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Karolinska Institutet, Stockholm, Sweden

THE PARADOX OF MICRONUTRIENTS
- *In vitro* and Human Studies -

Therese Woodhill

Karolinska Institutet

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To my dear family

ABSTRACT

Are you wanting a more popular scientific summary? Please turn to page 68.
Vill du hellre läsa en populärvetenskaplig sammanfattning? Bläddra då till sid. 70.

Many essential micronutrients found in fruits and vegetables exhibit antioxidant properties, protecting against oxidation and oxidative stress. Their ability to react with electrons also enables them to act as *pro*-oxidants, causing damage opposed to preventing it. While fruit and vegetables rich in micronutrients generally protect against oxidative damage and related diseases, supplementation studies with micronutrients have not been verified to have the same health beneficial effect. In fact, even harmful consequences have been seen. The reason for the ambiguous results is still not fully understood and this thesis investigates some of the factors believed to be involved. The focus is on the effect of micronutrients on DNA lesions, potential contributors to cancer development. The thesis takes you into chemical properties of micronutrients (papers I and II), it also explores their health effects in two human studies (papers III and IV).

When investigating pro-oxidant properties of thirteen micronutrients in this thesis, two of them stood out oxidising the DNA nucleoside deoxyguanosine in a dose-dependent manner – namely vitamin A and vitamin C. Compounds permitted in supplements as either 'vitamin A' (four compounds) or 'vitamin C' (five compounds) also differed in their potencies to act as pro-oxidants. The combination of vitamin C and copper particularly stood out, inducing oxidation to pure deoxyguanosine, but also to the DNA of cells in culture, a potential concern since both are commonly found in multivitamin supplements.

In an observational study, the impact of folate/folic acid status, intake or genes on DNA lesions (uracil misincorporation, oxidative DNA lesions, DNA breaks) in mononuclear cells of 36 infertile women was explored. Folate and related parameters were found not to correlate with DNA lesions in these women with adequate folate status. Our results add to the carefully accumulating evidence indicating that, even though folate status has been associated with DNA lesions, the effect seems to be more evident in subjects with folate deficiency. An increased interest has been drawn towards micronutrient supplements imitating the natural content of fruit, vegetables and berries, as for example with berry extracts. This thesis report on a crossover intervention study where chronic kidney disease patients were administered a supplement containing oil extracts from the sea buckthorn berry rich in micronutrients and fatty acids. An eight-week administration did not affect their levels of DNA lesions (oxidative DNA lesions, DNA breaks) in minor salivary glands, nor mouth dryness or disease specific parameters.

Taken together, the results within this thesis add to the somewhat confusing evidence on micronutrient supplements and their properties and health effects. It emphasises that each micronutrient has unique properties, some with strong pro-oxidant activity dependent on factors in their immediate surrounding. The results also add to the growing evidence of a lack of effect on health from micronutrient supplements. It might be that supplementation with micronutrients provides best benefit in those with initially low nutrient levels.

LIST OF PUBLICATIONS[1]

I. Bergström T, Bergman J and Möller L. Vitamin A and C compounds permitted in supplements differ in their abilities to affect cell viability, DNA and the DNA nucleoside deoxyguanosine. *Mutagenesis*, 2011; 26(6): 735-744.

II. Bergström T, Ersson C, Bergman J and Möller L. Vitamins at physiological levels cause oxidation to the DNA nucleoside deoxyguanosine and to DNA – alone or in synergism with metals. *Mutagenesis*, 2012; 27(4): 511-517.

III. Ersson C[2], Woodhill T[2], Altmäe S, Murto T, Stavreus-Evers A, Wanhainen C, Poortvliet E, Böttiger A, Yngve A and Möller L. DNA damage and lack of correlation to folate status and metabolism in women with unexplained infertility – a study investigating uracil misincorporation into DNA, oxidative DNA lesions and DNA strand breaks. *Manuscript*, 2012.

