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Lipid Peroxidation *in vivo*

Evaluation and Application of Methods for Measurement

BY

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ABSTRACT

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Lipid peroxidation is thought to be an important factor in the pathophysiology of a number of diseases and in the process of ageing, but its measurement *in vivo* has been difficult. The aim of this thesis was to evaluate methods for measurement of lipid peroxidation *in vivo* that are suitable for clinical investigations, and to apply these methods in animal and human studies investigating basal conditions and situations associated with increased lipid peroxidation.

The ferrous oxidation in xylenol orange assay for quantification of total plasma lipid hydroperoxides was re-evaluated regarding sample handling and storage. It was shown to be a useful tool for analyses of fresh but not stored plasma samples.

A methodology for measurement of the total amount (sum of free and esterified) of an F_2-isoprostane, 8-iso-prostaglandin $F_{2\alpha}$, in tissues using alkaline hydrolysis in combination with an existing radioimmunoassay was developed. High levels of 8-iso-prostaglandin $F_{2\alpha}$ in rat liver tissue were quantified by this technique both at basal conditions and in an experimental model of increased lipid peroxidation induced by carbon tetrachloride.

Supplementation with vitamin E to rats decreased both non-enzymatic and enzymatic lipid peroxidation as measured by 8-iso-prostaglandin $F_{2\alpha}$ and a major prostaglandin $F_{2\alpha}$ metabolite. This was verified both in the urine at basal conditions, and in the urine and liver tissue after carbon tetrachloride induced lipid peroxidation.

In a randomised cross-over study in humans, a rapeseed oil-based diet with an increased proportion of easily oxidised polyunsaturated fatty acids was compared to a control diet rich in saturated fats. The rapeseed oil-based diet did not seem to increase the degree of lipid peroxidation in plasma and urine as measured by 8-iso-prostaglandin $F_{2\alpha}$, hydroperoxides and malondialdehyde, presumably due to a sufficient content of antioxidants in the rapeseed oil diet.

In conclusion, the simultaneous measurement of several biomarkers of lipid peroxidation is a promising approach for future studies investigating the possible role of lipid peroxidation *in vivo* under basal conditions and in the pathology of diseases.

Keywords: lipid peroxidation, hydroperoxides, F_2-isoprostanes, MDA, prostaglandins, measurement, carbon tetrachloride, vitamin E, dietary fat quality, rapeseed oil.

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To Martin, my family and the memory of my father

PAPERS DISCUSSED

This thesis is based on the papers listed below, which are referred to in the text by their Roman numerals:

I. Södergren E, Nourooz-Zadeh J, Berglund L, Vessby B. Re-evaluation of the ferrous oxidation in xylenol orange assay for the measurement of plasma lipid hydroperoxides.
J Biochem Biophys Methods 1998; 37: 137-146.

II. Södergren E, Vessby B, Basu S. Radioimmunological measurement of F_2-isoprostanes after hydrolysis of lipids in tissues.
Prostaglandins Leukot Essent Fatty acids 2000; 63: 33-36.

III. Södergren E, Cederberg J, Basu S, Vessby B. Vitamin E supplementation decreases basal levels of F_2-isoprostanes and prostaglandin $F_{2\alpha}$ in rats.
J Nutr 2000; 130: 10-14.

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CONTENTS

ABBREVIATIONS

AOC	antioxidative capacity
BHT	butylated hydroxytoluene
BMI	body mass index
CCl_4	carbon tetrachloride
CHD	coronary heart disease
CI	confidence interval
COX	cyclooxygenase
E%	energy per cent
EIA	enzyme immunoassay
FOX2	ferrous oxidation in xylenol orange version 2
GC	gas chromatography
HDL	high-density lipoprotein
8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$
15-K-DH-$PGF_{2\alpha}$	15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$
LDL	low-density lipoprotein
MDA	malondialdehyde
GC-MS	gas chromatography-mass spectroscopy
MUFA	monounsaturated fatty acid
PUFA	polyunsaturated fatty acid
RIA	radioimmunoassay
SAFA	saturated fatty acid
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
VLDL	very low-density lipoprotein
XO	xylenol orange

INTRODUCTION

Oxidation of lipids

Lipids is a heterogeneous group of compounds having several important functions in the body such as being an efficient source of energy, constituents in cell membranes and nerve tissues, thermal and electrical insulators and acting as local hormones etc. [1]. They are also important dietary constituents not only because of their high-energy value but also because of the fat-soluble vitamins and essential fatty acids that are contained in the fat of natural foods. Energy is produced from lipids in the body when lipids are β-oxidised and oxygen is reduced to water in the respiratory chain by mitochondria in different tissues [1]. About 85 to 90% of the oxygen consumed by humans is utilised by the mitochondria for energy production, and the remaining 10 to 15% is used in direct chemical non-enzymatic reactions and by various oxidase and oxygenase enzymes in the body [2, 3].

When lipids are oxidised without release of energy, unsaturated lipids go rancid due to oxidative deterioration when they react directly with molecular oxygen [3-9]. This process is called lipid peroxidation and the insertion of an oxygen molecule is catalysed by free radicals (non-enzymatic lipid peroxidation) or enzymes (enzymatic lipid peroxidation) [5, 7]. This type of lipid oxidation has been recognised since antiquity as a problem in the storage of fats and oils, and peroxidation of lipids has long been studied by food chemists, polymer chemists and even museum curators interested in the oxidative degradation of valuable paintings. The mechanism of free radical-induced lipid peroxidation was established in the 1940s by Farmer and his collaborators working at the research laboratories of the British Rubber Producers Association [3, 4]. Later in the 1950s, the relevance of lipid peroxidation to biological systems and medicine began to be extensively explored.

Free radicals, antioxidants and oxidative stress

Free radicals and other reactive species

What is a free radical?
A free radical may in simple terms be defined as an atom or molecule that contains one or more unpaired electrons and is capable of independent existence [5, 6, 10-13]. An unpaired electron is an electron that occupies an orbital alone

(indicated in the text by $^{\bullet}$), but electrons usually associate in pairs in orbitals of atoms and molecules. Free radicals are generally more reactive than non-radicals due to their unpaired electron, but different types of free radicals vary widely in their reactivity [6, 10-13]. The oxygen molecule (O_2) qualifies as a free radical because it contains two unpaired electrons, but is not particularly reactive due to a special electron arrangement that makes the reactions with oxygen spin restricted [5]. However, when oxygen is partly reduced, several different reactive oxygen species, both radicals and non-radicals, may be produced [6, 10-13]. Examples of reactive oxygen species are hydroxyl radicals ($OH^{\bullet}$), superoxide anion radicals ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). The hydroxyl radical is an extremely reactive free radical. It is very unstable and attacks a large array of molecules in the nearby environment. Examples of other free radicals are the two gaseous radicals nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$), the carbon-centred ($R^{\bullet}$), alkoxyl ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) formed during peroxidation of lipids and the trichloromethyl radical ($CCl_3^{\bullet}$) formed by the metabolism of carbon tetrachloride (CCl_4) in the liver.

Radical reactions

Radicals can react with other molecules in several ways [6, 10, 12, 13]. When two free radicals meet, their unpaired electrons can form a shared electron pair in a covalent bond and both radicals are lost. When a radical gives one electron to, takes one electron from or simply adds on to a non-radical, that non-radical becomes a radical. Since most molecules present in living organisms are non-radicals, any free radical produced in the body will most likely react with a non-radical and generate a new radical. Hence, free radical reactions *in vivo* tend to proceed as chain reactions.

The Janus face of free radicals

Free radicals and various reactive oxygen species are continuously produced in the body [6, 11-13]. They can be formed as a by-product in the mitochondrial respiratory chain due to leakage of electrons from the electron transport chain or by reactions catalysed by transition metal ions such as iron and copper ions. They may also be derived from external sources such as cigarette smoke, radiation, UV light, pollution and from the metabolism of certain drugs. The free radicals formed can react with DNA, proteins and lipids in the body and cause extensive oxidative damage [6, 11-13].

Free radicals are not only produced as an unwanted product; they are also formed deliberately in the body for useful purposes and have important physiological functions [6, 11-13]. A well-defined role for free radicals is when activated phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) produce superoxide anion radicals and hydrogen peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses [14]. In addition,

free radicals are also produced by an array of enzymes e.g. pyruvate metabolising enzymes, oxidases, carboxylases, hydroxylases, peroxidases, fruit ripening enzymes and radical enzymes [3].

Methods for measurement

Free radicals and other reactive species are difficult to detect and measure due to their short lifetime [3, 15, 16]. One of the few techniques that detect free radicals directly is electron spin resonance with spin trapping, which allows a radical to react with a trap molecule to give a more stable product to monitor [3, 4, 17-19]. Trapping methods have been proven very useful *in vitro* and in animal studies, but their usefulness has been limited in human studies [3, 17, 18]. An alternative to trapping methods are fingerprinting methods based on the measurement of products of free radical-damaged DNA, proteins and lipids [3, 4].

Antioxidants

The function of antioxidants

Organisms have evolved sophisticated antioxidant defence systems and repair systems for protection against free radicals and free radical damages at different sites [7, 11-13, 20-23]. There are a number of antioxidants present in the body and derived from the diet. Based on their location in the body, they can be divided into intracellular and extracellular antioxidants [7, 11, 22].

Antioxidants in the body and from the diet

Superoxide dismutase, catalase and glutathione peroxidase are intracellular antioxidant enzymes that convert potential substrates (superoxide anion radicals and hydrogen peroxide) to less reactive forms in the body [7, 11-13, 20-23]. Several extracellular antioxidants such as proteins (transferrin, lactoferrin, albumin, ceruloplasmin) and urate prevent free radical reactions in the body by sequestering transition metal ions by chelation. Albumin, bilirubin and urate may also scavenge free radicals directly [7, 11].

Antioxidants from dietary sources include lipid soluble vitamins such as vitamin E and carotenoids as well as the water-soluble vitamin C [7, 11-13, 20, 22, 23]. Selenium from the diet is important for the functioning of the antioxidant enzyme glutathione peroxidase [7, 12, 13, 22]. The identification and study of antioxidants from dietary sources is a fast expanding field of research and several other important antioxidants have been investigated such as flavonoids and other plant phenolics, taurine and α-lipoic acid [23].

Vitamin E inhibits lipid peroxidation
Vitamin E appears to be one of the most important free radical-scavenging antioxidants within membranes and lipoproteins [12, 21, 24, 25]. It is an effective chain-breaking antioxidant that protects polyunsaturated lipids from peroxidation by scavenging peroxyl radicals. When vitamin E acts as a chain breaker, it donates a hydrogen atom to the free radical, gets oxidised itself and converted to a radical. The resulting vitamin E radical is suggested to be regenerated to vitamin E by other antioxidants, primarily vitamin C [12, 21]. The regeneration of vitamin E by other antioxidants is one part of the intricate co-operation that exists between different antioxidants in the antioxidant defence system. α-Tocopherol is the biologically most active form of vitamin E *in vivo* [26]. In supplementation studies, synthetic forms of vitamin E are often used such as all-rac-α-tocopheryl acetate and all-rac-α-tocopheryl succinate. Rich natural sources of vitamin E are vegetable oils, nuts and whole grain and the main dietary intake derives from table margarines and vegetable oils.

Oxidative stress

Definition
Oxidative stress has been defined as a disturbance in the balance between antioxidants and prooxidants (free radicals and other reactive species), with increased levels of prooxidants leading to potential damage [21, 27-29]. This imbalance can be an effect of depletion of endogenous antioxidants, low dietary intake of antioxidants and/or increased formation of free radicals and other reactive species.

Free radical damage
Oxidative damage to DNA, proteins and lipids can ultimately lead to outcomes such as disorganisation, dysfunction and destruction of membranes, enzymes and proteins [10, 12, 21]. Specifically, peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity, inactivation of membrane-bound receptors and enzymes, increased permeability to ions and possibly eventually membrane rupture [4, 7]. If the oxidative stress is particularly severe, it can produce cell death [21, 30]. Death can occur by necrosis, but in a number of cell types, such as neuronal cells, a mild oxidative stress can trigger the process of apoptosis, activating the intrinsic suicide pathway present within all cells [31, 32].

Free radicals and antioxidants in disease
Free radical damages can accumulate over time and may thereby contribute to cell injury and development of human diseases. Free radicals have been implicated in the development of several diseases including atherosclerosis, diabetes, cancer, chronic inflammatory diseases and neurodegenerative diseases

as well as in the process of ageing [3, 5, 33-37]. In most cases, free radicals are believed to be a component of the pathology of the disease and arise to a major or minor extent as a consequence of the underlying disease.

The potential role of dietary antioxidants in prevention and treatment of these diseases, especially atherosclerosis and cancer, have been extensively studied during the last two decades [38-45]. Epidemiological data indicate that a high intake of foods rich in antioxidant vitamins (mainly β-carotene, vitamin C and E) or high blood concentrations of antioxidant vitamins are associated with a reduced risk for cardiovascular disease and cancer at several sites. Findings from human intervention studies testing the potential of antioxidant supplementation are inconsistent and do not allow firm conclusions. Recommendations on antioxidant intakes for disease prevention must await evidence from further controlled intervention trials, some currently under progress. However, based on the available data, protection against oxidative damage seems to be best served by a diet rich in a variety of antioxidants found in fruits and vegetables. Definitive evidence for the association between free radical generation and development of disease, and evidence for the efficiency of antioxidants in disease prevention have been difficult to obtain partly because of limitations and shortcomings with methods to assess oxidative stress *in vivo* [4, 42].

Lipid peroxidation

Non-enzymatic lipid peroxidation

Lipid peroxidation – a free radical fingerprinting method
Lipid peroxidation is probably the most extensively investigated free radical-induced process [3-9]. One of the earliest descriptions of the different stages of lipid peroxidation was given in the late 1820s by de Saussure, who used a simple mercury manometer to study the uptake of oxygen by a layer of walnut oil on water (reviewed in [3]). Polyunsaturated fatty acids (PUFAs) are particularly susceptible to peroxidation and once the process is initiated, it proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination [7].

