to the oxidised lipoproteins found in circulation. However, studies on both the effect of oxidised dietary lipids on lipoprotein lipid oxidation and the reactions that modify oxidised fat after ingestion have been scarce. Studies on the effects of dietary antioxidants on the lipid oxidation *in vivo* and the risk of atherosclerosis have been inconclusive. More clinical trials are needed to test the importance of lipoprotein oxidation as a cardiovascular risk factor in humans.

In the recent years, various methods have been optimised and applied to the analysis of lipid oxidation products *in vivo*, and information on the molecular structures of oxidised lipids in plasma, lipoproteins and atherosclerotic plaques has started to accumulate. However, specific structures of oxidised TAG molecules present in these tissues and lipoprotein fractions have not been investigated earlier. In the orginal research in this thesis, an approach based on high-performance liquid chromatography-electrospray ionisation-mass spectrometry (HPLC-ESI-MS) and baseline diene conjugation (BDC) methods was used in order to investigate lipid oxidation level and oxidised TAG molecular structures in pig and human lipoproteins after dietary interventions.

The approach was optimised with human LDL samples, which contained various oxidation products of TAG. LDL particles of hyperlipidaemic subjects contained an elevated amount of conjugated dienes. In the pig studies, several oxidised TAG structures with hydroxy, keto, epoxy or aldehydic groups were found in chylomicrons and VLDL after diets rich in sunflower seed oil. Also, the results showed that oxidised sunflower seed oil increased the oxidation of lipoprotein lipids and their TAG molecules. TAG hydroperoxides could be detected neither in the small intestinal mucosa of the pigs fed on the oxidised oil nor in their chylomicrons or VLDL.

In the clinical studies, dietary flavonol aglycones extracted from sea buckthorn berries did not have an effect on lipoprotein lipid oxidation and other potential risk factors of atherosclerosis, but their absorption was demonstrated. Oil supplementation seemed to increase the bioavailability of the flavonols. Oxidised TAG molecules were detected in LDL particles of the subjects after both flavonol and control diets.

ABBREVIATIONS

ACN:DB	acyl carbon number:number of double bonds
ALD	(core) aldehyde
AUC	areas under the concentration-time curve
apoB	apolipoprotein B-100
BDC	baseliene diene conjugation
CI	chemical ionization
DEAE	diethylaminoethanol
DNPH	dinitrophenylhydrazine
EI	electron impact ionisation
ELSD	evaporative light scattering detector
ESI	electrospray ionisation
GC	gas chromatography
GPx	glutathione peroxidase
HDL	high density lipoprotein
HETE	hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
HPLC	high-performance liquid chromatography
HPODE	hydroperoxyoctadecadienoic acid
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LOX	lipoxygenase
MDA	malondialdehyde
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NP	normal-phase
oxLDL	oxidised LDL
PAPC	palmitoylarachidonoyl glycerophosphocholine
PC	glycerophosphocholine
PCOOH	phosphatidylcholine hydroperoxide
PLPC	palmitoyllinoleoyl glycerophosphocholine
RP	reversed-phase
sn	stereospecific numbering
SOD	superoxide dismutase
TAG	triacylglycerol
TBARS	thiobarbituric acid-reactive substances
TLC	thin-layer chromatography
TMS	trimethylsilyl
Tyr$^{\cdot}$	tyrosyl radical
vit C	vitamin C
VLDL	very low density lipoprotein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers referred to in the text by the Roman numerals I–V.

I Suomela, J.-P., Ahotupa, M., Sjövall, O., Kurvinen, J.-P., & Kallio, H. (2004) New approach to the analysis of oxidized triacylglycerols in lipoproteins. *Lipids* 39 (5): 507–512.

II Suomela, J.-P., Ahotupa, M., Sjövall, O., Kurvinen, J.-P., & Kallio, H. (2004) Diet and lipoprotein oxidation: analysis of oxidized triacylglycerols in pig lipoproteins. *Lipids* 39 (7): 639–647.

III Suomela, J.-P., Ahotupa, M., and Kallio, H. (2005) Triacylglycerol hydroperoxides not detected in pig small intestinal epithelial cells after a diet rich in oxidized triacylglycerols. *Lipids* 40 (4): 349–353.

IV Suomela, J.-P., Ahotupa, M., and Kallio, H. (2005) Triacylglycerol oxidation in pig lipoproteins after a diet rich in oxidized sunflower seed oil. *Lipids* 40 (5): 437–444.

V Suomela, J.-P., Ahotupa, M., Yang, B., Vasankari T., and Kallio, H. (2006) Absorption of flavonols derived from sea buckthorn (*Hippophaë rhamnoides* L.) and their effect on emerging risk factors for cardiovascular disease in humans. *J. Agric. Food Chem.* 54 (19), 7364–7369.

1 INTRODUCTION

Several studies suggest that oxidised low density lipoprotein (LDL) particles have an important role in the development of atherosclerosis (1–4). Based on the results various research groups have obtained during the last 20 years, oxidised chylomicrons/chylomicron remnants and very low density lipoprotein (VLDL) particles may have a similar role as potent contributors to atherogenesis (5–8). Most studies have concentrated on the analysis of oxidised cholesterol, glycerophospholipid and protein species found in the surface layers of the lipoprotein particles. There are very few studies on the oxidised triacylglycerol (TAG) species, which may also affect the physicochemical characteristics of lipoproteins, but which are found in the core of the particles. In TAGs, a fatty acyl group is esterified to each of the three hydroxyl groups of a glycerol molecule. Like in glycerophospholipids and cholesteryl esters, unsaturated fatty acid residues within the molecule are susceptible to both non-enzymatic and enzymatic oxidation, which may be of physiological importance.

Effects of dietary antioxidants on lipid oxidation *in vivo* and the risk of atherosclerosis have been widely studied, but the results have often been confusing and inconclusive (9). Several epidemiological studies, however, have indicated some degree of inverse association between flavonoid intake and coronary heart disease (10). While there has been a major focus on their antioxidant properties, flavonoids and their *in vivo* metabolites may also exert modulatory effects in cells through actions at protein kinase and lipid kinase signalling pathways. Some of these effects may be anti-inflammatory (11, 12). Some animal and human studies suggest that sea buckthorn flavonoids may lower blood viscosity and enhance cardiac function (13–18). In the present research, the effects of flavonols extracted from sea buckthorn (*Hippophaë rhamnoides* L.) on the potential risk factors of cardiovascular disease were studied.

Generally, studies of the dietary effects on lipoprotein oxidation at molecular level have been scarce. Also, there are only a few useful methods for direct measurement of lipoprotein oxidation *in vivo* (19). In the present studies, a novel approach based on high-performance liquid chromatography-electrospray ionisation-mass spectrometry (HPLC-ESI-MS) and baseline diene conjugation (BDC) methods has been used in order to investigate lipid oxidation in pig and human lipoproteins after dietary interventions.

2 LITERATURE REVIEW

2.1 OXIDATION HYPOTHESIS OF ATHEROSCLEROSIS

The narrowing and blockage of the arteries by a plaque (lesion) that consists of cholesterol, calcium, clotting proteins and other substances, is called atherosclerosis. When this process occurs in the arteries leading to the heart, the result is coronary heart disease. The hypothesis that oxidative modification of LDL plays an important role in the progression of atherosclerosis has been widely accepted (20), although some researchers suggest that there is a possibility that oxidative events are merely an injurious response to atherogenesis or a secondary event related to it (21). The oxidation hypothesis is supported by various *in vitro* and animal studies, as well as observational studies in man. Supporting clinical trial results, using antioxidant intervention, have been more elusive (22–24). These inconsistent results may not be surprising since LDL oxidation is induced by several oxidants and proceeds by several mechanisms, and the efficacy of antioxidants may depend on these factors (25). However, appropriate clinical trials are needed to properly test the importance of oxidation in the pathogenesis of atherosclerosis in humans (22).

Studies have demonstrated that several cell types can modify LDL *in vitro*, but the exact mechanisms by which they do so remain unsettled. The site and mechanisms whereby lipoproteins (**Table 1, Figure 1**) are oxidised have not been fully resolved (24, 26). In addition to the oxidation products formed endogenously by enzymatic and non-enzymatic reactions, also diet contributes to the oxidised lipoproteins found in circulation. It seems that oxidised dietary lipids are absorbed from the small intestine and/or they initiate a cascade of subsequent reactions that result in the increased oxidation of plasma lipoproteins (26).

Oxidised lipoproteins have been identified in atherosclerotic lesions in both animals and humans (27). Oxidised lipids found in human lesions include many of the products also generated during *in vitro* oxidation of LDL, such as oxidised fatty acids attached to glycerol/cholesterol backbone, oxysterols and oxidation products of α-tocopherol. Protein oxidation products are also present, particularly in more advanced lesions (28).

Table 1. Average composition and characteristics of human plasma lipoproteins (29, 30).

	Chylomicrons	**VLDL**[c]	**IDL**[d]	**LDL**[e]	**HDL**[f]
Origin	Intestine	Liver	VLDL	VLDL	Liver/intestine
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Density (g/mL)	<0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Composition (weight%)					
Triacylglycerols[a]	88	56	29	13	15
Cholesterol esters[a]	3	15	34	48	30
Free Cholesterol[b]	1	8	9	10	10
Phospholipids[b]	8	20	26	28	45
Protein	1-2	6-10	11	21	45-55
Apolipoproteins	B-48, A-I, A-IV, C1, C2, C3, E	B-100, C1, C2, C3, E	B-100, C1, C2, C3, E	B-100	A-I, A-II, E

[a] core lipids
[b] surface lipids
[c] very low density lipoprotein
[d] intermediate density lipoprotein
[e] low density lipoprotein
[f] high density lipoprotein

2.1.1 Oxidised LDL and the development of atherosclerotic plaques

Atherosclerosis is a disease that develops slowly over decades. Key events in early lesion development are the accumulation of plasma lipoproteins in the intima (the innermost layer of a blood vessel), increased expression of adhesion molecules, and the migration of monocytes into the intima. Monocytes are transformed into macrophages and they accumulate large deposits of lipoprotein-derived cholesterol. The accumulation of lipids leads to the formation of swollen macrophages, so called “foam cells”, which are the hallmark of early atherosclerotic lesions (28). Atherosclerotic lesions can be classified as initial lesions (classes I–II), fatty streak lesions (II–III), fibro-fatty lesions (IV–V) and complex/ulcerated lesions (V–VI), representing the spectrum of atherosclerosis. A lesion that is potentially symptom-producing is called an atheroma (31).

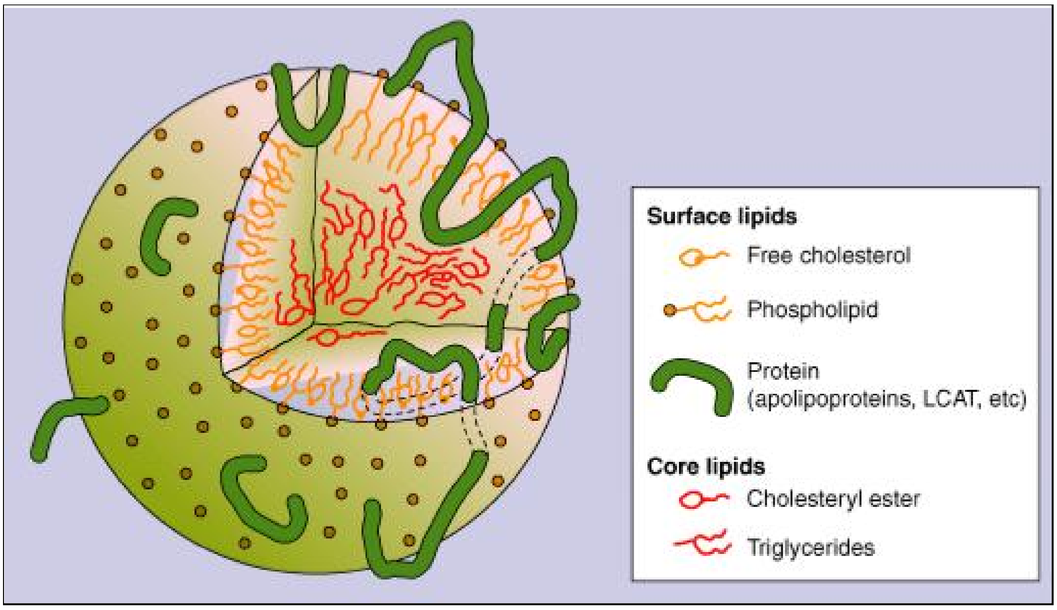


Figure 1. General lipoprotein structure. LCAT, lecithin:cholesterol acyltransferase. Reproduced with permission from Durrington PN. Hyperlipidaemia: Diagnosis and Management. 2nd edition. London: Butterworth-Heinemann; 1995 (32).

According to the oxidation hypothesis, LDL is subject to oxidative modifications of the lipid and protein components of the particle. Initial modification of LDL results in mildly oxidised particles that are no longer recognised by LDL receptors. Hydroperoxides of cholesteryl esters, glycerophospholipids and TAGs are found in these particles. Greater oxidation results in the accumulation of more advanced oxidation products like free and esterified lipid aldehydes, and in the modification of apolipoprotein B-100 (apoB) (4, 28). Receptor-mediated uptake of LDL, the major carrier of plasma cholesterol, is normally suppressed through the down-regulation of LDL-receptor expression in response to an increasing intracellular cholesterol level. The modified LDL particle is still taken up by macrophages, but via receptors whose expression is not controlled by cholesterol loading. These receptors include scavenger receptor CD36 and scavenger receptor class A. The uncontrolled LDL uptake leads to the formation of the foam cells, which are present in the earliest detectable lesions (**Figure 2**) (4, 28, 33).

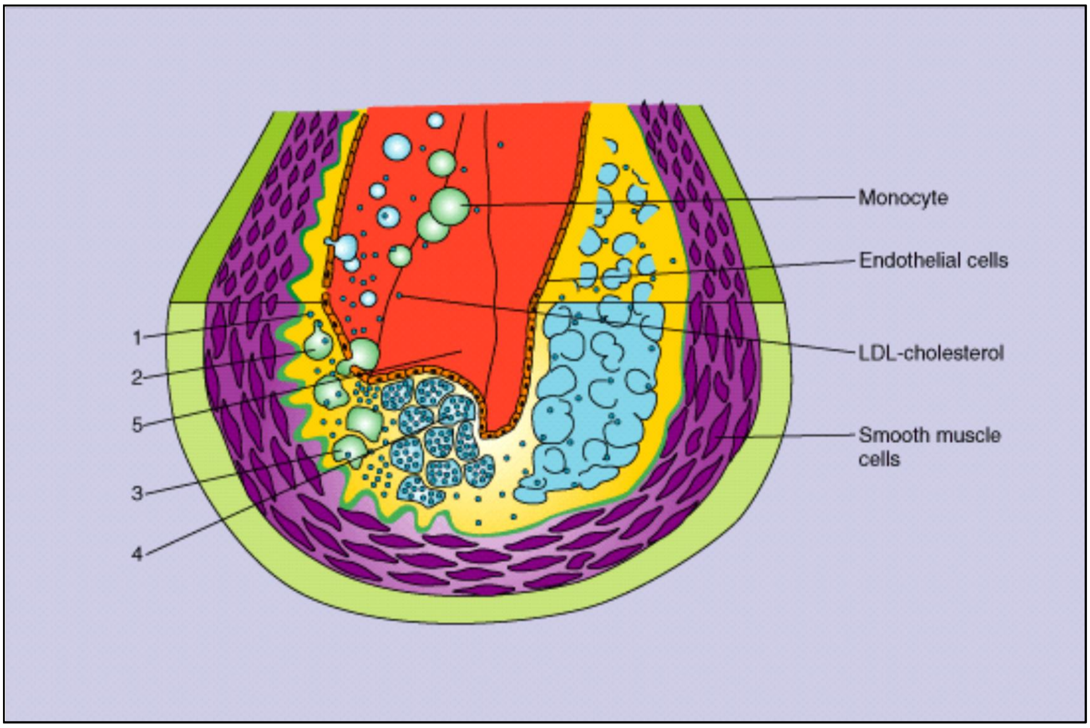


Figure 2. The atherosclerotic process based on the oxidation hypothesis. 1, low density lipoproteins (LDL) become oxidised. 2, circulating monocytes are recruited to the arterial intima by chemoattractants, including oxidised LDL. 3, these monocytes undergo differentiation, becoming macrophages, which are scavenger cells that recognise and accumulate oxidised LDL. 4, the lipid-laden macrophages then become foam cells, which cluster under the endothelial lining to form a bulge into the artery. 5, this bulge is called a fatty streak and is the first overt sign of atherosclerotic change (21).

2.1.1.1 Potentially proatherogenic effects of oxidised LDL

In addition to the promotion of foam cell formation, oxidised LDL (oxLDL) induces the expression of chemotactic factors that attract inflammatory cells into the arterial wall, and is also chemotactic by itself (4, 28, 34). OxLDL seems to inhibit cholesterol export from macrophages (28). At high levels, the oxidation products may promote cell death (35, 36) and thus contribute to the formation of the necrotic core characteristic of an advanced atherosclerotic plaque. OxLDL promotes the expression of matrix metalloproteinases, which are important factors in plaque instability and rupture, phenomena that lead to the clinical symptoms of atherosclerosis (37). Oxidation of LDL also increases its sensitivity to aggregation and to modification by sphingomyelinase, promoting further the likelihood of its intimal modification and uptake by macrophages (28). A pro-inflammatory state may be stimulated by the modulation of the synthesis of a variety of cytokines and growth factors. All of these changes may promote lipid accumulation and inflammation, processes contributing to the development of atherosclerosis (4).

2.1.1.2 Oxidants and oxidation mechanisms involved in LDL oxidation

Lipoproteins can be oxidised by many kinds of oxidants, and by different pathways and mechanisms, both enzymatic and non-enzymatic (**Figure 3**). Some of the oxidants may originate from cells such as macrophages, endothelial cells and smooth muscle cells. Other oxidants may be derived from exogenous sources, such as diet and smoking. Polyunsaturated fatty acids are often the primary targets of oxidation, and they are present in cholesteryl esters, glycerophospholipids and TAGs. The fatty acids are initially oxidised to hydroperoxides. These acyl hydroperoxides may then be further reacted to e.g. alcohols, or they can undergo C–C bond cleavage to form short-chain (3–9 carbons) aldehydes, such as malondialdehyde, hexanal and 4-hydroxyhexanal. Aldehydic products formed within esterified lipids are called core aldehydes. Non-enzymatic peroxidation of arachidonic acid generates prostaglandin-like compounds, isoprostanes and epoxyisoprostanes. Epoxyisoprostanes may undergo rearrangement to isolevuglandins. Reactive oxidation products such as aldehydes and isolevuglandins form adducts with apoB, which leads to the increased uptake of the LDL particle by macrophages. Cholesterol oxidation leads to the formation of hydroperoxy-, keto- and hydroxycholesterols (25, 28, 38).

