Effects of Antioxidants and Pro-oxidants on Oxidative Stress and DNA Damage using the Comet Assay

Studies on Blood Cells from Type 2 Diabetes Subjects and Mouse Lymphoma Cells

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Abstract

Diet and oral supplements comprise two distinct sources of antioxidants known to prevent oxidative stress. Beneficial effects from antioxidants have been seen for patients at risk for type 2 diabetes.

The aim of this thesis was to evaluate the positive effects of antioxidants against oxidative stress and DNA damage in type 2 diabetes subjects. We also used antioxidants as tools to determine the mechanisms behind genotoxicity induced by mutagenic pro-oxidative agents in mouse lymphoma cells. Several techniques were used to measure oxidative stress and DNA damage, but the main technique used was alkaline comet assay.

The results showed that the fruit and vegetable intake was inversely related to oxidative stress in type 2 diabetes subjects. However, oral supplementary intake of 20 antioxidants did not decrease oxidative stress biomarkers.

In studies on mouse lymphoma cells, using the alkaline comet assay, DNA damage was induced by catechol and o-phenylenediamine (OPD), while 4-nitro-o-phenylenediamine (4-NOPD) induced only oxidative damage, showing different mechanisms of action behind the mutagenicity of the compounds. Also, oxidative stress was induced by catechol and 4-NOPD, whereas imbalances in the nucleotide pool were seen after exposure to OPD or 4-NOPD. Addition of antioxidants together with these pro-oxidants showed that β-carotene was able to reduce DNA damage at low concentrations of catechol, but increased DNA damage at high concentration. In comparison, addition of α-tocopherol slightly decreased catechol-induced DNA damage at all concentrations of catechol. However, no effect of α-tocopherol was seen on OPD- or 4-NOPD-induced DNA damage.

In conclusion, antioxidants from fruits and vegetables, but not from oral supplements, reduced oxidative stress in type 2 diabetes patients, suggesting fruits and vegetables being a healthier source for antioxidant-intake, as compared to oral supplements. Different mechanisms of action for mutagenic pro-oxidants were shown in mouse lymphoma cells, introducing the nucleotide pool as an interesting target for oxidative stress. Reduction of catechol-induced DNA damage by β-carotene or α-tocopherol was shown, with a pro-oxidative action of β-carotene at high concentration of catechol. Interestingly, α-tocopherol was not able to decrease OPD- or 4-NOPD-induced DNA damage, supporting different mechanisms of action behind the genotoxicity from the three pro-oxidants.

Keywords: metabolic syndrome, fruit and vegetable intake, plasma antioxidants, beta-carotene, alpha-tocopherol, inflammation, oxidative DNA damage, lipid peroxidation, mouse lymphoma assay, ROS, nucleotide pool, viability, DNA dye

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List of Papers

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


IV Åsgård R, Håkansson S, Lundin R, Hellman B. Evaluation of α-tocopherols effect on catechol and o-phenylenediamine induced DNA damage: An in vitro study using two different staining techniques in the comet assay. *Submitted manuscript*


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Additional publications (not included in the thesis)


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Contents

Introduction ................................................................................................... 15
  Background .............................................................................................. 15
  Oxidative stress ........................................................................................ 16
  Reactive oxygen species ........................................................................ 16
  General and oxidative DNA damage ................................................... 18
  Repair of oxidative DNA damage ....................................................... 18
  Repair of oxidised bases in the nucleotide pool .................................. 19
  General and oxidative DNA damage as biomarkers for disease .......... 19
  Lipid peroxidation ............................................................................... 20
  Protein oxidation .................................................................................. 20
Antioxidants ............................................................................................. 21
  Dietary antioxidants ............................................................................. 21
  Effects of dietary antioxidants on type 2 diabetes ................................ 21
  β-Carotene ........................................................................................... 22
  α-Tocopherol ....................................................................................... 23
Lifestyle related diseases .......................................................................... 24
  Obesity and metabolic syndrome ......................................................... 24
  Type 2 diabetes .................................................................................... 26
  Cancer .................................................................................................. 27
  Genetic aspects in relation to antioxidants, oxidative stress and lifestyle related diseases ........................................... 27

Research Aims .............................................................................................. 29
  General aim ............................................................................................ 29
  Specific aims .......................................................................................... 29

Materials and methods .................................................................................. 30
  Comet assay (Paper I-V) .......................................................................... 30
    An assay of general and oxidative DNA damage ................................ 30
    Further development of the comet assay method ............................... 31
    Discussion of the comet assay protocols ............................................. 33
    Protocol for the alkaline comet assay with FPG (Paper I-II) ............... 34
    Protocol for the alkaline comet assay with hOGG1 (Paper III and V) ........................................................................ 35
    Protocol for the alkaline comet assay, without enzymes (Paper IV) ........................................................................ 36
Studies on type 2 diabetes subjects (Paper I and II).................................37
Subjects ................................................................................................37
A three day food diary .........................................................................38
Blood sampling for analyses of oxidative DNA damage.....................38
Methods in studies on type 2 diabetes subjects ...................................39
Studies on mouse lymphoma cells (Paper III-V) .....................................40
Cell lines and culture conditions ..........................................................40
Treatment with pro-oxidants .................................................................40
Methods in studies on mouse lymphoma cells ....................................41

Results and discussion ..................................................................................42
Effects of antioxidants from fruit and vegetable intake on oxidative stress in type 2 diabetes subjects (Paper I) .........................................................42
Effects of antioxidants from oral supplements on oxidative stress in type 2 diabetes subjects (Paper II) ..............................................................45
Effects of β-carotene on general and oxidative DNA damage in vitro induced by a pro-oxidant (Paper III) .................................................................47
Effects of α-tocopherol on DNA damage in vitro induced by pro-oxidants (Paper IV) ..........................................................................................50
Effects of mutagenic pro-oxidants on oxidative stress as an explanation of mutagenicity in vitro (Paper V) .................................................................54

Conclusions ...................................................................................................59
General conclusion ...................................................................................59
Specific conclusions ..................................................................................59

Concluding remarks and future perspectives .............................................61

Popular Scientific Summary .........................................................................62

Acknowledgements .......................................................................................64

References .....................................................................................................67
Abbreviations

AGE products  Advanced glycation end products
AHA  American heart association
BER  Base excision repair
BMI  Body mass index (kg/m^2)
BrdU  Bromodeoxyuridine
BSA  Bovine serum albumin
Catechol  1,2-dihydroxybenzene
CHD  Coronary heart disease
CIAP  Calf intestinal alkaline phosphate protocol
CM-H_2DCFDA  Probe for detection of ROS
CPT-Vacutainer  Cell preparation tubes with sodium heparin
CRP  C-reactive protein
CVD  Cardiovascular disease
Cu^+  Copper ion
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DSB  Double strand breaks
dG  Deoxyguanosine
E %  Energy percentage
EDTA  Ethyl-enediamine-tetraacetic acid
EI  Energy intake
ELISA  Enzyme-linked immunosorbent assay
EtBr  Ethidium bromide
FAPyG  2,6-diamino-4-hydroxy-5-formamidopyrimidine
FPG  Formamido pyrimidine glycosylase
FPG-sites  Part of DNA-damage induced from FPG
fapy-Ade  4,6-diamino-5-formamidopyrimidine
fapy-Gua  2,6-diamino-4-hydroxy-5-formamidopyrimidine
Fe^{2+}  Iron ion
fMN-Trf-Ret  Micronucleated transferrin + reticulocytes
HOCL  Hypochlorous acid
\cdot HO_2  
HbA1c  Long-term biomarker for blood glucose
HDL  High density lipoprotein
hOgg1  Human oxoguanine DNA-glycosylase 1
HPLC  High pressure liquid chromatography
H₂O₂  Hydrogen peroxide
IDF  International diabetes federation
IL-6  Interleukin 6
LDL  Low density lipoprotein
MDA  Malondialdehyde
MLA  Mouse lymphoma assay
NHLBI  National heart lung and blood institute
NO  Nitrogen oxide
OH⁻  Hydroxyl anion
‘OH  Hydroxyl radical
ONOÖ⁻  Peroxynitrite
OPD  ortho-Phenylenediamine
¹O₂  Singlet oxygen
‘O₂⁻  Superoxidium anion
O₃  Ozone
PAL  Physical activity level
PGF  Prostaglandin factor
PrI  Propidium iodide
P-53 gene  protein 53 gene (a tumour suppressor gene)
PUFA  Poly unsaturated fatty acids
R10P  Cell culture medium including 10 % serum
RIA  Radio immunoabsorbent assay
RNA  Ribonucleic acid
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
‘RO  Alkoxyl radical
‘RO₂  Peroxyl radical
SSB  Single strand breaks
S9-mix  Organ tissue homogenate from rat liver
TFT  Trifluorothymidine
TRIS  Tris(hydroxymethyl)aminomethane
4-NOPD  4-Nitro-ortho-phenylenediamine
4-NQO  4-Nitroquinoline-N-oxide
8-OH-Gua  8-hydroxyguanine
8-iso-PGF₂α  8-iso-prostaglandin factor₂α
8-oxodA  8-oxo-2′-deoxyguanosine
8-oxo-dG  8-oxo-7,8-dihydro-2′-deoxyguanosine
15-keto-dihydro-PGF₂α  15-keto-dihydro-prostaglandin factor₂α
Populärvetenskaplig sammanfattning


Ett mål med denna avhandling var att belysa hur intag av antioxidanter från frukt, grönsaker eller kapslar/piller kan påverka typ 2 diabetiker, ett annat att studera mekanistiska aspekter av oxidativ stress och DNA-skada i muslymfomaceller, med hjälp av pro-oxidanter och antioxidanter.

Den första studien (delarbete I) visade att ett högt intag av frukt och grönsaker hos typ 2 diabetiker kunde minska nivån av oxidativ stress. Detta resultat stöds även av andra studier i litteraturen. En teori är att det är det höga innehållet av antioxidanter i frukt och grönsaker som kan sänka oxidativ stress i våra celler. Eftersom typ 2 diabetiker har höga nivåer av oxidativ stress, kan effekten bli ännu starkare hos dessa än hos friska individer. I delarbete I visade vi också att plasmanivåer av β-karoten (pro-vitamin A) var en bra biomarkör för intag av frukt och grönsaker.

Många studier har gjorts för att studera effekter efter extra tillskott av en eller flera antioxidanter. Dessa studier har utförts i kemiska system, i celler liksom på människor. En del studier visar en antioxidativ effekt medan många inte kan påvisa någon effekt alls. I delarbete II såg vi ingen effekt hos typ 2 diabetiker på fem biomarkörer för oxidativ stress efter tillskott av 20 olika antioxidanter i kapslar, från frukt- och grönsaker, under 12 veckors tid.

I delarbete III visade vi att tillsats av β-karoten i muslymfomaceller gav en sänkning av katekol-inducerad oxidativ stress. Däremot vid höga koncentrationer av den DNA-skadande kemikalien katekol, så höjde β-karoten...
nivåerna av både generell- och oxidativ DNA-skada. Vitamintillskott gavs dels under 18 timmar innan exponering med katekol, dels samtidigt med katekol under 3 timmar. Båda behandlingar uppvisade liknande mönster när det gällde antioxidanteffekt, medan den pro-oxidativa effekten var större vid 18 timmars förbehandling. I detta sammanhang är det intressant att notera att tillskott av β-karotén på manliga rökare och asbestarbetare, gav upphov till en ökad förekomst av lungcancer och död från hjärtkärllsjukdom, i två stora kliniska studier. Det skulle kunna visa samma pro-oxidativa effekt från β-karoten som i vår cellstudie.

α-Tokoferol (vitamin E) är en annan antioxidant som förekommer i frukt och grönsaker. Frön, nötter och vegetabilisk olja är dock bättre källor för vitaminet. Detta vitamin har upphämt antioxidanteffekt i kemiska system och i cellsystem. Hos människan har tillskott av vitaminen visat sig motverka både oxidativ stress och hjärtinfarkt, medan några studier inte har visat någon effekt. I delarbete IV studerade vi effekten av tillskott med α-tokoferol på DNA-skador som uppstod efter exponering av modellsubstanserna katekol, o-phenylenediamine (OPD) eller 4-nitro-o-phenylenediamine (4-NOPD) i muslymfomaceller. Vid samtidig exponering för α-tokoferol och katekol förhindrades DNA-skador från katekol av vitaminet. Däremot hade α-tokoferol inte någon effekt på skador orsakade av OPD eller 4-NOPD. Dessa resultat är ytterligare ett bidrag till litteraturen som visar att antioxidanter kan ha varierande effekt beroende på vilka ämnen och koncentrationer som används, samt i vilken miljö de verkar. I delarbete IV ingick även metodutveckling i form av att vi visade att den mindre farliga DNA-färgen GelRed vid mätning av DNA-skador i comet assay, kunde ersätta DNA-färgen etidium bromid (som misstäcks kunna orsaka mutationer och cancer hos människa).

I delarbete V undersökte vi i muslymfomaceller ifall oxidativ stressrelaterade mekanismer kunde ligga bakom mutationer orsakade av OPD eller 4-NOPD, samt ifall det förekom en tröskeldoseffekt eller inte. Resultaten visade att en generell oxidativ stress och även DNA-oxidation hade samband med mutationer orsakade av 4-NOPD, medan störningar i nukleotidpoolen hade samband med mutationer från både OPD och 4-NOPD. I båda fallen fanns det en tröskeldos, där nivåerna stämde överens för DNA-skador och mutationer. Studien visade också att OPD ger både generell DNA-skada och mutationer i lägre koncentration än 4-NOPD, och var därför mer potent genotoxisk och mutagen.

De slutsatser som kan göras utifrån studierna i denna avhandling är att människor, speciellt typ 2 diabetiker, kan skyddas mot oxidativ stress vid intag av frukt och grönsaker. Därför kan ett högt intag av frukt och grönsaker rekommenderas. Extra tillskott av samma antioxidanter som finns i frukt och grönsaker, men i form av piller eller kapslar, har inte någon entydig effekt på oxidativ stress i människa. Speciellt gäller detta typ 2 diabetiker. I muslymfomaceller visar kostrelaterade antioxidanter, som β-karotén och α-
Introduction

Background

Oxidative stress and DNA damage play an important role in the pathogenesis of lifestyle-related diseases (Lesgards et al 2002) and relevant levels of antioxidants and an efficient repair capacity are important protective factors against disease (Slater 1994). Lifestyle-related diseases like obesity, metabolic syndrome, type 2 diabetes and cardiovascular disease (CVD) are diseases where diet and oxidative stress can have a significant impact on the onset and the progression of the disease. Lifestyle modifications can be effective in delaying or preventing the development of the metabolic syndrome (Zhu et al. 2004, Foreyt 2006), type 2 diabetes (Barrera et al. 2006), CVD (Lesgards et al. 2002) and also cancer (Mamede et al. 2011, Greenberg et al. 2013). In Swedish studies with participants at risk of developing CVD and type 2 diabetes, positive health effects have been seen in response to lifestyle intervention. Reductions of weight and blood pressure (Sjöström et al 1999), decrease of body weight, blood lipids and lower rates of all coronary events (Sundin et al. 2003, Lisspers et al. 2005), improved blood lipid profile, insulin sensitivity and blood pressure values (Adamsson et al. 2010) and a significant decrease of oxidative DNA damage (Åsgård et al. 2008) have been reported. Diet is a lifestyle factor that can be changed on an individual basis, in contrast to genes and environmental factors.

