Measurement and Evaluation of Antioxidant Status and Relation to Oxidative Stress in Humans

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Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 131
Dissertation presented at Uppsala University to be publicly examined in Sal IX, Universitetshuset, Uppsala, Wednesday, April 26, 2006 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Numerous diseases are associated with reduced antioxidant defence and oxidative stress. The antioxidant defence includes dietary and endogenous antioxidants and involves complex interactions between them. The effects of dietary factors on antioxidant status and oxidative stress of healthy humans were investigated in the studies described in this thesis. Assays of plasma antioxidant capacity encompass interactions between various antioxidants. Although uric acid has an unclear function as an antioxidant, it is a major determinant of antioxidant capacity. We measured antioxidant capacity in the presence and absence of uric acid to provide more information on the application of measures of antioxidant capacity. Individuals with high dietary intakes of various antioxidants and antioxidant rich foods, especially when combined, had higher plasma antioxidant capacities than those with lower antioxidant intakes. However, there were no associations between dietary intake of antioxidants or antioxidant rich foods and the plasma concentration of F2-isoprostanes, which is considered a reliable biomarker for oxidative stress. Intakes of various doses of a mixture of bilberry juice and black tea, rich in flavonoids for four weeks, increased antioxidant capacity in some groups, but urine levels of F2-isoprostanes were not affected. There were substantial individual variations in responses to the drinks related to baseline antioxidant capacity. Supplementation with eicosapentaenoic acid and docosahexaenoic acid decreased the plasma levels of F2-isoprostanes, but not prostaglandin F2α formation or antioxidant capacity.

It was concluded that a high intake of foods rich in antioxidants is related to improved antioxidant status. After intake of foods rich in antioxidants, the antioxidant status may increase, but with considerable individual variation in the responses, which warrants further investigation. Lipid peroxidation in vivo is not easily affected by dietary antioxidants in healthy humans. Although n-3 fatty acids are highly unsaturated, they reduce nonenzymatic free radical-catalyzed lipid peroxidation, but not enzymatic lipid peroxidation.

Keywords: antioxidant, antioxidant status, antioxidant capacity, oxidative stress, lipid peroxidation, F2-isoprostanes, dietary factors, vitamin E, n-3 fatty acids, human

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ISSN 1651-6206
ISBN 91-554-6519-6
urn:nbn:se:uu:diva-6742 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-6742)
To William
List of Papers

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


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<th>Description</th>
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<tbody>
<tr>
<td>AOC</td>
<td>antioxidant capacity</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>GAE</td>
<td>gallic acid equivalents</td>
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<tr>
<td>HB/LT</td>
<td>high bilberry and low tea</td>
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<tr>
<td>HB/HT</td>
<td>high bilberry and high tea</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>LB/LT</td>
<td>low bilberry and low tea</td>
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<tr>
<td>LB/HT</td>
<td>low bilberry and high tea</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MB/MT</td>
<td>medium bilberry and medium tea</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidized LDL</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>prostaglandin F$_{2\alpha}$</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>SAFA</td>
<td>saturated fatty acid</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox equivalent antioxidant capacity</td>
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</table>
Introduction

Humans with high intakes of fruit and vegetables have reduced risks of several diseases such as cardiovascular diseases and certain forms of cancers (1-5). Numerous diseases are associated with reduced antioxidant defence and increased oxidative stress (6, 7). Antioxidants are assumed to constitute the protective agents in fruit and vegetables as they inhibit reactions with reactive free radicals and other reactive species that otherwise might lead to damage in the body. However, clinical intervention studies using large doses of antioxidants do not support this assumption. This disparity may be caused by complex interactions between various types of antioxidants in the defence against oxidative stress. The antioxidant defence system is complex and includes both antioxidants from the diet and antioxidants formed in the body, each of which has a specific mechanism of action.

Antioxidants

Antioxidants are defined as (8):

any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate

An oxidizable substrate might be a lipid, DNA, protein or any molecule found in vivo.

The body is endowed with complex antioxidant systems, which include exogenous antioxidants derived from the diet and endogenous antioxidants formed in the body. The mechanisms by which various antioxidants protect against oxidative stress differ and antioxidants can be divided into three main groups (9):

- Antioxidant enzymes - catalyse the breakdown of free radical species
- Chain breaking antioxidants - small molecules such as vitamin C and vitamin E that can donate or receive an electron to stop free radicals to generate more radicals in a chain reaction that otherwise
is the situation when a free radical reacts with another molecule, as in the lipid peroxidation process

- Metal binding proteins – prevent metal ions to form free radicals

The mechanism of antioxidant defence also varies between different cells and tissues, and between extracellular and intracellular compartments (10). There is probably no single “superior” antioxidant: antioxidants differ in the type of free radical or reactive species targeted and in the cellular environment to which they are best suited.

Dietary antioxidants

Vitamin C
Vitamin C is a powerful water-soluble, chain-breaking antioxidant that has several antioxidant effects including the ability to donate electrons to most water-soluble radicals and oxidants such as superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals, and singlet oxygen (11, 12). Ascorbic acid is also able to regenerate \( \alpha \)-tocopherol at the aqueous-lipid interface (13). Ascorbic acid is probably enzymatically regenerated.

Vitamin E
There are eight forms of Vitamin E: \( \alpha \)-, \( \beta \)-, \( \gamma \)- and \( \delta \)-tocopherols and \( \alpha \)-, \( \beta \)-, \( \gamma \)- and \( \delta \)-tocotrienols (10). All forms have antioxidant functions, but \( \alpha \)-tocopherol is suggested to be the most potent lipid-soluble chain-breaking antioxidant within cell membranes and lipoproteins (14).

Carotenoids
Over 600 carotenoids have been described (10). This large group comprising coloured pigments in plants includes carotenoids such as \( \beta \)-carotene, lycopene, lutein and \( \alpha \)-carotene (10). \( \beta \)-carotene is considered as one of the most important carotenoids present in lipoproteins and membranes. These lipid-soluble antioxidants are effective scavengers of singlet oxygen, but also of peroxyl radical at low oxygen pressure (15).

Flavonoids
Occurring naturally in foods and beverages from plant sources such as fruits, vegetables, berries, tea, and wine, flavonoids are polyphenolic compounds that provide an important dietary source of antioxidants (16-18). Flavonoids have the chemical structure C6-C3-C6, which includes two benzene rings linked by three carbons (19). Flavonoids can be classified according to the variations in the C3-ring as flavonols, flavones, flavanols (or catechins), flavanones, anthocyanins, and isoflavonoids. More than 6000 different fla-
vonoids have been identified, having properties related to their chemical structures (20).

Tea is rich in catechins, and contains the flavonols quercetin, kaempferol, and myricitin (21). Catechins are oxidized during the processing of black tea, which involves fermentation of tea leaves and forms fermentation products such as thearubigens and theaflavins. Blueberries have attracted interest because of their high anthocyanin content (17), which is reflected in their high antioxidant capacity (22). Lowbush “wild” blueberries, bilberries (Vaccinium myrtillus) have been reported to have a higher anthocyanin content and antioxidant capacity than cultivated highbush blueberry (22).

Endogenous antioxidants
Antioxidants synthesized in the body include antioxidant enzymes and metal-binding proteins. Cells contain several antioxidant enzymes, the three major classes of which are superoxide dismutase, glutathione (GSH) peroxidase and catalase. There is also a number of specialized antioxidant enzymes (23). Because glutathione peroxidase contains selenium at its active site, severe selenium deficiency can result in deficiency of this enzyme (24).

Metal binding proteins include proteins such as iron-binding proteins transferrin and lactoferrin, and the copper-binding protein caeruloplasmin that bind free transition metal ions that otherwise participate in the formation of free radicals (10).

Uric acid
In humans, uric acid is an end-product of purine metabolism. Mammals other than humans and higher primates produce little or no uric acid since they synthesize uricase, the enzyme which catalyses the oxidation of uric acid to allantoin (25). Plasma levels of uric acid have increased during human evolution. This may be a consequence of a gene mutation in which loss of the ability to synthesize ascorbate synthetase coincided with loss of the ability to synthesize uricase, in which case uric acid levels could have increased to compensate for some of the antioxidant functions of ascorbic acid (26). Some studies have shown that uric acid has antioxidant properties, as it scavenges peroxyl and hydroxyl radicals, and binds metal ions which otherwise catalyse free radical reactions (26-28). In addition, administration of uric acid during high-intensity exercise, as a model of oxidative stress, reduces the levels of F2-isoprostanes (29). Recently, Hayden and Tyagi suggested that uric acid functions as an antioxidant when present at normal concentrations, but functions as a pro-oxidant at higher concentrations and accelerates atherosclerosis (30). A J-shaped curve between plasma levels of uric acid and all-cause mortality in haemodialysis patients has been reported, which may indicate that antioxidant capacities in these cases are low (31).
Furthermore, uric acid is shown to become a pro-oxidant *in vitro*, when other antioxidants are reduced and it has been suggested that the pro-oxidant potential of uric acid is high compared to that of other antioxidants (32). Several, but not all, epidemiological studies have reported an association between hyperuricemia and cardiovascular diseases (33), and increased levels of uric acid are also associated with diseases such as gout (33) and renal disease (32). Furthermore, uric acid is increased directly after physical activity (29), in obesity (33) and with age (34).

**Albumin**

Albumin may be an important antioxidant since it contains SH (sulphydryl)-groups that can react with hydrogen peroxide and peroxyl radicals (10, 35).

**Bilirubin**

Bilirubin has been shown to scavenge peroxyl radicals at least *in vitro* (10). Bilirubin can protect albumin-bound fatty acids against oxidative damage.

**Interactions between antioxidants**

That most randomized controlled trials have failed to demonstrate protective effects of supplementation of antioxidants, often in high doses, may indicate that the antioxidant defence system requires a perfect balance for optimal function. There may be a need for a combined action of a number of antioxidants or it may be that less well-known antioxidants are more important. Generally, dietary plants often contain several hundred types of antioxidants (36), a minor proportion of which is comprised of well-known antioxidants.

Results from *in vitro* studies have demonstrated interactions between \( \alpha \)-tocopherol and ascorbic acid (37-41), and between \( \alpha \)-tocopherol and flavonoids (42). The tocopheroxyl radical, which is formed as a result of the antioxidant action, is re-converted to \( \alpha \)-tocopherol by acceptance of a hydrogen ion from ascorbic acid or flavonoids. In animal studies, a high intake of vitamin C has been reported to increase tissue vitamin E concentration in some (43, 44), but not in all studies (45, 46). However, interactions between antioxidants in humans are more unclear. An increased intake of vitamin C has been shown to improve vitamin E concentrations in plasma supporting an *in vivo* interaction between vitamin C and E (47), but supplementation with vitamin C and vitamin E alone or in combination reduced lipid peroxidation to a similar extent (48).
Antioxidant capacity

Measurement of antioxidant capacity

The failure of randomized controlled trials to demonstrate protective effects of antioxidants may indicate that interactions between antioxidants, and the balance between them, are of critical importance. In an approach to understand this area better, measurement of antioxidant capacity could provide information. By measuring the antioxidant capacity, an integrated picture of the antioxidant defence may be achieved, taking known as well as unknown antioxidants into account and interactions between various antioxidants. Moreover, it obviates the need to measure the concentrations of individual antioxidants. Several antioxidants such as tocopherols, ascorbic acid, carotenoids, uric acid, bilirubin, and sulfhydryl groups contribute to plasma and serum antioxidant capacity (49), but unknown antioxidants certainly also make a significant contribution (50).