Free-radical-mediated lipid peroxidation proceeds by chain mechanism where the peroxyl radicals act as chain-carrying species. The mechanism and products are the same regardless of whether the oxidation is initiated by attack of hydroxyl radicals, peroxynitrite ($ONOO^-$), tyrosyl radical or other radical species. In LDL, linoleate is the major fatty acid, whose non-enzymatic oxidation proceeds by a mechanism that produces racemic 9- and 13-hydroperoxyoctadecadienoates (HPODEs) in equal amounts as the primary oxidation products. Both *Z*,*E* and *E*,*E* stereoisomers are formed (25).

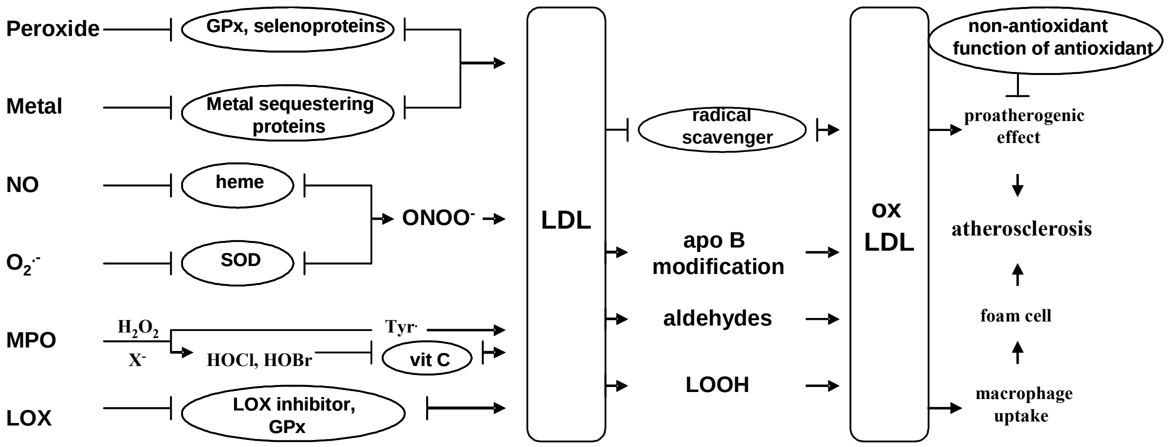


Figure 3. Oxidation of LDL potentially leading to atherogenesis, and its inhibition by antioxidants. The ovals represent antioxidants. GPx, glutathione peroxidase; LOX, lipoxygenase; MPO, myeloperoxidase; SOD, superoxide dismutase; Tyr·, tyrosyl radical; vit C, vitamin C (25).

The enzymatic oxidation, on the other hand, gives regio-, stereo- and enantio-specific lipid hydroperoxides. As an example, oxidation of linoleic acid in LDL by rabbit reticulocyte 15-lipoxygenase selectively gives 13(S)-9*Z*,11*E*-HPODE (39). 15-lipoxygenase type 1 and 5-lipoxygenase pathways are the prime candidates within the enzyme class implicated in atherosclerosis, although several details remain to be investigated (40). Oxidation by myeloperoxidase proceeds by both radical and non-radical mechanisms. Recent clinical trials support a role for myeloperoxidase in atherogenesis (41). Myeloperoxidase uses hydroperoxide and halide ions to give hypochlorous (HOCl) and hypobromous (HOBr) acids and tyrosyl radicals, which may oxidise LDL by several mechanisms. Tyrosyl radicals promote lipid peroxidation and cross-link protein tyrosine residues (25). 3-chlorotyrosine has been found to be markedly elevated in LDL isolated from human atherosclerotic intima (42). HOCl is increasingly implicated in atherosclerosis (28). It reacts with hydrogen peroxide, hydroperoxides as well as thiol and amino groups of proteins to give various radical species. HOCl reacts with the amino groups also to give chloramines, which may break down to aldehydes (25). Although products of LDL oxidation are potentially atherogenic, some of them, such as lysophosphatidylcholine, may give rise to compensatory beneficial responses, such as as an upregulation of antioxidant enzyme activity (24).

The oxidative modification of apoB may be even more important than lipid oxidation *per se* for recognition by scavenger receptors. ApoB may be oxidised by both radical and non-radical mechanisms. The modifications of apoB by aldehydes formed by lipid peroxidation, glycation and amino acid oxidation may play an important role (25, 43).

Some potentially atherogenic compounds may also serve as anti-atherogenic agents. Nitric oxide (NO) reacts rapidly with superoxide to give peroxynitrite, which oxidises LDL to an atherogenic form. However, NO is also known to act as a radical-scavenging antioxidant (25). Interestingly, in some animal studies, both overexpression and deficiency of lipoxygenase have led to decreased atherosclerosis, although the evidence in general supports the role of certain lipoxygenases in atherogenesis (44). Some lipid oxidation products, like certain eicosanoids, may also have anti-atherogenic effects, e.g. through control of platelet activation (45).

2.1.2 Oxidation of other lipoproteins than LDL

Oxidation of other lipoprotein classes than LDL may also contribute to atherogenesis. The mechanisms that lead to the oxidation of LDL are likely to affect other classes as well. Some large TAG-rich lipoprotein particles may be less likely to enter the arterial wall than LDL, but when oxidised are likely to deliver a greater oxidant load to the arterial wall. Also, according to Mamo and Wheeler (46), chylomicrons and their remnants may associate with arterial tissue with even greater efficiency than LDL. Oxidised chylomicrons and their remnants seem to be potentially atherogenic (5–8). Oxidation of chylomicrons results in particles that may serve as a substrate for scavenger receptors (8). However, chylomicron remnants and VLDL may induce foam cell formation even in their native forms (4, 47). Oxidised VLDL has been identified in atherosclerotic lesions and may have a contribution to cardiovascular risk when plasma TAG-levels are elevated (4).

Lipoprotein remnant particles can be derived either from chylomicrons or from VLDL (4). These particles penetrate the arterial wall more easily than VLDL, and once there they may undergo oxidation, which may be more likely if they already carry diet-derived lipid oxidation products (4, 48). Although chylomicron remnants seem to be accumulated efficiently into macrophages even without oxidative modification, the effects may be different between the oxidised and non-oxidised particles. There appears to be greater similarity to the effects of oxLDL after oxidation of the particles, which suggests that the effects of oxidation products predominate in these conditions (47).

Most studies looking at the relative susceptibility of lipoproteins to oxidation have found high density lipoprotein (HDL) to be more susceptible than LDL (44, 49). The low content of antioxidants in HDL relative to LDL is believed to explain this difference. The research on LDL oxidation in human atherosclerotic lesions has provided insight into the most likely physiologic mediators of lipoprotein oxidation. The oxidisability of HDL and its presence in extracellular fluid suggest that the mediators of LDL oxidation are likely to be the most relevant for studying the potential effects of oxidation on HDL function. For example, studies suggest that HDL is readily oxidised *in vitro* by peroxidase-generated tyrosyl radical as well as by peroxynitrite. In addition to its potentially harmful effects, oxidised HDL may also enhance some mechanisms induced by native HDL that prevent the accumulation of cholesterol in the artery wall (44).

2.2 EFFECT OF DIET ON THE LIPID OXIDATION IN LIPOPROTEINS

2.2.1 Oxidised dietary lipids

Mechanisms for the oxidation of lipids in food include enzymatic oxidation, photo-oxidation, autoxidation and thermal oxidation (**Figure 4**). Thus, the storage and the methods of food processing play a role in the process. Oxidised fatty acids can be found in most lipid classes including TAGs, glycerophospholipids and sterol esters. The hydroperoxides formed may also be transformed into molecules such as aldehydes, alcohols and ketones. These structures can be present attached to the original lipid (glycerol or sterol) backbone, or as free molecules (50–53). In addition, oxysterols, *e.g.* 7β-hydroxycholesterol and 7-ketocholesterol, may be formed from sterols by non-enzymatic and enzymatic mechanisms (47).

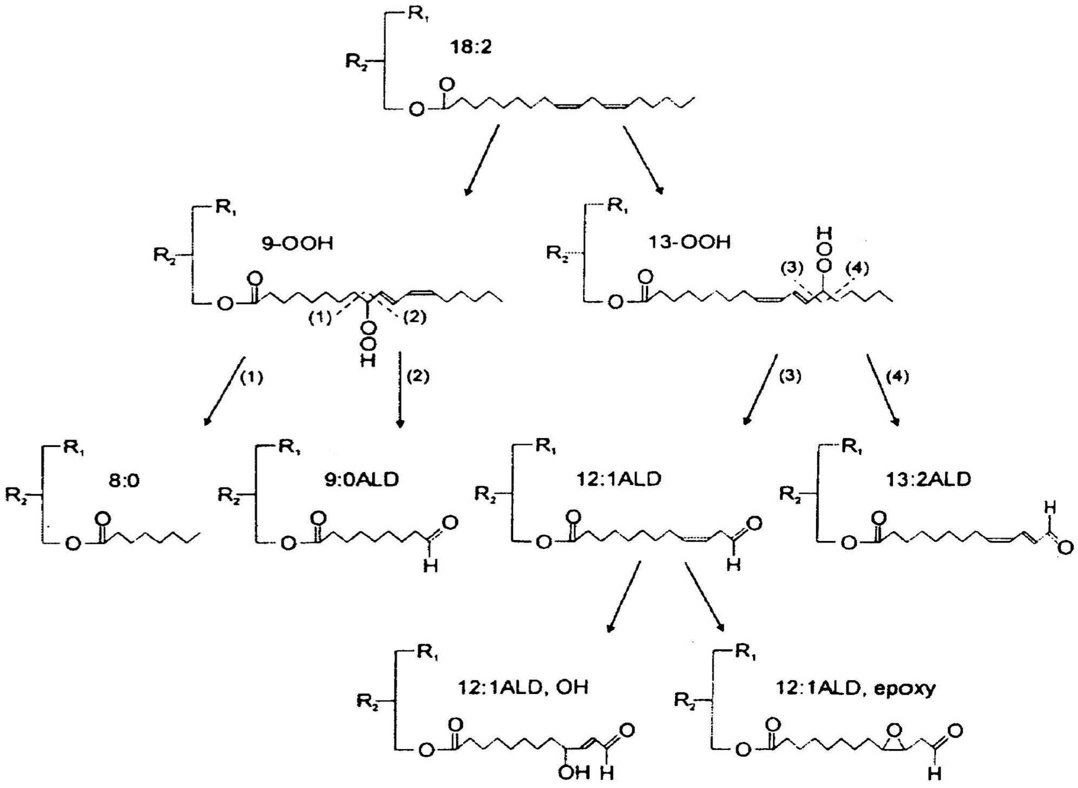


Figure 4. A scheme of postulated non-enzymatic formation of non-volatile triacylglycerol oxidation products from linoleic acid. ALD, core aldehyde.

There is evidence that both peroxidised fatty acids (54–57) and oxysterols (58–61) are absorbed in chylomicrons and are delivered to liver in chylomicron remnants, where they may be incorporated into endogenous lipoproteins (47). Alternatively, instead of absorption as such, oxidised dietary lipids may initiate a cascade of subsequent reactions in small intestinal mucosa that result in the increased oxidation of chylomicron lipids. Potentially atherogenic oxidised lipoproteins are thus at least partially derived from oxidised lipids in the diet.

In a postprandial study in humans by Wilson et al. (62), dietary fat containing TAG molecules with either two ^{13}C-labeled hydroxystearic acid residues or two ^{13}C-labeled dihydroxystearic acid residues increased the plasma concentration of ^{13}C-labeled hydroxy fatty acids. The amounts of these labeled hydroxy fatty acids that were absorbed were estimated to be on the average 21.0% and 4.5%, respectively. The absorption of naturally occurring hydroxy fatty acids in the diet (from walnut oil) was estimated to be even higher, 38.7%.

In a human study by Staprans et al. (56), the quantity of oxidised fatty acids found in chylomicron/chylomicron remnant lipoprotein fraction was proportional to the amount of oxidised fatty acids in the heated corn oil in the test meal (measured as conjugated dienes). Over the 8 h time period it takes to clear chylomicrons/chylomicron remnants from the circulation, there were considerably higher levels of oxidised fatty acids in the chylomicrons of the subjects that were fed the oxidised meal compared to those that were fed the control meal. There was no increase in the oxidation of other lipoproteins. In a study made on rats (55), intragastrically adminstered oxidised linoleic acid was shown to be delivered to liver via chylomicrons. After this, the oxidised fatty acid was shown to be incorporated into VLDL lipids (tested in a rat liver perfusion). Also, in a study made on rabbits (63), the amount of conjugated dienes, an indicator of lipid oxidation, increased in β-migrating VLDL (β-VLDL) after a diet containing oxidised corn compared to control diet. The rabbit model has, however, been criticised in that it fails to mimic human lipoprotein metabolism. In a study made on rats by Garrido-Polonio et al. (64), a diet containing thermally oxidised sunflower seed oil increased the oxidation of liver, serum, VLDL, LDL and HDL, measured as thiobarbituric acid-reactive substances (TBARS) at the end of a diet period of 27 days.

When α-epoxy cholesterol was fed to human subjects, the compound was found in chylomicrons/chylomicron remnants and also in VLDL, LDL and HDL, and remained in the circulation for 72 h (60). In another study (59), rabbits were fed with oxidised cholesterol or non-oxidised cholesterol. Oxysterols in TAG-rich lipoprotein fraction and remnant/LDL lipoprotein fraction seemed to be more abundant in the animals fed with oxidised cholesterol, although they were also found in the samples of the group fed with non-oxidised cholesterol. In rabbit aortas, adding oxidised cholesterol (65) or oxidised corn oil (63) to diet increased fatty streak formation compared with diets with non-oxidised cholesterol or non-oxidised corn oil, respectively.

2.2.2 Dietary antioxidants

The presumable requirement of oxidative modification of LDL preceding lipid accumulation and foam cell formation has led to the hypothesis that antioxidants may have a preventive effect in atherosclerosis. In particular, vitamin E, a lipophilic radical-scavenging antioxidant, has been the subject of many studies, discussion, reviews and debates (25). The possible antiatherogenic effect of vitamin C, a hydrophilic radical scavenger, is also interesting (66). Many epidemiological studies have indicated that diets rich in natural antioxidants may be beneficial in the prevention of cardiovascular disease, and *in vitro* studies support this view (21, 47). However, some animal experiments and recent large-scale dietary supplementation clinical trials with β-carotene and vitamin E have failed to show significant protective effects (67, 68). Supplementation with vitamin C does not seem to provide a general benefit, either, but it may be beneficial to certain subpopulations of patients. Combination of vitamin E with other antioxidants does not seem to consistently convey cardiovascular benefit (21).

The effects of dietary flavonoids on biomarkers of oxidative stress and cardiovascular disease risk factors in humans have not been well studied, and the results are still inconclusive (9). As with other antioxidants, the dose may have a major effect on the antioxidant potential of flavonoids (69). Most prospective cohort studies have indicated some degree of inverse association between flavonoid intake and coronary heart disease (10). In several *in vitro* and *ex vivo* studies (70–77), but not in all (69, 78, 79), quercetin (a major dietary flavonol), its glycosides and its biological conjugates have decreased the susceptibility of LDL to oxidation. Additive and synergistic effects with other phenolic compounds or other antioxidants on the prevention of LDL oxidation have been reported (75, 80–82). The results of studies by Hayek et al. (83) and Enkhmaa et al. (84) suggest that dietary consumption by apolipoprotein E-deficient mice of quercetin, or by LDL receptor-deficient mice of quercetin-3-(6-malonylglucoside), leads to attenuation in the development of the atherosclerotic lesions, and that this effect is associated with reduced susceptibility of their LDL to oxidation. In another study (85), aortic fatty streak area was significantly reduced in hypercholesterolaemic hamsters receiving quercetin at nutritional doses, suggesting that quercetin might prevent the development of atherosclerosis.

In healthy humans, consumption of flavonoids (mostly quercetin glycosides) in the form of onions and black tea did not appear to have an effect on F_2-isoprostanes and autoantibodies to oxidized LDL (86), and thus did not seem to inhibit lipid peroxidation. However, supplementation with lyophilised grape powder containing various flavonols and other polyphenolic compounds significantly reduced whole-body oxidative stress in women as measured by urinary F_2-isoprostanes (87). The levels of plasma tumor necrosis factor-alpha, which plays a major role in the inflammation process, were also decreased. Through alterations in lipoprotein metabolism, oxidative stress and inflammatory markers, the intake of lyophilised grape powder seemed to beneficially affect some risk factors for coronary heart disease.

The inconsistent results on the effects of antioxidants, as well as some studies suggesting dissociation of atherosclerosis and lipoprotein lipid oxidation (21, 88), have cast some doubts on the oxidation hypothesis. It is noteworthy, however, that there seems to be a lack of evidence for a deficiency of vitamin E in humans, and therefore major effects of the antioxidant might not be expected (21). Also, a particular antioxidant may be effective against some oxidants and oxidation mechanisms, but not for others. For example, vitamin E, the major antioxidant in LDL particles, has a potent antioxidant effect against free-radical-mediated oxidation, but is not capable of inhibiting oxidation by non-radical mechanisms, such as direct oxidation by lipoxygenase, HOCl, peroxynitrite and aldehydes. It does not seem to be efficient against tyrosyl radical-initiated oxidative cross-linking, either. Vitamin E fails to inhibit myeloperoxidase pathways *in vitro*. It is important to evaluate antioxidant potency in relation to the type of oxidants and mechanisms (24, 25, 41).