General aims in his thesis were to examine if antioxidants from the diet or from oral supplements could affect biomarkers of oxidative stress and DNA damage in type 2 diabetes subjects, and also to study effects from β-carotene and α-tocopherol, two antioxidants present in fruits and vegetables, on oxidative stress and DNA damage induced by pro-oxidants in mouse lymphoma cells. Oxidative stress-related mechanisms and potential threshold mechanisms behind the mutagenesis of two aromatic amines were also studied in the same cell system.
Oxidative stress

Reactive oxygen species

Oxidative stress is an imbalance between oxidative pressure and efficiency of the defence system (antioxidant capacity and DNA repair). Reactive oxygen species (ROS) are common denominators for radicals and non-radicals involved in oxidation of biological molecules (Table 1). In living organisms, induction of ROS makes antioxidants become active (Halliwell and Gutteridge 1990).

Table 1. Examples of reactive oxygen species (ROS), radicals and non-radicals.

<table>
<thead>
<tr>
<th>Radicals</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide, ( \cdot O_2^- )</td>
<td>Hydrogen peroxide, H(_2)O(_2)</td>
</tr>
<tr>
<td>Hydroxyl, ( \cdot OH )</td>
<td>Hypochlorous acid(^a), HOC(_)I</td>
</tr>
<tr>
<td>Peroxy, ( \cdot RO_2 )</td>
<td>Ozone, O(_3)</td>
</tr>
<tr>
<td>Alkoxy, ( \cdot RO )</td>
<td>Singlet oxygen, ( ^1 )O(_2)</td>
</tr>
<tr>
<td>Hydroperoxy, ( \cdot HO_2 )</td>
<td>Peroxynitrite(^b), ONOO(^-)</td>
</tr>
</tbody>
</table>

\(^a\)Can also be called reactive chlorinated species, \(^b\)Can also be called reactive nitrogen species

Radicals can be defined as any species containing one or more unpaired electrons. As such, they are very reactive and important agents in lipid-, protein- and DNA-oxidation (Halliwell and Gutteridge 1999). In addition, non-radicals can also induce these oxidative processes. In Table 1 examples of ROS are shown. Neither superoxidium anion \( \cdot O_2^- \) or hydrogen peroxide \( H_2O_2 \) are particularly reactive in aqueous solution, but in other media they can act as either oxidising or reducing agents. The highly reactive \( \cdot OH \) can be generated in biologically relevant systems by multiple reactions. Two such examples are the Fenton reaction (Figure 1) and UV-induced hemolytic fission of the O-O bond in H\(_2\)O (Halliwell and Gutteridge 1999). Transition metals, such as iron and copper, will increase oxidation through the Fenton reaction (Halliwell and Gutteridge 1999). In the Fenton reaction \( (M^{2+} + H_2O_2 \rightarrow M^{3+} + OH^- + \cdot OH) \) the highly reactive hydroxyl radical \( \cdot OH \) is produced from the conjunction of a transition metal ion (e.g. Fe\(^{2+}\), Cu\(^{+}\)) and H\(_2\)O\(_2\).
Figure 1. Schematic presentation showing formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) in the human metabolism. Oxygen ($O_2$) forms superoxide anion (‘$O_2^-$‘). In presence of nitrogen oxide (NO), peroxynitrite (ONOO$^-$) can be formed. Superoxide dismutase (SOD) can utilise superoxide anions to form hydrogen peroxide ($H_2O_2$). In the presence of iron or copper, $H_2O_2$ can form hydroxyl radicals (‘OH) through the Fenton reaction, or otherwise will undergo enzymatic conversion to water and oxygen.

Generally, increased free radical formation accelerates DNA damage whereas ‘$O_2^-$’, nitrogen oxide (NO) or $H_2O_2$, at physiologically relevant levels, do not react with any of the DNA or RNA bases, or with the ribose or deoxyribose sugars, at significant rates (Halliwell and Gutteridge 1999). The highly reactive OH‘ reacts with organic compounds, capable of adding to double bonds of heterocyclic DNA bases and abstracting H atoms from the methyl group of thymines or from each of the C-H bonds of 2’-deoxyribose. Further reactions of C- or N-centred radicals of DNA bases and C-centred radicals of the sugar moiety will give a variety of final products (Evans et al. 2004).

Exposure of DNA to the ‘OH radical, generates a multitude of products, since it attacks sugars, purines and pyrimidines (Breen and Murphy 1995). ‘OH can add to guanine at position 4, 5 and 8 in the purine ring. Addition to C-8 produces a C-8 OH-adduct radical that can be reduced to 8-hydroxyguanine, or undergo opening of the imidazole ring, followed by one-electrone reduction and protonation, to give FAPyG, another biomarker for oxidative DNA damage (Breen and Murphy 1995). Type 2 diabetes patients have been shown to have an increased formation of ‘OH attacking DNA, but no increase in damage to DNA by hypochlorous acid (HOCl) or peroxynitrite (ONOO$^-$) was seen (Rehman et al. 1999).

Induction of ROS, induce oxidative stress which might increase inflammation and further progression of lifestyle related disease. Mutations in the DNA will sometimes be a result of this process, which might lead to apoptosis, non-functional proteins or even carcinogenesis.
General and oxidative DNA damage

General DNA damage, as measured by the alkaline comet assay, consists of double-strand breaks, single-strand breaks and alkali-labile sites. Oxidative DNA damage, as measured by the alkaline comet assay with addition of DNA repair enzymes, is described as repair products from oxidative DNA lesions. Induction of ROS will increase both general (Tice et al. 2000) and oxidative (Smith et al. 2006) DNA damage. Radical-induced DNA damage includes a large variety of mechanisms and final products (Dizdaroglu and Jaruga 2012). Antioxidants reduce mainly oxidative (Lowe et al. 1999) but also general (Singh et al. 2008) DNA damage. An inter-laboratory validation of measurement of the oxidative DNA damage product 8-oxodG in human lymphocytes, as measured with HPLC, showed that the median concentration was 4.24 per 10^6 guanines, and that the levels were elevated during oxidative stress (Gedik et al 2005). Because of this a large amount of blood is needed for analyses of 8-oxodG with HPLC in mononuclear blood cells from humans. In contrast, in the alkaline comet assay a very small amount of blood is needed for detection of oxidative DNA damage, either in mononuclear blood cells from humans or in various cultured cells. This was one of the reasons for choosing comet assay in the studies of this thesis.

Repair of oxidative DNA damage

DNA-repair enzymes will cut out oxidised DNA bases and replace them with undamaged equivalents to avoid further mutations. Oxidised DNA base lesions are removed by two types of enzymatic activities: base excision repair (BER) involving removal of single lesions by a glycosylase action; or nucleotide excision repair (NER), a process involving removal of a lesion-containing oligonucleotide (Cooke et al. 2003).

DNA strand-breaks and alkali-labile sites can be determined by the comet assay protocol without DNA repair enzymes. Single strand break (SSB) rejoining takes a few minutes, while repair of Double strand breaks (DSB) takes more than 1 h, and repair of oxidised bases is more difficult to study (Collins et al. 1995). Human lymphocytes are less sensitive to damage by H_2O_2, but less efficient at removing the damage compared with HeLa cells, and endonuclease was not able to repair oxidative damage at all in human lymphocytes (Collins et al. 1995).

With the addition of DNA repair enzymes oxidative DNA damage can also be measured. Formamidopyrimidine glycosylase (FPG) (Paper I-II) primarily removes 8-oxodG, 8-oxoA, 8-OH-Gua, fapy-Gua and fapy-Ade (Boiteux et al 1992, Tchou et al 1994), but can possibly also remove a number of other oxidised purines and pyrimidines. Because of this broad detection of oxidised bases, addition of FPG in the comet assay can detect a broad measurement of oxidative DNA damage.
The DNA repair enzyme human oxoguanine DNA-glycosylase (hOGG1) (Paper III and V) mainly removes 8-OH-Gua, 8-oxodG and fapy-Gua (Boiteux and Radicella 2000), with a higher specificity than FPG (Smith et al. 2006). hOGG1 participates in base excision repair (BER) (Evans et al. 2004). 8-oxodG is a well-studied oxidative DNA damage product, produced when ROS react with deoxyguanosine (dG) (Kasai and Nishimura 1983) and that is rather specifically removed by the hOGG1 enzyme (Michaels et al 1992). Therefore addition of hOGG1 in the comet assay can allow detection of 8-oxodG. This was also used in papers III and V for the detection of oxidative DNA damage.

Repair of oxidised bases in the nucleotide pool

8-oxo-dG is the most common oxidatively base modification by reaction of `OH on C8 position of dG (Kasai and Nishimura 1983) in DNA or free guanine in the nucleotide pool (Hayakawa et al. 1995). The free guanine nucleotide in the nucleotide pool is more prone for `OH -attacks compared to dG in DNA (Svoboda and Harms-Ringdahl 1999), and much of biologically relevant oxidations occur in the nucleotid pool (Russo et al. 2004). Repair mechanisms in mammalian cells works through 8-Oxo-dGTPase recognising and dephosphorylating 8-oxo-dGTP to 8-oxo-dGMP and prevents incorporation to DNA. 8-oxo-dGMP is changed into 8-oxo-dG and is exported out of the cell where it can be used as a biomarker for oxidative stress (Hagdoost et al. 2006).

General and oxidative DNA damage as biomarkers for disease

Since antioxidants have shown to protect against general and oxidative DNA damage, and antioxidants also have shown to protect against disease in epidemiological studies, general and oxidative DNA damage are traditionally assumed to be biomarkers for disease. To actually prove that general and oxidative DNA damage are biomarkers for disease are however very difficult. For example, DNA damage was previously accepted as a consequence of, and marker for, the development and progression of atherosclerosis, a common consequence of type 2 diabetes or CVD, in epidemiological studies (Malik and Herbert 2012). However, recent observations that high blood glucose and angiotensin II levels induce oxidative DNA damage in human vascular cells in vitro revise the old picture, suggesting that DNA damage is an active promoter of atherosclerosis (Malik and Herbert 2012).

When studying oxidative DNA damage, one must consider that a great deal of work remains to define the exact roles of oxidative DNA damage in the pathogenesis of disease (Cooke et al. 2003). The oxidative DNA damage in diabetes might contribute to the pathogenesis of the complications from the diabetes, and 8-oxodG in urine and mononuclear cells serves as a useful
biomarker for the evaluation of oxidative stress in type 2 diabetes patients (Hinokio et al. 1999). Patients with type 2 diabetes have significantly higher levels of oxidative DNA damage, measured as 8-oxodG in mononuclear cell DNA, muscle DNA and in the urine compared to control subjects (Evans et al. 2004). Appropriate levels in the cells of antioxidants and antioxidant enzymes are suggested to protect from induction of oxidative DNA damage in these patients, which might otherwise promote further disease or even result in increased frequency of mutations and carcinogenesis.

Lipid peroxidation

Lipid peroxidation has been defined by A.L. Tappel as “the oxidative deterioration of polyunsaturated lipids” (Halliwell and Gutteridge 1999). Lipoproteins and fatty acids are targets of oxidative stress. Initiation of lipid peroxidation is caused by the attack on lipids of any species that can abstract a hydrogen atom from a methylene (=CH₂) group. Polyunsaturated fatty acids (PUFAs) are sensitive to these attacks. The membranes that surround cells and cell organelles contain large amounts of PUFAs side chains and the major constituents of biological membranes are lipids, such as lecithin, cholesterol and phospholipids. Peroxidation of linoleic acids leads to two hydroperoxides. Peroxidation of arachidonic acid will lead to six lipid hydroperoxides as well as cyclic peroxides and other products, including the isoprostanes (Halliwell and Gutteridge 1999). Dietary antioxidants, such as α-tocopherol and β-carotene, can prevent lipid peroxidation through mechanisms such as scavenging or quenching (Sies 1992).

A number of biomarkers are available to quantify lipid peroxidation, among them 8-iso-PGF₂α, an oxidation product of prostaglandins. Isoprostanes are prostaglandin derivatives mainly formed by peroxidation of arachidonic acid catalysed by free radicals. Free or total 8-iso-PGF₂α can be quantified with different analysis techniques including gas chromatography-mass spectrometry, liquid chromatography, enzyme immunoassays and radioimmunoassay, the latter used in Paper I and II for detection of 8-iso-PGF₂α (Basu 1998b).

Protein oxidation

Proteins in our bodies can also be oxidised which may lead to an increased oxidative stress. The concentration of plasma nitrotyrosine can be measured by an immunological assay as a biomarker for oxidative stress in proteins (Bioxytech, OxisResearch).
Antioxidants

Dietary antioxidants

Antioxidants are substances able to decrease oxidation of DNA, lipids and proteins. One common mechanism is scavenging of ROS, which means searching for and inactivating ROS (Sies 1992). Another mechanism is quenching, by suppressing the oxidative action of singlet oxygen (Sies 1992). Fruits, berries, vegetables, nuts and seeds are rich in antioxidants, such as α-carotene, β-carotene, lycopene, lutein, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol, tocotrienols, ascorbic acid, selenium, anthocyanidines, flavanones, flavones, quinic acid and cynarin. Intake of foods rich in these antioxidants may decrease oxidative damage to DNA, lipids or proteins and thereby prevent the onset of and the progression of various diseases, such as type 2 diabetes (Loft and Paulsen 2000).

Previous studies on healthy individuals have shown that a high intake of fruits and vegetables can decrease oxidative DNA damage as measured by the alkaline comet assay (ESCODD 2002a, Hofer et al. 2006). Consumption of oral antioxidant supplements has become popular, and these are assumed to decrease oxidative stress. However, there is no clear proof that oral supplements of antioxidants will reduce lifestyle-related diseases in humans. Surprisingly, antioxidant-induced pro-oxidative effects have also been reported in large clinical studies such as The Alpha-Tocopherol, Beta-Carotene cancer prevention study (ATBC) and the Carotene And Retinol Efficacy Trial (CARET) (The ATBC group 1994, Omenn et al. 1996).

It is well known that fruits and vegetables are beneficial for health (Becker and Hagman 1999), but the intake of fruits and vegetables in Sweden is on average lower than the recommendations. The average intake is 362 g/person/day for females and 311 g/person/day for males (Riksmaten-vuxna 2010-2011); compared to the recommended 500 g/person/day (NNR 2004). The positive health effects from fruit and vegetable intake is probably due to the high content of micronutrients, such as vitamins and minerals. Some of the vitamins are considered to be antioxidants, protecting us from oxidative stress, and thereby protecting us against lifestyle related diseases.

Effects of dietary antioxidants on type 2 diabetes

Consumption of foods rich in antioxidants, can favourable influence markers of oxidative stress in type 2 diabetes subjects, whereas no clear association is seen with oral antioxidant supplement consumption (Stocker 1999, Mann et al 2004). Intake of antioxidants from the diet has been proposed to be part of the strategy to prevent the complications from type 2 diabetes (Jialal et al. 2002, Scott and King 2004, Segal 2004, Davi et al. 2005). Based on results in the literature, antioxidants is a potential preventative treatment in metabol-
ic syndrome patients before the onset of diabetes (King and Loecken 2004). On the other hand, advice against the use of antioxidants upon fully-developed diabetes is also available in the literature (King and Loecken 2004).

β-Carotene

Figure 2. Chemical structure of β-carotene

β-Carotene (Pro-vitamin A) is the vitamin in vegetables (carrots, paprika and pumpkins) and fruits (mangoes and papayas) that give them their yellow, orange or red colours. It is also used as a food additive (E160a). It is present in lipoproteins and in cellular membranes, and works through scavenging of ROS by searching for and inactivation of the radicals. It also functions as a physical quencher by suppressing the oxidative action of singlet oxygen (Sies 1992). Plasma levels of β-carotene in humans are on average 0.3 - 0.6 µM, while total carotenoid levels on average are 1.0 - 2.1 µM (Sies et al. 1992). The plasma level of β-carotene has been shown to be a good biomarker for fruit and vegetable intake (Tucker et al. 1999, Block et al. 2001).