Several methods have been developed during last decade to measure the antioxidant capacity of plasma, serum, and of other biological samples and in foods (51-56). Most of these methods measure the extent to which free radical generation is inhibited by antioxidants present in the sample. The methods are based on different technologies using different free radical generators, target molecules and endpoints. Therefore it is possible that the response of various antioxidants will depend on the particular assay used. Antioxidant capacity assays can provide information on absorption and bioavailability of dietary antioxidants in intervention studies, which could be especially important if an assay for a specific compound with antioxidant properties is not available. Antioxidant capacity is suggested to be part of a tightly regulated homeostatic mechanism, as stable antioxidant capacities have been measured over long time periods using various assays (50). The relative advantages and disadvantages of the various antioxidant capacity assays vary according to the situation and the type of study. However, opinions regarding various antioxidant capacity assays vary considerably, probably depending on the complexity of antioxidants. Recently, it was proposed that the procedures and applications of the ORAC assay, and possibly those of the TEAC assay, should be standardised (56). In most of the antioxidant capacity assays, uric acid is a major contributor, with clear correlations between antioxidant capacity and the uric acid content (49, 50, 57) but the function of uric acid as an antioxidant is unclear. Sulphydryl groups found on proteins such as albumin also contribute to the antioxidant capacity (50). Ascorbic acid, tocopherols and carotenoids are only modest contributors to plasma antioxidant capacity (57, 58).
Furthermore, these assays can provide data about the antioxidant capacities of different compounds. Both ORAC and TEAC values of many compounds and food items are reported (36, 59).

In this thesis, antioxidant capacity was measured using a chemiluminescence antioxidant capacity (AOC) assay, the widely used oxygen radical absorbance capacity (ORAC) assay, and the Trolox equivalent antioxidant capacity (TEAC) assay.

The chemiluminescence antioxidant capacity (AOC) assay
This chemiluminescence assay is considered to be sensitive because of its ability to distinguish between efficient and less efficient antioxidants (60). It has also been argued that this assay may be unrepresentative of oxidative stress *in vivo* (52). Results derived using this assay correlate with those derived using the total radical trapping antioxidant parameter (TRAP) assay (52), one of the first antioxidant capacity assays. The AOC assay is described in further detail in the Methods section.

The oxygen radical absorbance capacity (ORAC) assay
The ORAC assay is considered to have a high specificity using a physiological important radical. (50). The use of various extraction techniques in the ORAC assay enables separate estimates of aqueous and lipid-soluble antioxidant capacities to be made. In addition, different sources of radicals can be used (61). An advantage of the ORAC assay is that it combines both time and degree of inhibition of radical generation because it takes the oxidation reaction to completion and uses the area under the curve to quantify the antioxidant capacity (53). ORAC estimates are significantly affected by protein concentrations, which may result in underestimation of other antioxidants (62). This method can be used to assess antioxidant capacities of samples in which proteins are present or absent.

The Trolox equivalent antioxidant capacity (TEAC) assay
The TEAC assay uses only the degree of free-radical inhibition at a fixed time to determine antioxidant capacity and does not take the duration of inhibition into account, which may result in underestimation of antioxidant capacity (54). This assay has been criticised because a non-physiological radical is used and because of dilution effects (50). However, commercial TEAC assay kits are available and the assay is relatively fast.

Several other assays such as the earlier mentioned TRAP assay, the ferric reducing ability (FRAP) assay and the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay have been used for determination of antioxidant capacity (56).
The TRAP assay, one of the first methods to be developed, was widely used in the past, but is complex and time-consuming (57). However, variations of the TRAP assay have been used (63). The ORAC assay is based on chemical principles similar to those of the TRAP assay (56). The FRAP assay measures the ability of antioxidants to reduce Fe$^{3+}$ to Fe$^{2+}$ (55). The principle of this assay differs from that of other antioxidant capacity assays because neither free radicals nor oxidants are used.

Plasma antioxidant capacity and dietary intake

A number of studies have assessed the effect of various antioxidant rich foods on antioxidant capacity, with conflicting findings (64-74). Immediate effects on antioxidant capacity after intake of black tea (65) and chocolate (75) have been observed. Other studies have reported no effects or mild immediate effects on antioxidant capacity after intake of black tea (67-69). Generally, an increase in antioxidant capacity is observed during the first or the second hour after intake, and subsequently a decrease in antioxidant capacity is observed four to five hours after intake (76).

Increased fasting plasma antioxidant capacities after two to eight weeks’ daily consumption of diets high in fruit and vegetables have been reported (70, 71, 74), but other studies failed to demonstrate effects of daily intakes of black tea (72), a high intakes of fruit and vegetables (77, 78) or intake of vegetable juice (79) on antioxidant capacity.

Plasma antioxidant capacity and diseases

Plasma antioxidant capacity is decreased in various clinical conditions associated with oxidative stress, such as in coronary heart disease (80), type 1 and type 2 diabetes (81-84) and is associated with diabetic complications (85).

Oxidative stress

Oxidative stress is a condition resulting from an imbalance between the production of free radicals or reactive oxygen and nitrogen species and antioxidant defence systems in which oxidation predominates (86). Each orbital of an atom usually contains a pair of electrons; free radicals are highly reactive because they contain one or more unpaired electrons and is capable of independent (“free”) existence (10). Examples of free radicals are the hydroxyl radical (OH$^\cdot$), the peroxyl radical (ROO$^\cdot$) and the superoxide anion (O$_2^\cdot$$^-\cdot$), where $^\cdot$ denotes one or more unpaired electrons. Oxidative stress might cause damage to lipids, protein and DNA, which may increase the risk of development of several diseases (86).
Free radicals and other reactive species are continuously produced in the body and have important functions for the immune system, as our white blood cells produce oxidants such as hydrogen peroxide and superoxide to kill bacteria.

Measurement of oxidative stress

Oxidative stress can be assessed by measuring lipid peroxidation in the body. Of the reactions relevant to oxidative stress, lipid peroxidation has been most frequently investigated (87). The lipid peroxidation process is initiated by a free radical attack on a polyunsaturated fatty acid. A lipid radical is formed that reacts with oxygen, leading to formation of a peroxyl radical that may further react with other lipids and produce a new lipid radical. Thereby the reaction leads to that a propagation reaction starts and is maintained, until a termination reaction occurs including for example a chain-breaking antioxidant (88). As lipid peroxidation is a complex process that occurs in several stages, it is difficult to estimate the overall lipid peroxidation process. Measurement of F2-isoprostanes (8-iso-PGF$_2\alpha$) seems to be the best method for measurement of lipid peroxidation in vivo as recent shown in a multilaboratory validity study organised at the NIH (89, 90).

Nonenzymatic lipid peroxidation in vivo

Isoprostanes, prostaglandin-like compounds that are formed in the body by free radical-mediated oxidation of arachidonic acid, were discovered in 1990 (91). One of the major F2-isoprostanes, 8-iso-prostaglandin F$_2\alpha$ (8-iso-PGF$_2\alpha$), is now considered as a golden biomarker of nonenzymatic lipid peroxidation in vivo (7, 91). Concentrations of F2-isoprostanes are elevated in clinical conditions associated with oxidative stress such as type 2 diabetes (92-96), hypercholesterolaemia (97-99), hyperhomocysteinaemia (100), rheumatic diseases (101), heart failure (102), ischaemia-reperfusion (103), atherosclerotic lesions (104, 105), and in smokers (106, 107).

Isoprostanes are formed in situ from esterified arachidonate in tissue phospholipids and are released by phospholipases in the free form into the plasma. They are then filtered from the plasma by the kidneys and excreted in the urine (108). Isoprostanes affect the integrity and fluidity of membranes, causing oxidative stress in tissues (7). Isoprostanes also act as vasoconstrictors in organs such as the kidney (91) and lung (109, 110).

The primary rate-limiting factor for formation of isoprostanes appears to be molecular oxygen and the availability of free radicals (111). F2-isoprostanes are continuously produced in a variety of tissues and are measurable in several biological fluids including plasma and urine (7). The levels of F2-isoprostanes in plasma and urine are correlated but there is a time lag between the appearance of F2-isoprostanes in plasma and urine (112), and
the levels of F2-isoprostanes in healthy subjects is about 30-40 times higher in urine (113). In addition, they are chemically stable and specific products of the free radical-mediated lipid peroxidation (7). Thus, the level of F2-isoprostanes reflects the peroxidation of arachidonic acid, but it is presumed that other lipids are also oxidised (88).

Techniques such as GC-MS (gas chromatography mass spectrometry), radioimmunoassay, and enzyme immunoassay have been used to measure concentrations of F2-isoprostanes in plasma, urine and tissues (7). A radioimmunoassay with a specific antibody was used in the studies described in this thesis. This method is considered to be sensitive and suitable for large numbers of samples (113). This radioimmunoassay was validated using GC-MS methods in an international study in which oxidative was induced by administration of CCl4 to rats (89, 90, 113).

Enzymatic lipid peroxidation in vivo

Lipid peroxidation can also be catalysed enzymatically, an important physiological process. Arachidonic acid is converted through the cyclooxygenase pathway to bioactive prostaglandins and thromboxanes (114). Prostaglandin F2α (PGF2α) controls several important physiological functions in the body (115) and is also involved in acute and chronic inflammation in various species (92, 116-119). Elevated levels of prostaglandin F2α have been observed in patients with type 1 and type 2 diabetes (92, 120) and rheumatic diseases (101), and in smokers (107). Since PGF2α has a very short half-life in vivo, a major metabolite of prostaglandin F2α, 15-keto-dihydro-PGF2α, that has a longer half-life and a higher concentration than that of PGF2α, is used as a biomarker of enzymatic cyclooxygenase-mediated lipid peroxidation in vivo and is detected in plasma and urine using radioimmunoassay (115, 121). 15-keto-dihydro-PGF2α has been shown to be a reliable indicator of cyclooxygenase-mediated inflammation in vivo (92, 116-119, 121). It has been suggested that F2-isoprostanes mediate the link between oxidative stress and inflammation (122).
Fatty acids and oxidative stress

The supply of antioxidants in dietary fat is related to the fatty acid composition. Unsaturated fatty acids are rich in antioxidants, but if they are highly unsaturated they are easily oxidized, which may deplete the antioxidants. The hydrogen atoms close to the double bonds in fatty acids have a weak energy of attachment and therefore easily react with free radicals (10). However, highly unsaturated fatty acids such as EPA and DHA have several beneficial health effects that affect risk factors associated with cardiovascular disease by reducing triglyceride levels (123), the risk of arrhythmia and thrombosis (124), and inflammation and by improving endothelial function (125). Supplementation with n-3 fatty acids also reduces cardiovascular mortality in coronary heart disease patients (33).

Antioxidants in health and disease

Epidemiological studies

Several epidemiological studies have shown that a high dietary intake of antioxidants is associated with a reduced risk of cardiovascular disease (2-5, 126, 127), when the well-known antioxidants such as vitamin E, vitamin C and β-carotene were studied, but also flavonoids and other antioxidants have been investigated. Epidemiological evidence for antioxidant effects is strongest for vitamin E, moderate for β-carotene and weaker for vitamin C (128). In general, humans with high intakes of vitamin E and β-carotene have 30-40 % less risk of developing cardiovascular disease than those with low intakes (128).