A study by Milne et al. (89) suggests that one reason for the uneffectiveness of vitamin E on the prevention of atherosclerosis may be because it may not effectively inhibit surface lipid oxidation of the LDL particle. Also, it has been suggested that dietary natural antioxidants may be effective only in selected subgroups of patients like those having high levels of oxidative stress (90). There is little evidence of overall depletion of vitamin E within atherosclerotic lesions, despite the presence of substantial amounts of advanced oxidation products, and this may be interpreted as indicating that peroxidation proceeds *in vivo* in the presence of vitamin E. It may be possible, however, that in a lesion there exists regions of relative vitamin E depletion where most oxidation could take place (4).

Moore et al. (91, 92) studied the effects of chylomicron remnant-like particles containing lycopene or probucol (a phenolic antioxidant drug) on lipid accumulation in THP-1 macrophages. The results showed that the remnant particles were protected from oxidation, but surprisingly lipid accumulation in the cells was enhanced. In contrast, in studies in which vitamin E or probucol were contained in modified LDL, macrophage foam cell formation has been reported to be suppressed (93, 94). It can be hypothesised that the potential beneficial effects of dietary antioxidants may be compromised when blood chylomicron remnant levels are raised, and this might provide a part of the explanation for the failure to demonstrate anti-atherogenic effects of dietary antioxidants in large scale intervention studies (47, 68).

Another potential oxidant leading to lipoprotein modification is α-tocopheroxyl radical, which is formed by the oxidation of the antioxidant α-tocopherol, the most common form of vitamin E. When LDL particles encounter mild oxidants or radicals with low frequency, tocopheroxyl radical may start to work as a peroxidation chain-carrying radical species (21). This raises the point that a co-antioxidant may be required to prevent vitamin E from being converted from an anti- to a pro-oxidant molecule *in vivo* (44, 95). Niki et al. (96) argue that α-tocopherol does not act as pro-oxidant in plasma, because vitamin C reduces the α-tocopheroxyl radicals rapidly *in vivo*. Niki (25) also questions the capability of vitamin C to act as a pro-oxidant *in vivo*.

2.3 LIPID OXIDATION PRODUCTS IN THE GASTROINTESTINAL TRACT

It is not well known in what degree different types of lipid oxidation products survive from mouth to small intestine and beyond, and whether all forms of oxidised lipids are absorbed into circulation. The studies by Kanner and Lapidot (97) suggest that human stomach fluid, having a low pH, may be a good medium for food lipid peroxidation in the presence of endogenous catalysts found in food, when dietary antioxidants are not available. In studies performed in rats by Kanazawa and Ashida (98, 99), linoleic acid hydroperoxides did not reach the small intestine when dosed intragastrically at moderate levels as such or as trilinoleoylglycerol hydroperoxides. Only when very high doses (200 μmol or more) of linoleic acid hydroperoxides were used, partial transport to the small intestine was observed. In the stomach, trilinoleoylglycerol hydroperoxides were broken down to linoleic acid hydroperoxides and hydroxides. Pure linoleic acid hydroperoxides were decomposed to hydroxides, epoxyketones and aldehydes. When moderate levels of hydroperoxides were used, only aldehydes entered the small intestine. Aldehydes also seemed to be partly absorbed into the body.

The small intestine is the primary site of nutrient absorption and a critical defense barrier against mutagens, carcinogens and oxidants of dietary origin. At present, the extent to which oxidative modifications of lipids influence their luminal solubility and their degree of penetration to plasma membrane is not known, nor is it known whether micellar formation is necessary for and critical to mucosal uptake of peroxidised lipids (100). In rats, glutathione peroxidase seems to effectively remove lipid hydroperoxides from the small intestine in normal conditions. However, the hydroperoxides may be transported into lymph in conditions where the supply of glutathione is limited (57, 101). In a study by Vilas et al. (102), feeding oxidised corn oil increased the weight of the mucosal cells of the rat intestine, suggestive of an inflammatory reaction. Total activity of glutathione peroxidase (NADPH oxidised/min) was unchanged or slightly increased in the intestinal mucosa. However, specific activity of the enzyme (NADPH oxidised/min/mg protein) was decreased. In the stomach, on the contrary, specific activity was increased.

Overall, evidence for transport of dietary lipid hydroperoxides into the circulation is controversial. While some researchers have documented the presence of unmodified lipid hydroperoxides in plasma following a fatty meal (103), others have provided evidence on the absorption of degradation products, such as hydroxyl fatty acids, conjugated dienes and aldehydes, into the body (99, 104, 105).

2.4 METHODS USED FOR THE ANALYSIS OF *IN VIVO* OXIDISED LIPIDS

In principle, many of the methods used for the analysis of lipid oxidation products in e.g. food can also be applied to biological samples. However, the quantities of various oxidation products are often limited in these samples, and therefore sensitive detection methods are needed. Also, the matrix of biological samples is challenging and various extraction and purification procedures are often needed during sample preparation in order to remove interfering compounds from the matrix. Nevertheless, sample preparation steps should be kept in minimum to avoid the formation of artefacts.

In the recent years, various methods have been developed and optimised for the sensitive, specific analysis of lipid oxidation products found *in vivo*. In the following paragraphs, analytical approaches used for the analysis of oxidised lipids in plasma, lipoproteins and atherosclerotic plaques are described. Total lipid oxidation, oxidised glycerophospholipids, cholesterol, cholesterol esters and fatty acids and are covered. Specific structures of oxidised TAG molecules present in plasma or lipoproteins *in vivo* have not been investigated before our present studies.

2.4.1 Analysis of total lipid oxidation in plasma and lipoproteins

Measurement of the formation of TBARS, different spectrophotometric and enzymatic techniques, and determination of autoantibodies have been used to estimate the oxidation of plasma and lipoproteins *in vivo* (23, 61, 106–108). Modern methods for the estimation include measurement of conjugated dienes at baseline level as well as determination of F_2-isoprostanes (see section 2.4.2) (23).

Measurement of TBARS is based on the formation of malondialdehyde (MDA) during lipid oxidation. MDA reacts with thiobarbituric acid, and the adduct formed can be measured by spectrophotometry or fluorometry (109, 110). Typically, samples are incubated with thiobarbituric acid and tricholoroacetic acid at an elevated temperature after which TBARS are measured (109). TBARS reflect secondary oxidation reactions, but MDA may be formed also by the breakdown of lipid hydroperoxides under the conditions used for the adduct formation (111). A number of compounds other than MDA also react with thiobarbituric acid to produce adducts which absorb at approximately the same wavelength. Thus, for more specific detection, chromatographic separation is needed and has been used for the measurement of TBARS in plasma (111–114). A second problem associated with the determination of TBARS is that oxidation products formed from other fatty acids than arachidonic acid may be neglected.

Lipoprotein lipid peroxides have been measured in chylomicrons, VLDL and LDL by a colorimetric method based on the reaction of lipid hydroperoxides with a leucomethylene blue derivative in the presence of haemoglobin. The amount of methylene blue formed is measured by its absorbance at 666 nm (54, 61, 115). Plasma, VLDL, LDL and HDL lipid hydroperoxides have also been measured using a colorimetric method that utilizes the oxidation of ferrous to ferric ion. Ferric ion binds to xylenol orange to produce a chromophore that can be measured at 560 nm (107, 116). The precision of this approach has been questioned (117). Another method used for the determination of lipid hydroperoxides in human plasma has been a tri-iodide spectrophotometric technique (106). In the method, lipids and lipoproteins are absorbed into a gel. A color reagent containing potassium iodide is added, the gel is centrifuged, and the absorbency of the supernatant is measured spectrophotometrically.

In addition to the colorimetric and spectrophotometric techniques, a sensitive enzymatic assay for lipid hydroperoxides has been applied to human plasma (118). The assay measures hydroperoxides directly by their stimulation of the cyclo-oxygenase activity of prostaglandin H synthase. The results obtained by the enzymatic assay indicated that the plasma concentration of lipid hydroperoxides in normal subjects is 0.5 μM., while values between 1.5–5.5 μM have typically been obtained by iodometric techniques, photon counting chemiluminescence and mesurement of ferrous ion oxidation in xylenol orange (116).

Chemiluminescence detection can also be used for the determination of lipid hydroperoxides *in vivo*. Zamburlini et al. (119) performed a specific analysis of lipid hydroperoxides in human plasma and lipoproteins by direct measurement using chemiluminescent single photon counting. In the method, the samples are added to a reaction mixture containing luminol, hemin (the Fe^{3+} oxidation product of heme) and Triton X-100 (a non-ionic surfactant). The photon emission by luminol is measured in a luminometer and quantification is performed.

Upon oxidation, LDL becomes immunogenic giving rise to the formation of autoantibodies. Studies with autoantibodies have demonstrated implication of oxidised LDL in human atherosclerosis and related conditions. Some studies seem to indicate that autoantibodies against oxLDL predict the development of atherosclerosis, but contradictory results have also been reported (23, 120). The applicability of the methodology for clinical purposes has been questioned particularly because of the difficulty of reproducible preparation of antigens (23). Autoantibody-based enzyme-linked immunosorbent assay (ELISA) has also been applied to the analysis of oxidised LDL in plasma in a study by Holvoet et al. (108). The results of the study suggest that plasma levels of oxLDL determined by ELISA are significantly elevated in ischemic heart disease patients and in heart transplant patients.

Since the method was introduced, measurement of conjugated dienes (including fatty acid hydroperoxides and hydroxides) has been widely used *ex vivo* to determine susceptibility of LDL to Cu^{2+}-induced oxidation (121). Later, the LDL baseline diene conjugation method (LDL-BDC) was developed to be used as an indicator of LDL lipid oxidation *in vivo* (122). The method measures the actual amount of conjugated dienes in native LDL at 234 nm, and is a specific indicator of circulating mildly oxidised LDL. The baseline levels of conjugated dienes have been measured in other lipoprotein classes as well in order to determine the degree of lipid oxidation (55, 56).

2.4.2 Analysis of different oxidised lipids in plasma, lipoproteins and atherosclerotic plaques

Several methods based on HPLC have been reported for the measurement of lipid oxidation *in vivo*. Lipid classes can be separated with normal-phase HPLC (NP-HPLC), and lipids differing in fatty acyl chain lengths, degree of unsaturation or type of oxidation by reversed-phase-HPLC (RP-HPLC). Chiral columns have been used to separate different enantiomers of lipid oxidation products (123–125). HPLC combined to chemiluminescence methods with isoluminol or luminol as well as to fluorimetry have been widely used to analyse lipid hydroperoxides *in vivo*. HPLC coupled to UV detection has been used e.g. in the analysis of conjugated dienes.

Electrospray ionisation-mass spectrometry (ESI-MS) is a soft ionisation technique that does not cause substantial fragmentation of biomolecules, and in most cases allows the detection of intact lipids without the requirement of derivatisation or extensive sample processing. ESI-MS can readily be coupled to HPLC. HPLC-ESI-MS and HPLC-ESI-MS/MS (tandem mass spectrometry) have been used in several studies to separate, identify and quantify various oxidised lipids found in e.g. atherosclerotic plaques (126).

2.4.2.1 HPLC-chemiluminescence

The group of Miyazawa (127) determined the phosphatidylcholine hydroperoxides (PCOOH) in human plasma by a NP-HPLC-chemiluminescence assay. In the chemiluminescence reaction used, lipid hydroperoxides react with cytochrome C-heme and produce oxidants. After this, luminol is oxidised under alkaline conditions and emits chemiluminescence. In addition to an ordinary detector measuring chemiluminescence emission, the researchers used a sensitive detector with a single photoelectron counting system. The results suggest that the plasma concentration of PCOOHs in healthy individuals is in the range from below 10 nM to 500 nM. The PCOOH peak chromatographed on chemiluminescence-HPLC of plasma lipids disappeared after reduction with $NaBH_4$. The peak intensities of PCOOHs with UV (234 nm) detection did not change before and after the $NaBH_4$ treatment, suggesting the reduction of hydroperoxides to hydroxides, another class of conjugated dienes absorbing at 234 nm. Thus, the results suggested that unmodified lipid hydroperoxides were indeed present in plasma.

Hui et al. (128) reported a RP-HPLC method for simultaneous measurement of hydroperoxides of cholesteryl ester and TAG in human plasma. The luminescent reagent consisted of cytochrome C and luminol in borate buffer. An internal hydroperoxide standard was synthesised for the analyses. Plasma cholesteryl ester hydroperoxide levels were in average 189 nM in healthy young and 210 nM in healthy elderly subjects. TAG hydroperoxides were not detected in the healthy individuals.

Yamamoto et al. (129) used a different chemiluminescence reagent in their study. The system was based on the detection of chemiluminescence emitted by isoluminol in the presence of hydroperoxide and microperoxidase. The researchers describe several HPLC conditions that may allow identification of different lipid hydroperoxides. Frei and Gaziano (130) determined the level of lipid hydroperoxides in native human LDL by RP-LC with chemiluminescence detection based on the reaction with isoluminol and micoperoxidase. With the same method, Bowry et al. (49) detected cholesteryl ester hydroperoxides in native human HDL.

Upston et al. (125) used RP-LC and the chemiluminescence reaction based on isoluminol (129) to determine cholesteryl ester oxidation in human atherosclerotic lesion, lipoprotein and plasma extracts. In another study, Upston et al. (31) analysed cholesteryl ester hydroperoxides/hydroxides and oxysterols of human aortic lesions by RP-HPLC and NP-HPLC, respectively, and found out that the oxidised lipids accumulated only at the later stages of lesion development. Chemiluminescence emitted by isoluminol was one of the detection methods used in the study.

Anion-exchange LC method was developed for the chemiluminescence assay of hydroperoxides in rabbit LDL by Yamaguchi et al. (131). The method involves anion-exchange HPLC separation using a diethylaminoethanol (DEAE)-glucomannan gel and direct detection of hydroperoxides in LDL particles (without extraction of the lipids) following post-column reaction with isoluminol, microperoxidase and Triton X-100. By this method, some hydroperoxides were detected in the native LDL of the Watanabe heritable hyperlipidaemic rabbits, even though the chromatographic peaks were relatively small.

2.4.2.2 HPLC-UV

Mowri et al. (132) studied by RP-HPLC-UV the cholesteryl esters extracted from atherosclerotic aorta. Two different oxidised fatty acids were detected. The fatty acids showed maximum absorption at 233 nm, suggesting that they contained a conjugated diene structure. One of the structures was identified as 13-hydroxyoctadecadienoic acid (13–hydroxylinoleic acid; 13-HODE).

Oxidised cholesterol and cholesteryl esters in human atherosclerotic plaques were determined by Suarna et al. (133). The researchers used RP-HPLC-UV at 234 nm to analyse the oxidised lipid species. The method could also be used to measure esterified forms of 7-ketocholesterol. For the analysis of different oxidised fatty acids within cholesteryl esters, separate rechromatography of oxidised cholesteryl linoleates was performed by NP-HPLC-UV. Approximately 30% of plaque cholesteryl linoleate was oxidised, with 17%, 12% and 1% present as fatty acyl hydroxides, ketones and hydroperoxides, respectively. Four isomers of both fatty acyl hydroxides and ketones were detected. 7-ketocholesterol was much less abundant in the samples.

Mashima et al. (134) analysed oxidised cholesteryl linoleate in human plasma by HPLC-UV at 234 nm. The sample preparation steps included lipid extraction by methanol/hexane and solid phase extraction. Fractions were collected by RP-HPLC and further separated by NP-HPLC. The researchers managed to separate *Z*,*E*/*E*,*Z* and *E*,*E* isomers of both 9- and 13-hydro(pero)xy linoleic acids. All of these structures were detected in the samples, and 9-hydro(pero)xy structures were more prominent. The results indicated the presence of free radical-mediated *in vivo* lipid peroxidation even in the young (aged 21–29) subjects. Artefactual oxidation was not detected during the analytical procedure.

Browne and Armstrong (135) determined the hydroperoxy and hydroxy structures of polyunsaturated fatty acids in plasma by RP-HPLC-UV at 236 nm after lipid extraction and alkaline hydrolysis of lipid esters. 9- and 13-HODEs constituted the main peaks. Other peaks representing different hydroperoxy and hydroxy structures were small.

Upston et al. (125) used NP- and chiral-phase HPLC coupled to UV detection at 234 nm to study the stereroisomers of cholesteryl HODEs in human carotid and aortic lesions, and in lesion lipoproteins (LDL and HDL). *Z*,*E*/*E*,*Z* configurations predominated over other products in each fraction. The content of *R* and *S* isomers of 13-HODE with *Z*,*E* configuration was equimolar in aortic lesions, indicating non-enzymatic, free-radical-mediated oxidation.

2.4.2.3 HPLC-ESI-MS(/MS)

By using HPLC, ESI-MS, and ESI-MS/MS, Watson et al. (33) showed that aortae of rabbits on an atherogenic diet contained increased levels of certain oxidation products of palmitoylarachidonoyl glycerophosphocholine (PAPC), namely 1-palmitoyl-2-(5-oxo)pentanoyl-*sn*-glycerophosphocholine, 1-palmitoyl-2-glutaryl-*sn*-glycerophosphocholine and a third product later identified as 1-palmitoyl-2-(5,6-epoxy)isoprostane E_2-*sn*-glycerophosphocholine (136). By using RP-LC-ESI-MS/MS, Hoff et al. (137) detected in human atherosclerotic lesions and LDL a different oxidation product of PAPC, where 5-hydroxy-8-oxo-6-octenoic acid is esterified to PC (HOOA-PC).

In addition to HOOA-PC, Podrez et al. (138) identified and quantified another structure, 5-keto-8-oxo-6-octenoic acid, esterified to *sn*-2 position of PC (KOOA-PC) in atherosclerotic rabbit aortas. Related 5-hydroxy and 5-keto structures esterified to glycerol backbone, where free carboxylic group was substituted for an aldehydic group, were also present in the oxidised PAPC molecules. All the corresponding structures formed from palmitoyllinoleoyl glycerophosphocholine (PLPC) were also present. In these molecules, esterified dodecenoic acid derivatives were present instead of octenoic acid derivatives. In addition to the abovementioned structures, 5-oxopentanoyl and glutaric acid esters derived from PAPC oxidation, and 9-oxononanoic acid and nonanedioic acid esters derived from PLPC oxidation, were also present. Other results obtained in the study suggest that this family of oxidized glycerophospholipids participates in CD36-mediated recognition of oxidised lipoproteins and foam cell formation *in vivo*.