The lipid peroxidation chain reaction
Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group upon a PUFA [3-9] (***Figure 1***). Since a hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-centred radical is

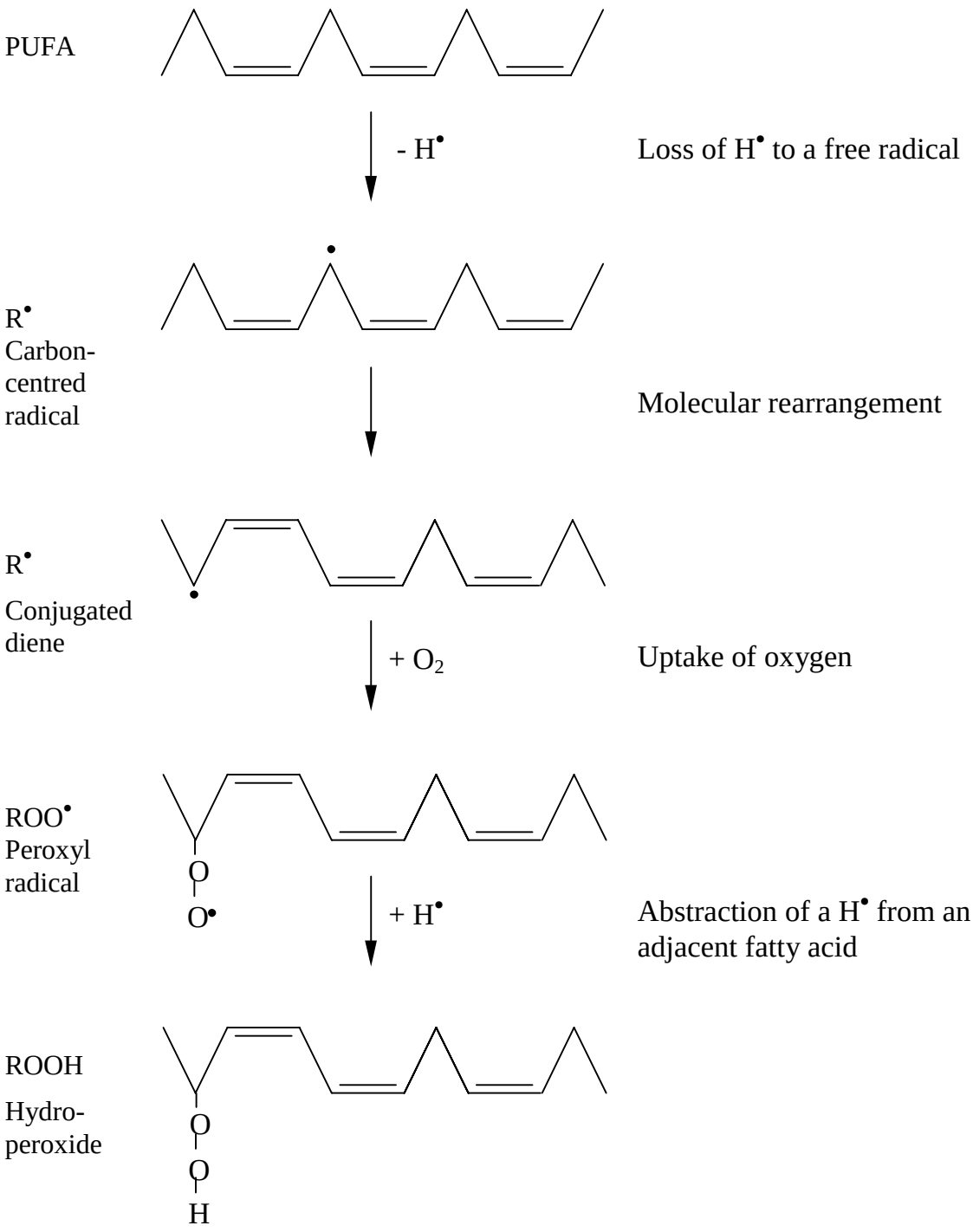

Figure 1 *Mechanism of non-enzymatic lipid peroxidation (modified from [7]).*

stabilised by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. Hence, a single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides. The length of the propagation chain before termination depends on several factors e.g. the oxygen concentration and the amount of chain-breaking antioxidants present. Hydroperoxides are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and copper ions). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as hydrocarbon gases (e.g. ethane and penthane) and aldehydes (e.g. malondialdehyde (MDA) and 4-hydroxynonenal).

The fatty acid structure determines the product

Another complexity of the lipid peroxidation process is that the initial abstraction of a hydrogen atom can occur at different points on the carbon chain of the fatty acid. Thus, peroxidation of arachidonic acid for example gives six different lipid hydroperoxides as well as cyclic peroxides and other products such as isoprostanes [3]. Further, the number of double bonds determines the susceptibility of a fatty acid to peroxidation [46, 47]. PUFAs are readily attacked by free radicals and become oxidised into lipid hydroperoxides, whereas saturated fatty acids (SAFAs) with no double bonds and monounsaturated fatty acids (MUFAs) with one double bond are more resistant to peroxidation. An adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom. Therefore, the greater the number of double bonds in a fatty acid chain, the easier the removal of a hydrogen atom, that is why PUFAs are more susceptible to peroxidation.

Enzymatic lipid peroxidation

Cyclooxygenase and lipoxygenase catalyses lipid peroxidation

The peroxidation of PUFAs can proceed not only through non-enzymatic free radical-induced pathways, but also through processes that are enzymatically catalysed [5, 7]. Enzymatic lipid peroxidation may be referred only to the generation of lipid hydroperoxides achieved by insertion of an oxygen molecule at the active centre of an enzyme [5, 7]. Free radicals are probably important intermediates in the enzymatically-catalysed reaction, but are localised to the

active sites of the enzyme. Cyclooxygenase (COX) and lipoxygenase fulfil the definition for enzymatic lipid peroxidation when they catalyse the controlled peroxidation of various fatty acid substrates. The hydroperoxides and endoperoxides produced from enzymatic lipid peroxidation become stereospecific and have important biological functions upon conversion to stable active compounds. Both enzymes are involved in the formation of eicosanoids, which comprise a large and complex family of biologically active lipids derived from PUFAs with 20 carbon atoms. Prostaglandins are formed by COX-catalysed peroxidation of arachidonic acid [48]. COX exists in at least two isoforms [49-51]. COX-1 is present in cells under physiological conditions, whereas COX-2 is induced in macrophages, epithelial cells and fibroblasts by several inflammatory stimuli leading to release of prostaglandins [49-53].

Methods for measurement of non-enzymatic lipid peroxidation

A variety of methods

Since free radical-induced lipid peroxidation is a complex process and occurs in multiple stages, there are many techniques available for the detection and measurement of lipid peroxidation products. Peroxidation of lipids can be assessed by measurement of the loss of unsaturated fatty acids, generation of primary peroxidation products or secondary degradation products [3, 4, 6-9, 54]. ***Table 1*** summarises various methods for lipid peroxidation measurements.

The approach to the detection of lipid peroxidation depends on whether the sample is a complex biological sample obtained *in vivo*, or if the sample is a relatively simple mixture obtained *in vitro* [8]. For clinical purposes, several practical considerations needs to be addressed such as storage of samples. The most accurate assays of lipid peroxidation are the most chemically sophisticated ones, however, they also require extensive sample preparation and great care has to be taken during sample handling and preparation to ensure that further peroxidation does not occur [4]. Possible confounding of lipid peroxidation products ingested from dietary sources needs also to be considered. Further, the chemical composition of the peroxidation products will depend on the fatty acid composition of the lipid substrate in question [4]. Consequently, selecting a single test to monitor peroxidation can give misleading results [3, 6, 8]. Various methods can have distinct advantages or disadvantages under different circumstances and employing a combination of several techniques is therefore probably the best approach to measure non-enzymatic and enzymatic lipid peroxidation.

Table 1. *Methods used to detect and measure non-enzymatic lipid peroxidation (modified from* [3, 4, 6]*).*

What is measured	**Method**
Loss of substrate	
Unsaturated fatty acids	GC / HPLC
Uptake of oxygen	Oxygen electrode
Primary products	
Total lipid hydroperoxides	FOX assay-Absorbance 550−600 nm [55, 56]
	Iodide oxidation-Absorbance 358 nm [57-62]
	Glutathione peroxidase [63]
	Cyclooxygenase [64, 65]
Individual lipid hydroperoxides	HPLC-chemiluminescence [66-69]
	GC-MS / HPLC
Conjugated dienes	Absorbance 230−235 nm [70]
	HPLC-Absorbance 230−235 nm [71, 72]
	Second derivative spectroscopy [73]
Secondary degradation products	
Hydrocarbon gases	GC
TBARS/MDA	TBA test-Absorbance 532−535 nm [74, 75]
	TBA test-Flourescence [76]
	TBA test-HPLC-Absorbance 532−535 nm [77-79]
	TBA test-HPLC-Fluorescence [80, 81]
Aldehydes	GC-MS / HPLC / Antibodies / Fluorescence
F_2-isoprostanes	GC-MS [82-85]
	Enzyme immunoassay [86, 87]
	Radioimmunoassay [88]

GC, gas chromatography; HPLC, high pressure/performance liquid chromatography; FOX, ferrous oxidation in xylenol orange; MS, mass spectroscopy; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde and TBA, thiobarbituric acid.

Conjugated dienes and the thiobarbituric acid test

Two of the most commonly used assays are based on the measurement of thiobarbituric acid reactive substances (TBARSs) or MDA by the thiobarbituric acid (TBA) test and conjugated dienes. The methods have been widely adopted since they are rapid and simple to perform, but have also been criticised for their lack of specificity.

Conjugated diene measurements for isolated lipids or lipoprotein fractions
Conjugated diene structures with a double-single-double bond (-C=C-C=C-) arrangement absorb UV light in the wavelength range 230–235 nm and can thus be detected by UV absorption spectrophotometry [4, 6, 73]. Conjugated diene measurements have successfully been used to study peroxidation in isolated lipoprotein fractions by applying continuous monitoring of conjugated diene formation during low density lipoprotein (LDL) oxidation to give an estimate of the susceptibility of the lipoprotein to oxidation [70, 89-91]. However, its application in body fluids e.g. plasma samples has been associated with serious confounding factors [6, 73, 92]. Many other substances than conjugated dienes such as haem proteins, purines and pyrimidines present in the plasma sample absorb light strongly at UV [3].

Greater sensitivity of this method can be achieved by extraction of the lipids into organic solvents, by combining HPLC with UV absorption or by applying second-derivative spectroscopy [3, 71, 73]. However, when using HPLC separation, most of the UV response in human serum was reported to consist of a non-oxygen-containing conjugated isomer of linoleic acid, 9-*cis*,11-*trans*-octadecadienoic acid [72, 93]. Dairy products and meat from ruminants are the major dietary sources of this conjugated linoleic acid isomer [94]. In human serum and tissues it appears to be derived mainly from the diet [95, 96], but has also been shown to be produced by endogenous conversion of dietary *trans* fatty acids [97] as well as by bacterial metabolism in the human cervix [98] and by bacterial lung pathogens *in vitro* [99]. The free radical-induced production of this isomer is controversial and Thompson and Smith showed that the 9-*cis*,11-*trans*-octadecadienoic acid was not found in the plasma of animals subjected to oxidative stress [100]. Consequently it follows that application of the conjugated diene method in animal or human body fluids and tissues has been questioned and needs careful consideration [6, 95, 98], but the method is still useful for isolated lipid or lipoprotein fractions such as LDL.

The TBA test for TBARS and MDA measurements
The TBA test is performed to measure the amount of MDA present in the sample. MDA is generated as a degradation product from peroxidised lipids [101] and as a side product of enzymatic metabolism of thromboxanes and

prostaglandins [102, 103]. It has been shown to be formed from PUFAs containing at least two double bonds [101], but has been proposed to be derived mainly from fatty acids with three or more double bonds [104, 105]. The basis of the TBA methods is the reaction of MDA with TBA at low pH and high temperature to form a coloured complex, the MDA-TBA complex, with an absorption maximum at 532–535 nm that can be measured by visible absorption spectrophotometry [74, 75]. The test works well in defined membrane systems (e.g. microsomes and liposomes), but its application to body fluids has produced a host of problems.

A major disadvantage of the TBA test is that it is non-specific and measures many parameters in addition to lipid peroxidation [79, 101, 105-108]. A substantial amount of the MDA detected may be generated from decomposition of lipid hydroperoxides during the acid heating stage of the TBA assay as an artefact [106, 109]. The amount of MDA formed is dependent on the lipid content of the sample, the amount of metal ion contamination in the reagents and antioxidants present in the sample. Addition of antioxidants such as butylated hydroxytoluene (BHT) or EDTA to the sample before analysis is an approach to suppress peroxidation during the test itself. Further, several other compounds (e.g. other aldehydes, carbohydrates, amino acids and bile pigments) react with TBA to form complexes that have absorption spectra overlapping that of the MDA-TBA complex [106, 110-112]. The specificity of the TBA assay is improved by detecting the MDA-TBA complex with fluorescence spectrophotometry at 553 nm [76]. Another means to achieve improved specificity is to separate the MDA-TBA complex by HPLC from other components absorbing at the same wavelength prior to the detection [77-79, 113]. A further approach that has successfully increased specificity is to combine HPLC separation with fluorescence detection [80, 81, 114]. Using HPLC-based methods adapted to avoid many of the artefacts mentioned, the TBA test is still a useful ‘screening’ method to use for examination of lipid peroxidation in large number of biological samples and for detection of peroxidation in defined systems *in vitro* [6].

Lipid hydroperoxides

Total or individual classes of lipid hydroperoxides

Several methods have been developed to measure lipid hydroperoxides in biological samples [3, 4, 8, 115]. They can be divided into two categories detecting either total or individual classes of lipid hydroperoxides and are generally dependent on the ability of hydroperoxides to oxidise other molecules. Total hydroperoxides are assayed using colorimetric, enzymatic or chemiluminescence (CL) techniques, while individual hydroperoxide classes are

measured by HPLC-linked microperoxidase-catalysed isoluminol chemiluminescence techniques. A problem regarding hydroperoxide measurements is the wide range of reported levels when using these different methods. The amount of hydroperoxides present at a given time will depend not only on the rate of initiation of peroxidation, but also on how quickly peroxides are decomposed to give other products [3, 8, 115]. Critical characteristics of all these assays are: (a) the influence of interfering factors, (b) the sensitivity of the method and (c) the efficiency of the oxidation process by different hydroperoxides [116].

HPLC-chemiluminescence based assays

Determinations of individual classes of lipid hydroperoxides by chemiluminescence are often based on microperoxidase conversion of hydroperoxides to alkoxyl radicals that reacts with isoluminol to produce light emission [66-68, 117-119]. Samples are extracted and then separated with HPLC prior to the microperoxidase catalysed reaction. Using this assay, the presence of cholesteryl ester hydroperoxides and phospholipid hydroperoxides can be detected. Reported plasma levels of phospholipid hydroperoxides range between 10 and 500 nmol/L [66-68] and cholesteryl ester hydroperoxides range between 3 nmol/L [66, 117] and 920 nmol/L [92, 120]. Another chemiluminescence based method for detection of total lipid hydroperoxides is the recently developed sophisticated 'single photon counting' chemiluminescence method [69, 121]. The sample is added to a mixture containing luminol and the photon emission is recorded. It has been applied to human plasma and lipoprotein fractions and native plasma has been reported to contain 1.5 to 5.5 µmol/L of total lipid hydroperoxides [69, 121].

The ferrous oxidation in xylenol orange assay

Total hydroperoxides may also be determined using the ferrous oxidation in xylenol orange (FOX) assay, which can be used for hydroperoxides present in the aqueous (FOX1) and in the lipid (FOX2) phases [55, 56, 122]. The FOX method is based on the oxidation of ferrous (II) to ferric (III) ions by hydroperoxides under acidic conditions (1). Ferric ions are detected by UV absorbance at 560 nm after reaction with the ferric ion indicator, xylenol orange (XO), generating a blue-purple complex with an absorbance maximum at 550–600 nm (2).