IV. Rodhe Y, Woodhill T, Thorman R, Möller L and Hylander B. The effect of sea buckthorn supplement on oral health, inflammation and DNA damage in hemodialysis patients: a double-blinded, randomized crossover study. *J Renal Nutrition*, 2012; in press.

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[1] Surname changed from Bergström to Woodhill in 2012.

[2] These authors contributed equally to this work.

Preface

The field of micronutrient supplements is a somewhat controversial field, with pro-supplement speakers on the one hand and con-supplement speakers on the other. Both sides are to some extent convinced that their view is correct. Since research has revealed both positive and negative health effects after micronutrient supplementation, it comes as no surprise that different views have arisen. And truthfully, who wouldn't like the idea of one fast and simple way to get the nutrients we need? In this thesis, results from my and others studies concerning antioxidants and other micronutrients, their properties and health effects are presented.

The world of research and science is a unique field; it is an exciting field where curiosity, learning and constant improvement is crucial parts in order to take the research in the direction of progression – and make a small contribution to something great. As for many before me, my time as a PhD student has been somewhat like a roller coaster ride, with both ups and downs with the project moving forward, standing still and even moving backwards (yes my friends, it is possible!), only to turn around and move forward again (luckily!). I've had the pleasure of having amazing colleagues who have inspired and guided me and with whom discussions have resolved many scientific issues.

I have also had the pleasure to teach, lecture and organise courses and events alongside my research. On a personal level, the time as a PhD student has given me the opportunity to open my eyes for the exciting and highly important world of environmental medicine and all the areas that it includes. I am now looking forward to continue the exciting learning journey.

The zigzag road of science will pervade as you read on in this thesis…

Therese Woodhill, Stockholm October 2012

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LIST OF ABBREVIATIONS

8-oxodG/8-oxodGua	8-Oxodeoxyguanosine
8-oxoG/8-oxoGua	8-Oxoguanosine
A	Adenine
AA	Ascorbic acid
ADME	Administration, distribution, metabolism and excretion
AlkA	3-Methyladenine glycosylase II
AP	Apurinic/apyrimidinic
ATBC	Alpha-Tocopherol, Beta-Carotene
BER	Base excision repair
C	Cytosine
CARET	The β-Carotene and Retinol Efficacy Trial
CE	Capillary electrophoresis
CKD	Chronic kidney disease
dG	Deoxyguanosine
DHAA	Dehydroascorbic acid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuracil monophosphate
EC	Electrochemical
ECVAG	European Comet Assay Validation Group
ELISA	Enzyme-linked immunosorbent assay
EndoIII	Endonuclease III
ESCODD	European Standards Committee on Oxidative DNA Damages
FapyA	Fapy-adenine
FapyG	Fapy-guanine
Folate MG	Folates in monoglutamate form
Folate PG	Folates in polyglutamate form
FPG	Formamido pyrimidine DNA glycosylase
FR	Folate receptor
G	Guanine
GC	Gas chromatography
GLUT	Glucose transporters
HL-60	Promyelocytic leukaemia cell line

hOGG1	Human 8-oxoguanine DNA glycosylase
HPLC	High performance liquid chromatography
MS	Mass spectroscopy
NER	Nuclear excision repair
OGG1	(Human) 8-oxoguanine DNA glycosylase
PBS	Phosphate buffered saline
PCFT	Proton-coupled folate transporter
PHS	Physician's health study
RBP	Retinol-binding protein
RCT	Randomised control trials
RDI	Recommended daily intake
RFC	Reduced folate carrier
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAM	s-adenosylmethionine
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SOD	Superoxide dismutase
SU.VI.MAX	Supplementation en Vitamines et Mineraux Antioxydants
SVCT	Sodium dependent vitamin C transporter
T	Thymine
TAC	Total antioxidant capacity
THF	Tetrahydrofolate (folate)
TTR	Transthyretin
U	Uracil
UDG	Uracil DNA glycosylase
UV	Ultraviolet
VITAL	VITamins And Lifestyle
μM	10^{-6} Molar (i.e. mol/L)