Ravandi et al. (139) investigated human atheroma by NP-HPLC coupled to ESI-MS. Hydroperoxides, hydroxides, epoxides, E_2-, F_2-, and epoxy isoprostanes as well as core aldehydes (5:0ALD and 9:0ALD/8:1(OH)ALD) attached to the *sn*-2 position of PC (with 16:0 or 18:0 in the *sn*-1 position) were detected and quantified. Interestingly, there was no clear relationship between the developmental stage of the plaque and whether the major oxidation products were early or advanced oxidation products. The early oxidation products (hydroperoxides and epoxdides) were found at the advanced stages of the plaques in nearly the same relative abundance as the more advanced oxidation products (core aldehydes and acids). In an earlier study (140), the researchers had detected various oxo-acyl esters also in plasma lipoproteins.

Kamido et al. (141) analysed lipids extracted from human atherosclerotic lesions by HPLC-ESI-MS. Core aldehydes were analysed as dinitrophenylhydrazine (DNPH) derivatives. 5:0 (pentanoyl) and 9:0 (nonanoyl) core aldehydes of PC were detected after phospholipase C digestion. Cholesteryl 9-oxo- and 5-oxo-alkanoyl esters and their aldol condensation dimers were also identified. Analysis of underivatised extract indicated the presence of mono- and di-hydroperoxides of PC and cholesterol esters.

In other studies by Kamido et al. (142, 143), the researchers identified and quantified PC core aldehydes with an alkyl group in the *sn*-1 position of the molecule in human atheromas. The analyses were performed by HPLC-ESI-MS and LC-direct liquid inlet interface-MS of DNPH derivatives after phospholipase C digestion. The derivatives gave $[M-1]^-$ ions corresponding to 1-*O*-hexadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol, 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol, 1-*O*-hexadecyl-2-(5-oxo)pentanoyl-*sn*-glycerol and 1-*O*-octadecyl-2-(5-oxo)pentanoyl-*sn*-glycerol. 1-*O*-hexadecyl-2-(5-oxo)pentanoyl-*sn*-glycerophosphocholine was shown to induce aggregation of rabbit platelets. 1-palmitoyl-2-(9-oxo)nonanoyl-*sn*-glycerophosphocholine, 1-stearoyl-2-(9-oxo)nonanoyl-*sn*-glycerophosphocholine, 1-palmitoyl-2-(5-oxo)pentanoyl-*sn*-glycerophosphocholine and 1-stearoyl-2-(5-oxo)pentanoyl-*sn*-glycerophosphocholine were also identified in the studies.

Mallat et al. (144) studied the lipids of human atherosclerotic plaques by RP-HPLC-ESI-MS/MS after ester hydrolysis. 5-, 8-, 9-, 11-, 12- and 15-hydroxyeicosatetraenoic acids (HETEs) as well as F_2-isoprostanes were identified and quantified. Also 5,6-epoxyeicosatetraenoic acid was found in some samples. The levels of all HETEs were significantly higher in the plaques retrieved from symptomatic patients (unstable plaques) compared with those retrieved from asymptomatic patients (stable plaques). 9-HETE was the most abundant compound found in the plaques. The researchers state that the molecule could not be derived from known enzymatic reactions, suggesting that non-enzymatic lipid oxidation may prominate in advanced atherosclerosis and may promote plaque instability.

Hoppe et al. (145) analysed oxidation products of cholesteryl linoleate found in human atherosclerotic lesions. The lipid extracts were treated with DNPH and the derivatives along with the unreacted lipids were subjected to RP-HPLC-ESI-MS. The researchers detected the following cholesteryl ester core aldehydes in the samples: 4:0ALD, 5:0ALD, 6:0ALD, 7:0ALD, 8:0ALD, 9:0ALD and 10:1ALD.

2.4.2.4 HPLC coupled with other detection methods

To determine TAGs and cholesteryl ester hydroperoxides in human plasma, Akasaka et al. (146) developed a method based on NP-HPLC coupled with fluorescence detection. Diphenyl-1-pyrenylphosphine was used as the postcolumn fluorescent reagent. HPLC eluent was monitored by UV absorbance at 268 nm prior to the post-column reaction. The reagent solution was added to eluent from UV detector, and the mixture reacted in a heated stainless-steel coil. Detection was performed by monitoring the fluorescence intensity at 380 nm with the excitation wavelength set at 352 nm. An internal standard was used for the quantification. Cholesteryl ester hydroperoxide level in the plasma samples was on average 24.5 nM. TAG hydroperoxides were not found. The researchers later developed a column-switching apparatus for the simultaneous determination of phosphatidylcholine, cholesteryl esters and TAG hydroperoxides (147). In the system, after separation of lipid classes by NP column, phophatidylcholine hydroperoxides are led to another NP column, whereas cholesteryl ester and TAG hydroperoxides are led to a RP column. In the study utilising the apparatus, the detection methods remained the same as in the earlier one. Hydroperoxides of both cholesteryl linoleate and phosphatidylcholine were detected in plasma.

Karten et al. (148) analysed cholesteryl ester core aldehydes of human LDL by RP-HPLC coupled with fluorescence detection. Lipid extracts were derivatised with 1,3-cyclohexanedione reagent before LC analysis. Detection was performed by monitoring the fluorescence intensity at 455 nm with the excitation wavelength set at 366 nm. In the study, LDL was oxidised *in vitro*, but 5-oxopentanoyl cholesterol and 9-oxononanoyl cholesterol seemed to be already present before the onset of the oxidation reaction.

Thomas et al. (149) studied the lipid hydroperoxides in LDL by HPLC coupled with mercury drop electrochemical detection, a method by which low detection limits have been achieved for cholesterol ester hydroperoxides. Fresh LDL, isolated carefully to avoid lipid autoxidation, was found to contain small amounts (6–12 pmol/mg protein) of cholesteryl ester hydroperoxides.

2.4.2.5 GC and GC-MS

Gas chromatography (GC) has been used in the analysis of oxidised cholesterol structures and fatty acid oxidation products, the latter after ester hydrolysis of original lipid molecules. For the identification of the molecular structures, MS detection coupled to preceding GC separation is valuable. When MS is not available, GC runs before and after acetylation of the hydroxyl group can be performed to gain some specificity to the otherwise difficult analysis of hydroxy fatty acids (150). Because of their thermal instability, lipid hydroperoxides cannot be analysed by GC before reduction.

In their study on the effect of oxidised dietary cholesterol on the development of atherosclerosis in rabbits, Staprans et al. (65) used GC to determine oxidised cholesterol levels in serum lipoprotein fractions of rabbits. In LDL fraction, 7α-hydroxycholesterol, 7β-hydroxycholesterol, α-epoxycholesterol, β-epoxycholesterol and 7-ketocholesterol were detected and quantified. 7α-hydroxycholesterol was not present in β-VLDL fraction.

Vine et al. (58) identified and quantified oxysterols in rat lymph lipid extracts by GC-MS with single-ion monitoring. The animals were gastrically infused with heat-treated cholesterol containing 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide. The incorporation of oxysterols into lymph chylomicrons differed over time with 7β-hydroxycholesterol having a peak absorption at 3 h, 7-ketocholesterol at 4 h and cholesterol 5α,6α-epoxide at 5 h. In a later study by the researchers (59), rabbits were fed with oxidised cholesterol or non-oxidised cholesterol. The concentration of 7β-hydroxycholesterol was similar in the TAG-rich lipoproteins of both groups, but the oxidised cholesterol-fed animals showed five times the concentration of cholesterol 5α,6α-epoxide and double the concentration of 7-ketocholesterol. The presence of 7-ketocholesterol in remnant/LDL lipoprotein fraction was exclusive to animals fed the oxidised cholesterol diet.

By using GC and GC-MS after saponification of the samples, Carpenter et al. (151) analysed 7β-hydroxycholesterol, 26-hydroxycholesterol, and HODEs in human atherosclerotic lesions at different stages of development. Hydroxycholesterols were determined as trimethylsilyl (TMS) ethers and HODEs as methyl esters. 26-hydroxycholesterol was significantly more abundant in advanced lesions than in intermediate or early lesions. Although HODE levels increased with the severity of a lesion, there were few statistically significant differences between the histological categories of the lesions. Waddington et al. (123) identified the fatty acid oxidation products from the hydrolysed lipid samples of human atherosclertotic plaques by GC-MS after RP-LC fractionation (UV detection at 235 nm and 270 nm). High levels of HODEs, 15-HETE and 11-HETE were detected. 9- and 13-keto derivatives of octadecadienoic acid and two previously unidentified compounds, 11-keto and 5,6-dihydroxy derivatives of eicosatetraenoic acid, were also detected. The analysis of fractions from RP-LC perfomed with chiral-phase LC column revealed that the major compunds, 9-HODE, 13-HODE, 11-HETE and 15-HETE, were racemic mixtures with an almost equal distribution of *R* and *S* isomers, indicating non-enzymatic lipid peroxidation.

Salonen et al. (110) determined oxysterols extracted from human serum by GC-isotope dilution-MS. The oxysterols were analysed as TMS ethers. 7β-hydroxycholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide and cholestane-3β,5α,6β-triol were measured. 7β-hydroxycholesterol concentration was associated with the progression of carotid atherosclerosis.

F_2-isoprostanes are produced by free radical oxidation of arachidonic acid and arachidonic acid containing phospholipids, and they can be analysed by GC-MS (111). F_2-isoprostanes are emerging biomarkers and potential mediators in cardiovascular diseases. Morrow et al. (152) reported their presence in plasma and their rapid increase during storage in -20 °C. The researchers used GC coupled with negative ion CI-MS to analyse the structures as pentafluorobenzyl ester/TMS ether derivatives. The measurement of F_2-isoprostanes in biological samples is challenging because they must be separated from other isoprostanes, prostaglandinds, and their metabolites (153).

Mori et al. (153) and Waddington et al. (124) used GC-electron capture negative ionisation-MS to analyse F_2-isoprostanes in human plasma and atherosclerotic plaques, respectively. Before the analysis by GC-MS as TMS ethers, solid phase extraction of the plasma hydrolysates with both RP and NP extraction cartridges was performed and the F_2-isoprostane fraction was collected by RP-LC. As in the earlier study (123), identification and quantification of oxidised fatty acids was also performed. The results were comparable to those of the earlier study. Thomas et al. (154) analysed the F_2-isoprostane levels in the lipids extracted from atheroslcerotic arteries of non-human primates. The researchers saponified the samples and converted them to pentafluorobenzyl esters. After separation by thin-layer chromatography (TLC), the residue containing F_2-isoprostanes was converted to TMS ethers and analysed by GC-MS.

2.5 SUMMARY

Numerous studies made during the last decades suggest that oxidatively modified lipoproteins are a major risk factor for atherosclerosis. Lipoproteins can be oxidised by several mechanisms, but the site and mechanisms of lipoprotein oxidation have not been fully resolved. In addition to the lipid oxidation products formed endogenously by enzymatic and non-enzymatic reactions, oxidised TAGs and oxysterols in the diet contribute to the oxidised lipoproteins found in circulation. It is not well known in what degree different types of lipid oxidation products survive from mouth to small intestine and beyond, and whether all forms of oxidised lipids are absorbed into circulation *per se*. In arteries, uncontrolled uptake of LDL particles by macrophages leads to the formation of foam cells. As a consequence, atherosclerotic plaques (lesions) are formed. Oxidised lipoproteins have been identified in atherosclerotic lesions in both animals and humans. In addition to their role in foam cell formation, oxidised lipoproteins may promote atherogenesis by several other mechanisms.

The inconsistent results on the effects of antioxidant supplementation on cardiovascular disease have cast some doubts on the oxidation hypothesis of atherogenesis. However, deficiency of vitamin E, the most studied antioxidant, may be a rare event in humans, and therefore major effects of the antioxidant might not be expected. Also, a particular antioxidant may be effective against some oxidants and oxidation mechanisms, but not for others. Still, appropriate clinical trials are needed to test the importance of oxidation in the pathogenesis of atherosclerosis in humans.

In recent years various methods have been developed and optimised for the analysis of lipid oxidation products found *in vivo*. Conjugated dienes at baseline level and F_2-isoprostanes are examples of indicators nowadays used for the determination of lipid oxidation level *in vivo*. HPLC and GC coupled with different detection methods have been widely used to analyse oxidised lipid structures in plasma, lipoproteins and atherosclerotic plaques. In addition to being higly sensitive detection methods, ESI-MS and ESI-MS/MS give the most detailed information on various molecular structures found in biological samples.

Glycerophospholipids with keto, hydroxy, or epoxy fatty acids, and/or core aldehydes, core acids, or isoprostanes attached to their *sn*-2 position have been identified in atherosclerotic lesions. Oxidised glycerophosphocholine structures with an alkyl group in the *sn*-1 position have also been detected. Cholesterol molecules esterified with hydroperoxy, hydroxy or keto fatty acids or with core aldehydes have been identified in plasma, lipoproteins and lesions. Also, double bond configurations and stereochemical structures of oxidised fatty acids found in cholesteryl esters have been deterimined in various studies. Studies on the structures of oxidised fatty acid residues within lipid molecules suggest a major role of non-enzymatic oxidation of the lipids found in atherosclerotic lesions. Oxidised cholesterol structures with hydroxy, keto and epoxy groups in various positions of the sterol molecule have been identified and analysed in plasma, lipoproteins and lesions. Specific structures of oxidised TAG molecules present in these tissues and lipoprotein fractions have not been investigated before the present studies.

3 AIMS OF THE STUDIES

The overall aim of the studies was to investigate the effects of oxidised dietary fat and flavonols on lipoprotein lipid oxidation, and to study their transfer from diet to the circulation. The emphasis was on the analysis of oxidised TAGs by combining modern analytical methods.

The detailed aims were:

- to optimise and apply an earlier developed HPLC-MS method to the analysis of oxidised TAG molecules in lipoproteins.
- to determine the effect of diet on the level of *in vivo* lipid oxidation in various lipoproteins by measuring the level of conjugated dienes in them.
- to determine the effect of diet on the level of *in vivo* TAG oxidation in various lipoproteins by TLC and HPLC methods.
- to investigate the effect of flavonols extracted from sea buckthorn, a berry rich in oil, vitamin C, tocopherols, carotenoids and flavonoids, on LDL oxidation and other potential risk factors of atherosclerosis.
- to investigate the absorption and bioavailability of the flavonols with and without oil supplementation.

4 MATERIALS AND METHODS

4.1 STUDY DESIGNS AND ETHICAL CONSIDERATIONS

4.1.1 Pre-study (**I**)

Two pooled serum samples each from 10 subjects of various ages were collected. With regard to total cholesterol level, one of the groups (group 1) consisted of normolipidaemic subjects (close to the lower limit of the normal range) and the other (group 2) of hyperlipidaemic subjects. Blood samples were obtained by venipuncture after an overnight fast.

4.1.2 Animal experiment 1 (**II**)

Nine growing pigs (castrated boars) from three different litters were used in the study. For two weeks, three groups of three pigs (groups 1, 2 and 3) were fed a diet containing 14% sunflower seed oil varying in oxidation levels. In each group, there was one animal from each of the three litters. The oil of group 1 was not oxidised, whereas the oils of groups 2 and 3 were oxidised in convection ovens at 60 °C until the appropriate peroxide values were reached. The peroxide values of the oils were as follows: group 1, 1 mequiv O_2/kg; group 2, 84 mequiv O_2/kg; group 3, 223 mequiv O_2/kg. The vitamin E contents of the oils were as follows: group 1, 566 mg/kg; group 2, 459 mg/kg; group 3, 1 mg/kg. The decrease in the vitamin E levels of groups 2 and 3 was caused by the heat treatments. Blood samples were obtained by venipuncture from the jugular vein at 3 and 4 hours (time points 1 and 2, respectively) after the last meal. The study plan was approved by the Test Animal Committee of MTT Agrifood Research Finland.

4.1.3 Animal experiment 2 (**III, IV**)

Twenty-six growing pigs (castrated boars) were used in the study. For 2 weeks, two groups of 13 pigs (groups 1 and 2) were fed a diet containing 16% sunflower seed oil varying in oxidation levels. The oil of group 1 was not oxidised (peroxide value 1 mequiv O_2/kg), whereas the oil of group 2 was oxidised in convection ovens at 60 °C until the peroxide value of 190 mequiv O_2/kg was reached. The vitamin E contents of the oils were as follows: group 1, 550 mg/kg; group 2, 103 mg/kg. The decrease in the vitamin E level of group 2 was caused by the heat treatment. Blood samples were obtained by venipuncture from the jugular vein at 3 and 4 h (time points 1 and 2, respectively) after the last meal. The study plan was approved by the Test Animal Committee of MTT Agrifood Research Finland.

4.1.4 Clinical trial 1 (V)

Fourteen healthy, non-smoking males (aged 35–53) with a slightly elevated total cholesterol level (5.3–7.2 mmol/L) were recruited for the randomised double-blind crossover study. The effects of oatmeal porridge (portion size 185 g; contained peeled apple and cinnamon as flavorings) supplemented with an aglyconic sea buckthorn flavonol extract were studied against a control porridge that did not contain the flavonols. The extract (0.4 g) added to the porridge contained a total of 78 mg flavonol aglycones, of which 54.1 mg isorhamnetin, 20.2 mg quercetin and 3.4 mg kaempferol. Flavonols were extracted from fruit pulp of sea buckthorn with an ethanol–water solution. The extract was purified by extraction of oil with hexane and removal of sugars with water.

During two study periods of 4 weeks, the subjects consumed daily a portion of oatmeal porridge with or without the sea buckthorn flavonol extract. The order of the study periods was randomised. The study periods were separated by a washout period of 4 weeks. Before and after each study period, after a 12-hour fast, blood samples were collected by venipuncture.

Exclusion criteria were hormonal, renal, haematological or hepatic dysfunction, a myocardial infarction, treatment with lipid-lowering or non-steroidal inflammatory drugs, cardiovascular medication, and an ongoing inflammatory state or disease. Each subject provided an informed written consent, and they were free to discontinue their participation in the study at any point without explanation. The study plan was approved by the Ethics Committee of the Hospital District of Southwest Finland.