(1) Fe^{2+} + hydroperoxides → Fe^{3+} alkoxyl radical + OH^-

(2) Fe^{3+} XO → blue-purple complex (560 nm)

In the FOX2 assay, the signal is authenticated using TPP, which specifically reduces lipid hydroperoxides. This procedure generates a proper control and a means to assess lipid hydroperoxides without interference from non-lipid

hydroperoxides [123]. Another advantage of this method is that no extraction step is necessary [56]. Possible lipid peroxidation chain reactions due to the alkoxyl radicals generated in the ferrous oxidation step (1) are prevented by addition of BHT to the FOX reagent. The assay is not influenced by diurnal variations or fasting [123]. Vitamin C, E and urate do not interfere with the FOX assay under physiological concentrations; however, a high concentration of vitamin C gives rise to a high background signal [122]. Samples collected in EDTA or DETAPAC (anticoagulants and metal-chelating agents) or plasma samples with hemolysis can not be used since they interfere with the assay [122]. The FOX2 assay in conjugation with the selective hydroperoxide TPP has been used to determine whole plasma lipid hydroperoxides in healthy volunteers and the concentrations range between 0.2 and 10.3 µmol/L [56, 123-126]. Hydroperoxide levels have also been shown to be elevated in patients with diabetes mellitus [123-126], after dialysis [127, 128] and after ischemia-reperfusion injury [129, 130].

The iodometric method

Another colorimetric method for total hydroperoxide quantification, the iodometric method, is based on the ability of lipid hydroperoxides to oxidise iodide (I^-) to iodine (I_2) [62]. Iodine can then be quantified by several methods. Continuous monitoring of UV absorption at or near 358 nm of the triiodide ion (I_3^-) chromophore formed from iodine in the presence of excess of iodide is often used [57, 59, 60]. Iodine can also be measured by UV absorbance at 365 nm after incubation with a commercially available colour reagent [58]. The iodine liberation method is one of the oldest and has been widely used in the food industry for bulk lipids [3]. It can be applied to extracts of biological samples provided that other oxidising agents are absent. Potential interfering factors are oxygen, coloured materials absorbing at the same wavelength, lipids and proteins that add iodine directly and hydrogen peroxide and protein peroxides that oxidises iodide [3, 60, 62]. The method is extremely oxygen sensitive, which can be prevented by removing oxygen from reagent solutions, by using anaerobic cuvettes and by adding cadmium ions to complex unreacted iodide [62]. Reported levels of lipid hydroperoxides determined with this method in human plasma range from 2.1 to 45.7 µmol/L [60, 61].

Glutathione peroxidase and cyclooxygenase assays

Glutathione peroxidase and COX are two enzymes used for enzymatic detection of total hydroperoxides. Glutathione peroxidase catalyses the oxidation of reduced glutathione to oxidised glutathione by hydroperoxides [3, 4, 63]. Oxidised glutathione can be determined directly by HPLC [63] or indirectly by consumption of NADPH. Fatty acid hydroperoxides within membrane or LDL lipids can not be measured unless phospholipases are first used [3, 63]. Levels of hydroperoxides in plasma have been reported to be approximately 1 µmol/L

[63]. Stimulation of the activity of COX by hydroperoxides can be used to measure trace amounts of total hydroperoxides in body fluids [3, 4, 64, 65]. The assay relates the presence of hydroperoxides to one of their potential biological actions, i.e. stimulation of eicosanoid synthesis. Reported plasma levels are about 0.5 µmol/L [64].

F_2-isoprostanes

F_2-isoprostanes – a promising indicator of lipid peroxidation in vivo
The discovery of the isoprostanes as products of lipid peroxidation has been a major advance in the ability to assess lipid peroxidation *in vivo* [3, 8, 9, 131-133]. The notion that prostaglandin-like compounds could be generated non-enzymatically by peroxidation of fatty acids was first demonstrated *in vitro* over 20 years ago. It was not until in the early 1990s that a group of prostaglandin F_2-like compounds, F_2-isoprostanes, was reported to be formed in human plasma by free radical-catalysed peroxidation of arachidonic acid [82].

Formation of F_2-isoprostanes
F_2-isoprostanes are initially formed *in situ* from esterified arachidonic acid in phospholipids and are then released in the free form into the circulation, presumably by phospholipases [134]. The free form represents only one pool of F_2-isoprostanes formed *in vivo* and the total generation of F_2-isoprostanes can be quantified after alkaline hydrolysis by measurement of the sum of free and esterified F_2-isoprostanes. By quantification of total amounts of F_2-isoprostanes in tissues it may be possible to investigate the location of oxidative injury in different diseases and to determine if some tissues are more prone to oxidation than others under certain pathological conditions [132]. F_2-isoprostanes are rapidly metabolised and excreted in the urine [135, 136]. Urinary F_2-isoprostanes or urinary metabolites of F_2-isoprostanes are of particular interest as indicators of oxidative injury because they offer a non-invasive parameter that reflects the production of F_2-isoprostanes over a period of time [132]. However, unmetabolised F_2-isoprostanes in urine may be confounded by a contribution of local F_2-isoprostane production in the kidney [133, 137]. Other forms of isoprostanes can also be formed in the body and may be interesting to detect, for example F_3-isoprostanes from eicosapentaenoic acid [138], F_4-isoprostanes from docosahexaenoic acid [139], D_2/E_2-isoprostanes [140] and neuroprostanes [141].

8-Iso-prostaglandin $F_{2\alpha}$ – a major F_2-isoprostane
Several different isomers of F_2-isoprostanes are formed (in total about 64) [3, 8, 9, 131-133]. 8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is one of the major F_2-isoprostanes formed *in vivo* [142, 143] and exhibits potent biological activity

e.g. as a vasoconstrictor in both the kidney and lung in rats and rabbits [82, 144-146]. In platelets and monocytes *in vitro*, 8-iso-$PGF_{2\alpha}$ has also been observed to be a minor product of COX-dependent formation [84, 147]. However, later it was concluded that this COX-dependent formation does not seem to contribute significantly to urinary levels of 8-iso-$PGF_{2\alpha}$ [148]. Further, levels of 8-iso-$PGF_{2\alpha}$ in normal human plasma and urine exceed the levels of COX-derived prostaglandins and thromboxanes by an order of magnitude [133, 137]. Levels of 8-iso-$PGF_{2\alpha}$ in human plasma and urine have also been shown to be unaffected by treatment with high doses of the COX inhibitors [82].

Detection of 8-iso-prostaglandin $F_{2\alpha}$

Sensitive gas chromatography-mass spectrometry (GC-MS) assays have been described to measure 8-iso-$PGF_{2\alpha}$ [82-85, 149] as well as enzyme immunoassays (EIAs) and radioimmunoassays (RIAs) [86-88]. The original method used for 8-iso-$PGF_{2\alpha}$ measurements described by Morrow et al [82, 149] is based on GC-MC with negative ion chemical ionisation. Various modifications of this method have been developed [83-85]. The GC-MS methods are highly sensitive and accurate, but also expensive and time consuming. Several immunoassays have therefore recently been developed including a commercially available EIA [86, 87] and a specific and sensitive RIA [88]. A common problem is that related compounds may interfere in binding to the antibody. The antibodies used have been tested for cross-reactivity with other major eicosanoids and isoprostanes, and the degree of cross-reactivity has been shown to be low [88]. To be fully evaluated, however, the accuracy and reliability of these immunoassays needs to be further validated against the GC-MS methods [132].

Levels of 8-iso-prostaglandin $F_{2\alpha}$ in vivo

8-Iso-$PGF_{2\alpha}$ has been suggested as a potential biomarker of non-enzymatic lipid peroxidation and oxidative injury because it can be specifically and accurately measured in normal biological samples, and the levels are increased under conditions of oxidative stress and modulated by antioxidants [133, 150]. Detectable levels of 8-iso-$PGF_{2\alpha}$ have been obtained in different body fluids including blood, urine, bile, pericardial fluid, lung condensates and cerebrospinal fluid as well as in different tissues [133]. Urinary 8-iso-$PGF_{2\alpha}$ excretion is not confounded by diurnal variation [151] or the lipid content of the diet, i.e. by 8-iso-$PGF_{2\alpha}$ from dietary sources [152, 153]. The levels of 8-iso-$PGF_{2\alpha}$ are increased in LDL by *in vitro* oxidative modification [154, 155]. In animals, the levels of 8-iso-$PGF_{2\alpha}$ are increased in models of oxidative stress [134, 156-165], increased with antioxidant deficiency [157, 166, 167] and reduced by dietary antioxidant supplementation [167-172]. Further, elevated levels of 8-iso-$PGF_{2\alpha}$ in human body fluids have also been observed in several conditions that are proposed to be associated with free radical induced oxidative

injury in humans such as smoking [173, 174], diabetes mellitus [175, 176], vascular reperfusion [177, 178], hypercholesterolemia [179], atherosclerotic lesions [180, 181] and liver cirrhosis[182].

Methods for measurement of enzymatic lipid peroxidation

Enzymatically derived lipid peroxidation is measured by analyses of eicosanoids, products of COX and lipoxygenase catalysed lipid peroxidation. Eicosanoids analyses are mainly performed by RIA, but may also be performed by bioassays or GC-MS based methods [183].

Prostaglandin $F_{2\alpha}$ metabolites

Enzymatic oxidation of arachidonic acid via the COX pathway leads to the formation of prostaglandins [48]. A major metabolite of the primary prostaglandin $F_{2\alpha}$, 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ (15-K-DH-$PGF_{2\alpha}$), can be used as an indicator of inflammation and enzymatic lipid peroxidation via the COX pathway [184]. In animal models of hepatotoxicity, endotoxaemia and reperfusion injury, 15-K-DH-$PGF_{2\alpha}$ has been shown to be increased [161-165, 184], as well as after supplementation with conjugated linoleic acid in humans [185]. The primary $PGF_{2\alpha}$ increases also during physiological control of luteolysis and parturition in various species [186, 187].

Carbon tetrachloride induced lipid peroxidation

Carbon tetrachloride was the first toxin from which it was shown that the injury it produces was largely or entirely mediated by free radicals [3, 188, 189]. Lipid peroxidation induced by CCl_4 is a commonly used experimental animal model for studying oxidative injury in biological systems [190, 191]. Cytochrome P-450 enzymes are believed to metabolise CCl_4 to trichloromethyl radicals that can initiate peroxidation of unsaturated fatty acids and start chain reactions of lipid peroxidation. The lipid-solubility of CCl_4 allows it to cross membranes and to be distributed to all organs. However, the liver is the major target organ of CCl_4-induced toxicity owing to its high content of cytochrome P-450 enzymes. Several antioxidants are recognised to scavenge free radicals and may therefore prevent propagation of the CCl_4-induced lipid peroxidation process. Vitamin E is a well-characterised chain-breaking antioxidant with the particular function of preventing lipid peroxidation in membrane systems [24, 25].

The CCl_4 model has been used to study responses in the levels of F_2-isoprostanes. Administration of CCl_4 to rats increases levels of 8-iso-$PGF_{2\alpha}$ in plasma, urine, bile and tissues [82, 156, 162, 192]. Elevated levels of 8-iso-$PGF_{2\alpha}$ in plasma increases even further in rats pre-treated with glutathione-depleting agents before CCl_4 administration [156]. Dietary vitamin E supplementation has been shown to suppress levels of 8-iso-$PGF_{2\alpha}$ in various rat models other than CCl_4 [167-172]. Whether vitamin E may also suppress elevated levels of 8-iso-$PGF_{2\alpha}$ after CCl_4 administration remains to be established.

Lipid peroxidation, atherosclerosis and dietary fat quality

Coronary heart disease (CHD) and other atherosclerotic diseases are the major cause of death in the Western world today. Elevated serum levels of total cholesterol, LDL cholesterol and an elevated ratio of LDL/HDL are established risk factors for CHD [193-195]. There is considerable evidence that oxidised lipoproteins play an important role in the development of atherosclerosis [196, 197]. The 'oxidised LDL hypothesis' suggests that oxidation of LDL by free radicals and other reactive species leads to modification of lysine residues on apo B [198-200]. The modified LDL particles are not recognised by the endogenous LDL receptor and bind to scavenger receptors on macrophages. The lack of down-regulation of the scavenger receptors allows continued uptake of LDL by the macrophages, which then develop into foam cells and fatty streaks. Atherosclerotic lesions are classified as fatty streaks, fibrous plaques and complex lesions, and the development of fatty streaks is an early key event in the initiation of the atherosclerotic lesion. Oxidatively modified LDL has a number of additional potential characteristics that may also contribute to its proatherogenic properties [197, 201]. The amounts of PUFAs and vitamin E in the LDL particle are important factors that may influence the propensity for oxidation of LDL.

It is well established that a high dietary intake of SAFAs is associated with high levels of total serum cholesterol and with an increased risk to develop CHD [202, 203]. On the contrary, epidemiological studies investigating the effects of dietary MUFA and PUFA on the outcome of CHD have been less conclusive [203]. Several intervention studies have demonstrated that the lipoprotein profile could be improved by substituting MUFA and PUFA for SAFA [204-207]. However, intervention studies with PUFA-rich diets have also shown that the susceptibility of lipoproteins to oxidation was increased and that very high intakes of PUFAs may carry non-favourable effects on the development of atherosclerosis [208, 209]. The oxidisability of fatty acids is considered to be

dependent on the degree of unsaturation [46, 47]. Therefore, dietary recommendations regarding fat intake and quality include a maximum level of the PUFA intake. Recent Nordic nutrition recommendations recommend a diet with a fat content that should not exceed 30 energy per cent (E%), an intake of SAFA of less than 10 E%, a PUFA intake of between 5-10 E% and the rest from MUFA [210].

There has been a pronounced change in the type of edible fats used in many countries, e.g. in northern Europe and Canada, with an increased consumption of low erucic acid rapeseed oil-based fats [211]. This development may lead to an increased dietary intake of MUFAs and PUFAs, especially α-linolenic acid (18:3 n-3). Several intervention studies with rapeseed oil-rich diets have shown favourable effects on the blood lipid profile in healthy volunteers [204-207] and in hyperlipidemic patients [212-214], with reduced serum levels of total and LDL cholesterol and unchanged serum levels of triglycerides and HDL cholesterol. However, the same rapeseed oil-rich diet may also potentially increase the degree of lipid peroxidation in the body, since rapeseed oil contains a considerable amount of easily oxidised PUFAs. However, rapeseed oil is also rich in both α- and γ-tocopherol, and it is not yet established how a rapeseed oil-based diet might influence the balance between lipid peroxidation and antioxidants present for protection *in vivo*.