Explanation of terms commonly used throughout the thesis.	
In vivo	In an organism. Often refers to animal or human studies.
In vitro	In glass/a test-tube. Refers to experimental work that is not conducted in an organism. For example, cell lines can be used.
Synthesise	Manufacture

1 INTRODUCTION

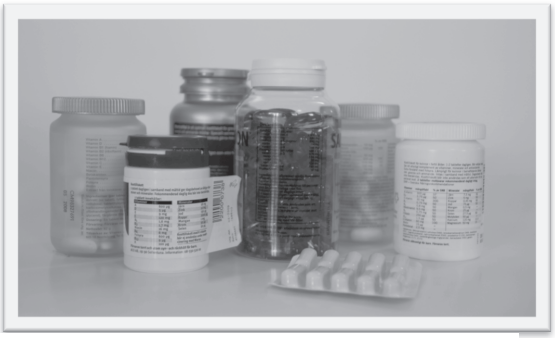


Figure 1. A selection of dietary supplements containing micronutrients. Photo by Lennart Möller.

Have you ever taken a dietary supplement? Maybe with the intention to deceive that approaching cold? Or increase your general health? Or reduce the risk of cancer from suddenly developing? Multivitamin supplements containing a mix of vitamins and metals ('minerals') are widely consumed in the general population. Recent reports from the national health and nutrition examination survey (NHANES) in US indicate that 33% of the adults consume multivitamin supplements containing both vitamins and metals regularly, or 'during the last month' [1]. Dietary supplements on the whole are consumed on a regular basis by 49% of the US adults [1]. To my knowledge no such figures are available for Sweden alone, but the prevalence is assumed to be similar here. In 2010, vitamin and mineral supplements were sold for 490 million SEK in Sweden, a figure that has been steadily increasing [2].

The wide use of dietary supplements is anticipated to be due to its advertised and accredited health beneficial effects, but how much is supported by scientific evidence? When considering their effect on cancer, the wide use of micronutrient supplements can actually be viewed as a concern. The use of supplements is currently debated, with a number of recent articles addressing the issue [3,4]. The bulk evidence is pointing towards a lack of health beneficial effects, in relation to cancer, from supplements when consumed by the general, well-nourished population. Even harmful effects have been observed, which emphasises the concern of the wide use of supplements as well as the need for clarification of the mechanisms behind micronutrients.

The thesis in your hand will bring you into the world of micronutrient supplements – discussing the (sometimes contradictory) properties of micronutrients, and their effects in humans, with a focus on their influence on the genetic make up, the DNA, and cancer.

2 FRUIT AND VEGETABLES VS. DIETARY SUPPLEMENTS

Are micronutrient supplements and their properties favourable or harmful to us? This thesis explores this issue. What do studies say about them and their content? This chapter aims to give a background to the field of micronutrient supplements, giving an overview of the complexity that this field of research faces. Later chapters will go deeper into specific micronutrients within the scope of this thesis. For reasons that will be made clear later on, the main focus in this thesis is on the effect of micronutrients on DNA and cancer.

2.1 FRUIT AND VEGETABLES

Fruit and vegetables are rich in antioxidants and a number of other micronutrients. A varied diet with plenty of fruit and vegetables has not only been *thought* to be beneficial but scientific research has also *verified* that they are good for many aspects of human health. Fruit and vegetables in the diet lower the risk of for example stroke, coronary heart disease, hypertension and cancer as summarised in Table 1 [5,6].