4.1.5 Clinical trial 2 (V)

Twenty-two healthy, non-smoking males (aged 34–59) were recruited for the study on the absorption of the aglyconic flavonol extract (same as that used in trial 1). The subjects were divided randomly into two groups. During each of two separate visits, after overnight fast, the subjects were given a single dose of oatmeal porridge (portion size 185 g; unflavored) containing the flavonol extract (group 1: 78 mg of total flavonols; group 2: 39 mg of total flavonols). During one of the visits, each subject was also given 3 g sea buckthorn oil mixed in the porridge (meal 1A, 78 mg flavonols; 1B, 78 mg flavonols and 3 g sea buckthorn oil; 2A, 39 mg flavonols; 2B, 39 mg flavonols and 3 g sea buckthorn oil). The oil had a standardised composition of a mixture of sea buckthorn seed oil and berry oil. The order of the meals (with or without oil) was randomised. The subjects were asked to restrain from flavonol-rich foods for two days preceding the meals. Blood samples were collected before and 1, 2 and 4 h after the meals for the determination of the levels of flavonols in circulation.

Exclusion criteria were the same as in study 1. The subjects provided an informed written consent before the study, and they were free to discontinue their participation in the study at any point without explanation. The study plan (a continuation of the earlier study) was approved by the Ethics Committee of the Hospital District of Southwest Finland.

4.2 PREPARATION OF REFERENCE COMPOUNDS (I-V)

The substrate TAGs for the oxidation reactions were commercially available. In preparing reference compounds from synthetic TAGs, the following methods were used: TAG epoxides were obtained using 3-chloroperoxybenzoic acid as a reagent; TAG hydroperoxides were prepared by photosensitised oxidation with methylene blue; TAG hydroxides were prepared by reduction from corresponding TAG hydroperoxides by triphenylphosphine; TAG ketones were obtained by reduction from corresponding hydroxides by manganese dioxide; synthetic TAG core aldehydes were made by triphenylphosphine reduction of ozonides and aldehyde hydrazones were prepared by reaction with DNPH.

4.3 CLINICAL ANALYSIS (V)

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG and glucose concentrations were measured by standard spectrophotometric methods. Paraoxonase activity was determined using paraoxon (O,O-diethyl-O-*p*-nitrophenylphosphate) as substrate, and measuring the formation of 4-nitrophenol spectrophotometrically. Determination of high-sensitivity CRP concentrations was performed from the serum using an ultrasensitive immunoassay. Plasma homocysteine level was analyzed by fluorescence polarization immunoassay. Antioxidant potential of the plasma samples was measured by their potency in resisting 2,2'-azobis(2-amidinopropane)dihydrochloride (ABAP) -induced peroxidation (TRAP-method).

4.4 SAMPLE PREPARATION

Plasma and serum were separated from cells by centrifugation (**I, II, IV, V**). Chylomicrons (Svedborg flotation > 400) and VLDL (Svedborg flotation 20–400) -rich fractions were isolated from plasma by ultracentrifugation (155) (**II, IV**). LDL was precipitated from serum and plasma by buffered heparin (156) (**I, II, IV, V**). Lipids were extracted from plasma and lipoproteins using chloroform/methanol (2:1, by vol).

Three pigs from each of the two groups in animal experiment 2 were killed 2–3 hours after the last meal (**III**). Approximately 40 cm length of small intestine was detached at a distance of 80–200 cm from the beginning of the small intestine, the distance depending on the progress of intestinal contents. The portion was split in longitudinal direction. The contents were removed and the piece was rinsed with tepid water. The epithelial cells were scraped off from the intestinal wall and the lipids were extracted using chloroform/methanol (2:1, by vol). Also, a small portion of adipose tissue was detached from the neck after slaughter. The lipids of the tissue were extracted using chloroform/methanol (2:1, by vol).

4.5 PURIFICATION OF TAGS AND THEIR OXIDATON PRODUCTS

For fatty acid analysis, the TAGs of chylomicrons and VLDL (**II, IV**), LDL (**I**), or small intestinal mucosa and adipose tissue (**III**) were purified using Sep-Pak® pre-packed silica columns (Waters, Milford, MA, USA). Normal-phase TLC was used to purify the oxidised TAGs with heptane/di-isopropyl ether/acetic acid (60:40:4, by vol) as the mobile eluent (157) (**I-V**). TAG and their oxidation products were detected in UV light after spraying with 2,7-dichlorofluorescein (**I-V**). Core aldehydes were also detected using a Schiff base reagent (**I, II**). Fractions containing the oxidised TAG molecules were scraped off the plates, and were recovered from the silica gel by extraction with chloroform/methanol (2:1, by vol) (**I-V**). The extracts were washed with distilled water.

4.6 FATTY ACID COMPOSITION OF TAGS (**I-IV**)

The fatty acid methyl esters of TAG were prepared by sodium methoxide-catalyzed transesterification (158). Methyl esters were dissolved in hexane and analyzed by GC (Perkin-Elmer AutoSystem, Norwalk, CT, USA) using a DB-23 column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies, Palo Alto, CA, USA). The instrument was equipped with a flame ionisation detector.

4.7 ANALYSIS OF OXIDISED TAGS (**I-V**)

TAGs and their oxidation products were separated by RP-HPLC. In some cases, core aldehydes were separated after derivatisation with DNPH reagent (**I, II**). Discovery® HS C18 column (250 mm × 4.6 mm i.d.; Supelco Inc., Bellefonte, PA, USA) was used in the system. The column was eluted at 0.85 mL/min and a linear gradient was used: 20% 2-propanol in methanol was changed to 80% 2-propanol in 20 min. The final composition was held for 10 min. Eighty-five percent of the effluent was led to a Sedex 75 (S.E.D.E.R.E., Alfortville, France) evaporative light scattering detector (ELSD) through a Shimadzu (Kyoto, Japan) SPD-6AV UV/VIS detector that was used for the analysis of DNPH derivatives of core aldehydes. Fifteen percent of the effluent was led to a Finnigan MAT TSQ 700 triple quadrupole mass spectrometer (Finnigan, San Jose, CA, USA) equipped with a nebulizer-assisted electrospray interface. Full-scan MS spectra (m/z 450–1100) were collected in positive ionisation mode. Negative ion full-scan spectra (m/z 600–1200) were collected when DNPH derivatives were analysed (**I, II**). The electrospray voltages used were +4.5 kV and -4.5 kV for positive and negative ions, respectively.

4.8 DETERMINATION OF LIPID OXIDATION LEVEL

For the estimation of *in vivo* lipid oxidation by the baseline level of diene conjugation in plasma and lipoproteins, extracted lipids of plasma (**II, IV**), chylomicrons (**II, IV**), VLDL (**II, IV**) or LDL (**I-V**) were dissolved in cyclohexane and analysed spectrophotometrically at 234 nm (122). The proportions of oxidised TAG molecules in the total lipids of lipoproteins were estimated by HPLC using the conditions described above (**II, IV**). The estimations were based on the ELSD chromatograms of the HPLC-runs in which an internal standard was used.

4.9 ANALYSIS OF FLAVONOL ABSORPTION (V)

To determine plasma levels of quercetin, kaempferol and isorhamnetin, the flavonol conjugates present in blood samples were hydrolysed by incubating plasma for 17 h at 37 °C with a crude enzyme preparation from *Helix pomatia* containing β-glucuronidase and sulfatase activity (159). After incubation, Bond Elut C18 solid phase extraction columns (Varian Inc., Palo Alto, CA), was used to separate the flavonols. The samples were immediately frozen and stored in -70 °C.

The flavonols were analysed by HPLC with fluorescence detection, where $Al(NO_3)_3$ was used as a chelating agent (160). The sample was dried and dissolved in methanol-water (1:1, by vol), and was injected onto Phenomenex Prodigy® ODS 5u (3) column (250 × 4.60 mm, 5 µm; Torrance, CA, USA) using acetonitrile/0.025 M phosphate buffer (pH 2.4), 31:69 (by vol) as the mobile phase at a flow rate of 1 mL/min. The column was placed in a column oven set at 30 °C. The column effluent was mixed with 0.4 mL/min 1.5 M $Al(NO_3)_3$ in methanol containing 7.2% (by vol) acetic acid in a postcolumn reactor placed in the column oven. The reactor consisted of a 15-m (0.25 mm internal diameter) Teflon tubing connected to the HPLC column. The fluorescence of the flavonol-metal complexes were measured at 485 nm using a Shimadzu RF-530 (Shimadzu Corporation, Kyoto, Japan) fluorescence detector with an excitation wavelength set at 422 nm. Areas under the concentration-time curve (AUC) were calculated using the trapezoidal rule. Areas under baseline concentration levels were ignored.

4.10 STATISTICAL ANALYSIS

All statistical analyses were carried out with SPSS 10.0 and SPPS 12.0 for Windows (SPSS, Chicago, IL, USA). Differences of $P < 0.05$ were considered statistically significant. Normal distribution of the data was tested with Shapiro Wilk's test.

The comparison of BDC values and fatty acid compositions was carried out with independent samples t-test and Mann-Whitney U-test (**I, III, IV**), or with the analysis of variance and Kruskal-Wallis test (**II**).

In the clinical studies (**V**), analysis of variance for repeated measures and Friedman test were used to compare the values of different parameters between different time points. T-tests for independent and paired samples as well as corresponding nonparametric tests were used to compare individual pairs between groups and time points. Appropriate Bonferroni corrections were used.

5 RESULTS AND DISCUSSION

The thesis is based on five original papers. Paper **I** describes the application of a new approach based on HPLC-ESI-MS and BDC methods to the analysis of oxidised TAG molecular structures and *in vivo* lipid oxidation level in lipoproteins. LDL samples of normolipidaemic and hyperlidaemic subjects were used in the study. The approach was used in all the later studies. In papers **II** and **IV** conjugated dienes, TAG oxidation level and oxidised TAG species found in chylomicrons and VLDL were studied in pigs after a diet rich in either non-oxidised or oxidised sunflower seed oil. Paper **III** describes the effects of the dietary oils on the oxidised TAG structures found in the small intestinal mucosa of pigs.

In paper **V** the effects of flavonols extracted from sea buckthorn on LDL lipid oxidation and other potential risk factors of atherosclerosis were studied in humans. The flavonol extract used was aglyconic. Also, the absorption of the flavonol aglycones was investigated by HPLC after the hydrolysis of flavonol conjugates formed *in vivo* back to aglycones.

5.1 ANALYSIS OF LIPID OXIDATION AND OXIDISED TAGS IN LIPOPROTEINS

5.1.1 Pre-study (I)

The validity for the present HPLC column and gradient program of the elution factors determined by Sjövall et al. (52) was tested with TAGs of different molecular species and with core aldehyde, hydroperoxide, hydroxide and epoxide reference compounds. The retention times obtained by the present HPLC column were longer than those of the column used by Sjövall et al. for the analysis of synthesised lipids (52). This is why the linear gradient was changed from the original 30 minutes to 20 minutes. However, the elution factors could be applied to the present column as such.

Differences were found between the oxidation levels of the samples. The LDL-BDC value was 22.2 μmol/L serum in the normolipidaemic group, and 88.1 μmol/L serum in the hyperlipidaemic group. The molar proportion of conjugated dienes in the LDL lipids of the hyperlipidaemic group was 1.7 times higher than in the LDL lipids of normolipidaemic group. Differences in dietary habits between the groups and, consequently, differences in the contribution of diet to oxidative stress in the body may partly explain the phenomenon. The amounts of oxidised TAG molecules detected were small. However, several species of oxidised TAGs were identified. These included TAG molecules with a keto or an epoxy group attached to a fatty acid, and TAG molecules with a fatty acid core aldehyde. No major differences were found between the oxidation products of the samples. In some TAGs of 52 acyl carbons, the keto/epoxy ratio was greater in the hyperlipidaemic group than in the normolipidaemic group. No hydroperoxides were found in the TAG. This is surprising, but in accordance with the hypothesis that only traces of unmodified hydroperoxy lipids are found in tissue and blood samples (161).

The oxidised structures were found by extracting peaks of a specific *m/z* value from MS chromatograms. Only $[M+Na]^+$ ions were formed from the molecules other than DNPH derivatives, which were detected as $[M-H]^-$ ions. If the retention time for a peak of an *m/z* value suggested a certain type of oxidation product and a particular fatty acid composition, and a homologous series of ions was found in the chromatogram, this was regarded as strong evidence of a specific molecular structure. It was estimated that, on average, at most 20 ng/mL of non-volatile oxidation products of LDL-TAG occurred in the serum of the subjects. The results showed that the HPLC-ESI-MS method is applicable to the research of TAG oxidation in lipoproteins.

5.1.2 Animal experiments (**II, IV**)

TAGs seem to have a contribution of almost 30% to the diene conjugation of LDL (Ahotupa M., and Viikari, J., unpublished results), a lipoprotein class containing considerably less TAG than chylomicrons and VLDL. Thus, oxidised TAG molecules would be expected to have a major contribution to the total lipid oxidation of lipoproteins, particularly chylomicrons and VLDL. The TAG molecular structures described below do not denote regioisomers.

In animal experiment 1 (**II**), the lipoprotein lipid oxidation level seemed to be higher and chylomicron TAGs seemed to be more oxidised in the pigs fed on the most oxidised oil (peroxide value: 223 mequiv O_2/kg) compared to those fed on non-oxidised oil. In animal experiment 2 (**IV**), lipoprotein lipid oxidation level was typically higher and lipoprotein TAGs were more oxidised in the pigs fed on the oxidised oil (peroxide value: 190 mequiv O_2/kg) (group 2) compared with those fed on the non-oxidised oil (group 1). There seemed to be less conjugated dienes in the VLDL fraction of group 2, but estimated proportions of oxidized TAG molecules in the total lipids of both chylomicrons and VLDL were higher in group 2. In the case of chylomicrons, the difference was considerable. It is noteworthy that in both animal experiments (**II, IV**), due to degradation during heat treatment, the oxidised test oils contained less vitamin E than the non-oxidised oils, resulting in lower total vitamin E content in the feed containing the oxidised oils compared to the feed containing the non-oxidised oil (in case of animal experiment 2, 177 *vs.* 249 mg/kg feed in groups 2 and 1, respectively). This may explain some, but not all, of the differences in lipid oxidation levels between different groups.

In both studies (**II, IV**), fatty acid compositions of the TAGs of chylomicrons and VLDL reflected to a large degree the fatty acid compositions of the test oils. In VLDL TAG, 18:3n-6 (γ-linolenic acid) was present in notable amounts, which indicated incorporation of endogenous γ-linolenic acid into VLDL. Several oxidised TAG structures were found in the chylomicron and VLDL samples. These products consisted of TAG molecules with a hydroxy, an epoxy or a keto group attached to a fatty acid, and of TAG molecules containing an aldehyde structure derived from a fatty acid. Typically, the oxidation products were formed from TAG with ACN:DB (acyl carbon numer:number of double bonds) 52:2, 52:3, 52:4, 54:3, 54:4, 54:5 and 54:6. Most of the oxidised structures were found in all samples at both time points. Typically, TAG molecules with oxidative modification in only one of the fatty acids were detected.

In animal experiment 2 (**IV**), TAG core aldehydes were identified as such, not DNPH derivatives. The aldehydic $[M + Na]^+$ ions were observed to be partly transformed to $[M + Na + 32]^+$ adduct ions in our ion source, possibly indicating formation of hemiacetal structures within the molecules (Sjövall, O., personal communication). The phenomenon helped in the detection and identification of the core aldehydes.

In animal experiment 1 (**II**), the peaks representing the TAG ketones of chylomicrons at time point 1 seemed to be slightly stronger in the pigs fed on the most oxidised oil compared to the other groups. In animal experiment 2 (**IV**), the peaks representing the TAG hydroxides/epoxides/ketones of 54 or 56 acyl carbons and the peaks representing the TAG core aldehydes of 45 carbons with a hydroxy/epoxy/keto group attached to the molecule were stronger in group 2 compared to group 1. An exception was the ion representing the structure 18:2 OH/18:1/18:1 (postulated), which was stronger in group 1. The ratio of the structure 18:2 keto/18:2/18:2 (ACN:DB 54:6) to the structures 18:2 keto/18:2/18:1+18:1 keto/18:2/18:2 (ACN:DB 54:5) seemed to be higher in group 2 compared to group 1.

Virtually no TAG hydroperoxides could be detected either in the chylomicron or VLDL samples (**II, IV**). This is in contrast with the oxidised test oils, where monohydroperoxides were the major class of oxidized TAGs. In the test oils, monohydroperoxides were formed from TAG with ACN:DB 52:2, 52:3, 52:4, 54:3, 54:4, 54:5 and 54:6 (like most of the oxidised TAG molecules in the lipoprotein samples). The hydroperoxy TAG molecules were partly fragmented in the ion source (cleavage of H_2O), which helped in the identification of such molecules. The product formed could be a ketone or an epoxide without a loss of any double bonds. Neff and Byrdwell (162) discovered that in atmospheric-pressure chemical ionization (APCI) ion source, TAG hydroperoxides formed epoxides by loss of the outer -OH from the hydroperoxy group, followed by cyclisation of the remaining oxygen. This reaction may also have taken place in our ESI source. The possibility that some decomposition of hydroperoxy structures had occurred during sample preparation cannot be excluded. However, in the present studies, TAG hydroperoxides had been relatively stable during extraction procedures, TLC, and HPLC runs.

Lipoproteins rich in TAG seem to be important factors in the induction of atherogenesis (163), which could at least partly be explained by oxidised TAG molecules. The results of the animal experiments (**II, IV**) show that the oxidation of dietary fat was reflected in the lipoprotein oxidation. Thus, it seems that the oxidised TAGs are absorbed in the small intestine and/or they initiate a cascade of subsequent oxidation reactions that result in the increased oxidation of lipoproteins and their TAG molecules.

5.2 ANALYSIS OF TAG OXIDATION IN SMALL INTESTINAL MUCOSA (III)

The TAGs of the mucosa samples represented mainly the reassembled molecules formed inside the epithelial cells. Minor amounts of the TAGs may also have originated from the luminal side of the cells, representing molecules not broken down by gastric and pancreatic lipases, and not detached by rinsing. The TAG fatty acid compositions reflected to a large degree those of the dietary sunflower seed oils. The results also indicated an effect of test oil autoxidation on the TAG fatty acid composition of adipose tissue. In the pigs fed on oxidised sunflower seed oil (group 2), but not in the pigs fed on non-oxidised oil (group 1), the relative amount of mucosal linoleic acid (18:2n-6) seemed to be lower compared to the corresponding test oil.