AIMS OF THE INVESTIGATIONS

The overall aim of this thesis was to evaluate some methods for measurement of lipid peroxidation in the body that are suitable for clinical investigations, and to apply these methods in animal and human studies investigating basal conditions and situations associated with increased lipid peroxidation. The specific aims were:

- to evaluate the effect of sample handling and storage conditions on the levels of total lipid hydroperoxides in human plasma measured with the ferrous oxidation in xylenol orange version 2 assay (**paper I**)

- to further develop a methodology for quantification of F_2-isoprostanes in tissues using alkaline hydrolysis of esterified F_2-isoprostanes and an existing radioimmunoassay for measurement of 8-iso-$PGF_{2\alpha}$, and to apply this methodology in a well known experimental model of carbon tetrachloride-induced lipid peroxidation in rats (**paper II**)

- to investigate the effects of supplementation with the antioxidant vitamin E on the basal levels of biomarkers of non-enzymatic and enzymatic lipid peroxidation in rats (**paper III**)

- to investigate the effects of supplementation with vitamin E on biomarkers of lipid peroxidation in an experimental model of carbon tetrachloride-induced lipid peroxidation in rats (**paper IV**)

- to compare the effects of a rapeseed oil-based diet with an increased proportion of easily oxidised PUFAs with a control diet rich in SAFA on the levels of biomarkers of *in vivo* lipid peroxidation in humans (**paper V**)

MATERIALS AND METHODS

Subjects and animals

Paper I: Thirty-two subjects (12 women, 20 men) were recruited from staff and from patients attending the metabolic unit at the Department of Geriatrics, Uppsala University Hospital, Sweden. Mean age was 52 ± 8 years (range 40-72 years). The group of subjects consisted of healthy persons, patients with type 2 diabetes mellitus and patients with other metabolic disorders (n = 12, 10, 10, respectively).

Paper II, Paper III and Paper IV: Male Sprague-Dawley rats were purchased from B & K Universal, Sollentuna, Sweden. In paper II control rats (n = 4) and rats treated with CCl_4 for different periods of time (n = 16, four time points with four rats in each group) were used. Two groups with control (n = 6) and vitamin E-supplemented rats (n = 8) were used in paper III. In paper IV, two additional groups with CCl_4-treated (n = 6) and vitamin E-supplemented CCl_4-treated rats (n = 8) were combined with the control rats in paper III to minimise the animals used. The rats had free access to water and food. They were subjected to a 12 h light/12 h dark schedule.

Paper V: Nineteen healthy subjects (6 women, 13 men) with mean age 50 ± 8 years and normal to moderately increased body weight and blood lipids were recruited from local companies by means of poster advertisement. Eligible for the study were men (30 to 65 years of age) and postmenopausal women (50 to 65 years of age) with serum cholesterol 5.4 to 8.0 mmol/L, serum triglycerides 1.3 to 5.0 mmol/L, fasting blood glucose 3.0 to 6.5 mmol/L, diastolic blood pressure < 95 mmHg and body mass index (BMI) <30 kg/m^2. All subjects were asked to abstain from dietary vitamin- and mineral supplements and acetyl salicylic acid during the diet periods and to maintain their habitual life style during the study.

Experiments and interventions

Paper I: Blood samples were collected from a mixed group of healthy volunteers and patients with metabolic disorders to obtain a wide range of detectable levels of plasma hydroperoxides.

Paper II and IV: CCl_4 was used to induce free radical-induced lipid peroxidation. Rats were gavaged with CCl_4 (2.5 mL/kg body weight) and blood, urine and liver samples were collected at different time intervals after CCl_4 administration. In paper II, liver tissues were collected at 0, 1, 2, 4, 6 hours, whereas blood and urine samples were collected at 4 hours in paper IV.

Paper III and IV: All rats received powdered food prepared from commercial food pellets (R36, Lactamin AB, Stockholm, Sweden) containing total lipids 4%, protein 18.5%, carbohydrates 55.7% and fibre 3.5% and vitamin E at 63 mg/kg for a period of three weeks. For vitamin E supplementation, all-rac-α-tocopheryl succinate (Merck, Darmstadt, Germany) was blended into the powdered food at a concentration of 20 g/kg diet resulting in a calculated daily intake of approximately 2 g/kg body weight.

Paper V: The study was designed to compare a rapeseed oil-based diet (RO) with an increased proportion of MUFAs and PUFAs, especially α-linolenic acid, and a control diet (SAT) with a high proportion of SAFAs. It was conducted with a randomised cross-over design including two consecutive four-week diet periods separated by a four-week washout period (***Figure 2***). Subjects were randomised to start with either of the two diets and were blinded to the type of diet they were following.

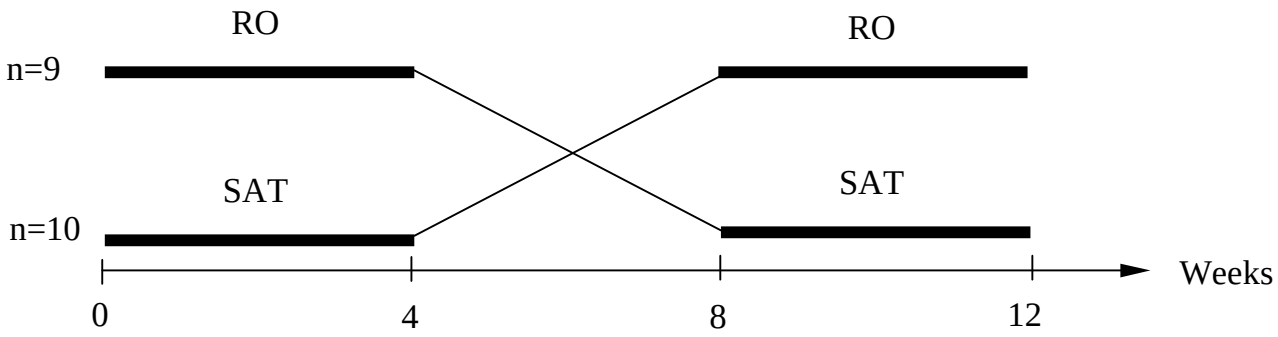

Figure 2. *Cross-over design of the study.*

The test diets were planned to contain 37 E% fat, 12 E% protein and 50 E% of carbohydrates. The composition of the RO diet was composed according to dietary recommendations [210], but with a fat content corresponding to the average intake of a Swedish population [215]. The control diet contained saturated fat products instead of rapeseed oil-based fat products. The fat quality of the two test diets (RO and SAT) was controlled by supplying fat products and

food items based on the fats tested in combination with dietary advice to avoid fats from other dietary sources such as fatty dairy and meat products. The subjects met a nutritionist before and during the diet periods for dietary advice and instructions on preparation of their diets to assure good adherence to the diet. Ten different lunch meals (five/week), wholemeal bread (three/day) and muffins (one/day) prepared with the fats tested were supplied weekly, as well as free amounts of cooking fat, table margarine (80% fat) and oil to use for cooking, spread and dressings. Double portions including the daily supply of food items and an approximated daily intake of supplied fat products were prepared. Two energy levels, 9 MJ and 12 MJ, were prepared and the energy requirement for each subject was approximated as 146 kJ/kg body weight. The body weight of the subjects was checked once a week to avoid weight changes and the energy level was adjusted together with dietary advice when needed. Dietary intake was monitored using three-day weighed food records (two weekdays and one weekend day) at five occasions, one at baseline and two during each diet period. The dietary food records were analysed by using the software program Stor MATs 4.0 (Rudans Lättdata, Västerås, Sweden) based on a food data base from the Swedish National Food Administration (PC-Kost 1996, SLV, Uppsala, Sweden). The compliance to the test diets was also controlled by analyses of the fatty acid compositions in serum phospholipids and cholesterol esters before and after the diet periods.

Blood, urine and tissue sampling

Paper I and V: Venous blood samples were drawn in the morning after an overnight fast. No smoking or heavy physical activity was allowed in the morning before sample collection. The blood samples were immediately placed on ice. In paper I, the blood samples were collected into heparinised tubes. To half of the plasma samples collected, BHT in methanol was added to a final concentration of 20 µmol/L. Fresh plasma samples were analysed within 1 to 8 hours after sample collection and samples that were stored frozen were analysed after 6 and 60 weeks. In paper V, blood samples were collected into serum tubes and heparin and dipotassium EDTA containing tubes. Heparin plasma was used for the analyses if nothing else is stated. Urinary samples were collected during 24 hours and three consecutive days in a special aliquot cup (Daisho Co. Ltd., Osaka, Japan). Serum, plasma and urine samples were stored at –70°C within 1 hour after sample collection until analysis.

Paper II, III and IV: The rats were weighed and surgical anaesthesia was induced with ether. During laparotomy, liver tissue was collected and the rats were killed by heart puncture. In paper III and IV, urine samples were collected in petri dishes before the rats were weighed and blood samples were drawn from

the abdominal aorta during ether anaesthesia. Blood samples were collected in heparinised glass tubes and plasma was prepared by centrifugation at 1930 × g for 8 min. All samples were immediately stored at -20°C during the experiment and thereafter at -70°C until analysis.

Measurement of clinical characteristics

Paper I and V: Body weight was measured on a digital scale with an accuracy of 0.1 kg and height was measured to the nearest cm. The BMI was calculated as the ratio of body weight (kg) to height squared (m^2). The fatty acid composition in serum phospholipids, serum cholesterol esters and the supplied food items were determined by gas liquid chromatography as described earlier by Boberg et al [216]. Very low-density lipoproteins, LDL, high-density lipoproteins (HDL) were isolated by a combination of preparative ultracentrifugation [217] and precipitation with a sodium phosphotungstate and magnesium chloride solution [218]. Cholesterol and triglyceride concentrations in serum and the isolated lipoprotein fractions were measured by enzymatic methods using the IL Test Cholesterol Triander's method 181618-10 and the IL Test Triglyceride Enzymatic-Colorimetric method 181610-60 in a Monarch 2000 centrifugal analyser (Instrumentation Laboratories, Lexington, MA, USA). The concentrations of serum apolipoprotein (apo) A-1 and apo B were determined by immunoturbidimetry (Orion Diagnostica, Espoo, Finland) in a Monarch apparatus. Lipoprotein (a) [Lp (a)] was measured by a Pharmacia apo (a) RIA (Pharmacia, Uppsala, Sweden). One unit/L of apo (a) corresponds to 0.7 mg/L Lp (a). Serum free fatty acids (FFA) were analysed with an enzymatic colorimetric method using a commercial kit (994-75409, Wako Chemical, Neuss, Germany) modified for use in a Monarch apparatus. Plasma glucose concentrations were measured in a Beckman Glucose analyser 2 (Beckman Instruments, Fullerton, CA, USA). Plasma insulin concentrations were determined by an enzyme-linked immunosorbent assay performed in an ES 300 automatic analyser (Boehringer Mannheim, Germany).

Hydrolysis and extraction of liver tissue

Paper II, III and IV: Liver samples were weighed, diluted with 3 volumes of phosphate buffer and homogenised under cold conditions. The homogenate was centrifuged at 1680 × g and 4°C for 10 min and the supernatant was stored at −70°C until further preparation within 1 week. The homogenate was first subjected to base hydrolysis by incubation with 3 volumes of 3 mol/L KOH at 37°C for 60 min, before acidification to pH 3-4 with 2 mol/L HCl and extraction

with 3 volumes of ethyl acetate. Extracted fractions were centrifuged at 1680 × g and 4°C for 10 min and the supernatant was evaporated under nitrogen. Samples were finally rediluted in less than 5% ethanol (total volume concentration) and phosphate buffer and stored at −70°C until analysis within 2 to 8 weeks. Hydrolysed and ethyl acetate extracted samples were used for quantification of the total amount of 8-iso-$PGF_{2\alpha}$ (sum of free and esterified 8-iso-$PGF_{2\alpha}$), whereas levels of free (unesterified) 8-iso-$PGF_{2\alpha}$ were measured after ethyl acetate extraction only.

Measurement of hydroperoxides

Total plasma hydroperoxide concentrations were measured using the FOX2 assay essentially as described by Nourooz-Zadeh et al [56] with minor modifications. The FOX2 reagent was prepared by dissolving 38 mg of XO and 440 mg of BHT in 450 mL HPLC-grade methanol with stirring. Ammonium ferrous sulphate (49 mg) was quickly dissolved in 50 mL of 250 mmol/L sulphuric acid and was added to the methanol solution. The final FOX2 reagent comprised of 100 µmol/L XO, 4 mmol/L BHT, 25 mmol/L sulphuric acid and 250 µmol/L ammonium ferrous sulphate in methanol (90 %, v/v). The extinction coefficient of the FOX2 reagent at 560 nm used was 3.86×10^4 $M^{-1}cm^{-1}$. It was routinely checked in hydrogen peroxide solutions of known concentrations and extinction coefficients lower than 3.72×10^4 $M^{-1}cm^{-1}$ were not accepted. For each plasma sample, 900 µL aliquots (quadruplicates) of plasma were incubated (30 minutes, 20-25°C) in 10 µL triphenylphosphine (10mmol/L) to remove hydroperoxides and to generate a blank, and 900 µL aliquots (quadruplicates) of plasma were incubated in 10 µL methanol to generate a test sample. Another incubation (30 minutes, 20-25°C) with the FOX2 reagent was performed. After centrifugation at 2400 × g for 10 min with a swing-out rotor (Hettich Rotenta/RP centrifuge, Hettich-Zentrifugen, Tuttlingen, Germany), the absorbance of the supernatants was determined at 560 nm in an Ultraspec 2000 spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). The hydroperoxide concentration of each sample was calculated from the difference of the absorbance of the blank and test samples.

Measurement of F_2-isoprostanes

Radioimmunoassay

Plasma, urine and liver samples were analysed for free and total (sum of free and esterified) 8-iso-$PGF_{2\alpha}$ using a newly developed RIA [88]. In brief, an

antibody was raised in rabbits by immunisation with 8-iso-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1´-carbonyldiimmidazole method. The cross-reactivity of the antibody with 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-K-DH-$PGF_{2\alpha}$, TXB_2, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$ and 8-iso-$PGF_{3\alpha}$ was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6%, respectively. The detection limit of the assay was about 23 pmol/L. Unextracted plasma and urine samples were used in the assay for measurement of free 8-iso-$PGF_{2\alpha}$ concentrations. Urinary levels of free 8-iso-$PGF_{2\alpha}$ were adjusted for creatinine concentrations measured by a colorimetric assay using a IL test creatinine kit (181672-00) in a Monarch 2000 centrifugal analyser (Instrumentation Laboratories, Lexington, MA, USA). Hydrolysed and extracted liver tissue preparations (as described above) were used in the assay for quantification of free and total amounts of liver 8-iso-$PGF_{2\alpha}$. In paper II, the levels were adjusted for protein content determined by a colorimetric assay using a Bio-Rad Protein Kit (500-0002, Bio-Rad Laboratories, Hercules, CA, USA).

Gas chromatography-mass spectrometry based method

Plasma samples were also analysed for total 8-iso-$PGF_{2\alpha}$ using a solid-phase extraction procedure and GC-MS as described by Nourooz-Zadeh et al [83]. Briefly, the samples were incubated with 0.5 ml of aqueous KOH (1 mol/L) to release esterified F_2-isoprostanes by hydrolysis, pH of the sample was re-adjusted to pH 2 by adding 0.5 ml of HCl (1 mol/L) and $PGF_{2\alpha}$-d_4 was added as the internal standard. F_2-isoprostanes were then isolated by solid-phase extraction on a C_{18} cartridge and a NH_4 cartridge. F_2-isoprostanes as pentafluorobenzyl ester/trimethyl ether derivates were analysed by GC on a Hewlett-Packard 5890 GC (Bracknell, UK) linked to a VG70SEQ MS (Fisons Instruments, Manchester, UK) using electron capture negative ion chemical ionisation (NICI) with ammonia reagent gas. Quantitative analysis was carried out by selective ion monitoring (SIM) of the carboxylate anion $[M-181]^-$ at *m/z* 569 and 573 for the F_2-isoprostanes and $PGF_{2\alpha}$-d_4 (internal standard), respectively.