Experimental studies

Oxidation of low-density lipoprotein (LDL) by free radicals or other reactive species in the vascular wall is considered to be involved in the development of coronary heart disease (129). Oxidized LDL is taken up by scavenger receptors on macrophages because the LDL receptors do not recognize modified forms of LDL. Macrophages are converted to foam cells, which results in fatty streaks in the vascular wall and contribute to development of atherosclerosis. Vitamin E is transported in the lipoproteins and together with β-carotene protects the fatty acids in LDLs from oxidation. Vitamin C on the other hand is a water soluble antioxidant with the ability to regenerate vitamin E after oxidation.
Randomized controlled trials

The protective effects of antioxidants demonstrated by experimental and epidemiological studies have not been confirmed by the majority of randomized controlled intervention trials. To date, meta-analyses of randomized controlled trials involving antioxidant supplements have not reported protective effects in primary or secondary prevention of coronary heart disease for any single antioxidant or combination of antioxidants (130-133). However, in the CHAOS study (Cambridge heart antioxidant study), in which coronary heart disease patients received 400 IU/d vitamin E, 800 IU/d vitamin E or placebo for two years, the incidence of cardiovascular death and non-fatal myocardial infarction was significantly decreased by vitamin E supplementation. A meta-analysis of the effects of vitamin E (50-800 mg/day) involving five large-scale trials (CHAOS, ATBC, GISSI, HOPE and PPP) and 29 000 patients found that there was no benefit from vitamin E supplementation in secondary prevention of coronary heart disease or the risk of cardiovascular events (128). In the CARET study (the beta-carotene and retinol efficacy trial), intake of \(\beta\)-carotene (30 mg/day) and vitamin A for four years by smokers and asbestos workers increased the incidence of cardiovascular disease and lung cancer, but in the meta-analysis, which included the CARET study and two other large-scale trials (ATBC and PHS), which were conducted on 70 000 healthy participants, no benefit or hazard was found to be associated with supplementation of 20-50 mg/day \(\beta\)-carotene (128).

Another meta-analysis (134), which included all causes of death, concluded that mortality rates are increased by supplementation of vitamin E at levels in excess of 400 IU/d and was slightly but nonsignificantly decreased by vitamin E dosages of less than 150 IU/d. When data for high and low doses of vitamin E were modelled together, vitamin E supplementation did not affect all-cause mortality. In most randomized controlled trials, the supplementation doses are high, which might have resulted in pro-oxidant effects.
Aims

The overall aim of this thesis was to contribute a piece of the puzzle in the complex antioxidant-oxidation area, by investigating how antioxidant status and oxidative stress are affected by dietary factors in healthy humans, and by studying various contributors to the antioxidant defence.

The specific aims were:

- to investigate plasma antioxidant capacity by a chemiluminescence assay and the importance of uric acid for antioxidant capacity by measuring antioxidant capacity with and without uric acid (Paper I)
- to study the correlations between plasma antioxidant capacity and various clinical variables to provide more knowledge on how the concept of antioxidant capacity could be applied (Paper I)
- to determine how dietary antioxidants (ascorbic acid, tocopherols and β-carotene) and foods rich in antioxidants affect plasma antioxidant capacity and nonenzymatic free radical-mediated lipid peroxidation in vivo (F₂-isoprostanes) (Paper II)
- to study the immediate and long-term (four weeks) effects of various doses and combinations of a drink naturally rich in flavonoids, consisting of bilberry and black tea, on plasma antioxidant capacity, serum tocopherols and F₂-isoprostanes (Paper III)
- to investigate whether supplementation with highly unsaturated EPA and DHA, with a controlled fat quality in the background diet, affects nonenzymatic lipid peroxidation (F₂-isoprostanes), enzymatic lipid peroxidation (measured as a metabolite of prostaglandin F₂α), plasma antioxidant capacity, and serum levels of tocopherols (Paper IV)
Subjects & methods

Study participants

Paper I and II
A total of 86 apparently healthy males with a mean age of 59.8 years (range 41.4-69.2 years) were recruited from an ongoing health survey. None of the subjects were taking dietary supplements and 19 were smokers.

Paper III
Sixty healthy subjects, 30 men and 30 women, who were aged 38-65 years and with moderately increased body weight, participated in the study. Subjects were mainly recruited by newspaper advertisement. Four subjects were smokers and five used snuff. The subjects were instructed to avoid nutritional supplements from two weeks before, and during, the study. They were asked not to change their lifestyle, usual diet, or physical activity patterns during the study.

Paper IV
A total of 162 healthy subjects (86 men and 76 women) aged 30-65 years with normal or moderately increased body weight participated in the study. The subjects were instructed to avoid nutritional supplements from two weeks before and during the study. They were asked not to change their lifestyle or physical activity patterns during the study.
Study design

Paper III

Subjects were randomized into five groups, which each received different doses and combinations of bilberry juice and tea in drinks. The experimental drinks were prepared by mixing a commercial bilberry (*Vaccinium myrtillus*) juice concentrate with black tea powder, sugar (17 g per 400 mL drink), and tap water to provide different doses and combinations of flavonoids. All drinks were prepared and frozen before the start of the study. The estimated daily doses of flavonoids from bilberry juice and tea, respectively, were 0.1, 0.3 and 0.5 g each in the low, medium and high groups. This gave a total supplementary intake of about 0.2 g flavonoids in the low bilberry- and low tea-content drink (LB/LT); 0.6 g flavonoids in the low bilberry- and high tea-content drink (LB/HT), the medium bilberry- and medium tea-content drink (MB/MT), and the high bilberry- and low tea-content drink (HB/LT); and 1.0 g in the high bilberry- and high tea-content drink (HB/HT).

The study was of parallel design and both the immediate and long-term effects of drinking bilberry-and-tea drinks were investigated. The immediate effect was studied the first day of the study after an overnight fast. The subjects consumed a single 400-mL bilberry-and-tea drink, and blood samples were collected before, and 60, 120, and 240 min after, intake. The long-term effects were investigated in the same subjects after they consumed 400 mL/day of a bilberry-and-tea drink for four weeks. Subjects were instructed to consume 200 mL of the drink with breakfast and 200 mL in the evening every day for four weeks except for the first day when the immediate effect was studied. Fasting blood samples were obtained before and at the end of the study. The LB/LT group was defined as the reference group, with which all other groups were compared, but differences in effect between all groups were also compared.

Paper IV

The KANWU study was a multicenter study involving Kuopio (Finland), Aarhus (Denmark), Naples (Italy), Wollongong (Australia) and Uppsala (Sweden). The participants were randomly assigned to either a diet containing a high proportion of saturated fatty acids (SAFA diet) or monounsaturated fatty acids (MUFA diet) for a study period of 90 days. Within each diet group there was a second random assignment to either supplements of capsules containing fish-oil (3.6 g (n-3) fatty acids/day containing 2.4 g EPA (20:5 (n-3)) and DHA (22:6 (n-3)), corresponding to three capsules twice daily of Pikasol, Lube A/S, Denmark) or to placebo capsules with the same amount of olive oil. Four groups were thus formed: SAFA + placebo (n=42);
SAFA + (n-3) (n=41); MUFA + placebo (n=40); and MUFA + (n-3) (n=39). The same assessments as at baseline were performed again at the end of the 90-d diet period.

Biochemical analyses

Measurement of antioxidant status

Measurement of antioxidant capacity

Antioxidant capacity with an enhanced chemiluminescence assay (AOC) (Paper I, II, IV)

AOC was measured using an enhanced chemiluminescence assay (52). This technique is based on the measurement of light emission from a chemiluminescent substrate such as luminol when it is oxidized in a reaction catalyzed by horseradish peroxidase. Because light emission is suppressed by radical scavenging antioxidants, the duration of light suppression is related to the amount of antioxidant present. Light emission was measured with a luminometer (1251 Luminometer, BioOrbit, Turku, Finland), and the sensitivity was set at 4. To initiate a chemiluminescent reaction, 890 μL of plasma (diluted in millipore water, 1:445, v/v) was dispensed into a cuvette in the luminometer. Subsequently, 10 μL horseradish peroxidase solution (diluted 1:200, v/v) (Amersham International, Amersham, UK) and 100 μL signal reagent consisted of luminol, enhancer and oxidant (Amersham International, Amersham, UK) were added, and light emission was measured for four minutes. AOC of samples were quantified by comparing the duration of light suppression to that induced by the tocopherol-analogue, Trolox (Aldrich Chemie, Steinheim, Germany). Thereafter, AOC was quantified relatively and expressed as μmol Trolox equivalents per litre plasma. Fresh horseradish peroxidase- and Trolox solutions were prepared daily.

AOC in the absence of uric acid (Paper I, II, IV)

Uric acid was removed from samples using uricase (Boehringer, Mannheim, Germany), which catalyses the oxidation of uric acid to allantoin:

\[
\text{uricase} \quad \text{uric acid} + O_2 + 2H_2O \rightarrow \text{allantoin} + CO_2 + H_2O_2
\]

One unit (U) of uricase catalysed the oxidation of uric acid to allantoin at a rate of 1 μmol/min at 25 °C. The uricase solution used in our assay con-
The oxygen radical absorbance capacity (ORAC) assay (Paper III)
The ORAC assay was performed in a Wallac 1420 Victor2 96 well plate reader (EG & Wallac, Finland) equipped with a fluorescence filter, as described by Cao and coworkers (53, 64), to measure antioxidant capacity in the plasma samples. Plasma was precipitated with perchloric acid to avoid influence from plasma proteins. The values obtained from the ORAC assay were calculated as µmol or mmol Trolox equivalents per liter. To measure antioxidant capacity in the different drinks, the ORAC assay was performed using a luminescence spectrometer (LS 50 B, Perkin Elmer, Beaconsfield, UK) equipped with a thermostat-controlled plate counter.

The Trolox equivalent antioxidant capacity (TEAC) assay (Paper III)
The TEAC assay was based on the assay of Miller and coworkers (54) and was performed using a commercial kit (NX 2332, Randox Laboratories Ltd., Ardmore, Crumlin, UK) in a Monarch centrifugal analyser (Instrumentation Laboratories, Lexington, MA, USA).

Uric acid concentration (Paper I-IV)
The concentration of uric acid in plasma was measured enzymatically (Instrumentation Laboratories, Lexington, MA, USA) in a Monarch centrifugal analyser. The coefficient of variation between duplicate plasma samples has been 1.4%.

Measurement of tocopherols (Paper I, III, IV)
Serum α-, β- and γ-tocopherol concentrations were determined using HPLC with fluorescence detection (135). Because tocopherols exist in the circulation combined with lipoproteins, we adjusted the tocopherol concentration relative to lipid concentration using the sum of cholesterol and triglyceride concentrations in serum (136).

Measurement of total phenols (Paper III)
Total phenols in plasma were analysed in a HP 8542A diode-array spectrophotometer (Hewlett Packard, Waldbronn, Germany) using the Folin-Ciocalteu reagent as described by Kähkönen and coworkers (137). Total phenols were calculated as mg gallic acid equivalents per liter (mg GAE/L).
Measurement of oxidative stress

Measurement of F2-isoprostanes (Paper II, III, IV)
Urine and plasma 8-iso-prostaglandin F2α (8-iso-PGF2α) was analysed by a specific and validated radioimmunoassay developed by Basu (113). The cross-reactivity between the 8-iso-PGF2α antibody was 1.7% with 15-keto-13,14-dihydro-8-iso-PGF2α, 9.8% with 8-iso-PGF2β, 1.1% with PGF2α, 0.01% with 15-keto-PGF2α, 0.01% with 15-keto-13,14-dihydro-PGF2α, 0.1% with TXB2, 0.03% with 11β-PGF2α, 1.8% with 9β-PGF2α, and 0.6% with 8-iso-PGF3α. The detection limit of the assay was 23 pmol/L. The levels of 8-iso-PGF2α in urine were adjusted for creatinine concentration, which was measured by a colorimetric method (Instrumentation Laboratories) in a Monarch centrifugal analyser.