Intake of the vitamin via fruits and vegetables has indicated positive health effects in several human studies (Ringer et al. 1991, Holick et al. 2002, Gaede et al. 2003, Mann et al. 2004, Guenegou et al. 2007). Antioxidant supplementation with β-carotene has shown positive effects in some human studies (Herraiz et al. 1998, Correa et al. 2000, Torbergsen and Collins 2000) but no effects in others (McLarty et al. 1995, Hennekens et al. 1996). In contrast, adverse effects were detected in male smokers and in asbestos workers who were supplemented with β-carotene - with increased incidence of lung cancer or cardiovascular deaths as a result (The ATBC group 1994, Omenn et al. 1996). Both the ATBC and the CARET studies were interrupted earlier than planned due to ethical aspects and, for the same reason, they cannot be repeated.

In vitro studies on β-carotene have shown equivocal results. In some studies the vitamin was acting as an antioxidant (DiMascio et al. 1991, Tsuchihashi et al. 1995, Miller et al. 1996, Weitberg and Corvese 1997, Woods et al. 1999, Muzando et al. 2005) and in other studies it was shown to act as a
pro-oxidant (Lowe et al. 1999, Woods et al. 1999, Murata and Kawanishi 2000, Bergström et al. 2011). It is dependent of the conditions in the system examined whether β-carotene gains antioxidant properties or not (Pryor et al. 1993). These contradictory results make it difficult to know if β-carotene is an antioxidant or not in humans.

α-Tocopherol

α-Tocopherol is the main source of vitamin E, in both food (mainly olive- and sunflower oils, but also in nuts, seeds as well as in fruits and vegetables) and oral supplements. It is used as an antioxidant additive in foods with the name E307. The human plasma level of α-tocopherol is 15 - 40 µM (Sies et al. 1992). Like β-carotene it is present mainly in lipoproteins and in cellular membranes. The vitamin is a scavenger of lipid peroxidation radicals, either through destruction of peroxyl radicals or through blocking the production of hydroperoxides from singlet oxygen (Ingold et al. 1987, Wang et al. 2001, Elmadfa and Wagner 2003), as shown in figure 1.

Some human studies demonstrate that vitamin E supplementation can prevent heart infarction (Stephens et al. 1996, Boaz et al. 2000) while others showed no effect (The ATBC study group 1994, Yusuf et al. 2000). By preventing oxidative stress, vitamin E was shown to be effective against atherosclerosis in a mouse model (Pratico et al. 1999). In contrast, in the absence of a co-antioxidant such as vitamin C, α-tocopherol might act as a pro-oxidant (Stocker 1999).

α-Tocopherol supplementation has beneficial effects on biomarkers of oxidative stress and inflammation in type 2 diabetes patients (Wu et al. 2007), and has been suggested as an additional therapy in diabetes (Jialal et al. 2002). Therapeutic use of the dietary α-tocopherol, especially at high doses, clearly shows a benefit against oxidative stress (LDL-oxidation and isoprostanes) and decreases inflammatory markers (CRP, IL-6) (Jialal et al. 2002).

When it comes to prevention of coronary heart disease (CHD), a meta-analysis of trials with vitamin E, did not show any benefits from the vitamin (Vivekananathan et al. 2003). In the HOPE Study around 10 000 patients who were at high risk for heart attack or stroke were followed for 4.5 years. The
subjects received 265 mg (400 IU) of vitamin E per day. No difference could be seen in cardiovascular events or hospitalisations for heart failure or chest pain compared to the control group (The HOPE study investigators 2000). However, a decrease in incidence of prostate cancer and in deaths from prostate cancer after supplementation with 50 mg α-tocopherol for 5-8 years in male smokers has been shown (Heinonen et al. 1998). This is an interesting finding, in contrast to the increased incidence of lung cancer, observed in the previous mentioned ATBC study and CARET study (The ATBC group 1994, Omenn et al. 1996), after supplementation with β-carotene.

One human study showed that an intake of food rich in α-tocopherol could decrease levels of DNA adducts (Ragin et al. 2010) and supplementation with the vitamin was shown to reduce oxidative stress (McCall and Frei 1999, Winklhofer-Roob et al. 2003) and even lower the risk for cardiovascular disease end-points (Stephens et al. 1996, Boaz et al. 2000). Numerous other human studies, did not show any effects on DNA damage or cardiovascular events of α-tocopherol (Fenech et al. 1997, The HOPE study investigators 2000, Sampson et al. 2001, Giovannelli et al. 2002, Elmadfa and Wagner 2003, Wollard et al. 2006, Sesso et al. 2008, Johansson et al. 2010, Müllner et al. 2013a) while one study showed an increase of oxidative stress from the vitamin (De Oliveira et al. 2012).


Lifestyle related diseases

Obesity and metabolic syndrome

Weight gain, excessive overweight and obesity are related to the lifestyle. Being overweight may lead to metabolic imbalances that induce metabolic syndrome. Overweight subjects have a body mass index (BMI) > 25 kg/m², and Obese subjects have a BMI > 30 kg/m². At the stage of metabolic syndrome, often with a high waist circumference, high blood pressure and high levels of glucose and lipids in the blood, lifestyle changes are even more important to prevent the onset of type 2 diabetes or CVD. Here dietary factors can be of importance in order to prevent oxidative stress which would accelerate the risk of disease. Studies showing associations between obesity, metabolic syndrome and type 2 diabetes and also with oxidative stress are shown in table 2.
Table 2. Studies showing that obesity will increase risk for metabolic syndrome which will increase risk for type 2 diabetes.

<table>
<thead>
<tr>
<th>Lifestyle-related disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>McLaughlin et al. 2004, Wannamethee et al. 2004</td>
</tr>
<tr>
<td>Obese subjects have an increased risk to develop type 2 diabetes</td>
<td>Cotran 1994</td>
</tr>
<tr>
<td>Obesity is associated with increased risk of CHD, stroke, type 2 diabetes, hypertension, dyslipidemia, musculoskeletal disorders and some cancers</td>
<td>Vega et al. 2006</td>
</tr>
<tr>
<td>A high energy intake from diet together with a low level of physical activity can increase weight and therefore lead to obesity and insulin resistance</td>
<td>Siddarth 2013, Robertson 2004</td>
</tr>
<tr>
<td>Obesity is associated with high levels of oxidative DNA damage and high levels of lipid peroxidation</td>
<td>Olusi 2002, De la Maza et al. 2006, Hofer et al. 2006, Vincent and Taylor 2006</td>
</tr>
</tbody>
</table>

Metabolic Syndrome

A high waist circumference and insulin resistance, will lead to high blood pressure, hyperlipidemia and high levels of insulin and glucose in the blood - which will cause an increased oxidative stress | Dekker et al. 2005 |

A high waist circumference together with two of the following - ↑triglycerides, ↑blood pressure, ↑fasting glucose, ↓HDL-cholesterol; are required to fulfill the IDF criteria for metabolic syndrome | Alberti et al. 2006 |

Effective lifestyle changes will reduce the metabolic risk factors, and therefore delay or prevent metabolic syndrome and lipid peroxidation | Zhu et al. 2004, Grundy et al. 2005, Foreyt 2006, Helmersson et al. 2004 |

Changes towards a more healthy diet, with more fruits and vegetables, and an increased level of physical activity during 17 days with two follow-up during 6 months, can decrease weight, waist circumference, body fat, and oxidative DNA damage, and also increase HDL-cholesterol, in metabolic syndrome patients | Åsgård et al. 2008 |

Obesity has been associated with a higher risk of colorectal-, pancreatic-, esophageal-, liver-, breast-, and renal cancer in epidemiological studies. | Forte et al. 2012 |

CVD = Cardio vascular disease, CHD = Cardiac heart disease, HDL = High-density lipoprotein, IDF = International diabetes federation.
Type 2 diabetes

Type 2 diabetes is a disease with insulin deficiency where subtle defects in the function of beta cells in the pancreas can be demonstrated, together with high blood glucose levels. Insulin resistance is often the second step in the disease, involving both impaired insulin release and end-organ insensitivity towards insulin. Insulin resistance, frequently associated with obesity, produces excessive stress on beta cells (Cotran 1994). Lifestyle modifications, such as diet and physical activity, can prevent the disease, or delay the development of diabetes (Barrera et al. 2006). Patients with type 2 diabetes have an elevated risk for oxidative stress and inflammation (Baynes 1991, Helmersson et al. 2004, Colak et al. 2005), a low capacity of antioxidant protection (Maxwell et al. 1997, Ruhe and McDonald 2001) and hence have increased levels of oxidative DNA damage (Leinonen et al. 1997, Hinokio et al. 1999, Dincer et al. 2002, Blasiak et al. 2004).

Type 2 diabetes is mainly characterised by high glucose levels in the blood. The high levels of glucose initiate formation of glycated proteins, glucose oxidation and increased fatty acids (Davi et al. 2005) which is important in the pathogenesis of diabetes complications (Ceriello 1997, Smit and Lutgers 2004). Positive correlations between levels of blood glucose and oxidative DNA damage in type 2 diabetes patients have been shown, and also with lipid peroxidation (Hinokio et al. 1999, Dincer et al. 2002, Robertson 2004, Song et al. 2007, Ryttter et al. 2009). Generation of ROS, largely due to hyperglycemia, will cause oxidative stress, and will continue to damage the insulin-producing cells (La Selva et al. 1993, Paolisso and Guigliano 1996, Ceriello 2000, King and Loeken 2004) and hasten the development and progression of diabetes and its complications (Schultz Johansen et al. 2005). It has previously been shown that glucose metabolism is related to oxidative stress (Ceriello 1997, Packer 2001). Furthermore, dietary intake of α-carotene, β-carotene and lycopene, as well as plasma β-carotene concentrations has been demonstrated to have beneficial associations with glucose metabolism in subjects at high risk of type 2 diabetes (Ylönen 2003).

Fruit and vegetable intake reduce the risk for type 2 diabetes, especially with a diet rich in root vegetables and green leafy vegetables (Cooper et al. 2012), and dietary intake of an antioxidant-rich diet (i.e. high in vegetables and PUFA-rich plant oil) in type 2 diabetes subjects has been shown to reduce DNA damage as measured by the alkaline comet assay (Müllner et al. 2013a), but not genome damage as measured by the micronucleus assay (Müllner et al. 2013b). Also, lower levels of DNA methylation resulting from increased DNA repair can be seen after regular intake of this diet (Switzeny et al. 2012). Antioxidants have been suggested for the treatment of diabetes (Schultz Johansen et al. 2005) and it seems appropriate to recommend subjects with diabetes type 2 foods rich in antioxidant nutrients. However, more conclusive evidence is required before the beneficial health
effects of oral antioxidant supplements upon diabetes can be robustly asserted (Mann et al. 2004).

As an indirect measure of an onset of oxidative stress, inflammatory markers, such as Interleukin-6 (IL-6), C-reactive protein (CRP), and 15-keto-dihydro-PGF\(_{2\alpha}\) can be measured as markers of ongoing inflammation. IL-6 is a commonly used biomarker for inflammation and also 15-keto-dihydro-PGF\(_{2\alpha}\), a metabolite of an oxidised prostaglandin, could be used as a biomarker for inflammatory response \textit{in vivo} (Basu 1998a). IL-6 is mainly formed by monocytes and macrophages and can start a systemic response to a local inflammatory damage (von der Thusen et al. 2003). This can result in production of acute phase proteins in the hepatocytes (e.g. CRP), that are excreted into the circulation and induce a systemic inflammation. Increased inflammation in diabetic subjects includes increased monocyte superoxide and pro-inflammatory cytokine release (IL1, IL-6, and TNF-\(\alpha\)), increased monocyte adhesion to endothelium and increased levels of CRP in plasma (Jialal et al. 2002).

**Cancer**

Since antioxidants, present in the diet, can decrease oxidative stress, and oxidative stress is an important risk factor not only in metabolic diseases, but also in other lifestyle-related diseases such as cancer, oral antioxidant supplements also have been suggested for prophylactic use against cancer (Mamede et al. 2011, Greenberg et al. 2013). It is known that higher levels of antioxidants in diet and in plasma are associated with lower risk of several kinds of cancers (Mamede et al. 2011, Aune 2012, Liu 2013). Some studies have associated oral antioxidant supplements with a lower risk of cancer, but it remains that no strong evidence establishes the usage of vitamin supplements in the prevention of cancer (Mamede et al. 2011, Misotti and Gnagnarella 2013, VANCE et al. 2013), and more studies are needed to clarify the mechanisms of action behind these effects (Mamede et al. 2011).

**Genetic aspects in relation to antioxidants, oxidative stress and lifestyle related diseases**

In respect to oxidative DNA damage the protective effects of antioxidants have been proved in various cases. Therefore, further research into antioxidant sources, levels, mechanistic pathways and kinetics of repair, as well as the interpretation of the expression of corresponding repair and antioxidant genes, is warranted (Loft and Paulsen 2000). Genetic variation in human genes is also known to relate to oxidative stress; underscoring that the study of genetic variation might be a useful approach in determining the impact of
oxidative stress in disease and ageing (Forsberg et al. 2001). For example, glutathione transferase P gene variants correlate to oxidative DNA damage; demonstrating that a genetic polymorphism has a measurable impact on a biomarker related to oxidative stress (Forsberg et al. 2001).

Cardiovascular events are associated with paraoxonase gene variants in subsets of diabetic subjects. Furthermore, it is known that some variants of the paraoxonase gene codes for proteins that can protect LDL-cholesterol from oxidation (Forsberg et al. 2001). Whilst it has been suggested that genotype and not diet is the predominant determinant of oxidative DNA damage (Halliwell 2002), established evidence of the impact of dietary antioxidants in the prevention of obesity and type 2 diabetes is sufficient to warrant further research. In this thesis though, we did not include any genetic aspects in the research. In further studies it would be interesting to also include relevant biomarkers to study genetic aspects.
Research Aims

General aim
The general aim of this thesis was to evaluate the effects and mechanisms of antioxidants with regard to (1) their role as biomarkers for protection against oxidative stress in type 2 diabetes subjects, and (2) their role in the genotoxicity induced by mutagenic pro-oxidants in mouse lymphoma cells. Several methods have been used, but the main technique was measurement of general and oxidative DNA damage using various versions of the alkaline comet assay, with or without addition of DNA repair enzymes.

Specific aims
- To study the influence from intake of fruits, vegetables and antioxidants from the diet on biomarkers for oxidative stress in type 2 diabetes subjects (Paper I).
- To identify useful biomarkers for fruit and vegetable intake in humans (Paper I).
- To determine the influence of antioxidant supplementation on biomarkers for oxidative stress in type 2 diabetes subjects (Paper II).
- To examine protective and hazardous effects from a physiological relevant concentration of β-carotene on general and oxidative DNA damage induced by catechol in mouse lymphoma cells (Paper III).
- To determine the effects of a physiologically relevant concentration of α-tocopherol on DNA damage induced by three pro-oxidants in mouse lymphoma cells (Paper IV).
- To evaluate if the less hazardous DNA-dye GelRed could replace ethidium bromide in the comet assay (Paper IV).
- To study the involvement of oxidative stress (intracellular oxidative stress, general and oxidative DNA damage, and effects on the nucleotide pool) as an explanation for the mutagenicity of two aromatic amines (Paper V).
Materials and methods

The main focus in this thesis has been to measure general and oxidative DNA damage, oxidative stress and mutations, as well as to measure dietary intake of and plasma levels of antioxidants. In all studies included in this thesis, the alkaline comet assay has been the main method and the three different protocols of this assay (Table 3) are extensively described below. Other methods of relevance for the results and discussion are listed in Table 4 and Table 5. The reader is referred to the material and method section in the specific papers for comprehensive details of methods, others than comet assay.