Table 1. Intake of fruit and vegetables lower the risk of… [5]

Convincing evidence for	Probable evidence for	Possible evidence for
Stroke	Cancer	Body weight gain
Coronary heart disease[1]		Certain eye diseases
Hypertension[2]		Dementia
		Osteoporosis

[1]cardiovascular disease
[2]high blood pressure

Since fruit and vegetables are rich in micronutrients (including both vitamins and antioxidants), antioxidants have to a large extent been accredited for the health beneficial effects of fruit and vegetables. What happens if we extract or synthesise (manufacture) individual or a few antioxidants/micronutrients and consume them on their own? Antioxidant supplements containing one or a few antioxidants have been synthesised with the aim to achieve the same health promoting effects as fruit and vegetables. But do they have the promised effects?

2.2 DIETARY SUPPLEMENTS

The micronutrients, vitamins and antioxidants that supplements contain have been added to the tablets or capsules in the hope to give the same health beneficial effects as those naturally found from fruit and vegetables. As will be discussed later the antioxidants and vitamins found in dietary supplements may be of synthetic origin, may be present in high doses, in unnatural forms and in combinations not naturally found in fruit and vegetables. This thesis is focused on the effects of micronutrient supplements

on DNA and DNA damage/lesions. As will be further explained in chapter 5, some DNA lesions can potentially lead to mutation and cancer. As you read on, the focus lies on the effects of micronutrient supplements and their effect on cancer prevalence. This section will give a selected overview of the scientific literature on the topic, schematically summarised in Figure 2. Can micronutrient supplementation prevent cancer?

The golden standard in micronutrient human studies is to conduct intervention studies which are randomised, large in size and placebo-controlled. These studies are also referred to as randomised controlled trials, RCTs. Other study designs are briefly described in chapter 6. Below, the attention is on well-established, large, randomised and placebo-controlled intervention studies conducted on micronutrient supplements in relation to cancer.

2.2.1 The Linxian General Population Nutrition Intervention Trial

Approximately 30 000 middle-aged men and women (n = 29 584, age 40-69 years) from the general population were given supplements containing different combinations of a number of micronutrients with the aim to monitor mortality and cancer frequency. The study was conducted between 1986 and 1991, and was carried out in the Linxian province in China, a region with a high incidence of oesophageal cancer and persistently low intake of several micronutrients. The participants were randomised into eight groups, receiving different combinations of vitamin A (retinol, 5000 IU), zinc (zinc oxide, 22.5 mg), vitamin B_2 (riboflavin, 3.2 mg), vitamin B_3 (niacin, 40 mg), vitamin C (ascorbic acid, 120 mg), molybdenum (molybdenum yeast complex, 30 μg), β-carotene (15 mg), selenium (selenium yeast, 50 μg) and vitamin E (α-tocopherol, 30 mg). The figures within brackets denote the daily intake. When compared to current recommendations by the Swedish National Food Agency (year 2012), depicted in Table 2, most of these vitamin doses are approximately twice the recommended daily intake (RDI). Supplements were taken daily during an average period of 5.25 years [7].

At the end of the trial, a 9% reduction of the total mortality rate and a 21% reduction of gastric (stomach) cancer incidence were observed in the group receiving a combination of β-carotene, vitamin E and selenium [7]. In a follow-up study published in 2009, the beneficial effects remained [8]. In the same area, another study was conducted in which patients diagnosed with oesophageal dysplasia in the same area were administered 14 vitamins and 12 minerals for six years. Again, positive results were obtained. The total mortality incidence was 7% lower in the supplemented group compared to controls, and the total cancer incidence was 4% lower [9].

2.2.2 The ATBC Lung Cancer Prevention Study

The aim of the Alpha-Tocopherol, Beta-Carotene (ATBC) prevention study was to determine whether supplementation of vitamin E (α-tocopherol) or β-carotene, or both, reduce lung cancer incidence in male smokers [10-12]. Initiated in 1985 in Finland, this study included 29 133 male habitual smokers (20.4 cigarettes/day) aged 50-69 years. They received vitamin E (α-tocopherol, 50 mg), β-carotene (20 mg), a combination of both or placebo for 5-8 years [12]. The dose of vitamin E corresponds to five times the

Table 2. Recommended daily intake (RDI) of micronutrients in Sweden[1]