Measurement of malondialdehydes (MDA) (Paper III)
Plasma MDA concentration was measured by high performance liquid chromatography (HPLC) with fluorescence detection (138).

Measurement of auto-antibodies against oxidized LDL (oxLDL) (Paper IV)
An enzyme-linked immunosorbent assay was used to determine auto-antibodies to oxLDL (139). The results are expressed as the ratio of binding to oxLDL to binding to native LDL (OxLDL/native LDL) after subtracting the mean background binding to the wells.

Measurement of prostaglandin F2α metabolites (Paper IV)
Plasma 15-keto-dihydro-prostaglandin F2α (15-keto-dihydro-PGF2α) was analysed by using a validated radioimmunoassay developed by Basu (121). The cross-reactivity between the antibody was 0.02% with PGF2α, 0.43% with 15-keto-PGF2α, <0.001% with PGE2, 0.5% with 15-keto-13,14-dihydro-PGE2, 1.7% with 8-iso-15-keto-13,14-dihydro-PGF2α, <0.001% with 11β-PGF2α, <0.001% with TXB2 and <0.01% with 8-iso-PGF3α. The detection limit of the assay was 45 pmol/L.
Other biochemical and clinical measurements

The concentrations of cholesterol and triglycerides in serum and in the isolated lipoprotein fractions were determined by enzymatic colorimetric methods (Instrumentation Laboratories) in a Monarch centrifugal analyser. LDL cholesterol was calculated according to Friedewald (140). Blood glucose concentration was analysed by a glucose oxidase assay (141). The concentration of insulin in plasma was determined using an enzymatic immunological assay (Boehringer Mannheim, Germany) in an ES 300 automatic analyser (Boehringer Mannheim). Blood pressure was measured in the supine position after a 10 min rest. Body weight was measured on a digital scale with an accuracy of 0.1 kg in indoor clothing and without shoes, and height was measured without shoes to the nearest 0.5 cm. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m²).

Dietary assessments

Paper II

For each participant, fourteen 24-h recalls were collected by telephone during a time period of one year. Each participant was interviewed approximately once a month and every day of the week was covered twice in a random order. An average daily intake of foods, food groups and beverages rich in antioxidants - fruits and berries, vegetables, root vegetables, leguminous plants, tea, coffee, red wine, white wine, juices, jams and marmalades - was calculated. The database from the Swedish National Food Administration (PC-Kost 1996, SLV, Uppsala, Sweden) was used for calculation of the nutrients in the 24-h recalls. Intake of ascorbic acid, tocopherols and β-carotene was calculated as well as density of these antioxidants per 1000 kcal.

Paper III

The subjects were asked to record their intake of all foods and beverages during three consecutive days (two weekdays and one weekend day) before and during the study. The amounts were reported in household measurements in numbers or specified as portion sizes as shown in a photograph provided to the participants. The intake of antioxidant-containing foods and beverages; fruits, berries, vegetables, root vegetables, leguminous plants, tea, coffee, red wine, white wine, juices, jams and marmalades, was calculated.
Paper IV
The subjects were asked to perform weighed food records during three consecutive days (two weekdays and one weekend day) once before and twice during the study. The intake during the study was calculated as the mean values of the two 3-d food records provided during the second and third month of the study. Local nutrient analysis software programs containing country-specific food databases, and data on margarines and other specially prepared foods used in the diets were used in the analysis.

Ethics
The studies were approved by the Ethics Committee of the Faculty of Medicine at Uppsala University.

Statistics
Statistical analyses were carried out using JMP version 3.2 or SAS version 8.0 (SAS Institute, Cary, NC, USA) statistical software. The values are expressed as mean ± standard deviation (SD). A p value < 0.05 was considered significant. Variables with a skewed distribution (Shapiro Wilk’s W test < 0.95) were log-transformed before statistical analysis. When the distribution was not normal after transformation, non-parametric tests were used.

The correlation coefficients (Pearson’s or Spearman’s coefficients, as appropriate) were calculated when correlations between variables were tested.

Student’s unpaired t test or Mann-Whitney’s test was used to analyse differences in antioxidant capacity between the mean of the three highest quartiles and the lowest one (Paper II).

Student’s paired t test or Wilcoxon test was used to analyse differences within the groups (III). Further in Paper III, ANOVA or Kruskal-Wallis test was used to analyse differences over time between the groups and to compare quartiles of the data of antioxidant capacity. Student’s unpaired t test or Mann-Whitney’s test was used to analyse differences between the LB/LT group and the other groups. When comparing the immediate response of antioxidant capacity between the groups, the incremental area under the curve (IAUC) was calculated according to the trapezoidal model.

In Paper IV, for each outcome variable the treatment effects were estimated from a statistical model in which treatment categories (SAFA diet/MUFA diet and the presence/absence of n-3 fatty acids) and their interaction were analyzed. Factors and treatment center, age, sex and the baseline value of the outcome variable were covariates. For outcome variables where the interaction between the treatment factors were non-significant, a limited
model was used excluding that term. The difference in effect between the presence and absence of n-3 fatty acids is presented with p-values combined over both diet groups, where all subjects supplemented with n-3 fatty acids were compared with all subjects given the placebo. Furthermore, the difference in effect between the two diet groups when combined over the presence/absence of n-3 fatty acids groups is presented with p-values. The difference in effect within groups over time is also presented.
Results

Plasma antioxidant capacity and the contribution of uric acid (Paper I)

The mean (SD) plasma antioxidant capacity was in the 86 men in the study 487.1 (80.5) μmol Trolox equivalents/L. In the absence of uric acid, the antioxidant capacity was 104.5 (23.2) and lipid-adjusted antioxidant capacity was 14.49 (2.75) μmol Trolox equivalents/L, respectively. AOC was correlated with clinical variables such as BMI, waist circumference and abdominal sagittal diameter and concentrations of insulin and triglycerides, all of which are associated with the metabolic syndrome as shown in Table 1. After elimination of uric acid, AOC was no longer correlated to these variables associated with the metabolic syndrome, but was correlated with the concentration of lipids.

In the absence of uric acid, and when AOC was adjusted for lipid concentrations, correlations with BMI, waist circumference, abdominal sagittal diameter and insulin were similar to the unadjusted associations, but correlations with serum lipid concentrations were of opposite sign.
Table 1. Correlations of AOC, uric acid and AOC without uric acid, respectively, with clinical variables (n=86)

<table>
<thead>
<tr>
<th></th>
<th>AOC</th>
<th>Uric acid</th>
<th>AOC without uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>r</td>
<td>-0.27</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>BMI</td>
<td>r</td>
<td>0.26</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.02</td>
<td>0.0007</td>
</tr>
<tr>
<td>Waist</td>
<td>r</td>
<td>0.31</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.004</td>
<td>0.0001</td>
</tr>
<tr>
<td>Abdominal sagittal diameter</td>
<td>r</td>
<td>0.22</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.04</td>
<td>0.0006</td>
</tr>
<tr>
<td>Insulin</td>
<td>r</td>
<td>0.22</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.04</td>
<td>0.0002</td>
</tr>
<tr>
<td>Serum triglycerides</td>
<td>r</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.0003</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td>r</td>
<td>0.10</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.37</td>
<td>0.82</td>
</tr>
<tr>
<td>Serum HDL cholesterol</td>
<td>r</td>
<td>-0.19</td>
<td>-0.33</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.08</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum LDL cholesterol</td>
<td>r</td>
<td>0.05</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.66</td>
<td>0.81</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>r</td>
<td>0.18</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.09</td>
<td>0.02</td>
</tr>
</tbody>
</table>
AOC was highly significantly correlated with the concentration of uric acid in the samples ($r=0.76$, $p<0.0001$). AOC also correlated with AOC without uric acid ($r=0.50$, $p<0.0001$).

There were significant positive correlations between AOC as well as AOC without uric acid with concentration of both $\alpha$- and $\gamma$-tocopherol as shown in Table 2.

Table 2. Correlations of AOC, uric acid and AOC without uric acid, respectively, with tocopherols (n=86)

<table>
<thead>
<tr>
<th></th>
<th>AOC</th>
<th>Uric acid</th>
<th>AOC without uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-tocopherol</td>
<td>r</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.007</td>
<td>0.13</td>
</tr>
<tr>
<td>Lipid adjusted $\alpha$-tocopherol</td>
<td>r</td>
<td>0.31</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.004</td>
<td>0.15</td>
</tr>
<tr>
<td>$\gamma$-tocopherol</td>
<td>r</td>
<td>0.22</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.016</td>
<td>0.11</td>
</tr>
<tr>
<td>Lipid adjusted $\gamma$-tocopherol</td>
<td>r</td>
<td>0.27</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.01</td>
<td>0.12</td>
</tr>
</tbody>
</table>
The importance of dietary antioxidants on plasma antioxidant capacity and lipid peroxidation \textit{in vivo} in middle-aged men (Paper II)

Plasma antioxidant capacity and dietary intake of ascorbic acid, tocopherols and \(\beta\)-carotene

When AOC was divided into quartiles, a higher dietary intake of ascorbic acid, tocopherols and \(\beta\)-carotene when summarised was observed in the three upper quartiles of AOC than in the lowest quartile as shown in Table 3. A higher dietary intake of ascorbic acid was related to higher levels of AOC, whereas the associations for tocopherols and \(\beta\)-carotene were weaker, when considered separately.

Table 3. Nutrient density of selected dietary antioxidants by quartiles of plasma antioxidant capacity (AOC) in the 86 men in the study

<table>
<thead>
<tr>
<th></th>
<th>Quartile 1 (lowest) of AOC n=21</th>
<th>Quartiles (2-4) of AOC n=65</th>
<th>P for difference between the lowest and 3 higher quartiles of AOC</th>
<th>Correlation with AOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (mg/1000 kcal)</td>
<td>27.8 (9.5)</td>
<td>38.1 (15.5)</td>
<td>0.006</td>
<td>(r=0.24) (p=0.026)</td>
</tr>
<tr>
<td>Tocopherols (mg/1000 kcal)</td>
<td>3.7 (0.8)</td>
<td>3.8 (0.5)</td>
<td>0.198</td>
<td>(r=0.19) (p=0.086)</td>
</tr>
<tr>
<td>(\beta)-carotene (mg/1000 kcal)</td>
<td>0.91 (0.61)</td>
<td>1.12 (0.77)</td>
<td>0.135</td>
<td>(r=0.12) (p=0.274)</td>
</tr>
<tr>
<td>Ascorbic acid+ tocopherols+(\beta)-carotene (mg/1000 kcal)</td>
<td>32.3 (9.7)</td>
<td>43.1 (15.8)</td>
<td>0.005</td>
<td>(r=0.25) (p=0.021)</td>
</tr>
</tbody>
</table>

Data are presented as means (SD).
In the absence of uric acid, the relationships between AOC and dietary intake of ascorbic acid, tocopherols and β-carotene, were similar, but with smaller and non-significant differences between the higher and lowest quartiles as shown in Table 4. However, there was a significant correlation between intake of β-carotene and AOC without uric acid, and a borderline significant correlation between intake of ascorbic acid and AOC without uric acid, and between the combined intake of ascorbic acid, tocopherols and β-carotene and AOC without uric acid as presented in Table 4.