Paper I and II include results from a clinical intervention study on type 2 diabetes subjects, with co-workers from five affiliations. Many methods were used in this study to evaluate the effects on oxidative stress and inflammation following intake of antioxidants from diet or from oral supplements. The effects from β-carotene and α-tocopherol on general and oxidative DNA damage induced by mutagenic pro-oxidants in cultured cells were evaluated in Paper III and IV. Finally, Paper V was a co-operation between three affiliations, and describes the effects of oxidative stress-related mechanisms upon the mutagenicity of two pro-oxidants.

Comet assay (Paper I-V)

An assay of general and oxidative DNA damage

Single cell gel electrophoresis (comet assay) was created by Östling and Johanson (Östling and Johanson 1984), with pH adjusted to 9.5, well below DNA unwinding pH, making it possible to detect double-strand breaks. It was shown to be applicable to cell cultures undergoing a certain treatment with toxic substances or irradiation, but they also showed that DNA damage in cell samples from humans undergoing certain treatments was possible to detect (Östling et al. 1987). 1988 was the start of the alkaline comet assay, where DNA unwinding and also electrophoresis in an alkaline solution (pH >13) was used (previously pH 9.5 was used), making it possible to detect not only double-strand breaks, but also single-strand breaks and alkali labile sites, as a biomarker for DNA damage (Singh et al. 1988).
General DNA damage is measured without addition of enzymes and consists of strand breaks and alkali-labile sites (Tice et al. 2000). Addition of DNA-repair enzymes, like FPG or hOGG1, is performed to add oxidative DNA damages to the general damages and thereby making it possible to also detect oxidative stress in DNA (Collins et al. 1997, Smith et al. 2006). The critical steps in all variants of the method are described in Figure 4. After mixing the cells with agarose and adding them to a microscope slide, lysis of the cells is performed in a high saline solution. After that DNA-repair enzymes such as FPG or hOGG1 are added, or a protocol without enzymes is performed. DNA-unwinding is then performed in a high alkali solution, following electrophoresis in the same solution. During the electrophoresis, shorter strands of DNA are dragged out of the “head”, wandering towards the anode forming a comet “tail”. While the “tail” consists of damaged DNA, the “head” consists of undamaged DNA. Staining of DNA with EtBr or GelRed makes it possible to detect the percentage fluorescence in the tail, out of the fluorescence of the whole comet (Figure 4).

Further development of the comet assay method

Generally, the alkali comet assay as a method to measure DNA damage in eukaryotic cells has stepwise been improved since it was first developed, and DNA repair enzymes have also been added in the method. The comet assay has been and is developed continuously and therefore the protocols in this thesis differ between the different papers. Large inter-laboratory validation studies have been performed. Two examples of this are ESCODD and ECVAG. ESCODD consisted of 27 European laboratories performing inter-laboratory validations (ESCODD 2002b, Riis 2002). In ESCODD collaborations were set up to examine background levels of 8-oxodG (an oxidative DNA damage) using the comet assay and HPLC with electrochemical detection (Gedik et al. 2005). ECVAG consisted of 14 laboratories performing comet assay validation studies (Forchhammer 2010, Johansson 2010). Ersson and co-authors have reported factors with an effect on DNA migration in the comet assay (Ersson et al. 2011).

One conclusion from ESCODD and ECVAG was that it is sometimes difficult to compare and repeat results between different laboratories. The consensus was that each laboratory should optimise the protocol to get reproducible results. Hence main comparisons should be performed within the same study in the same laboratory, and it is important to do properly designed studies with triplicates and three repeated experiments. During drug development though, within the pharmaceutical industry, it is common to do triplicate but only one experiment in the standard genotoxicity testing of drug candidates. Here the protocols have been optimised for running large volumes and the comet assay is always compared to other tests performed at the same time on the same cells or animals.
Figure 4. The alkaline comet assay protocol, the critical steps and what happens with cells and DNA. When the cells are mixed with agarose and added to a microscope slide, the cell will form a hemisphere in the agarose (1). At the lysis of the cell in high saline solution (2), the cell membrane is lysed and the cell organelles and matrix are rinsed out of the cell. The nucleus membrane is also lysed and the DNA alone will after this step fill up the hemisphere (nucleoid). At step 1-6 in the figure the DNA is coloured green. Running the standard alkaline comet assay protocol, step 3 and 4 are excluded. Running the FPG- or the hOGG1-protocol, the slides are incubated in enzyme buffer (3) and after that incubated with either enzyme buffer or FPG-enzymes or hOGG1-enzymes (4). In this step the enzymes (yellow) will cut out oxidised DNA bases, adding oxidative DNA damage to the general damage at the formation of the comets in the electrophoresis step. DNA unwinding is performed in high alkaline buffer (5) at pH >13, where the supercoiled DNA-strands are unwound from the histones and free DNA-strands are further treated with electrophoresis in the same high alkaline buffer (6). During the electrophoresis short DNA-strands created from single-strand breaks, double-strand breaks or strand breaks at alkali-labile sites are transported out from the “nucleoid” towards the anode (+), while undamaged long DNA-strands remains inside the “nucleoid”. The cells with damaged DNA will form a “comet” with a “head” consisting of undamaged DNA and a “tail” consisting of damaged DNA. The cells with no damaged DNA will simply form a “head” with no “tail”. Staining of the DNA in ethidium bromide or GelRed gives the DNA a red colour (7). The fluorescence intensity of the tail, compared with the head, is detected with a fluorescence microscope (8) and analysed with an image analysis program. The percentage damaged DNA out of the total DNA is expressed as % Tail Intensity. When using the protocols with addition of FPG- or hOGG1-enzymes the oxidative DNA damage can be calculated by subtraction of the general DNA from the total DNA damage.
Discussion of the comet assay protocols

Three different protocols for the alkaline comet assay were used in this thesis (Table 3). In Paper I and II lymphocytes and monocytes were isolated from blood, taken from type 2 diabetes patients before and after oral antioxidant supplementation, alkaline comet assay with FPG-enzymes was used for the frozen human blood cells, which were carefully thawed and directly added to slides. Here EtBr was used for staining DNA.

The alkaline comet assay without enzymes was used in paper IV and with hOGG1-enzymes in paper III and V. The protocols in Paper III-V follow the guidelines for in vitro SCG (comet assay) suggested by the expert panel of the International Workshop on Genotoxicity Test Procedures (IWGTP), which has been used within the pharmaceutical industry for many years (Tice et al. 2000). Following this protocol the cells were thawed and cultured for four days, followed by a 3h treatment with a mutagen before analysis via the comet assay. In the present studies EtBr was used for staining of the DNA, and in Paper IV replacement of EtBr with the less hazardous dye GelRed was evaluated.

There are differences between the FPG-protocol and the other two protocols when it comes to handling, times, some solutions and the times for the denaturation and the electrophoresis. Previous studies have shown that the time of the denaturation in a high alkali solution and also the time for the electrophoresis in the same solution is of importance for the result. An optimisation of the denaturation time to 20-40 min has been found to be enough to unwind the DNA and prepare the DNA for the electrophoresis step. Previous results indicate that not all alkali labile sites have been converted to strand breaks after 20 or 40 min of denaturation (Ersson and Möller 2011). The FPG-protocol in this thesis had a denaturation time of 40 min compared to 30 min for the other protocols. For the electrophoresis, the strength of the electric field seems to have no impact on DNA migration of control cells (Ersson and Möller 2011), but when DNA damage is present, the sensitivity of the comet assay can be improved by altering the electric field (Ersson and Möller 2011). The FPG-protocol in this thesis had an electrophoresis time of 30 min compared to 20 min for the other protocols.

The time used for incubation with FPG- or hOGG1-enzymes, has shown to be important for the results. It is important not to use too short incubation time (Johansson et al. 2010). The studies in this thesis have used the same optimised incubation times (30 min in 37 °C), so the enzymes work properly. Note that the extra incubation time for 30 min in 37 °C when using enzymes might have an effect on the result, but this is not studied in detail. The protocols are described in detail to allow for the reader to compare the protocols and also to repeat them in experiments. The critical steps of all three protocols are described in Figure 4 and the methods have also been generally described elsewhere (Andersson & Hellman 2005, Smith et al. 2006).
Table 3. Three different protocols of the alkaline comet assay used in the studies

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Application</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline comet assay, with FPG*</td>
<td>Studying oxidative DNA damage in blood cells from diabetes subjects.</td>
<td>Thawed cells were added to the slides, 40 min denaturation, 30 min electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Staining with EtBr.</td>
<td></td>
</tr>
<tr>
<td>Alkaline comet assay, with hOGG1**</td>
<td>Studying general and oxidative DNA damage in mouse lymphoma cells.</td>
<td>4-day cell culturing before addition to slides, 30 min denaturation, 20 min electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Staining with EtBr.</td>
<td></td>
</tr>
<tr>
<td>Alkaline comet assay***</td>
<td>Studying general DNA damage in mouse lymphoma cells.</td>
<td>4-day cell culturing before addition to slides, 30 min denaturation, 20 min electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Staining with EtBr or with GelRed.</td>
<td></td>
</tr>
</tbody>
</table>

*Paper I and II, **Paper III and V, ***Paper IV. EtBr = ethidium bromide, GelRed = alternative DNA-dye. FPG = Formamido pyrimidine glycosylase. hOGG1 = human oxoguanine DNA-glucosylase.

Protocol for the alkaline comet assay with FPG (Paper I-II)

The cells were kept on ice and in darkness during the whole procedure to prevent auto-oxidation. Lymphocytes and monocytes in 200 µl aliquots were thawed gently in a 37 °C water bath and kept on ice. One ml of (4 °C) RPMI 1640 medium containing L-glutamine with 10 % heat-inactivated fetal bovine serum was added drop-wise, immediately followed by centrifugation at 200 x g for 3 min (4 °C). The supernatant was discarded and the cells were washed in PBS and centrifuged at 200 x g for 3 min (4 °C). The cell pellet was mixed in low melting point agarose (SIGMA, Germany) on 3-well microscope slides (Menzel, Germany). Thereafter the cells were lysed in a pH 10 buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris with 1 % Triton X-100 (MP Biomedicals, USA) for 1 h at 4 °C, leaving mainly super coiled DNA embedded in the agarose.

Incubation was performed in enzyme buffer with pH 8 (0.1 M KCl, 0.5 mM EDTA, 40 mM Hapes, 0.2 mg/ml bovine serum albumin) for 2x7 min at 4 °C. The cells were then treated with Formamido pyrimidine glycosylase (FPG) 500 units/100 µl (Trevigen, USA) diluted 1:120 in the enzyme buffer. Diluted FPG (30 µl), or enzyme buffer (30 µl), were added to each fields on the slides following incubation in a humidity chamber at 37 °C for 30 min. During this incubation FPG cut out oxidised DNA-bases, increasing the strand breaks. DNA-unwinding in 0.3 M NaOH with 1 mM EDTA at pH>13 for 40 min prepared the samples for the following electrophoresis, which was performed in a Sub-Cell GT unit (BIO-RAD, USA) for 30 min in the same high alkali solution at 25 V (0.86 V/cm). DNA, which is negatively charged, moved as smaller single-stranded fragments towards the anode and formed a tail of damaged DNA. The longer strands of undamaged DNA stayed in the round “comet head”. The more damaged DNA, the larger the
Neutralisation was performed in 0.4 M Tris pH 7.5 for 2 x 5 min, after which the samples were washed for 5 min in water, dried over-night, and then fixed in methanol for 5 min.

Staining of DNA was performed in 10 µg/ml ethidium bromide in Tris-acetate-EDTA (TAE, pH 7.8) for 5 min and thereafter the excess of ethidium bromide was washed away during 5 min in a TAE buffer. The intensity from the “tail” out of the whole comet (head + tail) was assessed with a fluorescence microscope (Olympus, Japan) with a 20 x lens, and a computerised image analysis program Komet 4.0 (Kinetic Imaging Ltd, UK) was used to calculate the percentage damaged DNA (% Tail). Cells (n=50) treated with FPG and cells (n=50) without FPG were analysed, per patient and time point. The oxidative DNA damage levels were calculated by subtraction of enzyme buffer treated samples from FPG treated samples. The protocol has previously been used in a large inter-laboratory validation study in the area of biomarkers for measuring oxidative DNA damages (ESCODD, 2002b).

Protocol for the alkaline comet assay with hOGG1 (Paper III and V)

After the exposure to mutagenic compounds the cells were mixed with 0.6 % low melting point agarose and applied to microscope slides, pre-coated with 0.6 % low melting point agarose. Slides with three round fields with Teflon around or normal microscope slides with three gels per slide were used (Menzel-Gläzer, Thermo Scientific, Deutschland). The slides were incubated for 1 h in a lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M TRIS, 1 % Triton X-100, 10% DMSO, pH 10) at +4 °C and protected from light.

After the lysis treatment, the slides were treated either with enzyme buffer alone or with enzyme buffer containing the enzyme hOGG1 (all ingredients from Trevigen, USA). Firstly, 0.6 ml of washing buffer; 1.8 ml of 25x FLARE buffer and 43.2 ml of H2O were added per slide, and the slides were incubated for 2 x 5 min. After that followed an incubation for 30 min at 37 °C in the dark, either in enzyme buffer alone (67.5 µl of 25x FLARE buffer, 16.9 µl of 100x BSA and 1604 µl of H2O), or in enzyme solution (844 µl of the enzyme buffer and 1.69 µl enzyme hOGG1). After that, the slides were transferred to an electrophoresis box at +4 °C dark where they were treated with an alkaline buffer (0.3 M NaOH, 1 mM EDTA [1 µM EDTA in paper V], pH >13) for 30 minutes before they were subjected to electrophoresis at +4 °C dark for 20 minutes at 25 V and 300 mA (0.7 V/cm).

After the electrophoresis the slides were washed for 15 min in a neutralisation buffer (0.4 M Tris, pH 7.5) in the same cold room, dried and stored in closed containers until the day of the image analysis. Before the image analysis the slides were stained with ethidium bromide (EtBr, Pharmacia Biotech, Sweden) with 30 µl/slide (200 mg/mL) and analysed with a fluores-
ence microscope using the image analysis software Comet Assay IV (Perceptive Instruments, UK). The mean tail intensity (showing the percentage of DNA that had moved from the nucleus towards the anode during the electrophoresis) was used as the indicator of the level of DNA damage. The comet assay protocol used in paper IV and V has previously been used (Smith 2006).

Protocol for the alkaline comet assay, without enzymes (Paper IV)

After the exposure, the cell viability was determined using the Trypan blue exclusion technique. Modified standard procedures for the comet assay under alkaline conditions [24,28], was used to evaluate DNA damage (single- and double-strand breaks and alkali-labile sites). Immediately after the exposure the cells were mixed with low-melting point agarose and applied to microscope slides (Menzel-Gläser Diagnostika, Germany), which had been pre-coated with normal melting point agarose. By using these slides it was possible to evaluate 3 gels (treatments)/slide. The slides were then incubated for 1 h in a lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M TRIS, 1 % Triton X-100, 10% DMSO, pH 10) at 4 °C and protected from light. The slides were transferred to an electrophoresis box at 4 °C where they were treated in the dark with an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13) for 30 min before they were subjected to electrophoresis in darkness at 4 °C for 20 minutes at 25 V and 300 mA (0.7 V/cm). After the electrophoresis the slides were washed for 15 min in neutralisation buffer (0.4 M Tris, pH 7.5), dried and stored in closed containers until the day of the image analysis.