Measurement of prostaglandin $F_{2\alpha}$ metabolites

Plasma and urine samples were analysed for a prostaglandin $F_{2\alpha}$ metabolite, 15-K-DH-$PGF_{2\alpha}$, using a newly developed RIA [184]. Briefly, an antibody was raised in rabbits by immunisation with 15-K-DH-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by the 1,1´-carbonyldiimmidazole method. The cross-reactivity of the antibody with $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, PGE_2,

15-keto-13,14-dihydro-PGE_2, 8-iso-15-keto-13,14-dihydro-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 9β-$PGF_{2\alpha}$, TXB_2 and 8-iso-$PGF_{3\alpha}$ was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001 and 0.01%, respectively. The detection limit of the assay was about 45 pmol/L. Unextracted plasma and urine samples of were used in the assay. Levels of 15-K-DH-$PGF_{2\alpha}$ in urine were adjusted for creatinine concentration determined as described above.

Measurement of malondialdehyde

MDA levels in plasma samples were measured using HPLC with fluorescence detection as described by Öhrvall et al [114] based on the methods of Wong et al [78] and Young and Trimble [80]. A TBA reaction was initiated by mixing 200 µL of dipotassium EDTA plasma with 750 µL of phosphoric acid (0.15 mol/L), 300 µL of water and 250 µL of TBA (42 mmol/L). The reaction mixture was incubated in a boiling water bath for 60 min, cooled on ice and then neutralised and precipitated by mixing 500 µl of the sample with 500 µl of a NaOH (1mol/L)-methanol solution (1:10, v/v). The MDA-TBA complex was separated by injecting 20 µL of the sample on a HPLC column (Lichrospher 100 RP-18, 250 x 4 mm). The mobile phase contained methanol-phosphate buffer (50 mmol/L) (2:3, v/v). Fluorescence was measured with an exitation wavelength of 532 nm and an emmission wavelength of 553 nm.

Measurement of antioxidants

Tocopherols

Serum/plasma tocopherols were determined using HPLC with fluorescence detection [219]. Serum/plasma (500 µL) was extracted with 500 µL of ethanol containing 0.05 g/L BHT and 2 mL of hexane. A 20-µL volume of supernatant was injected into a HPLC column (LiChrospher 100 NH_2, 250 x 4 mm). Fluorescence was measured with an excitation wavelength of 295 nm and an emission wavelength of 327 nm. In paper V, the levels of serum tocopherols were adjusted for the sum of serum cholesterol and triglyceride concentrations as suggested by Thurnham et al [220].

Antioxidative capacity

Plasma antioxidative capacity (AOC) was measured by a chemiluminescence assay by Whitehead et al [221] as described by Öhrvall et al [222]. The assay is based on measurement of light emission when a chemiluminescent substrate, luminol, is oxidised by hydrogen peroxide in a reaction catalysed by horseradish

peroxidase. Suppression of the light output by antioxidants is related to the AOC of the sample. The suppression was compared with the quenching activity of trolox, a tocopherol-analogue, and the concentration was expressed as trolox equivalents. The light emission was measured in a luminometer (1251, BioOrbit, Turku, Finland). In paper III and IV, uricase was used to eliminate the urate content in the sample before the AOC was measured resulting in an AOC value without the response from urate.

Ethics

The human and animal studies were approved by the Ethics Committee of the Faculty of Medicine at Uppsala University and all subjects gave their informed consent before entering the study.

Statistics

Data are presented as means ± SD, except in paper V with results expressed as least square means ± SD taking imbalance into account. All variables were continuous and on an interval scale. Variables with skewed distributions according to Shapiro Wilk's test ($W < 0.95$) [223] were log-transformed. Variables that were normally or log-normally distributed were tested with parametric methods and other variables were tested with non-parametric tests. All tests were two-tailed and $P < 0.05$ was regarded as statistically significant. The statistical analyses were performed using the statistical software packages Stata for Windows, version 4.0 (Stata Corporation, College Station, TX, USA) and JMP, version 3.2 (SAS Institute, Cary, NC, USA).

Associations between variables were examined with Pearson product-moment correlations or the non-parametric Spearman rank correlations. In paper I, the intra-assay SDs of the hydroperoxide measurements were calculated by pooling the SDs of the quadruplicates obtained with and without TPP treatment for each sample. One outlier (defined as deviating more than 3 SDs from the mean) was not used in the analyses of associations between variables. Agreements between hydroperoxide analyses of fresh and stored samples were also evaluated using the Bland-Altman method [224]. In paper II, differences between groups were tested using unpaired Student's t-test with Bonferroni correction considering four comparisons between the groups. In paper IV, differences between the three groups were first tested in an overall test using either analysis of variance or the non-parametric Kruskal-Wallis test [225]. In case of a significant overall test, pair-wise comparisons were made using unpaired Student's t-test or

Mann-Whitney's non-parametric test. In paper V, the analyses take into account the cross-over design of the experiment [226]. An analysis of variance model with factors for intervention sequence, subject and time was used. Values obtained after the two diets were compared with each other and with the baseline value. A test for carry-over effect was performed and if this test was significant, only data from the first diet period was used for comparisons of the two diets. Differences between and within diets are presented with mean effects and P values. For variables used to evaluate the primary aim, results are also presented with 95% confidence intervals (95% CIs) for the difference between, or for log-transformed variables for the ratio of (95% CI ratio) the effects of the two test diets.

RESULTS

Paper I

Evaluation of sample handling and storage conditions on plasma lipid hydroperoxides

In clinical studies, analysis of samples that have been stored frozen and thawed is often a prerequisite for practical reasons. The FOX2 assay for measurement of plasma lipid hydroperoxides was evaluated for its suitability for measurement of fresh and stored plasma samples. Lipid hydroperoxides were analysed in plasma samples from 32 healthy volunteers. Samples were analysed fresh and after storage for 6 and 60 weeks at –70°C with and without addition of the chain breaking antioxidant BHT (***Table 2***).

Table 2. *Hydroperoxide concentrations (µmol/L) in fresh and stored human plasma samples.*

	Fresh		**Stored**		
			6 weeks		**60 weeks**
	(n = 30)	**+ BHT** (n = 31)	(n = 32)	**+ BHT** (n = 29)	(n = 21)
Mean (SD)	8.35 (3.09)	8.59 (2.82)	6.00 (2.23)	5.93 (2.15)	6.43 (2.45)
Range	4.03 – 19.5	3.85 – 18.2	2.88 – 13.5	2.11 – 12.0	1.65 – 11.7
Intra-assay CV (%)	7.6	10.6	12.1	11.1	-

Inclusion of BHT immediately after sample collection had no effect on the levels of hydroperoxides neither in samples analysed fresh nor after storage at –70°C for 6 weeks compared to samples without BHT addition ($P = 0.14$ and $P = 0.62$, respectively). There was a positive correlation between hydroperoxides in fresh samples with and without BHT ($r = 0.89$, $P < 0.001$) and in stored samples with and without BHT addition ($r = 0.70$, $P < 0.001$).

Storage of samples at –70°C for 6 weeks without BHT was associated with large individual variations in the loss or formation of hydroperoxides. The changes in hydroperoxide levels after storage compared to samples analysed fresh ranged from a loss of 78% to an increase of 52%, with a mean change represented by of a loss of 23% of the hydroperoxide content. The time period of storage (6 or 60 weeks) did not seem to affect the content of hydroperoxides and the mean level of hydroperoxides in samples stored for 60 weeks was not different when compared to samples stored for 6 weeks (P = 0.77). Intra-assay coefficients of variation were <12%, with the lowest variation in fresh samples without BHT addition (7.6%).

There were no significant correlations between hydroperoxide levels in fresh and stored plasma samples in the presence or absence of BHT (r = 0.33, P = 0.09 and r = 0.32, P = 0.09, respectively). The agreement between hydroperoxides analysed in fresh and stored samples were also analysed according to Bland-Altman [224], which supported the conclusions of a poor agreement with a difference in detectable levels of fresh and stored samples. The Bland-Altman analysis showed (a) that there were wide limits of agreement between the analyses of fresh and stored samples (–6.93 to 2.63 µmol/L), (b) that the levels of hydroperoxides in fresh samples were higher than in stored samples with a mean difference of –2.15 µmol/L (95% CI –3.08 to –1.22) (c) that the difference was not dependent on the concentration since there was no linear association between the differences and the mean (r = 0.24, P = 0.21).

Comparison of the levels of hydroperoxides with other indicators of lipid peroxidation and antioxidants

In the same plasma samples, other indicators of lipid peroxidation (8-iso-$PGF_{2\alpha}$ as measured by GC-MS and MDA) as well as biomarkers of antioxidants (α- and γ-tocopherol and AOC) were also detected and compared to each other (***Table 3***). The hydroperoxide levels from analysis of fresh plasma samples were significantly correlated with the levels of 8-iso-$PGF_{2\alpha}$, but were not correlated with any of the other indicators for lipid peroxidation or antioxidants. However, the levels of 8-iso-$PGF_{2\alpha}$ and MDA were both inversely correlated with the concentrations of lipid adjusted α-tocopherol.

Table 3. *Correlations of plasma levels of hydroperoxides in fresh samples (µmol/L) by other biomarkers of lipid peroxidation and antioxidants.*

	r	**P**
8-iso-$PGF_{2\alpha}$ (nmol/L)	0.41	0.028
MDA (µmol/L)	–0.087	0.65
α-Tocopherol (µmol/L)[a]	–0.25	0.20
γ-Tocopherol (µmol/L)[a]	–0.22	0.26
α/γ-Tocopherol ratio	0.15	0.44
AOC (µmol trolox equivalents/L)	–0.21	0.27
Urate (µmol/L)	–0.32	0.095
AOC/urate	–0.34	0.067

[a] Lipid adjusted by division with the sum of serum triglycerides and cholesterol.
MDA = malondialdehyde, AOC = antioxidative capacity.

Paper II

Quantification of F2-isoprostanes in liver tissue

Analysis of total amounts (sum of free and esterified) of an F_2-isoprostane, 8-iso-$PGF_{2\alpha}$, allows a direct measurement of the extent of oxidative injury in key tissues of interest. The total amount of 8-iso-$PGF_{2\alpha}$ was analysed using alkaline hydrolysis of tissue lipids and a specific RIA for measurement of 8-iso-$PGF_{2\alpha}$. The methodology was evaluated in an experimental rat model of CCl_4-induced lipid peroxidation (***Figure 3***).

Basal levels of total 8-iso-$PGF_{2\alpha}$ in hydrolysed liver tissue of control rats were 6.5 times higher than the levels of free 8-iso-$PGF_{2\alpha}$. In rats treated with CCl_4 to induce lipid peroxidation, the dynamics of formation of free and esterified 8-iso-$PGF_{2\alpha}$ in the liver tissue differed. The maximum level of total 8-iso-$PGF_{2\alpha}$ in liver tissue of rats treated with CCl_4 was found at 2h after CCl_4 administration. At this time point, the total 8-iso-$PGF_{2\alpha}$ level was almost 13 times higher than the free 8-iso-$PGF_{2\alpha}$ level. The generation of free 8-iso-

$PGF_{2\alpha}$ in liver tissue of CCl_4-treated rats was increasing continuously after CCl_4 administration with the highest detectable level at the last time-point at 6h.

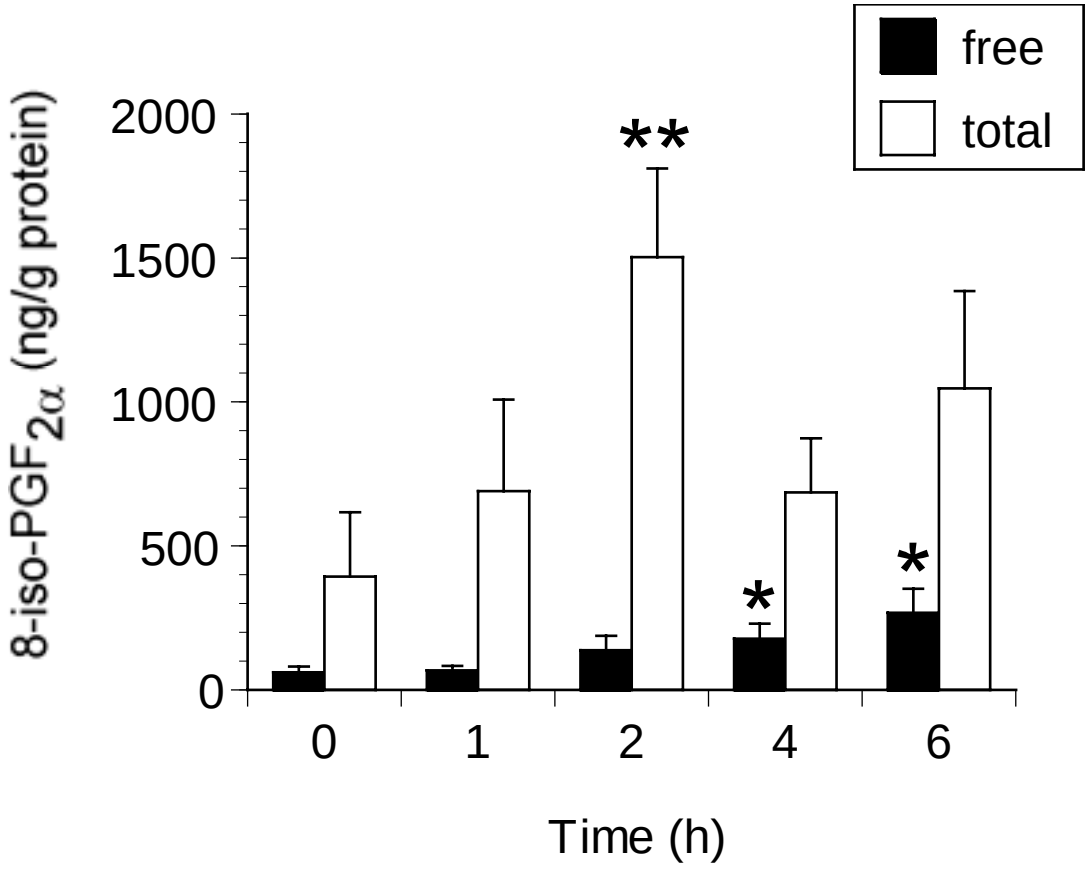

Figure 3. *Levels of free and total 8-iso-$PGF_{2\alpha}$ in liver tissue at different time intervals after carbon tetrachloride treatment (*P< 0.05 and **P < 0.01 for difference from controls at 0 time).*

Paper III and IV

Vitamin E supplementation reduces both basal levels and carbon tetrachloride induced levels of F_2-isoprostanes and prostaglandin $F_{2\alpha}$ in rats

The effect of vitamin E supplementation on the basal levels (Paper III, ***Table 4***) and CCl_4-induced levels (Paper IV, ***Table 5***) of both non-enzymatic and enzymatic lipid peroxidation was investigated. 8-Iso-$PGF_{2\alpha}$, a prostaglandin $F_{2\alpha}$ metabolite (15-K-DH-$PGF_{2\alpha}$), MDA and antioxidants were measured in control rats and rats supplemented with vitamin E, rats treated with CCl_4 and rats supplemented with vitamin E prior to CCl_4 treatment.