Table 4. Nutrient density of selected dietary antioxidants by quartiles of plasma antioxidant capacity (AOC) without uric acid in the 86 men in the study

<table>
<thead>
<tr>
<th></th>
<th>Quartile 1 (lowest) of AOC without uric acid n=21</th>
<th>Quartiles (2-4) of AOC without uric acid n=65</th>
<th>P for difference between the lowest and 3 higher quartiles of AOC without uric acid</th>
<th>Correlation with AOC without uric acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (mg/1000 kcal)</td>
<td>34.8 (16.0)</td>
<td>36.1 (14.5)</td>
<td>0.676</td>
<td>r=0.20 p=0.065</td>
</tr>
<tr>
<td>Tocopherols (mg/1000 kcal)</td>
<td>3.7 (0.9)</td>
<td>3.8 (0.5)</td>
<td>0.401</td>
<td>r=0.16 p=0.145</td>
</tr>
<tr>
<td>β-carotene (mg/1000 kcal)</td>
<td>0.93 (0.52)</td>
<td>1.07 (0.69)</td>
<td>0.505</td>
<td>r=0.25 p=0.023</td>
</tr>
<tr>
<td>Ascorbic acid+tocopherols+β-carotene (mg/1000 kcal)</td>
<td>39.6 (15.9)</td>
<td>41.0 (15.1)</td>
<td>0.714</td>
<td>r=0.21 p=0.056</td>
</tr>
</tbody>
</table>

Data are presented as means (SD).

Plasma antioxidant capacity and intake of antioxidant rich foods

A higher combined intake of fruit and berries, vegetables, and root vegetables as well as the total intake of selected foods excluding tea and coffee was found in the three upper quartiles of AOC than in the lowest quartile as presented in Table 5.
Table 5. Daily consumption of antioxidant rich foods by quartiles of plasma antioxidant capacity (AOC) in the 86 men in the study

<table>
<thead>
<tr>
<th></th>
<th>Quartile 1 (lowest) of AOC n=21</th>
<th>Quartiles (2-4) of AOC n=65</th>
<th>P for difference between the lowest and 3 higher quartiles of AOC</th>
<th>Correlation with AOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits and berries (g)</td>
<td>96 (55)</td>
<td>132 (83)</td>
<td>0.097</td>
<td>r=0.04 p=0.740</td>
</tr>
<tr>
<td>Vegetables (g)</td>
<td>46 (27)</td>
<td>56 (33)</td>
<td>0.133</td>
<td>r=0.09 p=0.420</td>
</tr>
<tr>
<td>Root vegetables (g)</td>
<td>9 (12)</td>
<td>12 (15)</td>
<td>0.763</td>
<td>r=0.09 p=0.478</td>
</tr>
<tr>
<td>Leguminous plants (g)</td>
<td>3 (8)</td>
<td>4 (18)</td>
<td>0.295</td>
<td>r=0.06 p=0.678</td>
</tr>
<tr>
<td>Fruits and berries+vegetables+root vegetables (g)</td>
<td>151 (72)</td>
<td>200 (95)</td>
<td>0.016</td>
<td>r=0.12 p=0.278</td>
</tr>
<tr>
<td>Black tea (g)</td>
<td>152 (165)</td>
<td>153 (250)</td>
<td>0.637</td>
<td>r=-0.13 p=0.225</td>
</tr>
<tr>
<td>Coffee (g)</td>
<td>507 (223)</td>
<td>448 (237)</td>
<td>0.069</td>
<td>r=-0.08 p=0.446</td>
</tr>
<tr>
<td>Red wine (g)</td>
<td>14 (26)</td>
<td>28 (42)</td>
<td>0.180</td>
<td>r=0.11 p=0.335</td>
</tr>
<tr>
<td>White wine (g)</td>
<td>10 (23)</td>
<td>24 (42)</td>
<td>0.602</td>
<td>r=0.27 p=0.134</td>
</tr>
<tr>
<td>Juices (g)</td>
<td>11 (21)</td>
<td>34 (66)</td>
<td>0.183</td>
<td>r=0.21 p=0.055</td>
</tr>
<tr>
<td>Jams/marmalades (g)</td>
<td>20 (19)</td>
<td>13 (12)</td>
<td>0.272</td>
<td>r=0.15 p=0.208</td>
</tr>
<tr>
<td>Total intake of selected foods excluding tea and coffee (g)</td>
<td>210 (87)</td>
<td>302 (153)</td>
<td>0.005</td>
<td>r=0.19 p=0.074</td>
</tr>
</tbody>
</table>

Data are presented as means (SD).
In the absence of uric acid, there were no significant differences or correlations in intake of different food groups, but lipid adjusted AOC without uric acid was significantly correlated with intake of vegetables, root vegetables and with total intake of selected foods excluding tea and coffee as shown in Table 6.
<table>
<thead>
<tr>
<th></th>
<th>Quartile 1 (lowest) of AOC without uric acid (lipid adjusted) n=21</th>
<th>Quartiles (2-4) of AOC without uric acid (lipid adjusted) n=65</th>
<th>P for difference between the lowest and 3 higher quartiles</th>
<th>Correlation with AOC without uric acid (lipid adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit and berries (g)</td>
<td>122 (68)</td>
<td>124 (81)</td>
<td>0.880</td>
<td>r=0.09 p=0.385</td>
</tr>
<tr>
<td>Vegetables (g)</td>
<td>42 (24)</td>
<td>57 (33)</td>
<td>0.048</td>
<td>r=0.23 p=0.031</td>
</tr>
<tr>
<td>Root vegetables (g)</td>
<td>11 (20)</td>
<td>12 (12)</td>
<td>0.180</td>
<td>r=0.34 p=0.004</td>
</tr>
<tr>
<td>Leguminous plants (g)</td>
<td>8 (20)</td>
<td>1 (4)</td>
<td>0.046</td>
<td>r=-0.12 p=0.284</td>
</tr>
<tr>
<td>Fruits and berries+vegetables+root vegetables (g)</td>
<td>175 (76)</td>
<td>193 (97)</td>
<td>0.500</td>
<td>r=0.19 p=0.073</td>
</tr>
<tr>
<td>Black tea (g)</td>
<td>172 (241)</td>
<td>146 (230)</td>
<td>0.273</td>
<td>r=0.01 p=0.922</td>
</tr>
<tr>
<td>Coffee (g)</td>
<td>443 (325)</td>
<td>469 (199)</td>
<td>0.393</td>
<td>r=0.10 p=0.371</td>
</tr>
<tr>
<td>Red wine (g)</td>
<td>22 (36)</td>
<td>26 (40)</td>
<td>0.897</td>
<td>r=-0.01 p=0.908</td>
</tr>
<tr>
<td>White wine (g)</td>
<td>10 (24)</td>
<td>24 (42)</td>
<td>0.101</td>
<td>r=0.09 p=0.616</td>
</tr>
<tr>
<td>Juices (g)</td>
<td>20 (48)</td>
<td>32 (62)</td>
<td>0.293</td>
<td>r=0.09 p=0.402</td>
</tr>
<tr>
<td>Jams/marmalades (g)</td>
<td>11 (8)</td>
<td>16 (16)</td>
<td>0.586</td>
<td>r=0.10 p=0.389</td>
</tr>
<tr>
<td>Total intake of selected foods without tea and coffee (g)</td>
<td>245 (82)</td>
<td>291 (159)</td>
<td>0.365</td>
<td>r=0.24 p=0.024</td>
</tr>
</tbody>
</table>

Data are presented as means (SD).
Plasma F<sub>2</sub>-isoprostanes and dietary intake of ascorbic acid, tocopherols and β-carotene

The levels of F<sub>2</sub>-isoprostanes were not associated with intake of ascorbic acid, tocopherols or β-carotene, neither when considered separately nor when combined.

Plasma F<sub>2</sub>-isoprostanes and intake of antioxidant rich foods

There were no associations, separately or combined, between the levels of F<sub>2</sub>-isoprostanes and intake of different food groups rich in antioxidants. However, subjects in the lowest quartile of F<sub>2</sub>-isoprostanes had a higher intake of white wine compared with subjects in the highest quartile (p=0.019 between the lowest and the highest quartile).

Correlations between plasma antioxidant capacity and plasma F<sub>2</sub>-isoprostanes

There was no significant correlation between AOC and F<sub>2</sub>-isoprostanes or between AOC without uric acid and F<sub>2</sub>-isoprostanes.
Effects of bilberry juice and black tea on plasma antioxidant capacity and lipid peroxidation in vivo: a dose-response study in healthy humans (Paper III)

Immediate effects after a single intake of a 400 mL bilberry- and tea drink

Plasma phenols: The total phenol concentration did not change after a single intake of the drinks in any group.

Plasma antioxidant capacity measured by the ORAC assay: Antioxidant capacity increased significantly in the LB/LT group from 541 ± 192 at baseline to 627 ± 179 μmol Trolox equivalents/L 120 min after intake of the drink (P = 0.039), whereas antioxidant capacity did not change significantly in the HB/HT group (584 ± 251 at baseline and 646 ± 265 μmol Trolox equivalents/L 120 min after ingestion; ns). Further, there were no differences in ORAC between the LB/LT and HB/BH groups, calculated as the incremental area under the curve. The immediate effects, measured by the ORAC assay, were determined only in the LB/LT and HB/HT groups.

Plasma antioxidant capacity measured by the TEAC assay: Antioxidant capacity increased significantly from baseline to 240 min in the MB/MT group (0.96 ± 0.08 at baseline to 0.99 ± 0.07 mmol Trolox equivalents/L at 240 min; P = 0.039) and in the HB/HT group (1.09 ± 0.11 at baseline to 1.14 ± 0.10 mmol Trolox equivalents/L at 240 min; P = 0.047). TEAC did not change in the other groups, and there were no significant differences between the groups or when the groups were compared with the LB/LT group, calculated as the incremental area under the curve.

Uric acid in plasma: The uric acid concentrations were not significantly changed after a single intake of the drinks (only measured in the HB/HT group).

Long-term effect after four weeks daily intake of 400 mL bilberry- and tea

The fasting antioxidant capacity measured by the ORAC assay increased significantly in the LB/LT, LB/HT and HB/LT groups (Table 7), but there were no significant differences between the groups or when the groups were compared with the LB/LT group. Total phenols, TEAC, uric acid concentrations, α- and γ-tocopherols, F2-isoprostanes and MDA did not change after four weeks as shown in Table 7.
Table 7. Effects of four weeks intake of the bilberry-and-tea drinks on the fasting concentrations of total phenols, antioxidant capacity, lipid adjusted tocopherols and uric acid, and markers of lipid peroxidation