In the oxidised test oil, TAG monohydroperoxides were the major class of oxidised TAG. Monohydroperoxides detected were formed from TAG with ACN:DB 52:2, 52:3, 52:4, 54:3, 54:4, 54:5 and 54:6. Dihydroperoxides were formed from TAG with ACN:DB 52:4, 54:6 and 54:5. However, these TAG hydroperoxides were not detected in the mucosa or the adipose tissue samples, neither as such nor as a combination of hydroperoxy and oxo/epoxy groups. This indicates an absence of these molecules in the tissue TAG or a considerable decrease in the level of them compared to the oxidized sunflower seed oil. Taken the sensitivity of the instrumentation into consideration, the decrease was estimated to be at least 80% in case of monohydroperoxides. The system was not optimised for the analysis of TAG trihydroperoxides and other equally polar TAG oxidation products.

TAG hydroxides, ketones and epoxides were detected in the tissue samples of both groups. No core aldehydes were detected. Same oxidised TAG molecular species were detected in both groups. It is interesting that TAG hydroxides, ketones and epoxides were also detected in both the non-oxidised and oxidised test oils, although only traces of hydroperoxides were observed in the non-oxidised oil. These findings indicate that our method sensitively detects the secondary oxidation products of TAG present in different matrices.

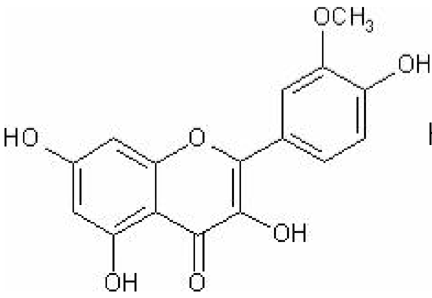
The results suggest an effective degradation of hydroperoxides before and/or during the reassembly of TAG molecules in the epithelial cells of small intestinal mucosa. The findings are in line with the results of Kanazawa et al. (98, 99), which showed a very limited transport of hydroperoxides into small intestine.

5.3. STUDIES ON SEA BUCKTHORN FLAVONOLS AND THEIR EFFECTS (V)

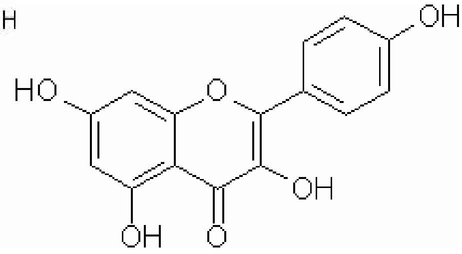
In recent years, a number of new potential risk factors or markers have been proposed as predictors of atherosclerosis and its complications. C-reactive protein (CRP) is a circulating acute-phase reactant that is increased many-fold during the inflammatory response to tissue injury or infection (164). An evolving body of work suggests that even small increases in CRP within the normal range are predictive of future vascular events (165). Homocysteine is a highly reactive amino acid formed as a by-product of the metabolism of the essential amino acid methionine (166). Numerous observational studies have reported on the association between homocysteine levels and vascular risk (165). Paraoxonase is an antioxidative enzyme that has a role in removing oxidised lipids from LDL and HDL. Studies suggest that low paraoxonase activity is a risk factor of cardiovascular disease (167). In clinical trial 1, the porridge with the sea buckthorn flavonol extract did not have clear effects on these parameters, nor on LDL-BDC values. However, HDL cholesterol was slightly decreased during the control period. There seems to have been a minor decrease in total cholesterol level as well during both periods, although this decrease was not significant.

In all subjects, some oxidised TAG molecules of 50 and 52 acyl carbons were detected in LDL after both study periods. The molecules were observed to contain keto and epoxy structures in their fatty acid residues. However, the consumption of test meals during the study periods did not have clear effects on the proportion of these structures in the LDL total lipids.

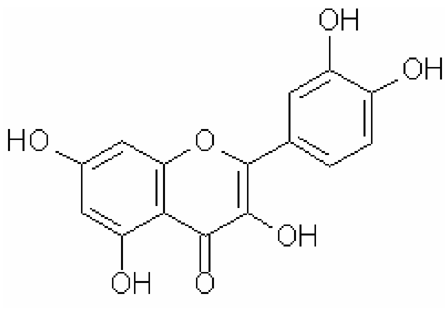
The flavonoids in sea buckthorn fruit pulp are mainly flavonols, of which isorhamnetin is typically found in largest amounts (**Figure 5**). Quercetin is also present, as well as small amounts of kaempferol. In the berries, flavonols are present mainly as glycosides. In the body, flavonols form conjugates with glucuronic acid and sulfate (168). In clinical trial 2, these conjugates were hydrolysed in order to simplify the analysis of flavonol absorption. Plasma flavonol concentrations were estimated by using standard compounds, which went through the same sample preparation procedures as the plasma samples. The flavonols added to porridge, both with and without added sea buckthorn oil, increased the plasma levels of isorhamnetin and kaempferol with both doses of the flavonol extract (78 or 39 mg of flavonols). The doses of total flavonols are not beyond the levels that could be obtained from normal diet that includes e.g. flavonol-rich berries or fruits.



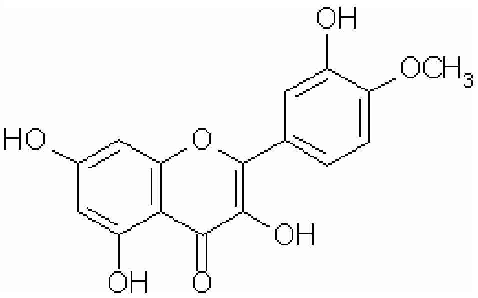
Isorhamnetin



Kaempferol



Quercetin



Tamarixetin

Figure 5. Molecular structures of some flavonol aglycones.

Although the trend was similar after all meals, the increase in the plasma quercetin concentration was only significant after meal 2B. The relatively high standard deviations and hence somewhat poor precision were not caused by HPLC separation or fluorescense detection, but rather by sample preparation steps. However, the results clearly demonstrate the absorption of the flavonol aglycones. The plasma concentrations of quercetin, kaempferol, and isorhamnetin peaked 1 h after the meal, and started to decrease after that.

This indicates absorption from the upper duodenum and perhaps even from the stomach. AUC-values were calculated, and both with and without added sea buckthorn oil, the results showed significantly ($P < 0.05$) higher plasma levels of kaempferol and isorhamnetin, when a higher dose of the extract was used.

Some of the isorhamnetin found in the plasma may have originated from quercetin methylated after absorption (3´-methylation) (169). The degree of 3´-methylation in humans compared to 4´-methylation is not known. However, it seems that most of the quercetin remains unmethylated (10, 168, 170). The HPLC method used does not efficiently differentiate between isorhamnetin (3´-methylquercetin) and tamarixetin (4´-methylquercetin). Thus, some tamarixetin formed from quercetin after absorption may have eluted along with isorhamnetin.

The addition of a small amount (3 g) of sea buckthorn oil seemed to have enhanced the absorption and bioavailability (determined as AUC) of flavonols in the group with a higher amount of dietary flavonols. This is in accordance with the study of Lesser et al. (169), in which higher fat concentration (17% *vs.* 3% in the diet) increased the bioavailability of quercetin. With the lower dose of flavonol extract, the increase was not shown in the present study.

The results give interesting new information on the absorption of different flavonol classes. The absorption of kaempferol and isorhamnetin aglycones, flavonols whose absorption and bioavailability have not been studied widely, was clearly shown.

6 CONCLUSIONS

During the last few years, various methods have been optimised and applied to the analysis of lipid oxidation products *in vivo*, and information on the molecular structures of oxidised lipids in plasma, lipoproteins and atherosclerotic plaques has started to accumulate. Methods based on HPLC-ESI-MS and HPLC-ESI-MS/MS have been particularly important in the field of structural research. Although TAGs are a major lipid class in lipoproteins, the analysis of oxidised TAG structures in the particles has been neglected.

In the present studies, a novel analytical approach based on HPLC-ESI-MS and BDC methods was applied to the analysis of lipoprotein lipids. The structural analysis of oxidised TAG molecules found in chylomicrons, VLDL and LDL is reported for the first time. Various oxidised TAG structures with hydroxy, keto, epoxy or aldehydic groups were found in lipoproteins and small intestinal mucosa. The oxidation levels of lipoprotein lipids and their TAG molecules were shown to be increased by oxidised dietary oil.

Dietary flavonols extracted from sea buckthorn did not affect LDL lipid oxidation, but their absorption was clearly demonstrated in the studies. Gastrointestinal absorption of isorhamnetin is reported for the first time. The results also suggest that the amount of ingested fat affects the bioavailability of the flavonols. Diet has an important contribution to oxidative stress in the body, and dietary components interact with each other both before and after absorption in the gastrointestinal tract. In order to fully benefit from dietary components with antioxidant activity, they should be ingested simultaneously with oxidisable components (e.g. fats) that are already oxidised to some degree or prone to oxidation in the body.

The present results encourage further research on the physiological consequences of dietary fat oxidation and antioxidant supplementation, as well as detailed studies on the effects of oxidised dietary lipids on lipid molecular structures found in various lipoproteins and tissues.

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REFERENCES

1. Witztum, J.L. (1994) The Oxidation Hypothesis of Atherosclerosis, *Lancet 344*, 793-795.

2. Berliner, J.A., and Heinecke, J.W. (1996) The Role of Oxidized Lipoproteins in Atherogenesis, *Free Radic. Biol. Med. 20*, 707-727.

3. Jialal, I., and Devaraj, S. (1996) Low Density Lipoprotein Oxidation, Antioxidants, and Atherosclerosis: A Clinical Biochemistry Perspective, *Clin. Chem. 42*, 498-506.

4. Young, I.S., McFarlane, C., and McEneny, J. (2003) Oxidative Modification of Triacylglycerol-Rich Lipoproteins, *Biochem. Soc. Trans. 31*, 1062-1065.

5. Grieve, D.J., Avella, M.A., Elliott, J., and Botham, K.M. (2000) The Interaction between Oxidised Chylomicron Remnants and the Aorta of Rats Fed a Normocholesterolaemic or Hypercholesterolaemic Diet, *J. Vasc. Res. 37*, 265-275.

6. Napolitano, M., Rivabene, R., Avella, M., Amicone, L., Tripodi, M., Botham, K.M., and Bravo, E. (2001) Oxidation Affects the Regulation of Hepatic Lipid Synthesis by Chylomicron Remnants, *Free Radic. Biol. Med. 30*, 506-515.

7. Naruszewicz, M., Wozny, E., Mirkiewicz, E., Nowicka, G., and Szostak, W.B. (1987) The Effect of Thermally Oxidized Soya Bean Oil on Metabolism of Chylomicrons - Increased Uptake and Degradation of Oxidized Chylomicrons in Cultured Mouse Macrophages, *Atherosclerosis 66*, 45-53.

8. Umeda, Y., Redgrave, T.G., Mortimer, B.C., and Mamo, J.C.L. (1995) Kinetics and Uptake *in vivo* of Oxidatively Modified Lymph Chylomicrons, *Am. J. Physiol. 31*, G709-G716.

9. Scalbert, A., Manach, C., Morand, C., Remesy, C., and Jimenez, L. (2005) Dietary Polyphenols and the Prevention of Diseases, *Crit. Rev. Food Sci. Nutr. 45*, 287-306.

10. Erlund, I. (2004) Review of the Flavonoids Quercetin, Hesperetin, and Naringenin. Dietary Sources, Bioactivities, and Epidemiology, *Nutr. Res. 24*, 851-874.

11. Williams, R.J., Spencer, J.P.E., and Rice-Evans, C. (2004) Flavonoids: Antioxidants or Signalling Molecules? *Free Radic. Biol. Med. 36*, 838-849.

12. Cho, S.Y., Park, S.J., Kwon, M.J., Jeong, T.S., Bok, S.H., Choi, W.Y., Jeong, W.I., Ryu, S.Y., Do, S.H., Lee, C.S., Song, J.C., and Jeong, K.S. (2003) Quercetin Suppresses Proinflammatory Cytokines Production through MAP Kinases and NF-Kappa B Pathway in Lipopolysaccharide-Stimulated Macrophage, *Mol. Cell. Biochem. 243*, 153-160.

13. Wang, B. (1993) Effect of Total Flavonoids of *Hippophaë rhamnoides* L. on Cardiac Performance and Blood Stream Dynamics of Rabbit, *Hippophaë 6*.

14. Wang, B. (1993) The Rheological Effect of Sea Buckthorn Total Flavonoids on the Blood of Normal Rats, *North-West J. Pharm. 8*.

15. Wang, B., and Wang, Y. (1993) Effect of Total Flavonoids of *Hippophaë rhamnoides* L. on the Cardiac Function of Guinea Pig, *Hippophaë 6*.

16. Wang, J., and Zaong, M. (1985) Observation on the Curative Effect of Sea Buckthorn Total Flavonoids on Ischemic Heart Disease, *Shanxi Med. Res. (Sea Buckthorn)*.

17. Wang, B. (1994) The Effect of Total Flavonoids of *Hippophaë rhamnoides* L. (TFH) on Cardiac Performance and Hemodynamics of Normal People, *J. Xian Med. Univ. 14*.

18. Wang, B., Li, K., and Qi, Y. (1994) Effect of Total Flavonoids of *Hippophaë rhamnoides* L. on Frog Heart *in vivo* and Toad Heart *in vitro*, *Hippophäe 7*.

19. Ahotupa, M., Marniemi, J., Lehtimäki, T., Talvinen, K., Raitakari, O.T., Vasankari, T., Viikari, J., Luoma, J., and Ylä-Herttuala, S. (1998) Baseline Diene Conjugation in LDL Lipids as a Direct Measure of *in vivo* LDL Oxidation, *Clin. Biochem. 31*, 257-261.

20. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. (1989) Beyond Cholesterol - Modifications of Low-Density Lipoprotein that Increase its Atherogenicity, *N. Engl. J. Med. 320*, 915-924.

21. Stocker, R., and Keaney, J.F. (2004) Role of Oxidative Modifications in Atherosclerosis, *Physiol. Rev. 84*, 1381-1478.

22. Witztum, J.L., and Steinberg, D. (2001) The Oxidative Modification Hypothesis of Atherosclerosis: Does it Hold for Humans? *Trends Cardiovasc. Med. 11*, 93-102.

23. Ahotupa, M., and Vasankari, T.J. (1999) Baseline Diene Conjugation in LDL Lipids: An Indicator of Circulating Oxidized LDL, *Free Radic. Biol. Med. 27*, 1141-1150.

24. Ferns, G.A.A., and Lamb, D.J. (2004) What does the Lipoprotein Oxidation Phenomenon Mean? *Biochem. Soc. Trans. 32*, 160-163.

25. Niki, E. (2004) Antioxidants and Atherosclerosis, Biochem. *Soc. Trans. 32*, 156-159.

26. Staprans, I., Pan, X.M., Rapp, J.H., and Feingold, K.R. (2005) The Role of Dietary Oxidized Cholesterol and Oxidized Fatty Acids in the Development of Atherosclerosis, *Mol. Nutr. Food Res. 49*, 1075-1082.

27. Ylä-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., and Steinberg, D. (1989) Evidence for the Presence of Oxidatively Modified Low-Density Lipoprotein in Atherosclerotic Lesions of Rabbit and Man, *J. Clin. Invest. 84*, 1086-1095.

28. Jessup, W., Kritharides, L., and Stocker, R. (2004) Lipid Oxidation in Atherogenesis: An Overview, *Biochem. Soc. Trans. 32*, 134-138.

29. Gotto, A.M., Pownall, H.J., and Havel, R.J. (1986) Introduction to the Plasma-Lipoproteins, *Methods Enzymol. 128*, 3-41.

30. Assmann, G. (1982) Apolipoproteins and Cardiovascular-Diseases, *Fette Seifen Anstr. 84*, 829.

31. Upston, J.M., Niu, X.W., Brown, A.J., Mashima, R., Wang, H.J., Senthilmohan, R., Kettle, A.J., Dean, R.T., and Stocker, R. (2002) Disease Stage-Dependent Accumulation of Lipid and Protein Oxidation Products in Human Atherosclerosis, *Am. J. Pathol. 160*, 701-710.

32. Durrington, P.N. (1995) *Hyperlipidaemia: Diagnosis and Management*, 2nd edition. Butterworth-Heinemann, London.

33. Watson, A.D., Leitinger, N., Navab, M., Faull, K.F., Hörkkö, S., Witztum, J.L., Palinski, W., Schwenke, D., Salomon, R.G., Sha, W., Subbanagounder, G., Fogelman, A.M., and Berliner, J.A. (1997) Structural Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally Oxidized Low Density Lipoprotein that Induce Monocyte/endothelial Interactions and Evidence for their Presence *in vivo*, *J. Biol. Chem. 272*, 13597-13607.

34. De Castellarnau, C., Sanchez-Quesada, J.L., Benitez, S., Rosa, R., Caveda, L., Vila, L., and Ordonez-Llanos, J. (2000) Electronegative LDL from Normolipemic Subjects Induces IL-8 and Monocyte Chemotactic Protein Secretion by Human Endothelial Cells, *Arterioscler. Thromb. Vasc. Biol. 20*, 2281-2287.

35. Chisolm, G.M., Ma, G.P., Irwin, K.C., Martin, L.L., Gunderson, K.G., Linberg, L.F., Morel, D.W., and Dicorleto, P.E. (1994) 7-Beta-hydroperoxycholest-5-en-3-beta-ol, a Component of Human Atherosclerotic Lesions, is the Primary Cytotoxin of Oxidized Human Low-Density-Lipoprotein, *Proc. Natl. Acad. Sci. U. S. A. 91*, 11452-11456.

36. Hakamata, H., Miyazaki, A., Sakai, M., Matsuda, H., Suzuki, H., Kodama, T., and Horiuchi, S. (1998) Isolation of Macrophage-Like Cell Mutants Resistant to the Cytotoxic Effect of Oxidized Low Density Lipoprotein, *J. Lipid Res. 39*, 482-494.

37. Xu, X.P., Meisel, S.R., Ong, J.M., Kaul, S., Cercek, B., Rajavashisth, T.B., Sharifi, B., and Shah, P.K. (1999) Oxidized Low-Density Lipoprotein Regulates Matrix Metalloproteinase-9 and its Tissue Inhibitor in Human Monocyte-Derived Macrophages, *Circulation 99*, 993-998.