The basal levels of urinary 8-iso-$PGF_{2\alpha}$ tended to be lower in vitamin E-supplemented rats compared to controls (***Table 4***). The basal levels of free 8-iso-$PGF_{2\alpha}$ in the liver were significantly lower in the vitamin E-supplemented group, as was urinary 15-K-DH-$PGF_{2\alpha}$. Supplementation with vitamin E did not affect the basal levels of plasma 8-iso-$PGF_{2\alpha}$, plasma 15-K-DH-$PGF_{2\alpha}$, liver total 8-iso-$PGF_{2\alpha}$ and plasma MDA. The basal plasma α-tocopherol concentration and the AOC were greater in the rats supplemented with vitamin E for three weeks compared to the control rats.

Table 4. *Effects of vitamin E supplementation on basal levels of biomarkers of lipid peroxidation and antioxidants.*

	Control (n = 6)	**Vitamin E (n = 8)**	**P for difference Control–Vitamin E**
Urinary free 8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.72 (0.40)	0.34 (0.19)	0.056
Plasma free 8-iso-$PGF_{2\alpha}$ (nmol/L)	0.43 (0.44)	0.49 (0.29)	0.59
Liver free 8-iso-$PGF_{2\alpha}$ (nmol/g liver)	0.47 (0.11)	0.18 (0.04)	<0.001
Liver total 8-iso-$PGF_{2\alpha}$ (nmol/g liver)	2.84 (0.59)	3.07 (1.44)	1.00
Urinary 15-K-DH-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.97 (0.38)	0.56 (0.21)	0.048
Plasma 15-K-DH-$PGF_{2\alpha}$ (nmol/L)	0.64 (0.22)	0.48 (0.28)	0.18
MDA (μmol/L)	2.54 (1.19)	2.64 (1.04)	0.90
Plasma α-tocopherol (μmol/L)	17.9 (1.7)	50.4 (10.4)	<0.001
Plasma AOC (μmol trolox equivalents/L)	181 (6)	275 (27)	<0.001

Values are mean (SD). MDA = malondialdehyde, AOC = antioxidative capacity.

CCl_4 treatment alone resulted in significantly higher levels of plasma, urinary and liver total 8-iso-$PGF_{2\alpha}$, and of plasma and urinary 15-K-DH-$PGF_{2\alpha}$ compared to the same controls as in paper III (***Table 5***). Rats supplemented with vitamin E prior to CCl_4 treatment had significantly lower levels of urinary and liver 8-iso-$PGF_{2\alpha}$, urinary 15-K-DH-$PGF_{2\alpha}$ and plasma MDA than rats treated with CCl_4 alone. However, plasma 8-iso-$PGF_{2\alpha}$ and plasma 15-K-DH-$PGF_{2\alpha}$ were not shown to be affected by vitamin E supplementation. Regarding antioxidants in the circulation, both α-tocopherol levels and the AOC in plasma were greater in vitamin E-supplemented rats after CCl_4 treatment when compared to CCl_4 treatment alone.

Table 5. *Effects of vitamin E supplementation (VitE) on levels of biomarkers of lipid peroxidation and antioxidants in carbon tetrachloride (CCl_4) induced lipid peroxidation.*

	Control (n = 6)	**CCl_4 (n = 6)**	**VitE+CCl_4 (n = 8)**	**P for diff CCl_4 – VitE+CCl_4**
Urinary free 8-iso-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.72 (0.40)	2.54 (0.94)**	1.57 (0.39)*	0.023
Plasma free 8-iso-$PGF_{2\alpha}$ (nmol/L)	0.43 (0.44)	0.81 (0.27)*	0.53 (0.30)	0.13
Liver total 8-iso-$PGF_{2\alpha}$ (nmol/g liver)	2.84 (0.59)	6.44 (1.05)**	2.44 (1.03)	0.002
Urinary 15-K-DH-$PGF_{2\alpha}$ (nmol/mmol creatinine)	0.97 (0.38)	5.26 (3.53)***	1.45 (0.77)	0.003
Plasma 15-K-DH-$PGF_{2\alpha}$ (nmol/L)	0.64 (0.22)	2.45 (0.64)***	2.04 (0.86)***	0.28
MDA (µmol/L)	2.54 (1.19)	2.44 (0.65)	1.56 (0.47)*	0.014
Plasma α-tocopherol (µmol/L)	17.9 (1.7)	15.5 (2.6)	29.2 (5.6)***	<0.001
Plasma AOC (µmol trolox equivalents/L)	181 (6)	212 (18)*	292 (29)***	<0.001

Values are mean (SD). *P < 0.05, **P < 0.001 and ***P < 0.001 for difference from controls. MDA = malondialdehyde, AOC = antioxidative capacity.

Paper V

A diet containing rapeseed oil-based fats does not increase lipid peroxidation in humans when compared to a diet rich in saturated fatty acids

To investigate if a rapeseed oil-based diet, containing an increased proportion of easily oxidised PUFAs such as α-linolenic acid, may affect the degree of lipid peroxidation *in vivo*, a rapeseed oil-based diet (RO) was compared with a control diet (SAT) rich in saturated fat in a cross-over designed dietary intervention study involving 19 subjects (6 women and 13 men).

No significant differences in plasma or urinary levels of free 8-iso-$PGF_{2\alpha}$ were observed between the RO and SAT diets (95% CI ratios 0.94 to 1.46 and 0.91 to 1.27, respectively)(***Table 6***). The 95% CI ratios for log transformed variables are considered significant if 1 is not included. The levels of plasma total 8-iso-$PGF_{2\alpha}$(95% CI ratio 0.56 to 1.36), plasma MDA (95% CI ratio 0.98 to 1.18) and plasma hydroperoxides (95% CI –0.81 to 0.86) did not differ between the RO and SAT diets either.

Table 6. *Biomarkers of lipid peroxidation in plasma and urine at baseline and after the rapeseed oil-based diet (RO) and the saturated diet (SAT).*

	Baseline	**RO**	**SAT**	**P value**[a]
Plasma (n = 19)				
Free 8-iso-$PGF_{2\alpha}$ (pmol/L)	111 (59)	125 (70)	111 (63)	0.17
Total 8-iso-$PGF_{2\alpha}$ (pmol/L)[b]	740 (530)	580 (640)	530 (340)	0.55
Hydroperoxides (µmol/L)	6.73 (1.59)	6.69 (1.92)	6.66 (2.63)	0.95
MDA (µmol/L)	0.69 (0.10)	0.66 (0.15)	0.61 (0.10)*	0.14
Urine (n = 17)				
Free 8-iso-$PGF_{2\alpha}$ (ng/mg creatinine)	–	3.31 (1.33)	3.10 (1.44)	0.39

Data are means (SD). [a] P values are for difference between the RO and SAT diets. [b] n = 14-17.
* Indicates difference between test diet and baseline (* $P < 0.05$). MDA = malondialdehyde.

A higher concentration of serum γ-tocopherol was detected after the RO diet compared to the SAT diet (95 % CI ratio 1.62 to 2.18), whereas the serum α-tocopherol concentration (95 % CI ratio 0.98 to 1.09) and plasma AOC (95 % CI –6.8 to 54.8) did not differ between the two test diets (***Table 7***).

Table 7. *Antioxidants in serum at baseline and after the rapeseed oil-based diet (RO) and the saturated diet (SAT).*

	Baseline	**RO**	**SAT**	**P value**[a]
α-Tocopherol (μmol/mmol)[b]	4.20 (0.64)	4.24 (0.45)	4.09 (0.36)	0.20
γ-Tocopherol (μmol/mmol)[b]	0.34 (0.14)	0.42 (0.09)**	0.23 (0.06)***	<0.001
AOC (μmol trolox equivalents/L)[c]	526.3 (87.5)	546.0 (93.6)	522.0 (89.8)	0.13

Data are means (SD); n = 19. [a] P values are for difference between the RO and SAT diets. [b] Lipid adjusted. [c] Without lipid adjustment. * Indicates difference between test diet and baseline (** P < 0.01, *** P < 0.001). AOC = antioxidative capacity.

The goal to compare a rapeseed oil-based diet (RO) with a control diet (SAT) rich in saturated fats was achieved (***Table 8***). The diets were comparable in their content of macronutrients. The two test diets differed in the amount of SAFAs, MUFAs and PUFAs as well as α-linolenic acid. The daily vitamin E intake was higher during the RO diet compared to the SAT diet due to a naturally high vitamin E content in rapeseed oil (15.3 vs. 11.5 mg α-tocopherol equivalents, P < 0.001). All subjects completed both diet periods and were weight stable during both diet periods. The dietary fat quality was reflected as expected in the fatty acid composition in serum phospholipids. Previously observed positive effects of RO diets on the blood lipid profile with lower serum cholesterol, LDL cholesterol, LDL/HDL ratio as well as lower serum concentration of apo B were also verified after the RO diet compared to the SAT diet (***Table 9***).

Table 8 *Daily dietary intake during the rapeseed oil-based diet (RO) and the saturated diet (SAT) calculated from two pooled 3-day weighed food records.*

Nutrients	**RO**	**SAT**	**P value**[a]
Energy (MJ)	8.9 ± 1.8	9.7 ± 2.1	0.070
Fat (E%)	35.9 ± 4.3	36.2 ± 3.7	0.70
SAFA (E%)	10.3 ± 1.7	17.6 ± 2.1	<0.001
MUFA (E%)	15.7 ± 2.3	10.9 ± 1.3	<0.001
PUFA (E%)	7.5 ± 1.0	4.6 ± 0.8	<0.001
18:2 n-6 (E%)	5.4 ± 0.7	4.0 ± 0.7	<0.001
18:3 n-3 (E%)	1.8 ± 0.3	0.4 ± 0.1	<0.001
Protein (E%)	14.8 ± 2.0	14.4 ± 2.0	0.19
Carbohydrates (E%)	47.3 ± 5.3	47.5 ± 4.1	0.86
Alcohol (E%)	2.0 ± 1.7	1.9 ± 1.9	0.57
Cholesterol (mg)	281 ± 98	364 ± 101	0.008
Dietary fibre (g)	17.1 ± 5.9	18.9 ± 6.4	0.11
Vitamin E (mg α-TE)	15.3 ± 2.8	11.5 ± 2.3	<0.001
Ascorbic acid (mg)	80 ± 43	87 ± 57	0.77
β-Carotene (mg)	2 ± 1	2 ± 1	0.36
Selenium (μg)	35 ± 8	36 ± 12	0.89

Data are means ± SD; n = 19. [a] P values are for difference between the RO and SAT diets. E% = percent of energy. α-TE = alfa-tocopherol equivalents.

Table 9. *Blood lipid profile at baseline and after the rapeseed oil-based diet (RO) and the saturated diet (SAT).*

	Baseline	**RO**	**SAT**	**P value**[a]
Total cholesterol (mmol/L)[b]	6.59 (1.02) / 5.73 (0.85)	5.85 (0.97)***	6.05 (1.14)	<0.001
Total triglycerides (mmol/L)	1.40 (0.61)	1.24 (0.65)	1.43 (0.96)	0.18
LDL cholesterol (mmol/L)	4.31 (0.94)	3.85 (0.81)***	4.33 (1.10)	<0.001
HDL cholesterol (mmol/L)[b]	1.55 (0.40) / 1.17 (0.27)	1.41 (0.30)*	1.15 (0.22)	0.26
LDL/HDL ratio	3.34 (0.97)	2.99 (0.80)**	3.47 (1.09)	<0.001
Apo A1 (g/L)	1.38 (0.19)	1.37 (0.16)	1.37 (0.18)	0.96
Apo B (g/L)	1.01 (0.15)	0.95 (0.20)	1.04 (0.19)	0.012
Lp (a) (mg/L)	196 (182)	205 (190)	191 (213)	0.42

Data are means (SD); n = 19. [a] P values are for difference between the RO and SAT diets. [b] Indicates a variable with carry-over effect; data are from the first diet period only; baseline value corresponds to the RO and SAT diet, respectively (RO/SAT); n = 10 for the RO diet and n = 9 for the SAT diet. * Indicates difference between test diet and baseline (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

DISCUSSION

Difficulties in assessment of lipid peroxidation

The scope of this thesis

The interest for oxidative stress in relation to the development of disease has gained large attention during the last decades. Lipid peroxidation is an example of oxidative injury that has been extensively studied. It is thought to be an important factor in the pathophysiology of a number of diseases and in the process of ageing, but its measurement *in vivo* has been difficult. The aim of this thesis was to evaluate some methods for lipid peroxidation measurement *in vivo* that are suitable for clinical investigations, and to apply these methods under basal conditions and in situations that are or may be associated with increased lipid peroxidation in animals and humans.

In the papers included in this thesis we have used assays for the measurement of F_2-isoprostanes, hydroperoxides and MDA. The methods for analysis of F_2-isoprostanes by RIA and hydroperoxides by the FOX2 assay require no extraction step and are simple, sensitive and specific. MDA detected by fluorescence after HPLC separation is a widely adapted method and was used for comparison. As a complementary information, antioxidant status was assessed by measurement of vitamin E and AOC.

The ideal method

Fingerprinting methods based on the measurement of lipid peroxidation products are the methods most often used to measure free radical damage *in vivo* [3-9]. The ideal assay for *in vivo* measurements of lipid peroxidation should be sensitive, reproducible, specific and based on a compound that is stable on storage for long periods of time. It should also be applicable for measurements of basal steady-state levels and increased levels due to oxidative stress. For the clinical situation and for routine purposes, it should also be simple, inexpensive and non-invasive. Further, sample preparation should be avoided or be simple, since generation of artefacts during sample preparation is a common problem. Finally, the biomarker should not be confounded by dietary sources and other interfering factors.

Strategy for choosing methods

Today there is no method that meets all these criteria discussed above. In addition, one major drawback for biochemists and clinicians is that most of the methods developed for measurement of lipid peroxidation damage *in vitro* are not always applicable for *in vivo* situations [227]. It has been concluded that choosing a combination of different methods for both primary and secondary lipid peroxidation products may be the best approach to assess oxidative injury *in vivo*. It is also important to specify the question raised and to choose suitable methods to investigate the specific question. Often, there are conflicting and diverging results reported in the literature. By the development of better methods this problem may be circumvented.

Mass spectrometry based methods are sensitive, specific and considered to be reliable [3]. However, they are also expensive, time consuming and requires well skilled personnel. Thus, the use of other methods validated against MS based methods may be an alternative approach. Validations may include identification of the compound measured with MS, comparison of detectable levels in healthy volunteers and verification of responses found with MS techniques in different animal models using prooxidants and antioxidants (e.g. CCl_4 and vitamin E).