<table>
<thead>
<tr>
<th></th>
<th>LB/LT n=12</th>
<th>LB/HT n=12</th>
<th>MB/MT n=12</th>
<th>HB/LT n=12</th>
<th>HB/HT n=12</th>
<th>P for diff. between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total phenols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg GAE/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2096 ± 177</td>
<td>2172 ± 167</td>
<td>2097 ± 164</td>
<td>2204 ± 180</td>
<td>2119 ± 147</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>2119 ± 199</td>
<td>2160 ± 172</td>
<td>2044 ± 213</td>
<td>2239 ± 188</td>
<td>2083 ± 150</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>ORAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol Trolox equivalents/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>541 ± 192</td>
<td>543 ± 85</td>
<td>575 ± 115</td>
<td>527 ± 149</td>
<td>586 ± 263</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>712 ± 249*</td>
<td>661 ± 150*</td>
<td>647 ± 107</td>
<td>618 ± 84**</td>
<td>703 ± 211</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>TEAC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol Trolox equivalents/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.10 ± 0.12</td>
<td>1.00 ± 0.11</td>
<td>0.96 ± 0.08</td>
<td>0.99 ± 0.06</td>
<td>1.09 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>1.14 ± 0.12</td>
<td>1.04 ± 0.07</td>
<td>0.98 ± 0.09</td>
<td>0.99 ± 0.07</td>
<td>1.12 ± 0.14</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>α-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/mmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.52 ± 0.17</td>
<td>1.58 ± 0.20</td>
<td>1.54 ± 0.15</td>
<td>1.50 ± 0.11</td>
<td>1.48 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>1.46 ± 0.23</td>
<td>1.56 ± 0.16</td>
<td>1.54 ± 0.18</td>
<td>1.53 ± 0.18</td>
<td>1.50 ± 0.19</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>γ-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/mmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.085 ± 0.022</td>
<td>0.087 ± 0.031</td>
<td>0.095 ± 0.033</td>
<td>0.086 ± 0.036</td>
<td>0.082 ± 0.019</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>0.088 ± 0.037</td>
<td>0.082 ± 0.031</td>
<td>0.095 ± 0.034</td>
<td>0.085 ± 0.029</td>
<td>0.081 ± 0.018</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Uric acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>296 ± 58</td>
<td>268 ± 71</td>
<td>282 ± 61</td>
<td>273 ± 66</td>
<td>272 ± 48</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>307 ± 69</td>
<td>277 ± 67</td>
<td>288 ± 47</td>
<td>278 ± 61</td>
<td>273 ± 50</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>8-iso-PGF₂α</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/mmol creatinine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>201 ± 65</td>
<td>205 ± 85</td>
<td>213 ± 59</td>
<td>208 ± 67</td>
<td>195 ± 53</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>188 ± 40</td>
<td>186 ± 64</td>
<td>204 ± 65</td>
<td>212 ± 84</td>
<td>193 ± 63</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>MDA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.60 ± 0.15</td>
<td>0.59 ± 0.14</td>
<td>0.59 ± 0.11</td>
<td>0.61 ± 0.14</td>
<td>0.61 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>After 4 wk</td>
<td>0.54 ± 0.12</td>
<td>0.57 ± 0.17</td>
<td>0.52 ± 0.11</td>
<td>0.58 ± 0.12</td>
<td>0.56 ± 0.09</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. Asterisks indicate different from baseline, * P < 0.05, ** P < 0.01.
Responders and nonresponders

There was a high degree of individual variability in antioxidant capacity, measured as ORAC, both at baseline and in the response (fasting levels) after four weeks. However, there was no dose-response effect. Baseline values of the subjects varied from 277 to 1180 μmol Trolox equivalents/L. The long-term changes from baseline values varied from a decrease of 316 to an increase of 721 μmol Trolox equivalents/L. The data were pooled and further analysed by reallocating subjects according to their responses to long-term supplementation. Subjects exhibiting an increase in antioxidant capacity more than 200 μmol Trolox equivalents/L after four weeks were reallocated to one group (responders). Subjects exhibiting a long-term increase in antioxidant capacity less than 200 μmol Trolox equivalents/L were reallocated to another group (nonresponders).

Subjects who exhibited an increase in ORAC after four weeks (‘responders’) also exhibited an increase in ORAC after a single dose of the bilberry-and-tea drink. The subjects who showed no change in ORAC after four weeks (‘nonresponders’) also showed no change in this variable after a single dose. There was a significant negative linear correlation between baseline antioxidant capacity and the change in capacity after four weeks as shown in Figure 2.

Figure 2. Correlations between changes in plasma antioxidant capacity measured by the oxygen radical absorbance capacity (ORAC) assay after four weeks’ daily intake of the bilberry-and-tea drinks and baseline ORAC values (n = 56).
Dietary n-3 fatty acids reduce plasma F2-isoprostanes but not prostaglandin F$_{2\alpha}$ in healthy humans (Paper IV)

Effects on lipid peroxidation

The nonenzymatic free radical mediated lipid peroxidation *in vivo* measured as F$_2$-isoprostanes in plasma was significantly decreased after three months of supplementation with n-3 fatty acids, whereas the enzymatic cyclooxygenase-mediated lipid peroxidation *in vivo* measured as prostaglandin F$_{2\alpha}$ in plasma did not change as presented in Table 8.

Antibodies against oxLDL were not affected by supplementation of n-3 fatty acids or by the diet.

Effects on antioxidant status

AOC was unchanged by supplementation of n-3 fatty acids or by diet, but lipid adjusted AOC without uric acid was improved by the diet as shown in Table 9. The serum concentrations of $\alpha$-tocopherols increased and $\gamma$-tocopherols decreased significantly after three months in subjects on both diets, but the concentrations were not affected by the n-3 supplementation (Table 9). The serum concentrations of $\beta$-tocopherols decreased in subjects that consumed the SAFA diet and increased in those that consumed the MUFA diet.
Table 8. Effects of consumption of a SAFA or a MUFA diet with supplemention of (n-3) fatty acids or placebo in the participants on markers of lipid peroxidation at baseline and after 3 months.

<table>
<thead>
<tr>
<th></th>
<th>SAFA diet</th>
<th>MUFA diet</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>placebo</td>
<td>n-3 fatty acids</td>
<td>placebo</td>
</tr>
<tr>
<td></td>
<td>n = 42</td>
<td>n = 41</td>
<td>n = 40</td>
</tr>
<tr>
<td><strong>Nonenzymatic lipid peroxidation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-iso-PGF2α, pmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>119 ± 145</td>
<td>80 ± 60</td>
<td>88 ± 97</td>
</tr>
<tr>
<td>After 3 months</td>
<td>97 ± 82</td>
<td>65 ± 45</td>
<td>91 ± 102</td>
</tr>
<tr>
<td><strong>Other oxidation products:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibodies against oxLDL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.31 ± 1.65</td>
<td>2.00 ± 1.16</td>
<td>2.01 ± 0.87</td>
</tr>
<tr>
<td>After 3 months</td>
<td>2.34 ± 1.87</td>
<td>1.99 ± 1.25</td>
<td>1.91 ± 0.95*</td>
</tr>
<tr>
<td><strong>Enzymatic lipid peroxidation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-keto-dihydro-PGF2α, pmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>133 ± 37</td>
<td>136 ± 65</td>
<td>119 ± 45</td>
</tr>
<tr>
<td>After 3 months</td>
<td>139 ± 45</td>
<td>128 ± 51</td>
<td>122 ± 51</td>
</tr>
</tbody>
</table>

¹ Values are means ± SD. Asterisks indicate significant within-group change, * P<0.05.
² There were no interactions between background diet and (n-3) fatty acids.
³ Differences in the change due to background diet adjusted for center, age, sex and the baseline value of the outcome.
⁴ Differences in the change due to n-3 fatty acids adjusted for center, age, sex and the baseline value of the outcome.
Table 9. Effects of consumption of a SAFA or a MUFA diet with supplementation of (n-3) fatty acids or placebo in the participants on antioxidant status at baseline and after 3 months\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>SAFA diet</th>
<th>MUFA diet</th>
<th>P-value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diet(^3) n-3 fatty acids</td>
</tr>
<tr>
<td></td>
<td>placebo</td>
<td>n-3 fatty acids</td>
<td>placebo n-3 fatty acids</td>
</tr>
<tr>
<td>AOC, (\mu\text{mol Trolox equivalents}/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>485 ± 107</td>
<td>495 ± 86</td>
<td>465 ± 97</td>
</tr>
<tr>
<td>After 3 months</td>
<td>490 ± 96</td>
<td>490 ± 94</td>
<td>471 ± 96</td>
</tr>
<tr>
<td>AOC without uric acid,(^2) (\mu\text{mol Trolox equivalents}/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>19.5 ± 6.1</td>
<td>19.1 ± 5.6</td>
<td>20.2 ± 6.5</td>
</tr>
<tr>
<td>After 3 months</td>
<td>19.3 ± 5.9</td>
<td>18.8 ± 4.4</td>
<td>20.6 ± 6.3</td>
</tr>
<tr>
<td>(\alpha)-tocopherol,(^3) (\text{mg/mmol})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.83 ± 0.26</td>
<td>1.80 ± 0.26</td>
<td>1.91 ± 0.28</td>
</tr>
<tr>
<td>After 3 months</td>
<td>1.90 ± 0.20</td>
<td>1.92 ± 0.24</td>
<td>2.12 ± 0.25</td>
</tr>
<tr>
<td>(\beta)-tocopherol,(^4) (\text{mg/mmol})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.020 ± 0.006</td>
<td>0.020 ± 0.005</td>
<td>0.020 ± 0.006</td>
</tr>
<tr>
<td>After 3 months</td>
<td>0.018 ± 0.005</td>
<td>0.019 ± 0.005</td>
<td>0.022 ± 0.006</td>
</tr>
<tr>
<td>(\gamma)-tocopherol,(^5) (\text{mg/mmol})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.09 ± 0.04</td>
<td>0.08 ± 0.05</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>After 3 months</td>
<td>0.06 ± 0.03</td>
<td>0.06 ± 0.03</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SD. Asterisks indicate significant within-group change, * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).

\(^2\) There were no interactions between background diet and (n-3) fatty acids.

\(^3\) Differences in the change due to background diet adjusted for center, age, sex and the baseline value of the outcome.

\(^4\) Differences in the change due to n-3 fatty acids adjusted for center, age, sex and the baseline value of the outcome.

\(^5\) Lipid adjusted.
Discussion

A number of diseases are associated with reduced antioxidant status and oxidative stress. Epidemiological studies have shown that a high dietary intake of antioxidants is associated with reduced risk of cardiovascular disease and some cancers. However, randomized controlled trials have not confirmed a positive effect of supplementation with antioxidants. Antioxidants exert their actions via an integrated network, and a specific balance between various antioxidants is probably needed for them to work optimally. This balance might have been disturbed in randomized controlled studies due to supplementation with high doses of a single or a few antioxidants. The effects of dietary factors and specific food items on antioxidant status and oxidative stress and the contributions to the antioxidant status of healthy humans, were investigated in this thesis. Antioxidant status was mainly measured as plasma antioxidant capacity that mirrors the combined action of antioxidants as well as interactions between them. Oxidative stress was mainly measured as F₂-isoprostanes, considered as a reliable and sensitive method for measurement of oxidative stress.

Plasma antioxidant capacity and the contribution of uric acid

The biological function of uric acid is unclear. Increased levels of uric acid appear to be as a consequence of oxidative stress in certain diseases and clinical conditions (142-144). Uric acid also has antioxidant properties because it scavenges peroxyl and hydroxyl radicals and binds metal ions to prevent them from catalysing free radical reactions (26, 27). This unclear function of uric acid as an antioxidant complicates the measurement of antioxidant capacity, since uric acid is a major determinant of the antioxidant capacity in plasma in most of these methods.

In Paper I, we showed that antioxidant capacity when uric acid was present in the sample, reflected the levels of uric acid and thus correlated with BMI, waist circumference and concentrations of insulin and triglycerides, all of which are associated with the metabolic syndrome (145). When uric acid was removed from samples before measurement of antioxidant capacity, subjects with a high antioxidant capacity exhibited a better metabolic condi-
tion compared with when uric acid was included in the analysis. After uric acid was eliminated, there was a correlation between antioxidant capacity and lipid concentration. This probably reflected tocopherols transported by lipid molecules. Therefore, antioxidant capacity measured without uric acid may need to be adjusted for lipids, especially if lipid concentration changes because of an intervention. In intervention studies, when a diet, food item, or supplement is tested and antioxidant capacity is measured at least twice in the same participant, measurement of antioxidant capacity in the presence of uric acid may be sufficient, if it is accompanied by measurement of uric acid levels to ensure that the level of uric acid has not changed during the study. When lipid concentrations changed during the intervention (Paper IV), antioxidant capacity was expressed as lipid adjusted antioxidant capacity without uric acid to compensate for the increased tocopherol concentration caused by increased lipid levels. If no adjustment for lipids has been done, the AOC value would have been misleading.