	Adult men	Adult women	Pregnant women	Nursing women
Vitamin A[2]	900 μg	700 μg	800 μg	1100 μg
B vitamins				
Thiamine	1.4 mg	1.1 mg	1.5 mg	1.6 mg
Riboflavin	1.7 mg	1.3 mg	1.6 mg	1.7 mg
Niacin[3]	19 mg	15 mg	17 mg	20 mg
Pantothenic acid[4]	-	-	-	-
Pyridoxine	1.6 mg	1.2 mg	1.5 mg	1.6 mg
Biotin[5]	-	-	-	-
Folate/folic acid	300 μg	300 μg, 400 μg[6]	500 μg	500 μg
Vitamin B_{12}	2.0 μg	2.0 μg	2.0 μg	2.6 μg
Vitamin C	75 mg	75 mg	85 mg	100 mg
Vitamin D[7]	7.5 μg	7.5 μg	10 μg	10 μg
Vitamin E[8]	10 mg	8 mg	10 mg	11 mg
Vitamin K[9]	-	-	-	-

[1]Recommendations by the Natural Food Agency in Sweden.
[2]1 retinol equivalent (RE) = 1 μg retinol = 12 μg β-carotene.
[3]1 niacine equivalent (NE) = 1 mg niacin = 60 mg tryptophan.
[4]No Nordic recommendations. 5 mg is mentioned on the National Food Agency's homepage (2012) as a norm in US.
[5]No Swedish recommendations. A varied diet should give 40 mg per day on average.
[6]300 μg: women above childbearing age, 400 μg: women who might become pregnant.
[7]10 μg for children under the age of two, and for elderly people with nonsufficient sun exposure.
[8]α-tocopherol equivalents, 1 α-tocopherol equivalent (αTE) = 1 mg RRR-α-tocopherol.
[9]Not part of the nutritional recommendations in the Nordic countries. The American Institute of Medicine considers 120 μg for men and 90 μg for women as an adequate intake.

recommended RDI in Sweden. The dose of β-carotene is of pharmacological (i.e. high) levels [11]. As a pro-vitamin A, β-carotene is included in the RDI for vitamin A, and 20 mg β-carotene is equivalent to twice the RDI for vitamin A.

The main finding of this intervention study came as a surprise to the authors. Amongst the subjects receiving β-carotene, the incidence of lung cancer was *increased* by 18% and mortality by 8%. Vitamin E supplementation significantly reduced prostate cancer [12]. The harm of β-carotene supplementation was diminished after the intervention was terminated, a follow-up after three years revealed a non-significant 17% increase of lung cancer incidence while the risk was absent six years after the intervention. The beneficial effect on prostate cancer was absent at the three-year follow-up [13].

2.2.3 CARET

The β-Carotene and Retinol Efficacy Trial, CARET, of US origin commenced in 1983 [14-16]. The participants were 18 314 smokers, former smokers and/or workers exposed to asbestos aged 45-74 years. They were either given a combination of vitamin A (retinyl palmitate, 25 000 IU) and β-carotene (30 mg) or placebo. The intervention prolonged for 4.0 years on average, and was terminated 21 months earlier than planned due to the preliminary findings and the outcome of the ATBC study. As in the ATBC study, CARET caused an increase in lung cancer incidence following supplementation, with 28%. Among the supplemented participants, there was an increase in incidence of total mortality by 17%, in mortality from lung cancer by 46% and in mortality from

cardiovascular disease by 26% [16]. A six-year follow-up concluded that the adverse effects by β-carotene and vitamin A on lung cancer incidence and all-cause mortality remained, although they were no longer statistically significant [14]. The increased risk of mortality from cardiovascular disease was no longer present.