Comparison of different methods for lipid peroxidation measurements

One interesting question regarding the methods for measurement of lipid peroxidation is how the responses obtained with different methods relate to each other. Therefore, the relationships between the different methods used to assess lipid peroxidation *in vivo* were investigated in paper I, III, IV and V. There was a significant correlation between the plasma levels of hydroperoxides analysed in fresh samples and 8-iso-$PGF_{2\alpha}$ measured by GC-MS in paper I ($r = 0.41$, $P = 0.028$). When data from all animals in the control and treatment groups in paper III and IV were combined ($n = 28$), no correlations were found between the different methods used. In paper V, the different methods were compared using values obtained before the intervention started and there were no significant correlations between 8-iso-$PGF_{2\alpha}$, hydroperoxides and MDA.

Agreement between methods used in this thesis was also investigated after supplementation with conjugated linoleic acid, a situation previously shown to have induced increased levels of urinary 8-iso-$PGF_{2\alpha}$ as measured by RIA [185]. Hydroperoxides in fresh plasma samples, MDA and 8-iso-$PGF_{2\alpha}$ as measured by RIA were analysed in samples from a group of overweight middle-aged men

with signs of the metabolic syndrome participating in a randomised controlled trial with supplementation with conjugated linoleic acid (unpublished data). Plasma and urine samples were taken after 8 weeks of intervention from subjects receiving placebo capsules (n = 7), capsules with a mixture of two isomers of conjugated linoleic acid (n = 7) and capsules with a 10-*trans*,12-*cis* isomer (n = 10). Plasma levels of total hydroperoxides analysed in fresh plasma samples were inversely correlated with the levels of plasma MDA ($r = –0.64$, $P < 0.05$). Further, there was a trend for a positive correlation between plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ measured with the RIA ($r = 0.40$, $P = 0.057$).

These results regarding 8-iso-$PGF_{2\alpha}$ and MDA are in accordance with previously described results from an animal study in which the plasma levels of 8-iso-$PGF_{2\alpha}$ measured with the same RIA and MDA were not correlated [162]. In contrast to the diverging results found *in vivo*, the levels of 8-iso-$PGF_{2\alpha}$ correlated well with other indicators of lipid peroxidation such as hydroperoxides, TBARS and conjugated dienes in isolated LDL during copper-mediated oxidation [154] and rabbit aortic endothelial cell-mediated oxidation [142]. However, the *in vitro* situation with oxidation of LDL differs from the *in vivo* measurements in many aspects, e.g. many interfering factors are absent and the oxidation of LDL is relatively extreme, which may explain some of the different results found in body fluids and in oxidised LDL.

On the contrary, when we investigated the relationships between the levels of biomarkers of lipid peroxidation and antioxidants, several strong inverse relationships were found in paper I, III, IV and V. The levels of 8-iso-$PGF_{2\alpha}$ measured by GC-MS and MDA were both inversely correlated with the levels of lipid corrected α-tocopherol ($r = –0.58$, $P < 0.001$ and $r = –0.40$, $P = 0.025$, respectively) in paper I. In paper III and IV, the levels of urinary, free liver and total liver 8-iso-$PGF_{2\alpha}$ as measured by RIA were inversely correlated with the levels of α-tocopherol ($r = –0.71$, $P < 0.001$; $r = –0.68$, $P < 0.001$ and $r = –0.40$, $P < 0.033$, respectively). Free liver 8-iso-$PGF_{2\alpha}$ measured by RIA and MDA were also negatively correlated with the AOC value in paper III and IV ($r = –0.74$, $P < 0.001$ and $r = –0.41$, $P = 0.030$, respectively). In paper V, the hydroperoxide levels were inversely correlated with the α-tocopherol and γ-tocopherol concentrations ($r = –0.60$, $P = 0.007$ and $r = –0.54$, $P = 0.016$, respectively). The inverse correlations between circulating tocopherols and biomarkers of lipid peroxidation are in agreement with the theory of oxidative stress with an imbalance between prooxidants and antioxidants. In addition, there was also a relationship between the free and total 8-iso-$PGF_{2\alpha}$ concentrations in the liver ($r = 0.51$, $P < 0.01$), as well as a trend for a correlation between plasma and urinary levels of 8-iso-$PGF_{2\alpha}$ ($r = 0.43$, $P = 0.054$) as measured by RIA in paper III and IV. However, the relationships

between the levels of 8-iso-$PGF_{2\alpha}$ in different compartments may be complicated to interpret due to the differences in the kinetics of their formation.

In a broader perspective, another interesting question is whether biomarkers for different targets of free radical attack are related to each other. In a study on healthy human subjects, England et al concluded that the steady-state levels of lipid peroxidation as measured with 8-iso-$PGF_{2\alpha}$ and oxidative DNA damage in plasma were not correlated [228].

The different responses between methods for lipid peroxidation assessment *in vivo* may partly be explained by the fact that these methods reflect different stages of the lipid peroxidation process. They may also derive their origin from different fatty acid substrates and consequently be affected by the fat quality of the diet. Another piece of explanation to the diverging responses could be underestimations of correlations due to technical measurement errors. Further, the range of data investigated in each study may also affect the correlations. A combination of correlations and the use of Bland-Altman analysis [224] may generally be preferred when comparing different methodologies for the measurement of one specific lipid peroxidation product. These data support the idea that the best approach may be to use a combination of several methods for the measurement of lipid peroxidation *in vivo*.

Hydroperoxides as an indicator of lipid peroxidation

Reported levels of total and individual classes of hydroperoxides

Hydroperoxide assays are generally divided into two categories detecting either total or individual classes of lipid hydroperoxides. Total plasma hydroperoxide levels in healthy individuals using four distinct techniques with varying complexity generate much the same answer. Total hydroperoxide levels have been estimated to be between 0.5 and 5.5 µmol/L [56, 60, 61, 69, 121, 123-125, 229]. Using HPLC-chemiluminescence assays, plasma levels of different individual classes of hydroperoxides has been reported to vary considerably. Phospholipid hydroperoxides have been reported to range between 10 and 500 nmol/L [67, 68, 92]. Cholesteryl ester hydroperoxides levels are reported to be as low as 3 nmol/L [66, 117] or as high as 920 nmol/L [120].

Hydroperoxide levels in paper I

In paper I, we re-evaluated the FOX2 assay for the assessment of plasma hydroperoxides in fresh and stored samples. We recorded 8.35 ± 3.09 µmol/L of total hydroperoxides in fresh plasma samples from a group consisting of both

healthy controls and patients with various metabolic disorders. There were no differences between the hydroperoxide levels in the control group, the diabetes group and the group with other metabolic disorders (8.47 ± 4.32 µmol/L, 7.97 ± 2.03 µmol/L, 8.56 ± 2.16 µmol/L, respectively). Our values for the controls are slightly higher than previously reported levels in controls [56, 123-125, 229]. On the other hand, the control groups in previous studies were mainly comprised of younger subjects, which may explain the higher hydroperoxide levels detected in plasma in our control group. Hydroperoxide levels in the groups with patients with diabetes and other metabolic disorders were in agreement with those reported for type 1- and type 2-diabetic subjects in earlier studies [123-125].

Sample handling and stability of hydroperoxides

The effects of sample handling and stability of hydroperoxides after freezing and thawing was the primary aim investigated in paper I. To our surprise, there was no significant relationship between plasma hydroperoxides analysed in fresh and stored plasma samples. There were large inter-individual differences in the change of hydroperoxide content after storage, which are difficult to explain. In most cases there was a loss of hydroperoxides after storage, but in a few cases an increase of hydroperoxides was observed. The loss of hydroperoxides may be explained by decomposition to secondary lipid peroxidation products, and the increase may be due to a generation catalysed by traces of transition metal ions. However, inclusion of BHT as a chain-breaking antioxidant had no effect on the content of hydroperoxides.

Information on the stability of hydroperoxides during storage from the literature is sporadic. Our results are in line with an investigation reported by Holley and Slater on the stability of eicosatetraenoic acid hydroperoxide using an HPLC-chemiluminescence technique [92]. They found that this hydroperoxide was stable for up to 2 weeks at –70°C and that there was a 53% loss of the eicosatetraenoic acid hydroperoxides when the samples were stored for one month, which was not affected by inclusion of BHT or desferal.

The generation or decomposition of products when freezing and thawing samples may be an important factor to consider not only regarding hydroperoxide measurements. Storage in liquid nitrogen may provide additional protection. Also, critical consideration of traces of hemolysis is important, especially for measurements with the FOX2 method in which hemolysis interferes with the assay. Different sample handling procedures could possibly explain some of the variations in the reported levels of individual classes of hydroperoxides as measured with different techniques [66-68, 92, 117, 120].

F_2-isoprostanes as an indicator of lipid peroxidation

Quantification of 8-iso-$PGF_{2\alpha}$ in tissue

There is a growing interest in quantification of tissue levels of 8-iso-$PGF_{2\alpha}$ for investigations of the susceptibility of different tissues to oxidative stress. Thus, it is valuable to analyse the total amount of 8-iso-$PGF_{2\alpha}$ present in tissues. The total formation of 8-iso-$PGF_{2\alpha}$ including both free and esterified 8-iso-$PGF_{2\alpha}$ can be quantified following release of the esterified 8-iso-$PGF_{2\alpha}$ by alkaline hydrolysis of the tissue. Thus, the analysis of total amounts of 8-iso-$PGF_{2\alpha}$ may allow a direct measurement of the extent of oxidative injury in tissues of interest.

In paper II, we employed a methodology for hydrolysis of liver tissue to analyse total 8-iso-$PGF_{2\alpha}$ using a recently developed RIA [88]. After alkaline hydrolysis of the liver tissue, levels of total 8-iso-$PGF_{2\alpha}$ were increased both in the basal state and in an animal model of CCl_4-induced lipid peroxidation compared to the levels of free 8-iso-$PGF_{2\alpha}$. The levels of total 8-iso-$PGF_{2\alpha}$ in the liver tissue of control rats were comparable to levels reported by Morrow and Roberts using a GC-MS technique [156].

8-Iso-$PGF_{2\alpha}$ are generated in the esterified form

In the mammalian body, 8-iso-$PGF_{2\alpha}$ is believed to be generated in the esterified form in tissue phospholipids and subsequently released into the circulation after hydrolysis of the esterified 8-iso-$PGF_{2\alpha}$ to the free form, presumably by phospholipases and other hydrolytic enzymes [134]. However, the nature of these enzymes responsible for the successive hydrolysis of esterified 8-iso-$PGF_{2\alpha}$ *in vivo* remains to be established. Endogenously formed 8-iso-$PGF_{2\alpha}$ is rapidly metabolised and efficiently excreted into the urine [135, 136].

In paper II, liver tissue was collected at different intervals after CCl_4 treatment. The maximum level of total 8-iso-$PGF_{2\alpha}$ in the liver was found at 2h after CCl_4 administration. The increase of total 8-iso-$PGF_{2\alpha}$ in the liver preceded the appearance of free 8-iso-$PGF_{2\alpha}$ in the circulation, since the liver is the main target organ of CCl_4-induced lipid peroxidation. The pattern of CCl_4-induced 8-iso-$PGF_{2\alpha}$ formation in the liver is in accordance with earlier reported patterns of CCl_4-induced 8-iso-$PGF_{2\alpha}$ formation in plasma and urine [162]. Levels of free 8-iso-$PGF_{2\alpha}$ in the liver increased similarly as the levels of free 8-iso-$PGF_{2\alpha}$ in the urine. These findings are in agreement with the findings reported by Morrow and Roberts [134, 156] and suggest that the free F_2-isoprostanes arose

from hydrolysis of peroxidised lipids. The proportions between free and total 8-iso-$PGF_{2\alpha}$ generated in this rat model of oxidative stress varied over time after CCl_4 administration and ranged from about 4 to 13 times higher total than free levels. This indicates that the formation of esterified 8-iso-$PGF_{2\alpha}$ and release of free 8-iso-$PGF_{2\alpha}$ by phospholipases and hydrolytic enzymes during the experiment is a dynamic process. The availability and activity of endogenous phospholipases and other hydrolytic enzymes that releases the esterified 8-iso-$PGF_{2\alpha}$ may thus vary over time, which can be a problem with regard to measuring tissue levels of total 8-iso-$PGF_{2\alpha}$.

F_2-isoprostanes are increased by prooxidants

The formation of 8-iso-$PGF_{2\alpha}$ in plasma and urine have been shown to be increased in rats after CCl_4 administration as measured with a specific RIA [88, 184] and in a series of previous experimental animal studies using methodologies based on GC-MS [134, 156, 230]. In paper II and IV, significantly increased levels of plasma, urinary and liver 8-iso-$PGF_{2\alpha}$ were detected in CCl_4-treated rats compared to controls. However, the increases after CCl_4 treatment were less pronounced than previously reported results irrespective of the method used [88, 134, 156, 162]. The smaller increases in the formation of 8-iso-$PGF_{2\alpha}$ after CCl_4 treatment in paper II and IV could possibly be caused by less gastrointestinal absorption due to differences in the conditions during administration of CCl_4 to the rats.

F_2-isoprostanes are suppressed by antioxidants

Only a few studies have investigated the effect of vitamin E on the formation of F_2-isoprostanes [166-168, 231]. Vitamin E supplementation was shown to reduce F_2-isoprostane generation in apolipoprotein E-deficient mice [168] and diabetic rats [167], whereas vitamin E deprivation has been related to increased levels of 8-iso-$PGF_{2\alpha}$ [166, 231].

The antioxidant vitamin E is a chain-breaking antioxidant preventing lipid peroxidation in membranes by scavenging of free radicals [24]. Vitamin E may therefore prevent propagation of the CCl_4-induced lipid peroxidation process through this mechanism. However, information on whether vitamin E also affects the inflammatory response through COX-mediated lipid peroxidation during hepatotoxicity is lacking.

In paper III and IV, rats were supplemented with vitamin E in the diet and the effects under normal conditions and after CCl_4-induced lipid peroxidation were investigated. In these studies, we used a high dose of vitamin E (2 g/kg body) to

counteract the severe liver damage induced by CCl_4 and the subsequent lipid peroxidation. The dose of vitamin E used in our study is in the upper range used in various animal studies. Safety studies of vitamin E intake (reviewed in [232]) reports that adverse effects were rarely observed with this dose in rats, and it was concluded that there were no evidence for adverse toxic effects nor mutagenic, carcinogenic or teratogenic effects even at high doses of vitamin E. Control rats and vitamin E supplemented rats in this study both gained weight at a similar rate and there was no difference in the final body weight between the groups. The antioxidative capacity and α-tocopherol level in plasma was greater in the vitamin E supplemented rats than in controls. The basal levels of urinary 8-iso-$PGF_{2\alpha}$ and the levels after CCl_4 treatment was lower in the vitamin E supplemented rats. Vitamin E supplementation was also shown to suppress the basal levels of free but not total 8-iso-$PGF_{2\alpha}$ as compared to controls. The cause for these different effects of vitamin E on 8-iso-$PGF_{2\alpha}$ levels in the liver is unclear. These results indicate that non-enzymatic lipid peroxidation at basal conditions and during experimental hepatic oxidative injury can be suppressed by dietary vitamin E supplementation in rats.