In contrast, in cross-sectional studies, antioxidant capacity should be presented with and without the contribution of uric acid and after adjustment of lipid levels. In these types of studies, antioxidant capacity is probably measured once and compared with that of other participants. Elevated uric acid concentrations could in some cases be caused by clinical and metabolic conditions such as kidney disease, gout, metabolic syndrome, or by strenuous physical activity and not better antioxidant defence, which complicates the interpretation of antioxidant capacity (29, 32, 33).

The results of Paper II indicate that the relationships between dietary intake and AOC, when uric acid was removed, were similar to those obtained when uric acid was present. However, the differences in antioxidant capacity between those who had higher intakes of antioxidants, or higher intakes of antioxidant rich foods, and those who had lower intakes were smaller. When uric acid was excluded, β-carotene, vegetables, and root vegetables were also associated with AOC, which may indicate that lipid-soluble antioxidants have a greater influence on AOC in the absence of uric acid. This conclusion is supported by the finding that the associations with serum concentrations of tocopherols were stronger when uric acid was excluded from the AOC measurement.

Other studies have reported increased levels of uric acid accompanied by increased plasma antioxidant capacity after intake of apples, and that this is related to the fructose content of apples (146, 147). However, this effect was not detected in any of our studies, neither as an immediate effect after the intake of bilberry-and-tea drinks nor after four weeks’ daily intake of bilberry-and-tea-drinks (Paper III).

Taken together, these results indicate that measurements of antioxidant capacity should always be accompanied by measurements of uric acid levels because differences in uric acid concentrations alone may explain between-
subject differences in antioxidant capacity and complicate the interpretation of estimates of antioxidant capacity.

Can we affect our antioxidant defence?

The antioxidant defence of the body is affected by several factors including intake of dietary antioxidants, levels of endogenous antioxidants, and ongoing oxidation processes in the body that determine the consumption of antioxidants.

The results in Paper II demonstrated that individuals with the highest intakes of antioxidant rich foods had better plasma antioxidant capacities, and that the associations between different antioxidant rich foods and plasma antioxidant capacity tended to be stronger when the antioxidant rich foods were considered together. High intakes of ascorbic acid, tocopherols and β-carotene were associated with greater plasma antioxidant capacity. In a previous study, fruit and berries were found to contribute most to the total intake of plant antioxidants in a Norwegian diet. However, beverages were not included in these calculations (36). In general, berries have high antioxidant capacities, but the difference in intake of fruit and berries between participants in the lowest quartile of antioxidant capacity and those in higher quartiles did not differ significantly (Paper II). When intakes of fruit and berries, vegetables and root vegetables were considered together, the difference in intake between the lowest and the higher quartiles was significant. Furthermore, beverages such as tea, wine and coffee are rich in polyphenols and other phenolic compounds. This is also reflected in their high antioxidant capacities (59) and increases in antioxidant capacity after intake (64, 65, 148). There were no associations between antioxidant capacity and intake of black tea, coffee, red wine or white wine in our study. Studies investigating the effect of food habits or long-term food intake on plasma antioxidant capacity are sparse (149, 150) but important because the bioavailability of dietary antioxidants is also considered. Others have estimated the antioxidant capacity of the diet, which does not account for the bioavailabilities of various antioxidants (70, 151, 152).

In paper IV, our results demonstrated that the participants improved the plasma antioxidant capacity after three months intake of a diet with a high proportion of MUFA. The concentrations of α-tocopherols increased and γ-tocopherols decreased after both the diet high in monounsaturated as well as high in saturated fatty acids, probably due to that the fat provided in the study contained a high amount of α-tocopherols. There were diverging effects in the diets on the concentrations of β-tocopherol, with decreased levels of β-tocopherol after intake of the diet high in saturated fatty acids and increased levels after the diet with a high proportion of monounsaturated fatty acids. Others have reported decreased concentrations of β-tocopherol after
intake of a diet rich in rapeseed oil or olive oil (153) and after intake of an almond-enriched diet (154).

Responders and nonresponders
The antioxidant capacities of various foods could differ enormously by more than 1000-fold (36). The results presented in Paper III showed that bilberry juice and black tea, two food items with high antioxidant capacities, increased the antioxidant capacities in some of the participants but not in others. Several studies have assessed the effects of various antioxidant rich foods on antioxidant capacity and the results have been conflicting (64-74). Our results from Paper III suggest that one explanation for the conflicting results is that there is a considerable individual variability in the antioxidant capacity response to antioxidant intake. Participants who exhibited an increase in ORAC after four weeks, the responders, also exhibited an increase in ORAC after 120 min after a single dose of the bilberry-and-tea drink, whereas the participants who showed no change in ORAC after four weeks, the nonresponders, also showed no change in this variable after a single dose. The high degree of individual variability in response to the drinks was related to the baseline antioxidant capacity. Antioxidant capacity increased only in subjects with low baseline antioxidant capacity. This individual difference in response may depend on that the plasma antioxidant capacity reaches a plateau, suggesting that especially individuals with low antioxidant capacity could improve the antioxidant status and might benefit from increasing their intake of antioxidant rich foods. Moreover, associations between genetic contribution to plasma antioxidant capacity have been reported (77). Interactions with other nutrients, such as milk addition to tea and chocolate have been shown in some, but not in all studies, to impair the antioxidant capacity (76, 155, 156). Previous studies have demonstrated the formation of bonds between flavonoids and proteins (157, 158). Further reasons for the differences in responses between individuals might be differences in absorption etc.

Are there interactions between antioxidants in vivo?
Studies in vitro have reported interactions between antioxidants (42). Cooperation between various antioxidants might provide greater protection against oxidative stress than any antioxidant alone. The results presented in Paper III showed that levels of tocopherols were not affected by four weeks’ daily intake of bilberry and tea drinks, suggesting that there was no sparing effect on tocopherols by flavonoids. Others have also failed to show interactions in vivo between tocopherols and flavonoids (159).
Of the randomized controlled trials, only a few have shown a protective effect of antioxidants in doses that might be achieved by dietary intakes. In the SUVIMAX study, a combination of 120 mg ascorbic acid, 30 mg vitamin E, 6 mg β-carotene, 100 μg selenium, and 20 mg zinc was administered (160). After 7.5 years, all-cause mortality and cancer was decreased in men but not in women.

In some of the randomized controlled trials, unexpected and even harmful effects of supplementation, were observed: there was increased risk of heart failure in patients with vascular disease or diabetes mellitus after a daily intake of 400 IU vitamin E for a median of seven years (161). These results support the recommendation for an intake of a balanced diet rich in different types of antioxidants, which facilitates interactions between antioxidants and does not disturb the balance between various antioxidants in the complex antioxidant network.

**Antioxidants and lipid peroxidation in vivo**

As both blueberries and tea are rich sources of flavonoids, have high antioxidant capacities, and are protective against LDL oxidation *in vitro*, it was hypothesized that they might decrease lipid peroxidation *in vivo*. However, despite increased antioxidant capacity (measured by the ORAC assay) in some of the groups, and a major contribution of flavonoids from the drinks, biomarkers of lipid peroxidation *in vivo* (measured as F2-isoprostanes and MDA) were not affected (Paper III). The lack of an effect on F2-isoprostanes and MDA might be because our participants were healthy with normal basal levels of these variables. If we had included participants with elevated levels of F2-isoprostanes, such as patients with diabetes or smokers, we might have observed an effect. Furthermore, higher doses or a longer study period might also have resulted in different findings. The results of our study agree with many other intervention studies that have also failed to detect an effect on F2-isoprostanes or MDA after intake of antioxidant rich food items, such as black or green tea (72, 162-164), blueberries (165) or a vegetable and fruit concentrate including blueberries (74) and after intake of fruit and vegetables (73).

A few intervention studies with healthy humans have shown decreased levels of F2-isoprostanes after intake of antioxidant rich food items, such 500 mL/d black tea for four weeks (166) or 500 mL/d orange juice for two weeks (167), after a daily intake of a diet rich in vegetables and fruit for two weeks (168) or with supplementation with garlic extract (169). Furthermore, daily intake of green tea extract decreased MDA concentrations but not the levels of F2-isoprostanes (170). Among smokers, intake of alcohol-free red wine decreased F2-isoprostane concentrations whereas red or white wine alone did not (171).
The findings reported in Paper II showed that there were no associations between intakes of various antioxidant rich foods or intakes of ascorbic acid, tocopherols or β-carotene and plasma F₂-isoprostanes. In contrast, a cross-sectional study reported an inverse correlation between plasma F₂-isoprostanes and fruit intake (172).

Supplementation with vitamin E or vitamin C generally does not affect levels of F₂-isoprostanes in healthy humans (173-176), but in patients with increased basal levels of F₂-isoprostanes, vitamin E reduces elevated F₂-isoprostane concentrations (94, 97). Supplementation with vitamin E (100-600 mg/d) decreased levels of F₂-isoprostanes in patients with type 2 diabetes (94) and hypercholesterolaemia (97, 177, 178), but not in smokers (179, 180). Supplementation with vitamin C (500-2000 mg/d) decreased levels of F₂-isoprostanes in smokers (180, 181), but not in patients with type 2 diabetes or hypercholesterolaemia (177, 178, 182). Furthermore, higher doses of α-tocopherol and carotenoids (111+1.24 mg/d, respectively) decreased F₂-isoprostanes, but not a lower dose (43±0.45 mg/d) in healthy humans (183). Animal studies with high doses of vitamin E have shown inhibition of F₂-isoprostanes formed in CCl₄ treated rats (184).

These discrepancies in the effects of intervention studies with antioxidant rich foods or antioxidant supplements on lipid peroxidation in vivo may depend on the clinical conditions of participants.

Moreover, the results from Paper II and Paper III indicate that lipid peroxidation in vivo is not easily affected by dietary antioxidants. Other reasons for the failure to detect an effect may be biological variation between individuals, and high day-to-day variations of levels of F₂-isoprostanes (185, 186).

Effect of n-3 fatty acids on nonenzymatic and enzymatic lipid peroxidation

Supplementation with n-3 fatty acids (DHA and EPA) decreased the nonenzymatic lipid peroxidation, measured as F₂-isoprostanes, but enzymatic lipid peroxidation, measured as PGF₂α-metabolites was unaffected (Paper IV). As n-3 fatty acids may compete with arachidonic acid for conversion to prostaglandins of the F3-series through the cyclooxygenase pathway, decreased formation of PGF₂α might be expected. However, as the formation of PGF₂α was unchanged, the mechanism by which n-3 fatty acids reduce F₂-isoprostanes seems to be by inhibition of free radical pathways. Free radicals are generated continuously under basal conditions in vivo and rapidly during inflammatory conditions (7). However, how n-3 fatty acids inhibit F₂-isoprostane formation is unknown. Moreover, decreased levels of F₂-isoprostanes in urine were reported in hyperlipidaemic subjects (187) and in
type 2 diabetic subjects (188, 189) after supplementation with EPA and DHA or after intake of fish. The author of those studies suggested that the anti-inflammatory effects of n-3 fatty acids, and reduced free radical formation of leukocytes, might explain the decreased urinary F2-isoprostanes.