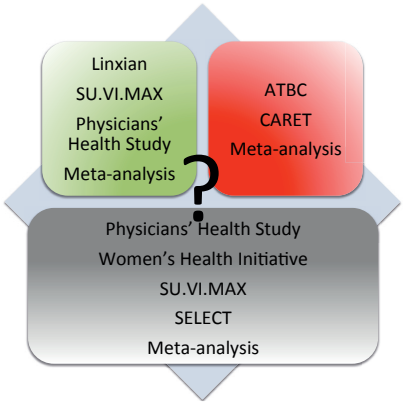


Figure 2. A schematic summary of large micro-nutrient supplement studies with observed health beneficial (green), harmful (red) or no effect (grey).

2.2.4 Physicians' Health Study I

This intervention was designed to test the effect of low-dose aspirin and β-carotene on cardiovascular and cancer disease in a healthy population [17,18]. It was initiated in 1982 when 22 071 US male physicians aged 40-84 years were given either aspirin (325 mg), β-carotene (50 mg), both or placebo to be consumed every second day. Due to clear beneficial effects of aspirin on myocardial infarction the aspirin intervention was terminated after five years while the β-carotene arm carried on [17]. After 12 years of β-carotene intake, no effect on cancer frequency or mortality was seen [18]. Neither among current nor former smokers, 11% and 39% of the study population, respectively, were there any effects in these outcomes [18].

2.2.5 Physicians' Health Study II

Following the physician's health study I (PHS I) a second study, PHS II, began in 1997 [19,20]. 14 641 male physicians aged 50 years or older were enrolled with the intention to investigate the effect of micronutrients on cardiovascular disease, total cancer and prostate cancer. A 2^4 factorial design was used in which the participants were randomised into 16 groups and administered different combinations of vitamin C (ascorbic acid, 500 mg), vitamin E (α-tocopherol, 400 IU), β-carotene (50 mg), multivitamin or placebo during an average period of eight years, or until 2003 for β-carotene. Vitamin E and β-carotene were taken on alternate days, while vitamin C and multivitamin were taken daily. The vitamin C and vitamin E daily doses are approximately six times and 20 times the Swedish RDI, respectively. β-carotene in PHS II has become subject to investigations beyond the scope of this thesis (i.e. on cognitive behaviour [21]). Neither vitamin C nor vitamin E either caused or prevented cardiovascular disease, prostate cancer or total cancer [19,20]. Results regarding the multivitamin supplementation were published while this thesis was at its final stage. These new results reveal a reduction in total cancer from multivitamin consumption in men with a baseline history of cancer [22].

2.2.6 The Women's Health Initiative

The Women's Health Initiative addresses the most common causes of death, disabilities and impaired quality of life in postmenopausal women, with focus on cardiovascular

disease, cancer and osteoporosis. The prevention study began in 1991, went on for 15 years and included randomised controlled clinical trials as well as an observational study. One of the trials investigated the effect of vitamin D and calcium supplementation on colorectal cancer risk and bone fractures [23,24]. 36 282 postmenopausal women from the US aged 50-79 years were administered a combination of calcium (calcium carbonate, 1 g) and vitamin D (vitamin D_3, 400 IU) or placebo daily, during an average period of 7 years. Treatment with vitamin D and calcium did not have any effect on the incidence of colorectal cancer [24].

2.2.7 The SU.VI.MAX study

The Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) study initiated in 1994 involved 13 017 French adults, both men (aged 45-60 years) and women (aged 35-60 years). On a daily basis during a period of 7.5 years, the participants were given capsules of vitamin C (ascorbic acid, 120 mg), vitamin E (30 mg), β-carotene (6 mg), selenium (selenium-enriched yeast, 100 μg) and zinc (20 mg), or placebo [25]. Overall, supplementation had no effect on total cancer incidence, cardiovascular disease or all-cause mortality. However, when analysing men and women separately, men in the supplementation group had lower cancer incidence (31% lower) as well as a lower mortality risk (37% lower) compared to the control group. The men also had lower levels of certain antioxidants at the start of the study (at 'baseline'). This reduced risk was no longer evident five years post-supplementation [26].