38. Waddington, E.I., Puddey, I.B., Mori, T.A., and Croft, K.D. (2002) Similarity in the Distribution of F-2-Isoprostanes in the Lipid Subfractions of Atherosclerotic Plaque and *in vitro* Oxidised Low Density Lipoprotein, *Redox Report 7*, 179-184.

39. Yamashita, H., Nakamura, A., Noguchi, N., Niki, E., and Kuhn, H. (1999) Oxidation of Low Density Lipoprotein and Plasma by 15-Lipoxygenase and Free Radicals, *FEBS Lett. 445*, 287-290.

40. Zhao, L., and Funk, C.D. (2004) Lipoxygenase Pathways in Atherogenesis, *Trends Cardiovasc. Med. 14*, 191-195.

41. Brennan, M.L., and Hazen, S.L. (2003) Amino Acid and Protein Oxidation in Cardiovascular Disease, *Amino Acids 25*, 365-374.

42. Hazen, S.L., and Heinecke, J.W. (1997) 3-Chlorotyrosine, a Specific Marker of Myeloperoxidase-Catalyzed Oxidation, is Markedly Elevated in Low Density Lipoprotein Isolated from Human Atherosclerotic Intima, *J. Clin. Invest. 99*, 2075-2081.

43. Uchida, K. (2000) Role of Reactive Aldehyde in Cardiovascular Diseases, *Free Radic. Biol. Med. 28*, 1685-1696.

44. Francis, G.A. (2000) High Density Lipoprotein Oxidation: *in vitro* Susceptibility and Potential *in vivo* Consequences, *Biochim. Biophys. Acta 1483*, 217-235.

45. Kobayashi, T., Tahara, Y., Matsumoto, M., Iguchi, M., Sano, H., Murayama, T., Arai, H., Oida, H., Yurugi-Kobayashi, T., Yamashita, J.K., Katagiri, H., Majima, M., Yokode, M., Kita, T., and Narumiya, S. (2004) Roles of Thromboxane A(2) and Prostacyclin in the Development of Atherosclerosis in apoE-Deficient Mice, *J. Clin. Invest. 114*, 784-794.

46. Mamo, J.C.L., and Wheeler, J.R. (1994) Chylomicrons or their Remnants Penetrate Rabbit Thoracic Aorta as Efficiently as Smaller Macromolecules Including Low Density Lipoprotein, High Density Lipoprotein and Albumin, *Coron. Artery Dis. 5*, 695-705.

47. Botham, K.M., Bravo, E., Elliott, J., and Wheeler-Jones, C.P.D. (2005) Direct Interaction of Dietary Lipids Carried in Chylomicron Remnants with Cells of the Artery Wall: Implications for Atherosclerosis Development, *Curr. Pharm. Des. 11*, 3681-3695.

48. Doi, H., Kugiyama, K., Oka, H., Sugiyama, S., Ogata, N., Koide, S., Nakamura, S., and Yasue, H. (2000) Remnant Lipoproteins Induce Proatherothrombogenic Molecules in Endothelial Cells through a Redox-Sensitive Mechanism, *Circulation 102*, 670-676.

49. Bowry, V.W., Stanley, K.K., and Stocker, R. (1992) High-Density-Lipoprotein is the Major Carrier of Lipid Hydroperoxides in Human Blood-Plasma from Fasting Donors, *Proc. Natl. Acad. Sci. U. S. A. 89*, 10316-10320.

50. Cohn, J.S. (2002) Oxidized Fat in the Diet, Postprandial Lipaemia and Cardiovascular Disease, *Curr. Opin. Lipidol. 13*, 19-24.

51. Byrdwell, W.C., and Neff, W.E. (1999) Non-Volatile Products of Triolein Produced at Frying Temperatures Characterized using Liquid Chromatography with Online Mass Spectrometric Detection, *J. Chromatogr. A 852*, 417-432.

52. Sjövall, O., Kuksis, A., Marai, L., and Myher, J.J. (1997) Elution Factors of Synthetic Oxotriacylglycerols as an Aid in Identification of Peroxidized Natural Triacylglycerols by Reverse-Phase High-Performance Liquid Chromatography with Electrospray Mass Spectrometry, *Lipids 32*, 1211-1218.

53. Sjövall, O., Lapveteläinen, A., Johansson, A., and Kallio, H. (1997) Analysis of Volatiles Formed during Oxidation of Extruded Oats, *J. Agric. Food Chem. 45*, 4452-4455.

54. Staprans, I., Pan, X.M., Miller, M., and Rapp, J.H. (1993) Effect of Dietary-Lipid Peroxides on Metabolism of Serum Chylomicrons in Rats, *Am. J. Physiol. 264*, G561-G568.

55. Staprans, I., Rapp, J.H., Pan, X.M., and Feingold, K.R. (1996) Oxidized Lipids in the Diet are Incorporated by the Liver into very Low Density Lipoprotein in Rats, *J. Lipid Res. 37*, 420-430.

56. Staprans, I., Rapp, J.H., Pan, X.M., Kim, K.Y., and Feingold, K.R. (1994) Oxidized Lipids in the Diet are a Source of Oxidized Lipid in Chylomicrons of Human Serum, *Arterioscler. Thromb. 14*, 1900-1905.

57. Aw, T.Y., Williams, M.W., and Gray, L. (1992) Absorption and Lymphatic Transport of Peroxidized Lipids by Rat Small-Intestine *in vivo* - Role of Mucosal Gsh, *Am. J. Physiol. 262*, G99-G106.

58. Vine, D.F., Croft, K.D., Beilin, L.J., and Mamo, J.C.L. (1997) Absorption of Dietary Cholesterol Oxidation Products and Incorporation into Rat Lymph Chylomicrons, *Lipids 32*, 887-893.

59. Vine, D.F., Mamo, J.C.L., Beilin, L.J., Mori, T.A., and Croft, K.D. (1998) Dietary Oxysterols are Incorporated in Plasma Triglyceride-Rich Lipoproteins, Increase their Susceptibility to Oxidation and Increase Aortic Cholesterol Concentration of Rabbits, *J. Lipid Res. 39*, 1995-2004.

60. Staprans, I., Pan, X.M., Rapp, J.H., and Feingold, K.R. (2003) Oxidized Cholesterol in the Diet is a Source of Oxidized Lipoproteins in Human Serum, *J. Lipid Res. 44*, 705-715.

61. Staprans, I., Rapp, J.H., Pan, X.M., and Feingold, K.R. (1993) The Effect of Oxidized Lipids in the Diet on Serum-Lipoprotein Peroxides in Control and Diabetic Rats, *J. Clin. Invest. 92*, 638-643.

62. Wilson, R., Lyall, K., Smyth, L., Fernie, C.E., and Riemersma, R.A. (2002) Dietary Hydroxy Fatty Acids are Absorbed in Humans: Implications for the Measurement of 'Oxidative Stress' *in vivo*, *Free Radic. Biol. Med. 32*, 162-168.

63. Staprans, I., Rapp, J.H., Pan, X.M., Hardman, D.A., and Feingold, K.R. (1996) Oxidized Lipids in the Diet Accelerate the Development of Fatty Streaks in Cholesterol-Fed Rabbits, *Arterioscler. Thromb. Vasc. Biol. 16*, 533-538.

64. Garrido-Polonio, C., Garcia-Linares, M.C., Garcia-Arias, M.T., Lopez-Varela, S., Garcia-Fernandez, M.C., Terpstra, A.H.M., and Sanchez-Muniz, F.J. (2004) Thermally Oxidised Sunflower-Seed Oil Increases Liver and Serum Peroxidation and Modifies Lipoprotein Composition in Rats, *Br. J. Nutr. 92*, 257-265.

65. Staprans, I., Pan, X.M., Rapp, J.H., and Feingold, K.R. (1998) Oxidized Cholesterol in the Diet Accelerates the Development of Aortic Atherosclerosis in Cholesterol-Fed Rabbits, *Arterioscler. Thromb. Vasc. Biol. 18*, 977-983.

66. Carr, A.C., McCall, M.R., and Frei, B. (2000) Oxidation of LDL by Myeloperoxidase and Reactive Nitrogen Species - Reaction Pathways and Antioxidant Protection, *Arterioscler. Thromb. Vasc. Biol. 20*, 1716-1723.

67. Noguchi, N. (2002) Novel Insights into the Molecular Mechanisms of the Antiatherosclerotic Properties of Antioxidants: The Alternatives to Radical Scavenging, *Free Radic. Biol. Med. 33*, 1480-1489.

68. Clarke, R., and Armitage, J. (2002) Antioxidant Vitamins and Risk of Cardiovascular Disease. Review of Large-Scale Randomised Trials, *Cardiovasc. Drug. Ther. 16*, 411-415.

69. Cirico, T.L., and Ornaye, S.T. (2006) Additive or Synergetic Effects of Phenolic Compounds on Human Low Density Lipoprotein Oxidation, *Food Chem. Toxicol. 44*, 510-516.

70. Chopra, M., Fitzsimons, P.E.E., Strain, J.J.T., Thurnham, D.I., and Howard, A.N. (2000) Nonalcoholic Red Wine Extract and Quercetin Inhibit LDL Oxidation without Affecting Plasma Antioxidant Vitamin and Carotenoid Concentrations, *Clin. Chem. 46*, 1162-1170.

71. Kostyuk, V.A., Kraemer, T., Sies, H., and Schewe, T. (2003) Myeloperoxidase/nitrite-Mediated Lipid Peroxidation of Low-Density Lipoprotein as Modulated by Flavonoids, *FEBS Lett. 537*, 146-150.

72. Aldred, S., and Griffiths, H.R. (2004) Oxidation of Protein in Human Low-Density Lipoprotein Exposed to Peroxyl Radicals Facilitates Uptake by Monocytes; Protection by Antioxidants *in vitro*, *Environ. Toxicol. Pharmacol. 15*, 111-117.

73. Janisch, K.M., Williamson, G., Needs, P., and Plumb, G.W. (2004) Properties of Quercetin Conjugates: Modulation of LDL Oxidation and Binding to Human Serum Albumin, *Free Radic. Res. 38*, 877-884.

74. Morand, C., Crespy, V., Manach, C., Besson, C., Demigne, C., and Remesy, C. (1998) Plasma Metabolites of Quercetin and their Antioxidant Properties, *Am. J. Physiol. 44*, R212-R219.

75. Arteaga, E., Villaseca, P., Rojas, A., Marshall, G., and Bianchi, M. (2004) Phytoestrogens Possess a Weak Antioxidant Activity on Low Density Lipoprotein in Contrast to the Flavonoid Quercetin *in vitro* in Postmenopausal Women, *Climacteric 7*, 397-403.

76. Zhang, X.F., Thuong, P.T., Jin, W., Su, N.D., Sok, D.E., Bae, K., and Kang, S.S. (2005) Antioxidant Activity of Anthraquinones and Flavonoids from Flower of Reynoutria Sachalinensis, *Arch. Pharm. Res. 28*, 22-27.

77. Da Silva, E.L., Tsushida, T., and Terao, J. (1998) Inhibition of Mammalian 15-Lipoxygenase-Dependent Lipid Peroxidation in Low-Density Lipoprotein by Quercetin and Quercetin Monoglucosides, *Arch. Biochem. Biophys. 349*, 313-320.

78. Filipe, P., Haigle, J., Silva, J.N., Freitas, J., Fernandes, A., Maziere, J.C., Maziere, C., Santus, R., and Morliere, P. (2004) Anti- and Pro-Oxidant Effects of Quercetin in Copper-

Induced Low Density Lipoprotein Oxidation - Quercetin as an Effective Antioxidant Against Pro-Oxidant Effects of Urate, *Eur. J. Biochem. 271*, 1991-1999.

79. Benito, S., Buxaderas, S., and Mitjavila, M.T. (2004) Flavonoid Metabolites and Susceptibility of Rat Lipoproteins to Oxidation, *Am. J. Physiol. 287*, H2819-H2824.

80. Chen, C.Y., Milbury, P.E., Lapsley, K., and Blumberg, J.B. (2005) Flavonoids from Almond Skins are Bioavailable and Act Synergistically with Vitamins C and E to Enhance Hamster and Human LDL Resistance to Oxidation, *J. Nutr. 135*, 1366-1373.

81. Yeomans, V.C., Linseisen, J., and Wolfram, G. (2005) Interactive Effects of Polyphenols, Tocopherol and Ascorbic Acid on the Cu^{2+}-Mediated Oxidative Modification of Human Low Density Lipoproteins, *Eur. J. Nutr. 44*, 422-428.

82. Meyer, A.S., Heinonen, M., and Frankel, E.N. (1998) Antioxidant Interactions of Catechin, Cyanidin, Caffeic Acid, Quercetin, and Ellagic Acid on Human LDL Oxidation, *Food Chem. 61*, 71-75.

83. Hayek, T., Fuhrman, B., Vaya, J., Rosenblat, M., Belinky, P., Coleman, R., Elis, A., and Aviram, M. (1997) Reduced Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice Following Consumption of Red Wine, or its Polyphenols Quercetin Or Catechin, is Associated with Reduced Susceptibility of LDL to Oxidation and Aggregation, *Arterioscler. Thromb. Vasc. Biol. 17*, 2744-2752.

84. Enkhmaa, B., Shiwaku, K., Katsube, T., Kitajima, K., Anuurad, E., Yamasaki, M., and Yamane, Y. (2005) Mulberry (*Morus alba* L.) Leaves and their Major Flavonol Quercetin 3-(6-Malonylglucoside) Attenuate Atherosclerotic Lesion Development in LDL Receptor-Deficient Mice, *J. Nutr. 135*, 729-734.

85. Auger, C., Rouanet, J.M., Vanderlinde, R., Bornet, A., Decorde, K., Lequeux, N., Cristol, J.P., and Teissedre, P.L. (2005) Polyphenols-Enriched Chardonnay White Wine and Sparkling Pinot Noir Red Wine Identically Prevent Early Atherosclerosis in Hamsters, *J. Agric. Food Chem. 53*, 9823-9829.

86. O'Reilly, J.D., Mallet, A.I., McAnlis, G.T., Young, I.S., Halliwell, B., Sanders, T.A.B., and Wiseman, H. (2001) Consumption of Flavonoids in Onions and Black Tea: Lack of Effect on F-2-Isoprostanes and Autoantibodies to Oxidized LDL in Healthy Humans, *Am. J. Clin. Nutr. 73*, 1040-1044.

87. Zern, T.L., Wood, R.J., Greene, C., West, K.L., Liu, Y.Z., Aggarwal, D., Shachter, N.S., and Fernandez, M.L. (2005) Grape Polyphenols Exert a Cardioprotective Effect in Pre- and Postmenopausal Women by Lowering Plasma Lipids and Reducing Oxidative Stress, *J. Nutr. 135*, 1911-1917.

88. Witting, P., Pettersson, K., Östlund-Lindqvist, A.M., Westerlund, C., Wagberg, M., and Stocker, R. (1999) Dissociation of Atherogenesis from Aortic Accumulation of Lipid Hydro(pero)xides in Watanabe Heritable Hyperlipidemic Rabbits, *J. Clin. Invest. 104*, 213-220.

89. Milne, G.L., Seal, J.R., Havrilla, C.M., Wijtmans, M., and Porter, N.A. (2005) Identification and Analysis of Products Formed from Phospholipids in the Free Radical Oxidation of Human Low Density Lipoproteins, *J. Lipid Res. 46*, 307-319.

90. Cherubini, A., Vigna, G.B., Zuliani, G., Ruggiero, C., Senin, U., and Fellin, R. (2005) Role of Antioxidants in Atherosclerosis: Epidemiological and Clinical Update, *Curr. Pharm. Des. 11*, 2017-2032.

91. Moore, E.H., Napolitano, M., Prosperi, A., Avella, M., Suckling, K.E., Bravo, E., and Botham, K.M. (2003) Incorporation of Lycopene into Chylomicron Remnant-Like Particles Enhances their Induction of Lipid Accumulation in Macrophages, *Biochem. Biophys. Res. Commun. 312*, 1216-1219.

92. Moore, E.H., Napolitano, M., Avella, M., Bejta, F., Suckling, K.E., Bravo, E., and Botham, K.M. (2004) Protection of Chylomicron Remnants from Oxidation by Incorporation of Probucol into the Particles Enhances their Uptake by Human Macrophages and Increases Lipid Accumulation in the Cells, *Eur. J. Biochem. 271*, 2417-2427.

93. Suzukawa, M., Abbey, M., Clifton, P., and Nestel, P.J. (1994) Effects of Supplementing with Vitamin-E on the Uptake of Low-Density-Lipoprotein and the Stimulation of Cholesteryl Ester Formation in Macrophages, *Atherosclerosis 110*, 77-86.

94. Yamamoto, A., Hara, H., Takaichi, S., Wakasugi, J.I., and Tomikawa, M. (1988) Effect of Probucol on Macrophages, Leading to Regression of Xanthomas and Atheromatous Vascular-Lesions, *Am. J. Cardiol. 62*, B31-B36.

95. Witting, P.K., Mohr, D., and Stocker, R. (1999) Assessment of Prooxidant Activity of Vitamin E in Human Low-Density Lipoprotein and Plasma, *Methods Enzymol. 299*, 362-375.

96. Niki, E., Yoshida, Y., Saito, Y., and Noguchi, N. (2005) Lipid Peroxidation: Mechanisms, Inhibition, and Biological Effects, *Biochem. Biophys. Res. Commun. 338*, 668-676.

97. Kanner, J., and Lapidot, T. (2001) The Stomach as a Bioreactor: Dietary Lipid Peroxidation in the Gastric Fluid and the Effects of Plant-Derived Antioxidants, *Free Radic. Biol. Med. 31*, 1388-1395.

98. Kanazawa, K., and Ashida, H. (1998) Catabolic Fate of Dietary Trilinoleoylglycerol Hydroperoxides in Rat Gastrointestines, *Biochim. Biophys. Acta 1393*, 336-348.

99. Kanazawa, K., and Ashida, H. (1998) Dietary Hydroperoxides of Linoleic Acid Decompose to Aldehydes in Stomach before being Absorbed into the Body, *Biochim. Biophys. Acta 1393*, 349-361.

100. Aw, T.Y. (2005) Intestinal Glutathione: Determinant of Mucosal Peroxide Transport, Metabolism, and Oxidative Susceptibility, *Toxicol. Appl. Pharmacol. 204*, 320-328.

101. Aw, T.Y., and Williams, M.W. (1992) Intestinal Absorption and Lymphatic Transport of Peroxidized Lipids in Rats - Effect of Exogenous Gsh, *Am. J. Physiol. 263*, G665-G672.