Before the image analysis the slides were stained with ethidium bromide (30 µl/slide; 20 µg/ml) and analysed with a fluorescence microscope using the image analysis software Comet Assay IV (Perceptive Instruments, UK). Fifty comets per gel were captured for image analysis. The mean tail intensity (showing the percentage of DNA that had moved from the nucleus towards the anode during the electrophoresis) was used as the indicator of the level of DNA damage. Ethidium bromide, EtBr (Pharmacia Biotech, Sweden) was added with 30 ul/slide or 10 ul/field (20 µg/ml) directly to each slide and the slides were kept in a humidity chamber until detection. Propidium iodide, PrI (Fluka, Switzerland) was added with 30 uL/slide (20ug/mL) directly to each slide and the slides were kept in a humidity chamber until detection. Gel Red (10000x, Biotium, USA) was added with 10 µl to 50 ml distilled water in a cuvette. The slides were incubated in the cuvette for 30 min, dark. The slides were held in neutralisation solution pH 7.5 for 30-60 s, in the dark, and after that kept in a humidity chamber until detection.
In the staining test all three dyes were used (Figure 6). In the combined tests with α-tocopherol and catechol, EtBr was used (Table 12). In the rest of the tests GelRed was used in the staining of DNA. After staining of the slides detection was performed with an Olympus BX60 fluorescence microscope with a Stingray camera (Allied vision technologies, Germany), a CoolLED pE-100 excitation light (536 nm) and the software Comet Assay IV (Perceptive instruments, UK). These instruments are shown in figure 5. Fifty cells per slide were examined and all tests (except for the pilot study) used duplicate or triplicate and 3 or 4 repeated experiments which gave 300, 450 or 600 examined cells per treatment.

Figure 5. The image analysis was performed using an Olympus BX60 fluorescence microscope coupled to a Stingray camera (Allied vision technologies, Germany) using a CoolLED pE-100 lamp (CooLED, UK) as the light source (excitation light of 535 nm). The software Comet Assay IV (Perceptive Instruments, UK) was used to evaluate the digitised images. The white spots on the screen in the picture are the comets that were detected. Photo by Malin Andersson.

Studies on type 2 diabetes subjects (Paper I and II)

Subjects

Patients with well-controlled type 2 diabetes participated in a randomised double-blind and placebo-controlled intervention study. Inclusion criteria were type 2 diabetes treated with either diet, or diet and oral hypoglycaemic medication. Other inclusion criterias were age of 40 to 75 years, BMI <35 kg/m², a stable body weight and HbA1c levels <10%. Exclusion criteria: insulin dependent diabetes and medication that could affect inflammation (including anti-inflammatory drugs). The patients were instructed not to use
antioxidant supplements at least one month before study-start, and also not to change the dietary intake or physical activity level during the intervention. Fifty-six patients were included in the cross-sectional studies (Paper I). Forty-seven of these subjects were randomly divided into three test groups: control group, single dose group, and double dose group and forty of the subjects completed the intervention study (Paper II). This is a high degree of participation in such clinical studies. A single dose (four capsules per day) contained antioxidant extracts corresponding to 500 g fruit, berries and vegetables, and a double dose corresponding to 1000 g. The oral supplementation with antioxidants lasted for 12 weeks.

A three day food diary
The dietary intake was calculated from a pre-coded 3-day food diary, which was a shorter variant of the 7-day food diary Menyboken (Becker et al. 1998), used in Riksmaten 1998, a nationwide dietary survey. Registration with the food diary was performed before the study start and after 3 weeks in the study. Data from the baseline dietary registrations, before study start, was used for comparisons. The subjects registered all foods and liquids they consumed for two weekdays and one weekend day (randomised). In the statistical analyses of the dietary record, we divided all calculated nutrient values with the energy intake for the same person (for example g/kJ) when testing possible correlations with plasma levels of antioxidants and also with levels of various biomarkers of oxidative stress- and inflammation, making the correlations more reliable (Willett et al. 1997). The macronutrients were presented in energy percentage (E%), which relates the intake to the individuals total energy intake (Table 7).

Blood sampling for analyses of oxidative DNA damage
The blood sampling was carried out in the morning with fasting patients. For the separation of mononuclear blood cells, a sample of 4 ml blood from each patient was collected in CPT Vacutainer cell preparation tubes (Becton Dickinson, USA). The tubes were kept cold and dark, to protect against oxidative stress, and were transported to the laboratory. Separation of mononuclear white blood cells was performed within four hours from blood sampling, by centrifugation of the CPT tubes at 1650 x g for 20 min at 12 °C. The plasma was collected and frozen at -80 °C. The cells were centrifuged in cold RPMI 1640 medium containing L-glutamine (Gibco, UK) in 700 x g for 15 min at 4 °C. The medium was removed and the cell pellet was resuspended in freezing medium, separated in 5 aliquots of 200 µl cell suspension for the comet assay (and the rest of the mononuclear blood cells for the 8-oxo-dG analyses) and after that slowly frozen to -80 °C and stored until further analysis. The freezing medium was made of heat-inactivated fetal bovine
serum (Gibco, UK), containing 10% dimethylsulfoxide (MERCK, Germany). This method has been validated in the interlaboratory collaboration ES-CODD, which attempted to provide standards and reliable protocol for analysis of 8-oxodG measured with HPLC and oxidative DNA damage measured with the alkaline comet assay with FPG (Gedik et al 2005).

Methods in studies on type 2 diabetes subjects

The methods used in the clinical intervention study (Paper I and II) are listed in Table 4. For details of the methods and endpoints, the reader is referred to the material and method sections in the specific papers.

Table 4. Methods used to determine food intake, levels of plasma antioxidants, anthropometry, clinical measurements and markers for oxidative stress and inflammation.

<table>
<thead>
<tr>
<th>Method</th>
<th>Endpoint</th>
<th>Paper(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The 3-day food diary</td>
<td>Intake of energy, macronutrients, fruits, vegetables and antioxidants</td>
<td>I</td>
</tr>
<tr>
<td>HPLC</td>
<td>Plasma antioxidants</td>
<td>I and II</td>
</tr>
<tr>
<td>Fluorometry</td>
<td>Plasma antioxidants</td>
<td>I and II</td>
</tr>
<tr>
<td>Anthropometry</td>
<td>Weight, BMI, waist circumference</td>
<td>I and II</td>
</tr>
<tr>
<td>Standard clinical measurements</td>
<td>HbA1c, glucose, insulin, s-cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol, blood pressure</td>
<td>I and II</td>
</tr>
<tr>
<td>ELISA</td>
<td>IL-6</td>
<td>I and II</td>
</tr>
<tr>
<td>ProSpec analyser</td>
<td>hsCRP</td>
<td>I and II</td>
</tr>
<tr>
<td>RIA</td>
<td>15-keto-dihydro-PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>I and II</td>
</tr>
<tr>
<td>Alkaline comet assay, with FPG</td>
<td>Fpg-sites (% Tail)</td>
<td>I and II</td>
</tr>
<tr>
<td>HPLC</td>
<td>8-oxodG</td>
<td>I and II</td>
</tr>
<tr>
<td>HPLC</td>
<td>MDA</td>
<td>I and II</td>
</tr>
<tr>
<td>RIA</td>
<td>8-iso-PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>I and II</td>
</tr>
<tr>
<td>Immuno assay</td>
<td>Nitrotyrosine</td>
<td>II</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Micronuclei-Trf-Ret</td>
<td>I and II</td>
</tr>
</tbody>
</table>

HPLC = High pressure liquid chromatography, FPG = Formamido Pyrimidine Glycosylase, HDL = High-density lipoprotein, BMI = kg/m², ELISA = Enzyme linked immunosorbent assay, IL-6 = Interleucin-6, hsCRP = human serum C-Reactive protein, PGF = Prostaglandin factor, MDA = Malondialdehyde, Trf-Ret = Trf-Reticulocytes. RIA = Radio immunsorbent assay.
Studies on mouse lymphoma cells (Paper III-V)

Cell lines and culture conditions

All experiments in Paper III-V were performed on mouse lymphoma L5178Y TK+/− cells, clone 3.7.2c (ML cells). In paper V, mycoplasma-free working cell-stocks, cleansed of pre-existing trifluorothymidine (TFT)-resistant mutants by growth in medium containing methotrexate, were prepared from the master culture. In paper III-V, the cells were cultured in R10P medium as described in the papers and maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. They were cultured four days before exposure and were used in the alkaline comet assay in paper III-V, and also in the other tests in paper V.

The mouse lymphoma cell line has been widely used in the pharmaceutical industry, and is regarded a relevant in vitro system to investigate genotoxicity and mutagenicity as well as the protection against oxidative stress and DNA damage from mutagenic compounds. It is a phenotypically stable cancer cell line due to the p53-/- phenotype mutation which results in less sensitivity in going into apoptosis and is also more prone for proliferation. It has a doubling time of 10 hours, an exogenous metabolic activation system (S9 activation) can be added with minimal toxicity and they also lack thymidine kinase (TK) activity (Mitchell et al. 1988). Also, the primary damages with following mutations could be detected within the same cells, using standard comet assay, mouse lymphoma assay and the ROS-test. Therefore they seem optimal to use in these well-established genotoxic endpoints.

Treatment with pro-oxidants

In Paper III-V, the mouse lymphoma cells were treated for 3 h with mutagenic pro-oxidants, to induce general- and oxidative DNA damage in the cells and also to evaluate the effects of antioxidants on DNA damage induced by these pro-oxidants.

Catechol (1,2-dihydroxybenzene, CAS Reg. No. 120-80-9) is a well-established mutagenic agent (Tsutsui et al. 1997, Wagenheim and Bolcsfoldi 1988), and it is also carcinogenic in rodents. Catechol induces both general DNA damage and oxidative DNA damage in the form of 8-oxodG (Oikawa et al. 2000, Andersson and Hellman 2005, Andersson and Hellman 2007, Demma et al. 2009). Catechol can be found in low concentrations in apples, onions and cigarette smoke (IARC 1986), and in a recently performed study catechol was used to induce DNA damage in glioblastoma cells (de Oliveira 2013).

The mutagenic substance ortho-phenylenediamine (OPD; CAS Reg. No. 95-54-5) has been shown to be both mutagenic (Ames et al. 1975, Voogd et al. 1980) and carcinogenic in rodents (Matsumoto et al. 2012). The sub-
stance induces DNA damage (Gichner et al. 2001) and increases the frequency of micronuclei in rodent studies (Wild et al. 1980).

4-Nitro-ortho-phenylenediamine (4-NOPD; CAS Reg. No. 99-56-9) has been found to be mutagenic (Gentile et al. 1987, Mitchell et al. 1988) but in contrast to the other studied compounds it does not induce cancer in rodents (NCI, 1979). Using the alkaline comet assay, 4-NOPD was shown to increase the level of DNA damage in human lymphocytes (Chye et al. 2008).

Methods in studies on mouse lymphoma cells

The methods used in cell studies (Paper III-V) are listed in Table 5. For details of the methods and the endpoints, in most cases with references, the reader is referred to the Material and method sections in the specific papers.

Table 5. Methods used to determine markers for oxidative stress and cell viability.

<table>
<thead>
<tr>
<th>Method</th>
<th>Endpoint</th>
<th>Paper(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline comet assay, without enzymes</td>
<td>% Tail Intensity</td>
<td>Paper IV</td>
</tr>
<tr>
<td>Alkaline comet assay, with hOGG1</td>
<td>% Tail Intensity</td>
<td>Paper III and V</td>
</tr>
<tr>
<td>CM-H$_2$DCFDA assay*</td>
<td>ROS fluorescence intensity</td>
<td>Paper V</td>
</tr>
<tr>
<td>Mouse lymphoma assay</td>
<td>Thymidine kinase forward mutants</td>
<td>Paper V</td>
</tr>
<tr>
<td>HPLC Nucleotide pool test**</td>
<td>Nucleosides (pmol/10 million cells)</td>
<td>Paper V</td>
</tr>
<tr>
<td>Trypan blue viability test</td>
<td>% cells alive</td>
<td>Paper III and V</td>
</tr>
</tbody>
</table>

Results and discussion

The main results in the thesis demonstrated that antioxidants from fruit and vegetable intake decreased oxidative stress, while oral antioxidant supplements did not change oxidative stress in type 2 diabetes subjects. Furthermore, it was shown that β-carotene and α-tocopherol, two antioxidants present in fruits and vegetables, modulated general and oxidative DNA damage induced by mutagenic pro-oxidative agents in mouse lymphoma cells. Finally, the results showed different mechanisms of action behind the mutagenicity of the three model pro-oxidants. Detailed results in the individual papers will be discussed below.

Effects of antioxidants from fruit and vegetable intake on oxidative stress in type 2 diabetes subjects (Paper I)

The levels of biomarkers for oxidative stress in type 2 diabetes subjects are high (Leinonen et al. 1997, Hinokio et al. 1999, Dincer et al. 2002, Blasiak et al. 2004). A high fruit and vegetable intake may however reduce risk for type 2 diabetes (Cooper et al. 2012, Müllner et al. 2013a). In addition, intake of antioxidants from diet, or from oral supplements, have been reported to change various biomarkers for oxidative stress and contribute to the prevention of diabetes complications (Jialal et al. 2002, Mann et al. 2004, Scott and King 2004, Segal 2004, Davi et al. 2005).

The aim of this study was to evaluate the importance of antioxidants from fruit and vegetable intake in type 2 diabetes subjects. In this study, a number of biomarkers for oxidative stress (Table 8) in type 2 diabetes subjects were measured. Also, the intake of antioxidants from diet and the levels in plasma, and how these were associated with the biomarkers for oxidative stress were determined. It was clearly shown that fruits and vegetables reduced the levels of oxidative DNA damage as determined by the comet assay and lipid peroxidation measurements of 8-Iso-PGF$_{2α}$ with HPLC (Table 6). This is the first study showing this effect, using all these biomarkers for oxidative stress.

It was also shown that high intake of vitamin C reduced the levels of lipid peroxidation (8-Iso-PGF$_{2α}$) and high plasma levels of ascorbate reduced the levels of oxidative DNA damage in mononuclear white blood cells (8-
oxodG) (Table 6). Plasma levels of all carotenoids (α-carotene, β-carotene, lutein and lycopene) were negatively correlated to IL-6, a biomarker for inflammation, and previous studies have also demonstrated an inverse relation between the levels of carotenoids in plasma and inflammation (Gao 2004). These results obtained indicate that vitamin C might reduce oxidative stress, while the carotenoids might reduce inflammation.

Table 6. Correlations at baseline between antioxidant levels measured with a 3-day food diary or in plasma and biomarkers for oxidative DNA damage or lipid peroxidation, in type 2 diabetes subjects.

<table>
<thead>
<tr>
<th>Antioxidant level (unit)</th>
<th>Oxidative stress biomarker (unit)</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated dietary intake level</td>
<td>Oxidative DNA damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit and vegetable intake (g/d)</td>
<td>Fpg-sites (% Tail)</td>
<td>-0.272</td>
<td>0.048*</td>
</tr>
<tr>
<td>Fruit and vegetable intake (g/d)</td>
<td>8-oxodG (8-oxodG/10^6 dG)</td>
<td>-0.094</td>
<td>0.512</td>
</tr>
<tr>
<td>Calculated dietary intake level</td>
<td>Lipid peroxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit and vegetable intake (g/d)</td>
<td>8-iso-PGF_2α (nmol/mmol creatinine)</td>
<td>-0.277</td>
<td>0.044*</td>
</tr>
<tr>
<td>Fruit and vegetable intake (g/d)</td>
<td>MDA (mmol/l)</td>
<td>0.224</td>
<td>0.110</td>
</tr>
<tr>
<td>Vitamin C (mg/d)</td>
<td>8-iso-PGF_2α (nmol/mmol creatinine)</td>
<td>-0.332</td>
<td>0.015*</td>
</tr>
<tr>
<td>Antioxidant level in plasma</td>
<td>Oxidative DNA damage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbate (µM)</td>
<td>8-oxodG (8-oxodG/10^6 dG)</td>
<td>-0.290</td>
<td>0.037*</td>
</tr>
<tr>
<td>Antioxidant level in plasma</td>
<td>Lipid peroxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Tocopherol (µg/ml)</td>
<td>MDA (mmol/l)</td>
<td>-0.287</td>
<td>0.037*</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient with Bonferroni–Holm correction; r = correlation coefficient, p-value with *p < 0.05. Both dietary intake levels and plasma levels were calculated per kJ for each person before statistical analyses. Fpg = Formamidopyrimidine glycosylase, dG = deoxyguanosine, PGF = Prostaglandine factor, MDA = Malondialdehyde.