2.2.8 SELECT

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) commenced in 2001 and included 35 533 men above 50 years of age from the US, Canada and Puerto Rico [27]. They were given vitamin E (α-tocopheryl acetate, 400 IU), selenium (selenomethionine, 200 μg), a combination of both, or placebo daily during an average period of 5.5 years. The main outcome investigated was prostate cancer, but also lung cancer, colorectal caner, other primary cancers and mortality. No significant reduction or increase in any of these cancer forms was observed. Initially a *non-significant* increase of 13% in prostate cancer was, however, seen amongst the participants receiving vitamin E [27]. In a follow-up this evolved to a significant increase of 17% [28]. The group receiving vitamin E had a statistically non-significant increase in prostate cancer of 13%. The initial absence of significant data in SELECT opened a debate in which both the micronutrient form used [29] and the common use of one or a few antioxidants for therapeutic purposes were questioned and challenged [30].

2.2.9 Meta-analysis

The attention of the current section is on large, randomised placebo-controlled intervention studies/trials. In order to obtain a comprehensive understanding of what the evidence from studies mean when they are put together, meta-analysis provides a helpful tool. In these studies, data from all available studies (within the set inclusion criteria) are considered in combination and conclusions drawn from all data.

A number of meta-analysis related to micronutrients and cancer or mortality have been conducted finding associations between for example β-carotene, vitamin A and vitamin E, singly [31,32] or in different combinations [31,33], and increased mortality risk. The same authors did, however, not find effects on mortality among the overall participants receiving vitamin C, selenium [31,32] or β-carotene [34]. Meta-analysis have concluded different micronutrients, alone or in combinations, to have either no effect on cancer [34-36], a protective effect [37] or a promoting effect [35].

2.2.10 Observational studies

A PubMed search on observational studies (case-control studies or cohort studies) and antioxidants (conducted using Mesh-terms, on the 28th September 2012) reveals 2 846 hits. In this section a selection of two large studies published during recent years are described within the scope of this thesis.

VITAL
In the VITamins And Lifestyle (VITAL) study 77 126 US men and women aged 50-76 years were followed in respect to vitamin use and its effect on lung cancer. Slatore *et al.* [38] found no evidence that either multivitamin, vitamin C or folate had any effect on lung cancer. Vitamin E, however, was associated with a statistically non-significant 5% increase in mortality risk, a risk that was more pronounced in smokers [38].

The Iowa Women's Health Study
This recent observational study from 2011, in which 38 772 women aged 55-60 years responded to food and supplement questionnaires concluded that a majority of the vitamin and mineral supplements consumed had no effect on mortality [39]. However, multivitamins, vitamin B_6, folic acid, magnesium and copper individually increased mortality risk in some of the statistical models, while the association between iron and mortality was strongest showing a correlation to mortality risk in several statistical models. Calcium supplementation was associated with decreased mortality risk [39].

2.2.11 What can explain the different effects from micronutrient supplements?

Taken together, studies have not shown micronutrient supplementation to be disease preventive, instead they have raised questions of why they often do not protect, and have sometimes even shown harmful health effect. The discrepancy is illustrated in Figure 2. As depicted in Figure 3, several factors have been, or can be, pinpointed as contributors to the different outcomes:

Nutritional status and health
The Linxian trial that reported beneficial effects from supplementation, was carried out in a high risk population. The general population of this area have a high incidence of oesophageal cancer accompanied by persistently low intake of several micronutrients [7]. The reduced cancer and mortality risk seen in supplemented men in the SU.VI.MAX study was suggested to be attributed to low baseline statuses of certain antioxidants [25]. In the intervention studies mentioned above, the participants showing no or harmful health effects did not report low nutrient status. However, the ATBC trial identified an inverse association between intake of α-tocopherol and β-carotene from

food at baseline and the risk of lung cancer during the trial [12]. These above studies suggest that the nutritional status at baseline seems to be important for the outcome of supplementation. Evidence suggests that deficient subject might benefit from supplementation of micronutrients, while healthy individuals might not. In addition, in the trials where healthy participants were studied, to which PHS I and II, the Women's Health Initiative, SU.VI.MAX and SELECT belong, no effect was observed on cancer incidence [18-20,24,25,27].