Metabolism of 8-iso-$PGF_{2\alpha}$ – plasma and urinary levels

In paper III and IV, the basal levels and the CCl_4-induced levels of 8-iso-$PGF_{2\alpha}$ in the urine were reduced by dietary supplementation with vitamin E, but not in the plasma. The main reason why plasma 8-iso-$PGF_{2\alpha}$ was not suppressed by vitamin E supplementation is probably because the kinetics of formation and availability of 8-iso-$PGF_{2\alpha}$ are different in the plasma and urine. The half-life of plasma 8-iso-$PGF_{2\alpha}$ is very short and 8-iso-$PGF_{2\alpha}$ metabolises rapidly and is efficiently excreted into the urine [136]. Urinary levels of 8-iso-$PGF_{2\alpha}$ may therefore reflect an earlier event of the biosynthesis and availability of 8-iso-$PGF_{2\alpha}$ in the body compared to plasma levels measured at the same time [136]. Consequently, plasma 8-iso-$PGF_{2\alpha}$ levels are determined both by their rate of formation and by their metabolism and urinary excretion, all of which could vary between individuals [228].

Depending on the situation that is investigated, the steady-state levels of a biomarker may be drastically increased for example in acute diseases or stressed tissues such as in myocardial infarction or severe burns, whereas the increase may be less dramatic in chronic diseases such as atherosclerosis, diabetes and neurodegenerative diseases. When using 8-iso-$PGF_{2\alpha}$ as a biomarker of oxidative injury, evidence must be interpreted considering both the type of oxidative injury and the metabolism of 8-iso-$PGF_{2\alpha}$.

Possible link between oxidative injury and inflammation

The use of recently developed RIAs for the measurement of both 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ is an interesting possibility for simultaneous measurement of non-enzymatic and enzymatic lipid peroxidation *in vivo* [88, 184]. Levels of both 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ in plasma and urine have been shown to be increased in animal models of CCl_4-induced hepatotoxicity [162], experimental septic shock [161, 163] and in reperfusion injury after cardiac arrest [165]. In the hepatotoxicity study [162], the oxidative injury, as measured by 8-iso-$PGF_{2\alpha}$, was increased before an increase in the inflammatory response, as measured by 15-K-DH-$PGF_{2\alpha}$, could be seen. This result suggests that the COX-dependent inflammatory response possibly could be a secondary effect of oxidative injury and a conceivable link between inflammation and oxidative stress [162].

In paper III, it was shown that basal conditions in normal rats compared to rats fed a vitamin E enriched diet differed. The basal levels of both urinary 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ were lower after vitamin E supplementation. In paper IV, the plasma and urinary levels of both 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ were also lower in vitamin E-supplemented rats treated with CCl_4 as compared to rats treated with CCl_4 alone. Thus, vitamin E was shown to have a suppressive effect on both non-enzymatic and enzymatic lipid peroxidation.

Dietary fat quality and oxidisability of different fatty acids

The contribution of different fatty acids to biomarkers of lipid peroxidation

The susceptibility of fatty acids to oxidation is thought to be dependent on their degree of unsaturation [46, 47]. However, little is known about the contribution of individual fatty acids to each of the different biomarkers of lipid peroxidation [233]. Some biomarkers are formed specifically from individual fatty acids and the formation of F_2-isoprostanes from arachidonic acid is one such example. When micelles of different fatty acids were oxidised *in vitro* it was shown that individual fatty acids yield different profiles of oxidation markers [233]. Studies concerning the contribution of different fatty acids to the formation of MDA is another example regarding the discussion about fatty acid substrates for different biomarkers. It has been suggested that MDA is formed mainly from fatty acids containing three or more double bonds [104, 105]. When various PUFAs were oxidised completely *in vitro*, the yields of free MDA on a molar basis were 0.5% of linoleic acid, 4.5% of α-linolenic acid, 4.9% of γ-linolenic acid, 4.7% of

arachidonic acid and 4.6% of docosahexaenoic acid [105]. The very low yield of MDA from linoleic acid agrees with the proposal that MDA is mainly formed from PUFAs with three or more double bonds [104]. The findings regarding the influence of different fatty acid substrates on the response in different biomarkers of lipid peroxidation is another argument for the use of more than one biomarker of lipid peroxidation in studies of lipid peroxidation *in vivo*.

Dietary fat quality and LDL oxidation

The fatty acid composition of the diet largely determines the fatty acid composition of plasma lipoproteins, which influences the rate and extent of LDL peroxidation [208]. Whereas SAFA- and MUFA-rich LDL particles are fairly resistant to oxidation, PUFA-rich LDL particles are more susceptible to lipid peroxidation *in vitro* [234-236]. However, only a few studies have investigated effects of dietary fat quality on biomarkers of *in vivo* lipid peroxidation instead of or in addition to *in vitro* oxidation of LDL particles, and an unanswered question is whether oxidation of LDL *in vitro* reflects relevant *in vivo* lipid peroxidation processes.

Does an increased consumption of rapeseed oil-based products lead to increased lipid peroxidation in the body?

Over the past two decades there has been an expansion of the production of low-erucic acid rapeseed oil (canola oil). Rapeseed oil is rich in unsaturated fatty acids and has been shown to have beneficial nutritional effects [204-207, 212-214], but little is known about the risk for an increased lipid peroxidation. From a public health perspective it is important to investigate the effects of a diet with rapeseed oil-based fats and consequently an increased intake of MUFAs and PUFAs, especially α-linolenic acid, compared with a diet composed in accordance with the reported intakes of fatty acids before rapeseed oil-based products were introduced.

Some studies have investigated effects on biomarkers of lipid peroxidation after intervention with diets containing a higher proportion of PUFA but a lower proportion MUFA than the rapeseed oil-based diet used in paper V. In these studies, increases in plasma TBARS formation [237], urinary excretion of F_2-isoprostanes [238] and urinary TBARS levels [239] were found. Intervention studies with n-3 fatty acid enriched diets have also shown increases of plasma MDA levels [240-243], which could be prevented by increasing the intake of vitamin E [240, 244].

In paper V, we compared a rapeseed oil-based diet with a control diet rich in saturated fats. There were no differences in the biomarkers of lipid peroxidation

(8-iso-$PGF_{2\alpha}$, hydroperoxides and MDA) and antioxidants (tocopherols and AOC) except for increased levels of serum γ-tocopherol after the RO diet compared to the SAT diet. The relatively narrow confidence intervals indicates a sufficient power of this study to detect clinically significant differences. However, according to the results in paper I, there are large inter-individual variations in the degree of loss or accumulation of plasma hydroperoxides after storage at –70°C. The analysis of hydroperoxides in paper V were performed on samples that had been frozen at –70°C and these results should therefore be interpreted cautiously. In conclusion, the results of the present study suggest that there is a balance between the content of antioxidants and PUFA in rapeseed oil-based fats when used in a natural mixed diet and that the amount of antioxidants naturally present is sufficient. The antioxidant content of the RO diet seems enough to increase circulating concentrations of γ-tocopherol and thus to possibly contribute to the protection of unsaturated fatty acids from oxidation.

The influence of a natural mixed rapeseed oil-based diet on lipid peroxidation has also been investigated in a study by Turpeinen et al [245]. There was considerable disparity between *in vitro* and *in vivo* indicators of lipid peroxidation. Plasma levels of MDA and conjugated dienes and the amount of TBARS and hydroperoxides in LDL were unchanged or slightly decreased, while the susceptibility of LDL to oxidation *in vitro* (lag time and time to maximum oxidation) was increased. Our findings in paper V are line with the *in vivo* findings of Turpeinen et al [245].

The composition of the test diets in paper V

In paper V, the dietary intake was monitored by weighed dietary records. The two test diets differed essentially in the E% of α-linolenic acid and in the amount of SAFA, MUFA and PUFA. The fat content of both test diets were in accordance with the reported intakes from a Swedish population study performed a decade ago i.e. before rapeseed oil products were as widely used as today [215]. The RO diet contained 1.8 E% of α-linolenic acid, which is similar to reported contents of α-linolenic acid in earlier studies investigating the effects of rapeseed oil [207, 246]. The proportions of SAFA, MUFA and PUFA in the RO diet were generally in agreement with today's dietary recommendations [210], but with a slightly higher intake of MUFAs due to the higher fat intake of about 36 E% compared to 30 E% as recommended. The content of α-linolenic acid in the SAT diet was 0.4 E%, which was in agreement with or somewhat lower than reported intakes from a recent Swedish population study [247]. The fatty acid composition of the SAT diet was similar to the intake of SAFA, MUFA and PUFA reported in the Swedish population study from a decade ago [215].

Beneficial effects of a rapeseed oil-based diet

In paper V, the estimated intake of vitamin E from the dietary records revealed a greater intake of vitamin E expressed as α-tocopherol equivalents (α-TE) during the RO diet compared to the SAT diet (15.3 and 11.5 mg α-TE per day). These data correspond to 13.9 and 11.0 mg α-tocopherol and approximately 14.1 and 5.6 mg γ-tocopherol per day during the RO and SAT diets, respectively. The γ-tocopherol content was approximated from the difference of the intake expressed as α-TE and α-tocopherol, with the assumption that β- and δ-tocopherols do not contribute significantly to the amount of α-TE. A conversion factor of 0.1 was used to calculated the approximated content of γ-tocopherol from this difference. The level of γ-tocopherol analysed in serum was higher after the RO diet compared to the SAT diet, whereas the serum α-tocopherol levels did not differ between the two diets. This leads to a lower ratio of α- to γ-tocopherol after the RO diet compared to the SAT diet. A low γ-tocopherol concentration and a high α- to γ-tocopherol ratio have been reported in patients with CHD [248, 249] and in a population with a high incidence of CHD [250]. These results suggest that a serum tocopherol profile with low γ-tocopherol levels and a high α- to γ-tocopherol ratio may be an indicator of an increased risk for CHD, and that a rapeseed oil-rich diet may be beneficial in helping to increase the levels of γ-tocopherol in the body. Further, the final results of the Lyon Diet Heart Study were recently reported and indicates that a Mediterranean diet rich in α-linolenic acid is beneficial in secondary prevention of CHD [251]. Surprisingly, data from the same study also suggest that the Mediterranean diet protects against CHD through mechanisms independent of traditional CHD risk factors such as blood lipid profile, blood pressure and smoking. The content of antioxidants and α-linolenic acid of several plant foods in the Mediterranean diet have been suggested to be critical mediators of the beneficial effects of this diet [251, 252]. These data further support possible positive effects of rapeseed oil with its high content of vitamin E and α-linolenic acid in addition to documented positive effects on the blood lipid profile [204-207, 212-214].

Future perspectives

The increased interest in the role of free radicals in the pathogenesis of a number of human diseases has led to an increased need for techniques to measure free radicals and their reactions *in vivo*. Oxidative injury can be assessed by the measurement of products of lipid peroxidation, DNA oxidation and protein

oxidation, and by measurement of depletion of antioxidants as a complementary approach. However, there are much to be learned about what steady-state levels and increased levels of these biomarkers mean and how they should be interpreted in relation to oxidative injury and the onset and progression of acute and chronic disease processes. Regarding methods for measurement of lipid peroxidation, there are many arguments that lead us to conclude that no single method is adequate by itself as an accurate indicator of lipid peroxidation *in vivo*, and that choosing a combination of suitable methods to try to answer a specific question raised may be a better way to study lipid peroxidation.

Better techniques for the assessment of free radical damage will also help us to understand more about antioxidants and to evaluate the potential role of antioxidants in the prevention of disease in future studies. The "antioxidant paradox" may represent an example of confusion due to inconsistent results. Epidemiological studies suggest a protective role of antioxidants, whereas intervention studies have not been able to support this finding. The discrepancies between the results from epidemiological and intervention studies may be explained by many factors. One factor might be the use of only disease outcome as an end-point or just one single method to evaluate the effects of antioxidants.

Today we do not know enough to answer if there is a causal relationship between plasma or tissue levels of antioxidants and chronic disease. Until more knowledge is gathered, we need to continue focus on recommending an increased intake of antioxidants from fruits and vegetables and other dietary sources. Few intervention trials have studied the antioxidant effects of diets rather than vitamin supplements. Findings from a recent trial that altered fruit, vegetable and fat intake in healthy adults support the hypothesis that changing dietary patterns may decrease the risk of atherosclerosis by favourably altering the balance between oxidant defence and damage [253]. In addition, interventions with e.g. Brussels sprouts [254] and tomatoes [255] have been shown to reduce oxidative DNA damage. The use of biomarkers of oxidative damage has also been proposed as a way of identifying which diets and other lifestyle factors could minimise oxidative damage and thus putatively delay the onset of chronic diseases.

Improved methods will help us in understanding the underlying mechanisms of free radical damage in the development of diseases. Recent research implicates that free radicals and antioxidants may be important in signal transduction and gene expression [256]. There are also possible genetic aspects in the way individuals may handle oxidative stress depending on differences in the levels of endogenous antioxidants, rates of repair and uptake and processing of antioxidants from dietary sources.

CONCLUSIONS

- The FOX2 assay is a useful tool for the measurement of total hydroperoxides in fresh plasma samples but not in stored plasma samples. Analysis of fresh plasma samples avoids the problem with large inter-individual variations in the change of hydroperoxides after storage. Little is known about the stability of individual classes of hydroperoxides during storage and further studies are required (**paper I**).

- A methodology based on alkaline hydrolysis of esterified F_2-isoprostanes and a specific radioimmunoassay for the quantification of total amounts of 8-iso-$PGF_{2\alpha}$ was successfully applied on rat liver tissue. The methodology for measurement of total levels of 8-iso-$PGF_{2\alpha}$ in tissues may be suitable for future investigations of the location of oxidative injury in the body and possibly also the susceptibility of different tissues to oxidative stress (**paper II**).

- Dietary supplementation with vitamin E was shown to decrease the levels of both 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ at basal conditions and after CCl_4-induced oxidative injury in rats. Thus, vitamin E supplementation may have an effect on both free radical-induced oxidative injury and cyclooxygenase-catalysed prostaglandin formation. The simultaneous measurement of 8-iso-$PGF_{2\alpha}$ and 15-K-DH-$PGF_{2\alpha}$ is a promising approach for studies investigating the possible roles of lipid peroxidation under normal conditions and in the pathology of various human diseases (**paper III and IV**).

- A rapeseed oil-rich diet does not seem to increase the degree of lipid peroxidation in the body compared to a diet rich in saturated fats. This is possibly due to a sufficient content of antioxidants in the rapeseed oil diet to increase circulating concentrations of antioxidants that may protect unsaturated fatty acids from oxidation (**paper V**).

- The results of this thesis support the proposal that selection of a combination of methods for simultaneous measurement of several biomarkers of both primary and secondary lipid peroxidation products as well as antioxidant levels is a promising approach for future studies investigating the possible role of lipid peroxidation *in vivo* under basal conditions and in the pathology of disease.

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