102. Vilas, N.N., Bell, R.R., and Draper, H.H. (1976) Influence of Dietary Peroxides, Selenium and Vitamin-E on Glutathione Peroxidase of Gastrointestinal-Tract, *J. Nutr. 106*, 589-596.

103. Ursini, F., Zamburlini, A., Cazzolato, G., Maiorino, M., Bon, G.B., and Sevanian, A. (1998) Postprandial Plasma Lipid Hydroperoxides: A Possible Link between Diet and Atherosclerosis, *Free Radic. Biol. Med. 25*, 250-252.

104. Kanazawa, K., Kanazawa, E., and Natake, M. (1985) Uptake of Secondary Autoxidation Products of Linoleic-Acid by the Rat, *Lipids 20*, 412-419.

105. Orada, M., Miyazawa, T., Fujimoto, K., and Kaneda T. (1987) Absorption and Metabolism of Autoxidation Products of Methyl Linoleate in Rat Liver, *J. Jpn. Soc. Nutr. Food Sci. 40*, 117-121.

106. Gorog, D.A., Ahmed, N., and Davies, G.J. (2002) Elevated Plasma Lipid Peroxide Levels in Angina Pectoris and Myocardial Infarction, *Cardiovasc. Pathol. 11*, 153-157.

107. Hennig, B., Toborek, M., Boissonneault, G.A., Shantha, N.C., Decker, E.A., and Oetgen, P.R. (1995) Animal and Plant Fats Selectively Modulate Oxidizability of Rabbit Ldl and Ldl-Mediated Disruption of Endothelial Barrier Function, *J. Nutr. 125*, 2045-2054.

108. Holvoet, P., Stassen, J.M., Van Cleemput, J., Collen, D., and Vanhaecke, J. (1998) Oxidized Low Density Lipoproteins in Patients with Transplant-Associated Coronary Artery Disease, *Arterioscler. Thromb. Vasc. Biol. 18*, 100-107.

109. Lu, Y.F., and Lu, S.W. (2002) Influence of Dietary Fat Saturation on Lipid Peroxidation of Serum and Low Density Lipoprotein in Rats, *Nutr. Res. 22*, 463-472.

110. Salonen, J.T., Nyyssönen, K., Salonen, R., Porkkala-Sarataho, E., Tuomainen, T.P., Diczfalusy, U., and Björkhem, I. (1997) Lipoprotein Oxidation and Progression of Carotid Atherosclerosis, *Circulation 95*, 840-845.

111. Kinter, M. (1995) Analytical Technologies for Lipid Oxidation-Products Analysis, *J. Chromatogr. B 671*, 223-236.

112. Young, I.S., and Trimble, E.R. (1991) Measurement of Malondialdehyde in Plasma by High-Performance Liquid-Chromatography with Fluorometric Detection, *Ann. Clin. Biochem. 28*, 504-508.

113. Richard, M.J., Guiraud, P., Meo, J., and Favier, A. (1992) High-Performance Liquid-Chromatographic Separation of Malondialdehyde Thiobarbituric Acid Adduct in Biological-Materials (Plasma and Human-Cells) using a Commercially Available Reagent, *J. Chromatogr. B 577*, 9-18.

114. Rabl, H., Khoschsorur, G., Colombo, T., Tatzber, F., and Esterbauer, H. (1992) Human Plasma-Lipid Peroxide Levels show a Strong Transient Increase After Successful Revascularization Operations, *Free Radic. Biol. Med. 13*, 281-288.

115. Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., and Yagi, K. (1985) A New Assay-Method for Lipid Peroxides using a Methylene-Blue Derivative, *Biochem. Int. 10*, 205-211.

116. Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., Ling, K.L.E., and Wolff, S.P. (1996) Low-Density Lipoprotein is the Major Carrier of Lipid Hydroperoxides in Plasma - Relevance to Determination of Total Plasma Lipid Hydroperoxide Concentrations, *Biochem. J. 313*, 781-786.

117. Pastorino, A.M., Maiorino, M., and Ursini, F. (2000) Kinetic Analysis of Lipid-Hydroperoxides in Plasma, *Free Radic. Biol. Med. 29*, 397-402.

118. Marshall, P.J., Warso, M.A., and Lands, W.E.M. (1985) Selective Microdetermination of Lipid Hydroperoxides, *Anal. Biochem. 145*, 192-199.

119. Zamburlini, A., Maiorino, M., Barbera, P., Roveri, A., and Ursini, F. (1995) Direct Measurement by Single-Photon Counting of Lipid Hydroperoxides in Human Plasma and Lipoproteins, *Anal. Biochem. 232*, 107-113.

120. Ylä-Herttuala, S. (1998) Is Oxidized Low-Density Lipoprotein Present *in vivo*? *Curr. Opin. Lipidol. 9*, 337-344.

121. Esterbauer, H., Striegl, G., Puhl, H., and Rotheneder, M. (1989) Continuous Monitoring of *in vitro* Oxidation of Human Low-Density Lipoprotein, *Free Radic. Res. Commun. 6*, 67-75.

122. Ahotupa, M., Ruutu, M., and Mäntylä, E. (1996) Simple Methods of Quantifying Oxidation Products and Antioxidant Potential of Low Density Lipoproteins, *Clin. Biochem. 29*, 139-144.

123. Waddington, E., Sienuarine, K., Puddey, I., and Croft, K. (2001) Identification and Quantitation of Unique Fatty Acid Oxidation Products in Human Atherosclerotic Plaque using High-Performance Liquid Chromatography, *Anal. Biochem. 292*, 234-244.

124. Waddington, E.I., Croft, K.D., Sienuarine, K., Latham, B., and Puddey, I.B. (2003) Fatty Acid Oxidation Products in Human Atherosclerotic Plaque: An Analysis of Clinical and Histopathological Correlates, *Atherosclerosis 167*, 111-120.

125. Upston, J.M., Terentis, A.C., Morris, K., Keaney, J.F., and Stocker, R. (2002) Oxidized Lipid Accumulates in the Presence of Alpha-Tocopherol in Atherosclerosis, *Biochem. J. 363*, 753-760.

126. Spickett, C.M., and Dever, G. (2005) Studies of Phospholipid Oxidation by Electrospray Mass Spectrometry: From Analysis in Cells to Biological Effects, *Biofactors 24*, 17-31.

127. Miyazawa, T. (1989) Determination of Phospholipid Hydroperoxides in Human-Blood Plasma by a Chemiluminescence-Hplc Assay, *Free Rad. Biol. Med. 7*, 209-217.

128. Hui, S.P., Murai, T., Yoshimura, T., Chiba, H., Nagasaka, H., and Kurosawa, T. (2005) Improved HPLC Assay for Lipid Peroxides in Human Plasma using the Internal Standard of Hydroperoxide, *Lipids 40*, 515-522.

129. Yamamoto, Y., Brodsky, M.H., Baker, J.C., and Ames, B.N. (1987) Detection and Characterization of Lipid Hydroperoxides at Picomole Levels by High-Performance Liquid-Chromatography, *Anal. Biochem. 160*, 7-13.

130. Frei, B., and Gaziano, J.M. (1993) Content of Antioxidants, Preformed Lipid Hydroperoxides, and Cholesterol as Predictors of the Susceptibility of Human Ldl to Metal Ion-Dependent and Ion-Independent Oxidation, *J. Lipid Res. 34*, 2135-2145.

131. Yamaguchi, Y., Kagota, S., Kunitomo, M., and Haginaka, J. (1999) High-Performance Liquid Chromatographic Assay of Hydroperoxide Levels in Oxidatively Modified Lipoproteins, *J. Chromatogr. B 731*, 223-229.

132. Mowri, H., Chinen, K., Ohkuma, S., and Takano, T. (1986) Peroxidized Lipids Isolated by Hplc from Atherosclerotic Aorta, *Biochem. Int. 12*, 347-352.

133. Suarna, C., Dean, R.T., May, J., and Stocker, R. (1995) Human Atherosclerotic Plaque Contains both Oxidized Lipids and Relatively Large Amounts of Alpha-Tocopherol and Ascorbate, *Arterioscler. Thromb. Vasc. Biol. 15*, 1616-1624.

134. Mashima, R., Onodera, K., and Yamamoto, Y. (2000) Regioisomeric Distribution of Cholesteryl Linoleate Hydroperoxides and Hydroxides in Plasma from Healthy Humans Provides Evidence for Free Radical-Mediated Lipid Peroxidation *in vivo*, *J. Lipid Res. 41*, 109-115.

135. Browne, R.W., and Armstrong, D. (2000) HPLC Analysis of Lipid-Derived Polyunsaturated Fatty Acid Peroxidation Products in Oxidatively Modified Human Plasma, *Clin. Chem. 46*, 829-836.

136. Watson, A.D., Subbanagounder, G., Welsbie, D.S., Faull, K.F., Navab, M., Jung, M.E., Fogelman, A.M., and Berliner, J.A. (1999) Structural Identification of a Novel Pro-Inflammatory Epoxyisoprostane Phospholipid in Mildly Oxidized Low Density Lipoprotein, *J. Biol. Chem. 274*, 24787-24798.

137. Hoff, H.F., O'Neil, J., Wu, Z.P., Hoppe, G., and Salomon, R.L. (2003) Phospholipid Hydroxyalkenals - Biological and Chemical Properties of Specific Oxidized Lipids Present in Atherosclerotic Lesions, *Arterioscler. Thromb. Vasc. Biol. 23*, 275-282.

138. Podrez, E.A., Poliakov, E., Shen, Z.Z., Zhang, R.L., Deng, Y.J., Sun, M.J., Finton, P.J., Shan, L., Febbraio, M., Hajjar, D.P., Silverstein, R.L., Hoff, H.F., Salomon, R.G., and Hazen, S.L. (2002) A Novel Family of Atherogenic Oxidized Phospholipids Promotes Macrophage Foam Cell Formation Via the Scavenger Receptor CD36 and is Enriched in Atherosclerotic Lesions, *J. Biol. Chem. 277*, 38517-38523.

139. Ravandi, A., Babaei, S., Leung, R., Monge, J.C., Hoppe, G., Hoff, H., Kamido, H., and Kuksis, A. (2004) Phospholipids and Oxophospholipids in Atherosclerotic Plaques at Different Stages of Plaque Development, *Lipids 39*, 97-109.

140. Ravandi, A., Kuksis, A., and Shaikh, N.A. (1999) Analysis of Core Aldehydes and Isoprostane Esters of Phospholipids in Plasma Lipoproteins and Human Atheroma by Electrospray Mass Spectrometry with Single Ion Monitoring. *Clin. Chem. 45*, A16.

141. Kamido, H., Nonaka, K., Kuksis, A., Marai, L., and Ravandi, A. (1996) Identification of Lipid Ester Core Aldehydes and Hydroperoxides in Human Atherosclerotic Lesions, *Circulation 94*, 4134.

142. Kamido, H., Nonaka, K., Moroi, M., Ravandi, A., and Kuksis, A. (1997) Alkyl Phosphatidylcholine Core Aldehydes Exist in Human Atheromas and Induce Platelet Aggregation through PAF Receptor, *Atherosclerosis 134*, 183.

143. Kamido, H., Eguchi, H., Ikeda, H., Imaizumi, T., Yamana, K., Hartvigsen, K., Ravandi, A., and Kuksis, A. (2002) Core Aldehydes of Alkyl Glycerophosphocholines in Atheroma Induce Platelet Aggregation and Inhibit Endothelium-Dependent Arterial Relaxation, *J. Lipid Res. 43*, 158-166.

144. Mallat, Z., Nakamura, T., Ohan, J., Leseche, G., Tedgui, A., Maclouf, J., and Murphy, R.C. (1999) The Relationship of Hydroxyeicosatetraenoic Acids and F-2-Isoprostanes to Plaque Instability in Human Carotid Atherosclerosis, *J. Clin. Invest. 103*, 421-427.

145. Hoppe, G., Ravandi, A., Herrera, D., Kuksis, A., and Hoff, H.F. (1997) Oxidation Products of Cholesteryl Linoleate are Resistant to Hydrolysis in Macrophages, Form Complexes with Proteins, and are Present in Human Atherosclerotic Lesions, *J. Lipid Res. 38*, 1347-1360.

146. Akasaka, K., Ohrui, H., Meguro, H., and Tamura, M. (1993) Determination of Triacylglycerol and Cholesterol Ester Hydroperoxides in Human Plasma by High-Performance Liquid-Chromatography with Fluorometric Postcolumn Detection, *J. Chromatogr. B 617*, 205-211.

147. Akasaka, K., Ohrui, H., and Meguro, H. (1993) Simultaneous Determination of Hydroperoxides of Phosphatidylcholine, Cholesterol Esters and Triacylglycerols by Column-Switching High-Performance Liquid-Chromatography with a Postcolumn Detection System, *J. Chromatogr. B 622*, 153-159.

148. Karten, B., Boechzelt, H., Abuja, P.M., Mittelbach, M., and Sattler, W. (1999) Macrophage-Enhanced Formation of Cholesteryl Ester-Core Aldehydes during Oxidation of Low Density Lipoprotein, *J. Lipid Res. 40*, 1240-1253.

149. Thomas, J.P., Kalyanaraman, B., and Girotti, A.W. (1994) Involvement of Preexisting Lipid Hydroperoxides in Cu2$^+$-Stimulated Oxidation of Low-Density-Lipoprotein, *Arch. Biochem. Biophys. 315*, 244-254.

150. Nikkari, T., Maloranta, U., Hiltunen, T., Jaakkola, O., and Ylä-Herttuala, S. (1995) Monitoring of Lipoprotein Oxidation by Gas-Chromatographic Analysis of Hydroxy Fatty-Acids, *J. Lipid Res. 36*, 200-207.

151. Carpenter, K.L.H., Taylor, S.E., Vanderveen, C., Williamson, B.K., Ballantine, J.A., and Mitchinson, M.J. (1995) Lipids and Oxidized Lipids in Human Atherosclerotic Lesions at Different Stages of Development, *Biochim. Biophys. Acta 1256*, 141-150.

152. Morrow, J.D., Harris, T.M., and Roberts, L.J. (1990) Noncyclooxygenase Oxidative Formation of a Series of Novel Prostaglandins - Analytical Ramifications for Measurement of Eicosanoids, *Anal. Biochem. 184*, 1-10.

153. Mori, T.A., Croft, K.D., Puddey, I.B., and Beilin, L.J. (1999) An Improved Method for the Measurement of Urinary and Plasma F-2-Isoprostanes using Gas Chromatography Mass Spectrometry, *Anal. Biochem. 268*, 117-125.

154. Thomas, M.J., Chen, Q.R., Sorci-Thomas, M.G., and Rudel, L.L. (2001) Isoprostane Levels in Lipids Extracted from Atherosclerotic Arteries of Nonhuman Primates, *Free Radic. Biol. Med. 30*, 1337-1346.

155. Ågren, J.J., Vidgren, H.M., Valve, R.S., Laakso, M., and Uusitupa, M. (2001) Postprandial Responses of Individual Fatty Acids in Subjects Homozygous for the Threonine- Or Alanine-Encoding Allele in Codon 54 of the Intestinal Fatty Acid Binding Protein 2 Gene, *Am. J. Clin. Nutr. 73*, 31-35.

156. Wieland, H., and Seidel, D. (1983) A Simple Specific Method for Precipitation of Low-Density Lipoproteins, *J. Lipid Res. 24*, 904-909.

157. Skipski, V.P., and Barclay, M. (1969) Thin-Layer Chromatography, *Methods Enzymol. 14*, 542-548.

158. Christie, W.W. (1982) A Simple Procedure for Rapid Transmethylation of Glycerolipids and Cholesteryl Esters, *J. Lipid Res. 23*, 1072-1075.

159. Erlund, I., Alfthan, G., Siren, H., Ariniemi, K., and Aro, A. (1999) Validated Method for the Quantitation of Quercetin from Human Plasma using High-Performance Liquid Chromatography with Electrochemical Detection, *J. Chromatogr. B 727*, 179-189.

160. Hollman, P.C.H. (2001) Determination of Flavonols in Body Fluids, *Methods Enzymol. 335*, 97-103.

161. Spiteller, G. (1998) Linoleic Acid Peroxidation - the Dominant Lipid Peroxidation Process in Low Density Lipoprotein - and its Relationship to Chronic Diseases, *Chem. Phys. Lipids 95*, 105-162.

162. Neff, W.E., and Byrdwell, W.C. (1998) Characterization of Model Triacylglycerol (Triolein, Trilinolein and Trilinolenin) Autoxidation Products Via High-Performance Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry, *J. Chromatogr. A 818*, 169-186.

163. Hamsten, A. (1993) Lipids as a Coronary Risk Factor - Analysis of Hyperlipemias, *Postgrad. Med. J. 69*, S8-S11.

164. Pepys, M.B., and Baltz, M.L. (1983) Acute Phase Proteins with Special Reference to C-Reactive Protein and Related Proteins (Pentaxins) and Serum Amyloid A-Protein, *Adv. Immunol. 34*, 141-212.

165. Hackam, D.G., and Anand, S.S. (2003) Emerging Risk Factors for Atherosclerotic Vascular Disease - A Critical Review of the Evidence, *J. Am. Med. Assoc. 290*, 932-940.

166. Mangoni, A.A., and Jackson, S.H.D. (2002) Homocysteine and Cardiovascular Disease: Current Evidence and Future Prospects, *Am. J. Med. 112*, 556-565.

167. Canales, A., and Sanchez-Muniz, F.J. (2003) Paraoxonase, Something More than an Enzyme? *Med. Clin. 121*, 537-548.

168. Williamson, G., and Manach, C. (2005) Bioavailability and Bioefficacy of Polyphenols in Humans. II. Review of 93 Intervention Studies, *Am. J. Clin. Nutr. 81*, 243S-255S.

169. Lesser, S., Cermak, R., and Wolffram, S. (2004) Bioavailability of Quercetin in Pigs is Influenced by the Dietary Fat Content, *J. Nutr. 134*, 1508-1511.

170. Manach, C., Morand, C., Crespy, V., Demigne, C., Texier, O., Regerat, F., and Remesy, C. (1998) Quercetin is Recovered in Human Plasma as Conjugated Derivatives which Retain Antioxidant Properties, *FEBS Lett. 426*, 331-336.

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