The dietary intake of fruits, vegetables and antioxidants was calculated using a 3-day food diary measurement of the study subjects before the study start. The data from the food diary calculations showed a positive correlation between the fruit and vegetable intake and plasma levels of α-carotene, β-carotene, lycopene, α-tocopherol and ascorbate. This confirms that plasma carotenoids might be predictors of fruit and vegetable intake, as shown also in previous studies (Granado et al. 1996, Campbell et al. 1994, Michaud et al. 1998, Tucker et al. 1999, Resnicow et al. 2000, Block et al. 2001, van Kappel et al. 2001, Al-Delaimy et al. 2005). Since the strongest significant association in the present study was seen between fruit and vegetable intake and plasma β-carotene (p<0.001), the result suggests that specifically plasma β-carotene measurement is a good predictor of fruit and vegetable intake, confirming previous studies (Tucker et al. 1999, Block et al. 2001).
The present study also showed a good correlation between dietary antioxidant levels and plasma levels of the same antioxidants. This was a validation of the effectiveness of the food diary to measure intake levels of antioxidants. The dietary intake of β-carotene, vitamin E, α-tocopherol, vitamin C and folate was also positively correlated to the dietary intake of fruit and vegetables (unpublished data), which confirms the effectiveness of the food diary to measure both fruit and vegetable intake and the intake of the antioxidants present in fruits and vegetables.

Table 7. Comparison between the calculated dietary intake in the type 2 diabetes subjects (28 females and 25 males) and the calculated dietary intake in two Swedish national surveys; Riksmaten (RM) 1998 (626 females and 589 males) and 2010-2011 (1005 females and 792 males), respectively.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>MJ</td>
<td>8.2</td>
<td>8.7</td>
<td>7.8</td>
<td>9.9</td>
<td>7.4</td>
<td>9.4</td>
</tr>
<tr>
<td>Proteins</td>
<td>E%</td>
<td>18</td>
<td>17</td>
<td>16</td>
<td>16</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Fats</td>
<td>E%</td>
<td>32</td>
<td>30</td>
<td>34</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>E%</td>
<td>48</td>
<td>48</td>
<td>47</td>
<td>46</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>µg</td>
<td>684</td>
<td>553</td>
<td>1874</td>
<td>1708</td>
<td>2265</td>
<td>1923</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>mg</td>
<td>9.8</td>
<td>8.5</td>
<td>6.8</td>
<td>7.9</td>
<td>11.7</td>
<td>13.2</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>mg</td>
<td>137</td>
<td>144</td>
<td>93</td>
<td>180</td>
<td>96</td>
<td>93</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>µg</td>
<td>7.8*</td>
<td>6.3</td>
<td>6.0</td>
<td>6.9</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Folate</td>
<td>mg</td>
<td>306</td>
<td>284</td>
<td>217</td>
<td>232</td>
<td>253</td>
<td>266</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>13</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Selen</td>
<td>µg</td>
<td>42</td>
<td>47</td>
<td>32</td>
<td>36</td>
<td>42</td>
<td>50</td>
</tr>
<tr>
<td>Fruit- and vegetable intake</td>
<td>g</td>
<td>490</td>
<td>516</td>
<td>261</td>
<td>261</td>
<td>362</td>
<td>311</td>
</tr>
</tbody>
</table>

The mean values per person and day are shown in the table. E% = percentage of the energy intake. Wilcoxon two-sample test, *p < 0.05 between females and males.

In table 7 the calculated intake of macronutrients and micronutrients (mainly antioxidants) in type 2 diabetes subjects are presented together with two national Swedish surveys on food intake, Riksmaten 98 (Becker 1998) and Riksmaten 2010-2011 (Riksmaten-vuxna 2010-2011). The values should be compared in relation to the average energy intakes, which are quite similar (8.2 – 9.9 MJ/person/day) between the studies. Comparing differences between genders in nutrient intake calculated from the food diary, the only statistical difference between males and females was for vitamin B12 (Table 7).

Whereas the average intake of α-tocopherol in type 2 diabetes subjects was similar to Riksmaten, the average level of β-carotene intake in type 2 diabetes subjects was lower, and the intake of fruits and vegetables was higher, and close to the recommended 500 g/day. This finding is difficult to explain since β-carotene was suggested to be a relevant biomarker for fruit
and vegetable intake in the present study. One explanation for inconsistency
could be that the group of type 2 diabetes subjects in our study had eaten
fruits and vegetables containing more of other antioxidants, such as vitamin
C and α-tocopherol, and not so much of β-carotene containing fruits and
vegetables. Another explanation could be that the β-carotene intake calculat-
ed from the food diary did not have such a strong correlation with fruit and
vegetable intake compared to the levels of β-carotene in plasma.

In summary, the intake of fruits and vegetables decreased the levels of a
number of biomarkers of oxidative stress, and vitamin C decreased the levels
of biomarkers for oxidative stress while the carotenoids decreased a bi-
omarker for inflammation, in type 2 diabetes subjects. It was also shown that
plasma level of β-carotene was a good biomarker for fruit and vegetable
intake in type 2 diabetes subjects.

Effects of antioxidants from oral supplements on
oxidative stress in type 2 diabetes subjects (Paper II)

The evidence for the usage of oral antioxidant supplements in the prevention
of type 2 diabetes and complications of the disease, is weak (Mann et al.
2004). The usage of oral antioxidant supplements may prevent the onset of
type 2 diabetes in metabolic syndrome patients, but the treatment of fully-
developed diabetes does not have enough evidence in the literature (King
and Loecken 2004).

Since oxidative stress was the main endpoint, the aim of the study was to
evaluate if the oral antioxidant supplementation for 12 weeks could affect
the level of oxidative stress in a group with diagnosed type 2 diabetes, non-
insulin dependent with a controlled weight and glucose metabolism and also
absence of a high inflammation level (CRP < 10).

The present study was unique by the use of 20 different antioxidants in
the oral supplementary capsules, and unusually many biomarkers for oxida-
tive stress and inflammation were determined. Forty-seven type 2 diabetes
subjects treated with diet with or without hypoglycaemic medication were
included in the study and 40 subjects fulfilled the intervention study, which
is a high degree of participation in such a clinical study. During 12 weeks the
subjects were supplemented with 16 capsules/person each day; either with
placebo capsules alone (control group), placebo and antioxidant capsules
(low dose group) or antioxidant capsules alone (high dose group). The anti-
oxidant capsules contained a mixture of natural extracts containing β-
carotene, α-tocopherol and 18 additional antioxidants (Table 1 in Paper II) in
doses giving the low dose group a daily recommended intake of antioxidants
present in fruits and vegetables, and the high dose group the double intake.
Data collection was performed at the start of the study and after 12 weeks of supplementation.

Table 8. Levels of biomarkers for oxidative stress in blood from type 2 diabetes subjects, at baseline and after 12 weeks of oral supplementation of antioxidants.

<table>
<thead>
<tr>
<th>Treatment group (dose)</th>
<th>Oxidative stress biomarker</th>
<th>Baseline Mean ± SD</th>
<th>12 weeks Mean ± SD</th>
<th>( p )-value between changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxidative DNA damage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>Fpg-site (% Tail)</td>
<td>29 ± 16</td>
<td>26 ± 8</td>
<td></td>
</tr>
<tr>
<td>Low dose group</td>
<td></td>
<td>32 ± 14</td>
<td>23 ± 6</td>
<td>ns</td>
</tr>
<tr>
<td>High dose group</td>
<td></td>
<td>31 ± 13</td>
<td>26 ± 6</td>
<td>ns</td>
</tr>
<tr>
<td>Control group</td>
<td>8-oxodG(^#) (8-oxodG/10(^6) dG)</td>
<td>1.0 ± 0.7</td>
<td>0.8 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Low dose group</td>
<td></td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>High dose group</td>
<td></td>
<td>0.8 ± 0.3</td>
<td>1.0 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Lipid peroxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>8-iso-PGF(_2\alpha) (nmol/mmol creatinine)</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Low dose group</td>
<td></td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>High dose group</td>
<td></td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Control group</td>
<td>MDA(^#) (µM)</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Low dose group</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>High dose group</td>
<td></td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Protein oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>Nitrotyrosine(^#) (nM)</td>
<td>349 ± 493</td>
<td>363 ± 491</td>
<td></td>
</tr>
<tr>
<td>Low dose group</td>
<td></td>
<td>197 ± 312</td>
<td>145 ± 186</td>
<td>ns</td>
</tr>
<tr>
<td>High dose group</td>
<td></td>
<td>198 ± 402</td>
<td>194 ± 404</td>
<td>ns</td>
</tr>
</tbody>
</table>

Control group \( n = 13 \), Low dose group \( n = 13 \), High dose group \( n = 14 \); \#35-39 subjects. No significant differences between the dose groups and the control group could be seen at comparisons of changes (from baseline to 12 weeks). Differences between dose groups and control group were examined with both one-way ANOVA and with Wilcoxon’s rank sum test followed by unpaired t-test. \( ns \) = not significant. Fpg = Formamidopyrimidine glycosylase, dG = deoxyguanosine, PGF = Prostaglandine factor, MDA = Malondialdehyde.

A significantly increased antioxidant level in plasma from the type 2 diabetes subjects was observed following the supplementation with antioxidants, both in the low- and high-dose groups \( p < 0.001 \), compared to the control group. There were increased plasma levels of \( \gamma \)-tocopherol, \( \alpha \)-carotene, \( \beta \)-carotene, lycopene, lutein and ascorbate, whereas \( \alpha \)-tocopherol was unchanged in plasma. The increase was significantly higher for the high-dose
group \( (p < 0.05) \) compared to the low-dose group for \( \gamma \)-tocopherol and lycopene, showing a dose-dependent uptake of the antioxidants.

Previous studies have shown an inverse relationship between oral antioxidant supplementation and oxidative DNA damage (Lean 1999) and inflammation (Upritchard 2000), even if the literature is somewhat inconclusive. In the present study, despite substantially increased plasma concentrations of antioxidants, there was no effect of the oral antioxidant supplementation on the five biomarkers for oxidative stress (Table 8) or on the three biomarkers for inflammation. This paper support data from other clinical studies, showing a lack of effect from oral antioxidant supplementation on oxidative stress (Sampson et al. 2001, Giovannelli et al. 2002, Johansson et al. 2008, Rytter et al. 2010).

The lack of effects on oxidative stress (Table 8) or inflammation from the antioxidant supplementation may be due to the study population who had well-controlled type 2 diabetes. Furthermore, the study group had a high fruit and vegetable intake, as calculated from the 3-day food diary, and high levels of plasma antioxidants, as measured with HPLC or Fluorometry. This is also reflected in that their levels of biomarkers for oxidative stress and inflammation were comparable to those found in healthy subjects, despite of the type 2 diabetes.

In summary, 12 weeks of oral antioxidant supplementation in type 2 diabetes subjects, did not affect the levels of biomarkers of oxidative stress and inflammation, despite substantially increased plasma concentrations of antioxidants.

**Effects of \( \beta \)-carotene on general and oxidative DNA damage *in vitro* induced by a pro-oxidant (Paper III)**


The antioxidative and pro-oxidative effects of \( \beta \)-carotene in mouse lymphoma cells exposed to catechol were examined using the alkaline comet assay. Catechol was used to induce general and oxidative DNA damage, and addition of hOGG1-enzymes in the alkaline comet assay was used to study
oxidative DNA damage in the cells. Two protocols were used; 18 h pre-
treatment with β-carotene followed by 3 h treatment with catechol, or simul-
taneous treatment with β-carotene and catechol for 3 h. β-Carotene did not
increase the basal level of DNA-damage at simultaneous treatment, but a
small increase was seen with the pre-treatment protocol (Table 9 and 10).
Addition of 0.5, 0.75 and 1.0 mM catechol raised the levels of general and
oxidative DNA damage in a dose-dependent manner (Table 9 and 10). Addi-
tion of β-carotene significantly decreased the level of catechol-induced DNA
damage at 0.5 mM and showed a tendency at 0.75 mM catechol (Table 9 and
10).

Unexpectedly, β-carotene increased the level of catechol-induced DNA
damage at the highest concentration (1.0 mM) of catechol, especially in the
pre-treated cells (Table 9 and 10). The level of catechol-induced DNA dam-
age seems to determine whether β-carotene will act as an antioxidant or a
pro-oxidant in mouse lymphoma cells, suggesting that the level of oxidative
stress determine whether β-carotene act as a pro-oxidant or and antioxidant.

Table 9. General and oxidative DNA damage (measured as % Tail Intensity) in mouse lym-
phoma cells after 3 h exposure to different concentrations of catechol, with or without simul-
taneous exposure to 2 µM β-carotene. General DNA damage was evaluated using the comet
assay without the DNA repair enzyme hOGG1. Oxidative DNA damage was evaluated using
the comet assay with hOGG1.

<table>
<thead>
<tr>
<th>Catechol concentration (mM)</th>
<th>Addition of hOGG1</th>
<th>Addition of β-carotene</th>
<th>% Tail Intensity (Mean ± 2 x SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>0.15 ± 0.13</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>-</td>
<td>3.08 ± 0.41***</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>4.58 ± 0.59***</td>
</tr>
<tr>
<td>0.00</td>
<td>-</td>
<td>+</td>
<td>1.05 ± 0.12</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>+</td>
<td>2.44 ± 0.25***</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>+</td>
<td>5.74 ± 0.75*** ^{b}</td>
</tr>
<tr>
<td>0.00</td>
<td>+</td>
<td>-</td>
<td>2.86 ± 0.24</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
<td>-</td>
<td>6.48 ± 0.45***</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
<td>-</td>
<td>6.86 ± 0.56***</td>
</tr>
<tr>
<td>0.00</td>
<td>+</td>
<td>+</td>
<td>2.98 ± 0.28</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
<td>+</td>
<td>5.43 ± 0.41*** ^{a}</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
<td>+</td>
<td>7.75 ± 0.74***</td>
</tr>
</tbody>
</table>

The number of cells analysed was 450 for each data point, and each data point represents the
mean value ± 2 x SEM after pooling the data from three independent experiments. ***p <0.001 (in comparison with vehicle control). \^{a}p <0.05; \^{b}p <0.01 (exposure with β-carotene in
comparison with exposure without β-carotene).
The obtained data could be considered as a possible pro-oxidant effect of β-carotene. This could be of interest when considering the results from the clinical studies where male smokers and asbestos workers had an increased incidence of lung cancer and cardiovascular deaths following supplementation with β-carotene (the ATBC study group 1994, Omenn et al. 1996).

Table 10. General and oxidative DNA damage (measured as % Tail Intensity) after 3 h exposure to different concentrations of catechol in mouse lymphoma cells that had been pre-treated for 18 h to either 2 µM β-carotene or vehicle before the exposure to catechol. General DNA damage was evaluated using the comet assay without the DNA repair enzyme hOGG1. Oxidative DNA damage was evaluated using the comet assay with hOGG1.