Negative results of randomized controlled trials

Epidemiological studies have shown that intake of fruit and vegetables is protective against cardiovascular diseases and some cancers (1-5). Experimental studies indicate that LDL oxidation is an important mechanism responsible for cardiovascular diseases (129). However, proof of the protective effects of antioxidants can only be demonstrated by randomized clinical trials. Most large trials have failed to prove this hypothesis. There are several possible reasons for this: individuals who consume a large quantity of fruit and vegetables might have more healthy lifestyles in general than those who do not, including dietary habits, physical activity, or smoking. Even if we can adjust for some of these variables, other unknown factors may be important. Supplementation with antioxidants has been shown to increase blood concentrations of the antioxidant (190) and to decrease levels of F2-isoprostanes, at least in subjects with elevated basal levels of F2-isoprostanes (94, 97, 177, 178, 180, 181).

Are plasma antioxidant capacity assays useful for measurement of overall antioxidant defence?

Use of different methods

The plasma antioxidant capacity assays measure the combined effect of many antioxidants in the sample, which are able to scavenge the free radicals generated in the assay. Interactions between antioxidants are also reflected in the assay value. In Paper III there was a discrepancy in the results in antioxidant capacity between the ORAC and the TEAC assay after four weeks daily intake of the bilberry-and-tea drink. This might relate to the different principles on which the methods are based, and therefore several methods may be used in parallel to elucidate the complex field of antioxidants and oxidation. Both the ORAC and the TEAC assays measure the inhibition of generated free radicals by the antioxidants in the sample, but different free radicals are generated and different technologies are used. The advantage of the ORAC assay is that it combines both inhibition time and inhibition degree of the radical generation, because it takes the oxidation reaction to completion.
uses the area under the curve to quantify the antioxidant capacity (53). The TEAC assay uses only the inhibition degree at a fixed point to determine antioxidant capacity (54).

Further studies are required to elucidate different contributors to the plasma antioxidant capacity, and especially the function of uric acid as an antioxidant. Better knowledge about biological relevance of different antioxidant capacity assays and standardisation for measurement of plasma antioxidant capacity is needed.

Should individuals at high risk take antioxidant supplements?
The results from randomized controlled studies of antioxidants in healthy individuals and in those at high risk are mainly negative. Since some randomised controlled trials have shown harmful effects of high doses of antioxidants, no recommendations about the intake of supplements of antioxidant can be made today. Further studies are required to provide more data on the optimum dose and type of antioxidants, and on which individuals who might benefit from supplementation of antioxidants. Therefore, only recommendations on high intakes of antioxidant rich foods, such as fruit and vegetables, can be made.

Concluding remarks
This thesis provides further knowledge on the complex antioxidant-oxidation field. The studies show how dietary factors and specific food items affect the antioxidant status and levels of oxidative stress in healthy humans, and how different contributors affect the antioxidant defence.

Antioxidant capacity, which takes into account both known and unknown antioxidants and their interactions, provides an integrated measure of the antioxidant defence system. Although uric acid is a major determinant of antioxidant capacity, its mode of action is unclear, which complicates interpretation of measurement of antioxidant capacity. In Paper I, we showed that it is possible to measure the plasma antioxidant capacity of samples from which uric acid is removed. Antioxidant capacity was correlated with uric acid concentration, but also with BMI, waist circumference and concentrations of insulin and triglycerides, all of which are associated with the metabolic syndrome, and reflecting the predominant effect of uric acid. There were no correlations between antioxidant capacity and variables associated with the metabolic syndrome when uric acid was eliminated from samples, but the remaining antioxidant capacity was correlated with plasma lipid concentration. This may partly reflect lipid soluble antioxidants that are transported by lipid molecules. The results indicate that the significance of the contribution of uric acid to antioxidant capacity differs according to the type
of study: it is more difficult to evaluate if antioxidant capacity, and the uric acid concentration, is measured only once, as is often the practice in cross-sectional studies, than if it was measured at least twice, as is the practice in intervention studies. Further studies are required to elucidate the antioxidant effect of uric acid.

The results from Paper II show that individuals with the higher intakes of antioxidant rich foods had plasma antioxidant capacities superior to those with the lowest intakes of antioxidant rich foods. The associations between different antioxidant rich foods and plasma antioxidant capacity tended to be stronger when the antioxidant rich foods were considered together. In addition, intakes of the well-known antioxidants ascorbic acid, tocopherols, and β-carotene were positively related to plasma antioxidant capacity. We conclude that optimal antioxidant status may be achieved by consumption of a balanced diet rich in a variety of antioxidants from different food sources, which would facilitate synergy between different types of antioxidants and integration of their effects. Our results also indicate that the lipid peroxidation in vivo is not easily affected by diet.

Although the bilberry-and-tea-drinks provided a large amount of flavonoids in some of the groups described in Paper III, increased plasma antioxidant capacity was not observed in all participants and a dose-response effect was not evident. There was a high degree of individual variability in responses to the drinks, which was related to baseline antioxidant capacity. Only participants with low baseline antioxidant capacities exhibited increased plasma antioxidant capacities 120 minutes after a single intake of the drink as well as after 4 weeks daily intake. These individuals might benefit from ingestion of foods rich in antioxidants. The reasons for the differences in responses between individuals are not clear and warrant further study. Despite an increase in plasma antioxidant capacity in some of the groups, lipid peroxidation in vivo was not affected. The levels of tocopherols were not affected after four weeks’ intake of the drinks, and this may indicate that flavonoids did not have a sparing effect on tocopherol.

EPA and DHA are highly unsaturated fatty acids and might therefore be readily oxidised. The results presented in Paper IV show that supplementation with n-3 fatty acids decreases nonenzymatic free radical-catalysed lipid peroxidation in vivo, but enzymatic cyclooxygenase-mediated lipid peroxidation was not affected. The mechanism by which EPA and DHA reduce nonenzymatic lipid peroxidation should be subject to further investigation. Furthermore, antioxidant capacity was not affected by supplementation with n-3 fatty acids, but was improved by the background diet containing a high proportion of monounsaturated fatty acids. In all groups, the concentrations of α-tocopherol increased and those of γ-tocopherol decreased; this was probably caused by the presence of fat containing high levels of α-tocopherol in the background diet. The concentrations of β-tocopherol decreased after consumption of the diet that contained a high proportion of satu-
rated fatty acids and increased after consumption of a diet with a high pro-
portion of monounsaturated fatty acids. The significance of these diverging
effects on the levels of β-tocopherol is unknown.
Conclusions

- Plasma antioxidant capacity can be measured both with and without uric acid (Paper I).

- Plasma antioxidant capacity correlated with uric acid concentrations, but also with BMI, waist circumference, and concentrations of insulin and triglycerides, all of which are associated with the metabolic syndrome. The correlations with variables associated with the metabolic syndrome were not evident after elimination of uric acid, but the remaining antioxidant capacity was correlated with lipid concentrations. This may partly reflect lipid soluble antioxidants that are transported by lipid molecules. If lipid concentrations change during an intervention, adjustment for lipid concentration may be necessary. The significance of the contribution of uric acid to antioxidant capacity could differ according to the type of study (Paper I).

- Individuals with high intakes of various antioxidant rich foods and ascorbic acid, tocopherols and β-carotene had plasma antioxidant capacities greater than those with low intakes of antioxidants (Paper II).

- Nonenzymatic free radical-catalysed lipid peroxidation in vivo, measured as F2-isoprostanes in plasma, was not associated with intake of antioxidant rich foods or dietary antioxidants (Paper II).

- Plasma antioxidant capacity increased in some groups both after a single intake of a bilberry-and-tea drink and after four weeks’ daily intake, but there were no dose-response effects. Only participants with low baseline antioxidant capacities exhibited increases in antioxidant capacity after a single intake of the drink as well as after 4 weeks daily intake (Paper III).

- F2-isoprostanes in urine were not affected after four weeks daily intake of a bilberry-and-tea drink (Paper III).

- Supplementation with EPA and DHA for three months decreased the concentrations of plasma F2-isoprostanes (an index of enzymatic lipid peroxidation), but it did not affect the concentrations of plasma prostaglandin F2α metabolites (an index of nonenzymatic lipid peroxidation) (Paper IV).
• Intake of a diet with a high proportion of monounsaturated fatty acids improved plasma antioxidant capacity (Paper IV).
Acknowledgements

Thank you to all who have contributed to this thesis in different ways, with special thanks to:

Bengt Vessby, my supervisor, for sharing your enormous knowledge, for excellent scientific guidance and for being a good model as a scientist.

Margareta Öhrvall, assistant supervisor, for good advices and for always reminded me to think in clinical terms.

Samar Basu, for introducing me to the world of isoprostanes and prostaglandins, and for support, helpfulness and enthusiasm.

Tommy Cederholm, head of the Section of Clinical Nutrition and Metabolism, for providing a creative and stimulating work environment, and for being a nice and positive boss.

Brita Karlström, for always providing a helping hand whenever needed even though you are extremely busy, and for much good advice.

Lars Lannfelt, head of the Section of Geriatrics, for creating a conductive, inspiring and generous work environment.

Inga-Britt Gustafsson, for support during my first years in the department.

Anders Sjödin, co-author, for many good ideas.

Afaf Kamal-Eldin, co-author, for sharing your enormous knowledge.

Norwegian co-authors, Grete Skrede, Anne Skivik Jorgensen, Grethe Iren Borge, Berit Karoline Martinsen, Kjersti Aaby and Jon Volden, for fruitful collaboration and great company at congresses.

All co-authors, for all valuable comments on the manuscripts.

All participants for interest and enthusiasm in the studies.

Erika Olsson, for being a dear friend and a ready listener in both good and difficult times, for encouragement, and for help with baby sitting.

Current and former colleagues at Clinical Nutrition and Metabolism Agneta Andersson, Achraf Daryani, Johanna Helmersson, Anette Järvi, Ulf Risérus, Elisabet Rytter, Annika Smedman, Eva Södergren, Eva Warensjö, Anja
Saletti, Wulf Becker and Ulf Holmbäck, for stimulating discussions, for reading my manuscripts and friendship through the years.

Barbro Simu, Siv Tengblad and Eva Sejby, for all help in the lab and enjoyable times during my laboratory days.

Lars Berglund and Rawya Moehsen, for support and for statistical advices.

The dieticians Helena Peterson, Marie von Post Skagegård, Agneta Nilsson, Susanne Fredèn, Marie Lemcke, Agneta Hedman, Karin Andersson, for interesting discussions and for being nice colleagues. Marina Spoverud-Ålvebratt, for being an excellent organiser in the metabolic kitchen during the diet studies.

Liisa Byberg, for being a good friend and for statistical advices.

Current and former colleagues at the Unit for Geriatrics, Ann-Cristin Åberg, Per-Erik Andersson, Hans Basun, Kristina Björklund, Merike Boberg, Kristina Dunder, Margareta Falkeborn, Klara Halvarsson-Edlund, Anu Hedman, Arvo Hänni, Erik Ingelsson, Hans-Erik Johansson, Lena Kilander, Maria Lindau, Karin Modin, Richard Reneland, Claes Risinger, Inger Stenström, Kristina Ström-Möller, Johan Sundström, Bernice Wiberg, Martin Wohlin, Björn Zethelius and Johan Årnlöv, for being nice colleagues.

All staff at former metabolic ward for taking care of the study participants.

All friends, for understanding that I have to work instead of spending time with you, especially, Annelie Wedebrand, for telephone conversations and support, and Christina Örjes, for movie nights and friendship through the years.

Kent, for invaluable support, encouragement, and good ideas.

My mother Inga-Lisa, my father Bengt-Olof; my older brother Johan and family, and my younger brother Fredrik; and especially my mother and my younger brother Fredrik for support, interest in my subject and baby sitting whenever needed, and my older brother Johan for help with old cars and other practical things.

My son, William, the sunshine in my life and the most important person for me, for making the days so much more fun, and for being such a good assistant at work. You reminded me of the world outside the department, and despite your age, understood why mum had to work so much.
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