<table>
<thead>
<tr>
<th>Catechol concentration (mM)</th>
<th>Addition of hOGG1</th>
<th>Addition of β-carotene</th>
<th>% Tail Intensity (Mean ± 2 x SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td>2.17 ± 0.50</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>-</td>
<td>1.63 ± 0.37*</td>
</tr>
<tr>
<td>0.75</td>
<td>-</td>
<td>-</td>
<td>3.98 ± 0.56***</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>3.41 ± 0.45***</td>
</tr>
<tr>
<td>0.00</td>
<td>-</td>
<td>+</td>
<td>5.48 ± 0.83</td>
</tr>
<tr>
<td>0.50</td>
<td>-</td>
<td>+</td>
<td>4.52 ± 0.72*</td>
</tr>
<tr>
<td>0.75</td>
<td>-</td>
<td>+</td>
<td>7.11 ± 1.02***</td>
</tr>
<tr>
<td>1.00</td>
<td>-</td>
<td>+</td>
<td>10.73 ± 1.27***</td>
</tr>
<tr>
<td>0.00</td>
<td>+</td>
<td>-</td>
<td>2.46 ± 0.45</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
<td>-</td>
<td>2.30 ± 0.42*</td>
</tr>
<tr>
<td>0.75</td>
<td>+</td>
<td>-</td>
<td>4.63 ± 0.68***</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
<td>-</td>
<td>4.58 ± 0.59***</td>
</tr>
<tr>
<td>0.00</td>
<td>+</td>
<td>+</td>
<td>6.62 ± 0.88</td>
</tr>
<tr>
<td>0.50</td>
<td>+</td>
<td>+</td>
<td>5.54 ± 0.74*</td>
</tr>
<tr>
<td>0.75</td>
<td>+</td>
<td>+</td>
<td>10.58 ± 1.12***</td>
</tr>
<tr>
<td>1.00</td>
<td>+</td>
<td>+</td>
<td>14.82 ± 1.74***</td>
</tr>
</tbody>
</table>

The number of cells analysed was 450 for each data point, and each data point represents the mean value ± 2 x SEM after pooling the data from three independent experiments. *p <0.05; ***p <0.001 (in comparison with vehicle control). c p <0.001 (exposure with β-carotene in comparison with exposure without β-carotene).

The smokers in the ATBC study were given β-carotene as an oral supplement, and the study demonstrated an increased incidence of lung cancer from the oral supplement. In the same study the smokers were asked to answer a food frequency questionnaire (FFQ). Complete dietary data were available for 27 111 participants. Calculated dietary nutrient intake was estimated by linking foods from the FFQ to food composition data available from the National Public Health Institute of Finland. The results showed that especially a diet rich in carotenoids (a high fruit and vegetable intake), was actually associated with a lower risk of lung cancer in the same cohort of
smokers (Holick et al. 2002), which is in agreement with our results. Furthermore, in a placebo-controlled, randomised, double-blind study a four-week oral supplementation with 60 mg/day of β-carotene increased the level of oxidative DNA damage, measured as 8-oxodG, in leucocytes from smokers, while the background level of DNA damage was decreased in leucocytes from non-smokers (Welch et al. 1999). This result indicates that oral supplementary antioxidants can have a pro-oxidative effect at high levels of oxidative stress (in the smokers).

In the literature several mechanisms have been proposed behind the dual actions of β-carotene when it comes to oxidative stress and DNA damage. Several mechanisms have been proposed including (i) an effect of β-carotene on DNA repair (Astley et al. 2004); (ii) pro-inflammatory effects of β-carotene generated ROS (van Helden et al. 2009), and (iii) the cleavage products of β-carotene during oxidative stress (Siems et al. 2002, Alija et al. 2006). More studies are needed to clarify the mechanisms in detail behind this dual effect of β-carotene.

In summary, it was shown that β-carotene did not increase the basal level of DNA damage at simultaneous treatment, but an increase was seen with the pre-treatment protocol. However, after treatment of the cells with a high concentration of catechol, β-carotene was found to increase the catechol-induced general and oxidative DNA damage, especially in the pre-treated cells. Interestingly, an opposite effect was observed at lower concentrations of catechol, statistically significant only for the procedure including oxidative DNA damage.

Effects of α-tocopherol on DNA damage in vitro induced by pro-oxidants (Paper IV)

The aim of this study was to evaluate the effects of α-tocopherol on DNA damage in mouse lymphoma cells, induced by pro-oxidants. The mutagenic pro-oxidants catechol, OPD and 4-NOPD were used to induce DNA damage. The pro-oxidants were chosen because they induce general and oxidative DNA damage, and also because of an indication of different mechanisms of action between the pro-oxidants (Paper III and V).

An additional aim was to evaluate if the less hazardous DNA-dye GelRed could replace ethidium bromide in the comet assay. In the pharmaceutical industry EtBr was previously replaced with PrI, a less hazardous dye. The relatively new dye GelRed has been claimed by the manufacturer to be a non-mutagenic substance (http://www.biotium.com). There is at least one published paper showing that GelRed is a simple and practical dye when staining DNA fragments during the electrophoresis, but only in large gels and not in the comet assay (Huang et al. 2010).
Every experiment was performed in duplicate or triplicate and repeated 3 or 4 times, except for the first pilot study (see Table 11). The cell viability was examined by the trypan blue technique, and the frequency of hedgehogs (comets without a distinct head, possibly representing dying cells) was low in all experiments (Table 11). The highest concentrations of the mutagenic pro-oxidants catechol, OPD and 4-NOPD had the highest frequency of hedgehogs (2-5 hedgehogs per 100 analysed cells).

Table 11. Cell viability as measured with trypan blue technique and hedgehogs as measured with the alkaline comet assay for all experiments in the study on mouse lymphoma cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>α-Tocopherol</th>
<th>DNA-dye</th>
<th>n (cells)</th>
<th>Viability (%) §</th>
<th>Hedgehogs#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>EtBr</td>
<td>100</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>10 µM</td>
<td>+</td>
<td>EtBr</td>
<td>100</td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>20 µM</td>
<td>+</td>
<td>EtBr</td>
<td>100</td>
<td>98</td>
<td>-</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>50 µM</td>
<td>+</td>
<td>EtBr</td>
<td>100</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>100 µM</td>
<td>+</td>
<td>EtBr</td>
<td>100</td>
<td>97</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>GelRed</td>
<td>450</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>10 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>98</td>
<td>3</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>20 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>99</td>
<td>11</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>50 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>99</td>
<td>7</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>100 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>200 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>500 µM</td>
<td>+</td>
<td>GelRed</td>
<td>450</td>
<td>99</td>
<td>8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>EtBr</td>
<td>300</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.0 mM</td>
<td>-</td>
<td>EtBr</td>
<td>300</td>
<td>96</td>
<td>2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>+</td>
<td>EtBr</td>
<td>300</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.0 mM</td>
<td>+</td>
<td>EtBr</td>
<td>300</td>
<td>97</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>GelRed</td>
<td>600</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>4-NOPD</td>
<td>1.2 mM</td>
<td>-</td>
<td>GelRed</td>
<td>600</td>
<td>98</td>
<td>4</td>
</tr>
<tr>
<td>OPD</td>
<td>0.24 mM</td>
<td>-</td>
<td>GelRed</td>
<td>600</td>
<td>99</td>
<td>22</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>+</td>
<td>GelRed</td>
<td>600</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>4-NOPD</td>
<td>1.2 mM</td>
<td>+</td>
<td>GelRed</td>
<td>600</td>
<td>98</td>
<td>8</td>
</tr>
<tr>
<td>OPD</td>
<td>0.24 mM</td>
<td>+</td>
<td>GelRed</td>
<td>600</td>
<td>99</td>
<td>10</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>EtBr</td>
<td>300</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.75 µM</td>
<td>-</td>
<td>EtBr</td>
<td>300</td>
<td>97</td>
<td>4</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.5 µM</td>
<td>-</td>
<td>EtBr</td>
<td>300</td>
<td>97</td>
<td>10</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>PrI</td>
<td>300</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.75 µM</td>
<td>-</td>
<td>PrI</td>
<td>300</td>
<td>97</td>
<td>4</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.5 µM</td>
<td>-</td>
<td>PrI</td>
<td>300</td>
<td>97</td>
<td>9</td>
</tr>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>-</td>
<td>GelRed</td>
<td>300</td>
<td>98</td>
<td>1</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.75 µM</td>
<td>-</td>
<td>GelRed</td>
<td>300</td>
<td>97</td>
<td>2</td>
</tr>
<tr>
<td>Catechol</td>
<td>1.5 µM</td>
<td>-</td>
<td>GelRed</td>
<td>300</td>
<td>97</td>
<td>13</td>
</tr>
</tbody>
</table>

§ mean value of 1-4 tests; # at detection of n cells
The result showed that the DNA damaging effect of 1 mM catechol was in agreement with previous studies (Oikawa et al. 2001, Andersson and Hellman 2005, Andersson and Hellman 2007, Demma et al. 2009). Furthermore, α-tocopherol did not increase the basal level of DNA damage in the cells. This is in agreement with other in vitro studies in the literature showing that α-tocopherol is without DNA damaging effect (Bergström et al. 2012, Hornsby and Harris 1987, Wang et al 1997).

Table 12. Effects of 20 µM α-tocopherol on DNA damage induced by the mutagens catechol, OPD and 4-NOPD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mM)</th>
<th>α-Tocopherol (20 µM)</th>
<th>% Tail Intensity (mean ± 2 x SEM)</th>
<th>Effect of α-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle for catechol test</td>
<td>0</td>
<td>A: No</td>
<td>1.20 ± 0.25</td>
<td>A versus B: p &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B: Yes</td>
<td>1.30 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>1.00</td>
<td>C: No</td>
<td>2.14 ± 0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D: Yes</td>
<td>3.01 ± 0.98***</td>
<td>C versus D: p &lt; 0.05</td>
</tr>
<tr>
<td>Vehicle for test of OPD and 4-NOPD</td>
<td>0</td>
<td>E: No</td>
<td>1.50 ± 0.25</td>
<td>E versus F: p &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: Yes</td>
<td>1.72 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>OPD</td>
<td>0.24</td>
<td>G: No</td>
<td>2.60 ± 0.48**</td>
<td>G versus H: p &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H: Yes</td>
<td>2.99 ± 0.52***</td>
<td></td>
</tr>
<tr>
<td>4-NOPD</td>
<td>1.20</td>
<td>I: No</td>
<td>1.93 ± 0.32</td>
<td>I versus J: p &gt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J: Yes</td>
<td>1.93 ± 0.37</td>
<td></td>
</tr>
</tbody>
</table>

The number of cells analysed was 300 (catechol) or 600 (OPD and 4-NOPD) for each data point, and each data point represents the mean value ± 2 x SEM after pooling the data from 3-4 independent experiments. *p <0.05, ***p <0.001 (in comparison with vehicle control). § exposure with β-carotene in comparison with exposure without β-carotene.

However, a significant decrease of catechol-induced (1 mM) general DNA damage was seen in the presence of 20 µM α-tocopherol (Table 12). This is in contrast to the pro-oxidative effect from β-carotene at 1 mM catechol in the previous study (Paper III) and indicates that α-tocopherol does not have the same ability to switch from an antioxidant to become a pro-oxidant. α-Tocopherol seems to be a more stable antioxidant than β-carotene. An antioxidative effect from α-tocopherol on pro-oxidant-induced DNA damage has also been reported in the literature (Kontush et al. 1996, Dabrosin and Ollinger 1998, Takahashi et al. 1998, Tao et al. 2004, Pazdro and Burgess 2012).

The effects of α-tocopherol on DNA damage induced by different pro-oxidants were evaluated and the mutagenic amines OPD and 4-NOPD were used to induce DNA damage in an experiment using GelRed in the staining
of DNA. The experiment showed no effect on DNA damage of 1.2 mM 4-NOPD, which confirmed the results in paper V using EtBr for staining of the DNA. OPD, on the other hand, showed an increase of DNA damage already at 0.24 mM after OPD treatment, confirming the results in paper V (Table 12).

To investigate whether the presence of α-tocopherol could affect the oxidative stress induced by OPD, a physiological relevant concentration of α-tocopherol (20 µM) was added simultaneously with OPD. No effect of α-tocopherol on the OPD-induced damage was seen (Table 12), suggesting different mechanisms of action behind the DNA damages induced by catechol and OPD. 4-NOPD did not induce DNA damage and addition of 20 µM α-tocopherol did not decrease the level of DNA damage at exposure for 4-NOPD.

While catechol is mainly working through the semiquinon radical pathway producing extra ‘OH (Hepel et al. 2012), α-tocopherol is probably able to trap this radical in the Fenton cascade, in the cellular membrane, the mitochondrial membrane or in the cytoplasm, preventing it to further increase DNA damage. OPD, on the other hand, will increase the production of ‘O₂⁻ and also ‘OH in the Fenton reaction or through the SOD-pathway where H₂O₂ will together with Cu⁺ form the DNA damaging radical ‘OH (Murata and Kawanishi 2011), and probably it is difficult for α-tocopherol to prevent DNA damage resulting from this pathway.

In paper IV we also tested the effect of three different DNA-dyes on % Tail Intensity and Total Intensity. The reason for this was to see if it was possible to replace the mutagenic and potentially human carcinogenic dye ethidium bromide (EtBr). In experimental studies EtBr was shown to interact with the DNA molecule by intercalating with double-stranded DNA, and thereby affecting the replication and transcription of DNA (Waring 1965). EtBr was mutagenic in Ames test (McCann et al. 1975) but it did not increase the level of DNA strand breaks in alkaline comet assay (Belyaev et al. 1999).

When it came to Total Intensity the signal was higher from EtBr compared to the other dyes, but the signals from the other dyes were high enough to detect catechol-induced DNA damage (not shown). As shown in figure 6, all three dyes were able to detect catechol-induced DNA-damage in a similar way and the three dyes did not differ at any concentration of catechol when it came to percentage DNA damage (% Tail Intensity). No statistical difference could be seen for the same dye at 0.75 compared to 1.5 mM catechol, and this was the same for all three dyes. Since the % Tail Intensity test did not show any differences between the dyes we decided to replace EtBr with the less harmful GelRed in our further comet assay experiments.
Figure 6. Level of DNA damage in L5178Y mouse lymphoma cells exposed for 3h to vehicle (DMSO) or different concentrations of catechol (0.75 or 1.5 mM). DNA damage was monitored as the % Tail Intensity after electrophoresis for 20 min in the alkaline comet assay using three different dyes when staining the DNA: EtBr (ethidium bromide); PrI (propidium iodide) or GelRed. Each data point represents the mean value ± 2 x SEM after pooling the data from 450 cells (3 slides/treatment; 50 cells per slide) from three independent experiments. ***p<0.001 (in comparison to vehicle control). No significant difference could be seen between different dyes at the same concentration of catechol.

In summary, α-tocopherol was without DNA damaging effects in mouse lymphoma cells. α-Tocopherol reduced the DNA damage induced by catechol, whereas it did not reduce the level of DNA damage induced by OPD or 4-NOPD. This suggests that the mechanisms behind DNA damage from the three compounds are different. In the present study it was also demonstrated that the more hazardous chemical EtBr could be replaced in the comet assay with the less hazardous chemical GelRed in the staining of the DNA before the image analysis.

Effects of mutagenic pro-oxidants on oxidative stress as an explanation of mutagenicity in vitro (Paper V)

Both 4-nitro-o-phenylenediamine (4-NOPD) and o-phenylenediamine (OPD) are mutagenic in mouse lymphoma cells but no DNA-adducts have been observed after exposure to these substances in mouse lymphoma cells, as measured with P³²-Postlabelling (M.R. O’Donovan, personal communication). Therefore indirect mechanisms can be suspected behind mutations. Since 4-NOPD previously was found in hair dyes and OPD was found in
pharmaceuticals, pesticides and plastics (Mitchell et al. 1988, Voogd et al. 1980), these substances are relevant to study because of their daily presence in the human environment. 4-NOPD was not carcinogenic in rodents, while OPD was shown to induce cancer in rodents (NCI 1979, Matsumoto et al. 2012).

The aim of this study was to evaluate the role of oxidative stress in the mutagenicity of 4-NOPD and OPD. Since the DNA is well-protected in the nucleus, ROS have to pass several protection mechanisms (membranes, enzymes and antioxidants) before actually oxidise bases in DNA. Therefore, oxidation of free bases in the nucleotide pool is an interesting additional biomarker for oxidative stress. We used mouse lymphoma cells to study the effects of the mutagenic compounds on the induction of ROS, general and oxidative DNA damage, as well as the effects on the nucleotide pool as possible mechanistic pathways behind the mutagenicity, measured with mouse lymphoma assay.

Table 13. Genotoxicity endpoints in L5178Y mouse lymphoma cells treated with 4-NOPD and OPD with or without S9.

<table>
<thead>
<tr>
<th>Genotoxicity test</th>
<th>4-NOPD without S9</th>
<th>4-NOPD with S9</th>
<th>OPD without S9</th>
<th>OPD with S9</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenicity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>OPD is a stronger mutagen than 4-NOPD</td>
</tr>
<tr>
<td>DNA-adducts*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No adducts from 4-NOPD or OPD</td>
</tr>
<tr>
<td>ROS induction</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Induction of ROS for 4-NOPD</td>
</tr>
<tr>
<td>General DNA damage</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Induction of general DNA damage for OPD, and 4-NOPD with S9</td>
</tr>
<tr>
<td>Oxidative DNA damage</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>Induction of oxidative DNA damage for 4-NOPD, but unclear for OPD</td>
</tr>
<tr>
<td>Nucleotid pool effect</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Decrease of dC, dT and dA in the nucleotid pool</td>
</tr>
</tbody>
</table>


We used 4-NOPD and OPD as model mutagenic pro-oxidants without any observations of DNA-adducts, and therefore possibly working through indirect mechanisms such as oxidative stress and effects in the nucleotide pool.
Methods measuring oxidative stress, such as ROS-test (Jakubowski and Bartosz 2000) and alkaline comet assay with hOGG1-enzyme (Smith et al. 2006) were used to study mechanisms behind the mutagenicity of the compounds, and also imbalances in the nucleotide pool were measured with a method previously described (Osterman Golkar 2013).

Both substances were found to be mutagenic in mouse lymphoma assay (Table 13). The mutagenic effect was especially pronounced for OPD, where even low concentration induced mutagenicity. Both substances induced general DNA damage, as also reported by others (Gichner et al. 2001, Chye et al. 2008), but in contrast to OPD, addition of S9-fraction, that contains phase I metabolising enzymes, was needed for 4-NOPD-induced DNA damage. 4-NOPD clearly induced oxidative DNA damage in a dose-dependent manner, while OPD showed oxidative DNA damage only for a few concentrations without a clear dose-response (Table 13).

Furthermore, ROS was clearly induced by 4-NOPD, while OPD did not induce ROS at all. For both substances the control level of ROS was 10-fold increased in the presence of the S9 metabolic activation (Figure 7). This means that the S9 treatment in all four methods partly contributed to the oxidative stress, and as a consequence also to the mutagenesis.

One explanation for this increase of ROS in the presence of the S9 metabolic activation, leading to an increase in oxidative DNA damage, as detected by the alkaline comet assay (see Paper V) is that the ROS-induced increase of pH will increase DNA damage, previously shown in human fibroblasts (Czene et al. 1997). The increase of ROS seen for 4-NOPD without addition of S9 might be explained by autoxidation. The probe in the ROS-test is oxidised mainly by \( \ddot{O}_2 \), \( \ddot{O} \)H and \( H_2O_2 \), which are always present in cellular samples, also explaining the background level at exposure for vehicle only.

In the nucleotide pool test we could notice a decrease of the bases (Table 13), except for dG. This effect was most obvious at the lower concentrations of OPD and 4-NOPD. The aromatic amines have the ability to get into the cell. Within the cell, cellular oxidative stress can increase (at least from 4-NOPD), and increase of cellular ROS can increase oxidation in the DNA, but might also increase oxidation in the nucleotide pool. In Paper V there are four possible pathways behind the induction of mutation from 4-NOPD and OPD; (1) direct attack on the DNA (2) induction of ROS will give rise to mutations (3) induction of oxidised DNA-bases, which results in mismatch repair or incorporation of oxidised bases in the DNA during replication (4) increased nucleotide pool imbalances, directly or after induction of ROS.
Figure 7. Effect of 4-NOPD (panel A) and OPD (panel B) on the level of ROS in mouse lymphoma cells after 3-h exposure with or without S9. The generation of ROS was measured as an increase in fluorescence emitted by the oxidation product CM-H$_2$DCFDA at 430 nm using a microplate reader.
Here we have shown that there is no direct attack on the DNA from the amines, but that 4-NOPD will give rise to cellular oxidative stress, which in turn will give rise to both oxidised DNA bases and imbalance in the nucleotide pool. OPD shows no tendency of ROS-induction but will affect the nucleotide pool, probably through direct attack or through the same pathway that increase the general DNA damage.

Considering the results of other studies in this thesis, a question of whether antioxidants like β-carotene or α-tocopherol might have the ability to stop oxidative stress or effects on the nucleotide pool from 4-NOPD or OPD arises. Since these lipophilic antioxidants are located within lipoproteins and in cellular membranes, they might be able to protect against lipid peroxidation or especially oxidative stress reactions from the mitochondrial respiration chain, probably working from the cellular membrane or within the cytoplasm.

Direct attacks from 4-NOPD, OPD or metabolites towards the DNA, should not be possible to stop with help from β-carotene or α-tocopherol, neither is attacks from free radicals in the DNA or in the nucleotide pool. This makes it more possible to believe that these antioxidants can stop the oxidative stress from 4-NOPD, but might not be able to stop more direct pathways from OPD. It would be interesting to study these antioxidants together with 4-NOPD and OPD in further in vitro tests, using the same biomarkers, possibly together with catechol, a mutagenic pro-oxidant used in Paper III and IV in this thesis.

In summary, as illustrated in Table 13, both OPD and 4-NOPD showed mutagenicity in the mouse lymphoma cells. Indirect effects on DNA, possibly related to an unbalanced nucleotide pool, mediated the mutagenicity of 4-NOPD and OPD to a large extent. Although induction of intracellular oxidative stress and oxidative DNA damage seemed to be a possible mechanism behind the mutagenicity of 4-NOPD, this pathway is of less importance for the more potent mutagen OPD.
Conclusions

General conclusion
The general conclusion from the studies in the thesis is that antioxidants from fruit and vegetable intake decreased oxidative stress, while oral antioxidant supplements did not change the level of oxidative stress in type 2 diabetes subjects, despite increased plasma levels of antioxidants. Another conclusion is that antioxidants could modulate oxidative stress as well as general and oxidative DNA damage induced by pro-oxidants in mouse lymphoma cells.

Specific conclusions

- High intake of fruits and vegetables was significantly related to low levels of biomarkers for oxidative stress in type 2 diabetes subjects (Paper I).

- Plasma level of β-carotene was demonstrated to be a good biomarker for fruit and vegetable intake in type 2 diabetes subjects (Paper I).

- Daily supplementary intake of 20 antioxidants, known to be present in fruits and vegetables, did not modulate any of the biomarkers for oxidative stress in type 2 diabetes subjects, despite increased levels of antioxidants in plasma (Paper II).

- A physiologically relevant concentration of β-carotene reduced catechol-induced oxidative DNA damage in mouse lymphoma cells. At a high level of catechol, β-carotene was found to increase both general and oxidative DNA damage. The same patterns were seen both at pre-treatment and at simultaneous treatment with catechol. β-Carotene alone did not cause DNA damage in these cells at simultaneous treatment with catechol, but an increase was seen with the pre-treatment protocol (Paper III).
• Both catechol and OPD caused DNA damage in cultured mouse lymphoma cells, while 4-NOPD did not. α-Tocopherol reduced catechol-induced DNA damage, but not OPD- or 4-NOPD-induced DNA damage, suggesting different mechanisms of action behind the DNA damages. α-Tocopherol alone did not cause DNA damage in these cells (Paper IV).

• The novel less harmful DNA-dye GelRed could replace the mutagenic EtBr in the alkaline comet assay (Paper IV).

• OPD was a more potent genotoxic and mutagenic agent than 4-NOPD in cultured mouse lymphoma cells. Mechanisms behind the 4-NOPD mutagenicity were related to induction of intracellular oxidative stress (ROS) and oxidative DNA damage, while OPD did not affect these markers. Imbalances in the nucleotide pool were part of the mechanisms for both OPD- and 4-NOPD-induced mutagenicity. (Paper V).
Concluding remarks and future perspectives

In the study on type 2 diabetes subjects an inverse relation was seen between intake of food rich in antioxidants and markers of oxidative stress, and also that plasma levels of antioxidants were correlated with oxidative stress markers (Paper I). This demonstrate that a high intake of fruits and vegetables can decrease levels of oxidative stress in type 2 diabetes subjects, and suggest that the development and progression of diabetes and also the secondary symptoms of the disease, may be delayed.

In the same study group an intake of oral antioxidant supplements, at levels resulting from a recommended intake of fruits and vegetables for 12 weeks, did not modulate markers of oxidative stress (Paper II). This indicates that an intake of antioxidants is more favourable when coming from food compared to oral supplements. More studies are however needed to clarify the effects of intake of antioxidants from fruits, vegetables or oral supplements in type 2 diabetes subjects, and related groups. Until more detailed evidence is obtained, the results in this thesis and also in the literature support the recommendation of a high intake of fruits and vegetables, especially for type 2 diabetes subjects, but do not really support the benefit of oral antioxidant supplements.

Two selected antioxidants, studied clinically in Paper I-II, showed both antioxidant and pro-oxidant effects in the DNA of mouse lymphoma cells in Paper III and IV. While both α-carotene and β-carotene show an antioxidant effect at lower levels of oxidative stress, β-carotene is able to switch to become a pro-oxidant, increasing the level of oxidative stress and DNA damage, at a higher level of oxidative stress. Despite a large number of studies in the field, little is known about the detailed mechanisms behind the effects of these antioxidants, and also of other antioxidants present in fruits, vegetables and oral supplements.

The results in Paper V identify several cellular targets for oxidative stress which also shows different explanations behind the effects of antioxidants. Threshold mechanisms were identified at certain concentrations of the model mutagens, either through oxidative stress in the cell and DNA or imbalances in the nucleotide pool. More research, on these and other oxidative stress-related targets, is needed to state which antioxidants, in which concentrations, are able to show either antioxidative or pro-oxidative effects, and also to relate this to the actual location of the antioxidants in the cell, for example working in the cellmembranes and cytoplasma or more close to the DNA.
Oxidative stress within cells might damage DNA, lipids and proteins. Oxidative stress is an important factor in lifestyle-related disease, such as type 2 diabetes, and intake of antioxidants from the diet, or from oral supplements, has shown to be able to reduce oxidative stress. Clinical- and epidemiological studies have shown positive health effects from intake of fruits, vegetables and antioxidants, especially in subjects with type 2 diabetes. The scientific results though, are contradictory.

An aim for this thesis was to evaluate effects from antioxidant intake from fruits and vegetables, or from oral supplements, in type 2 diabetes subjects. Another aim was to study mechanistic aspects of oxidative stress and DNA damage in mouse lymphoma cells, studied after exposure to mutagenic pro-oxidants and antioxidants.

The first study in this thesis (Paper I) showed that a high intake of fruits and vegetables in type 2 diabetes subjects can decrease the levels of oxidative stress. This is supported by other studies in the literature. One theory is that it is the high content of antioxidants in fruits and vegetables that are responsible for decreased oxidative stress. In this study we also showed that β-carotene in plasma can be a biomarker for fruit and vegetable intake.

Many studies have been performed to study whether oral supplementation with one or a few antioxidants, in the form of a pill or a capsule can lower oxidative stress as effectively as high intake of fruits and vegetables. These studies have been performed in chemical systems, in cell systems as well as in humans. In paper II we did not see any effects in type 2 diabetic subjects on five different biomarkers for oxidative stress after oral supplementation with as many as 20 antioxidants, from fruits and vegetables, at recommended daily intake levels (or double) and a treatment duration for 12 weeks. This is supported by earlier studies showing no effect from the antioxidant supplementation, even though some studies show an antioxidative effect and a few studies even show a pro-oxidative effect.

In paper III we performed a study with addition of β-carotene in mouse lymphoma cells, showing a tendency to protect against DNA damage induced by catechol, a mutagenic pro-oxidant. However, at the highest concentration of catechol we saw a significant increase of the catechol-induced DNA damage (both general and oxidative) after addition of the antioxidant β-carotene. The addition of β-carotene was administered either 18 hours before the exposure of catechol or simultaneous with catechol. Both treat-
ment protocols showed similar results, but the pro-oxidative effect was larger after pre-treatment with the vitamin. This result suggests the same phenomenon as seen in two human studies on male smokers and asbestos-exposed patients that surprisingly showed an increased incidence of lung cancer and cardiovascular deaths after supplementation with β-carotene. In these studies it was probably the increased load of oxidative stress in the patients that made the antioxidant β-carotene switch to be a pro-oxidant.

α-Tocopherol is another well-studied antioxidant present in fruits and vegetables, but seeds, nuts and vegetable oils are better sources for this vitamin. This antioxidant previously showed antioxidative effects in chemical systems, cell systems and in humans. In paper IV, we have studied the effect of this vitamin in mouse lymphoma cells, with DNA damage as an end-point after exposure to the model substances catechol, o-phenylenediamine (OPD) and 4-nitro-o-phenylenediamine (4-NOPD). Simultaneously supplementation with α-tocopherol decreased the catechol-induced DNA damage but not the OPD- or 4-NOPD-induced damage. This suggests there are different mechanisms of action behind the DNA damage from the three agents. In paper IV we also showed that GelRed can replace the DNA-dye ethidium bromide in the alkaline comet assay.

We also wanted to study mechanisms, related to oxidative stress, behind the mutations induced by OPD or 4-NOPD, two mutagenic pro-oxidative substances present in hair-dyes, cigarette smoke, pharmaceuticals, pesticides and plastics. A mechanistic study on cells was performed according to the standard protocols for genotoxicity and mutagenicity testing on cells within the pharmaceutical industry, together with “in-house protocols” for testing of general oxidative stress and changes in the nucleotide pool. There was an increase of general oxidative stress and oxidative DNA damage after exposure of 4-NOPD indicating that oxidative stress was the main mechanism behind the mutagenicity of this substance. We could also show an effect on the nucleotide pool behind the mutagenicity of both OPD and 4-NOPD, where OPD showed to be the more potent genotoxicant and mutagen of the two aromatic amines (Paper V).

The general conclusions from the studies in this thesis are that humans, especially type 2 diabetes subjects, might be protected against oxidative stress from intake of fruits and vegetables. Intake of oral antioxidant supplements, present in fruits and vegetables, in relevant doses did not have any effects on oxidative stress in type 2 diabetic subjects. In cell-systems, antioxidants such as β-carotene or α-tocopherol, can show a slight anti-oxidative effect, indicating a protective effect from the vitamins. In contrast, in an environment with a high load of oxidative stress, β-carotene can have a pro-oxidative effect increasing the level of general and oxidative DNA damage, which we could show in the cell system. Furthermore, different mechanisms of action for the three studied pro-oxidants were shown using biomarkers associated with oxidative stress.
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