Supplement composition

Supplements often contain one or a few micronutrients present in combinations that differ from those naturally found in fruit and vegetables. Many antioxidants co-operate with one another and one antioxidant might therefore require the presence of another in order to function properly. For example, vitamin C and E cooperate in preventing oxidation of cell membranes. Vitamin E scavenges peroxyl radicals, protecting the membrane from lipid oxidation. Vitamin C recycles (reduces) vitamin E back to its original form, enabling further protection [40]. Without vitamin C, the protection from vitamin E would be less efficient. Similarly, expected properties of supplements can be speculated to be absent if the naturally cooperating 'partner(s)' are not present.

Micronutrient dose

Furthermore, it may not be enough that the correct micronutrients are present; they may need to be present at a specific dose. Micronutrient supplements often contain high doses of micronutrients, which might also hamper their function. Some properties of antioxidants are affected by the antioxidant's own concentration. β-Carotene and vitamin C have both been observed to *cause* oxidation instead of protecting against it when present at high concentrations [41]. The ATBC trial, CARET as well as PHS I had pharmaceutical (i.e. high) levels of β-carotene, the two former causing harmful effects (in smokers) while the latter did not (in mostly non-smokers) [12,16,18].

Micronutrient form

Many micronutrients found in supplements are not of natural origin, instead many have been synthesised, i.e. chemically manufactured. Vitamin E constitutes a classic example of the important implications of this. Vitamin E is naturally found in eight isomeric forms, but is often only present in one form (α-tocopherol) in supplements. During recent years the importance of another vitamin E compound, γ-tocopherol, has been stressed, highlighting the importance of the choice of micronutrient forms [42]. As mentioned above, the SELECT opened a debate of the form of selenium used in supplement studies [29]. The European Parliament and Council regulate which specific compounds that are allowed in supplements (directive 2002/46/EC). For vitamin C this directive comprises five different compounds, and for vitamin A four different compounds. These are compared and discussed in paper II and chapters 7 and 8.

Milieu

Environmental factors also influence the action of many micronutrients. For example, under a high oxygen pressure or high temperature, β-carotene can act as a pro-oxidant instead of an antioxidant (pro-oxidants will be explained later in this and later chapters). In the ATBC trial as well as in CARET, smokers had an increased risk of developing lung cancer after β-carotene supplementation [12,16]. The lung of a smoker

is recognised for having a high oxygen pressure, a high level of free radicals and low antioxidant levels, especially of vitamin C and E [43]. These conditions can promote pro-oxidant behaviour of β-carotene but also oxidation of β-carotene. Resulting oxidation products have been proposed to be involved in the adverse health effects in smokers [43,44].

Antioxidants – pro-oxidants

Micronutrients with antioxidant properties generally help to protect biomolecules against oxidation and oxidative damage. Under certain conditions they can, however, start to act as oxidants instead of antioxidants, causing oxidative damage as opposed to preventing it. Antioxidants thus have the ability to also act as *pro-oxidants*. β-carotene, vitamin C and vitamin A have all been documented to be able to act as pro-oxidants [45-47]. In addition, the phenomenon of pro-oxidant properties is extensively studied in papers I and II and further described and discussed in chapters 5, 7 and 8.

Oxidative stress

When the antioxidants act as pro-oxidants, the balance between oxidising agents and the protective systems of the cell, can become disturbed and a condition referred to as oxidative stress occur. In all constituent articles, papers I-IV, oxidative stress plays an important role.

This thesis investigates and discusses properties and health effects of micronutrients. Much focus lies on pro-oxidant properties of antioxidants, oxidative stress and DNA damage/lesions.

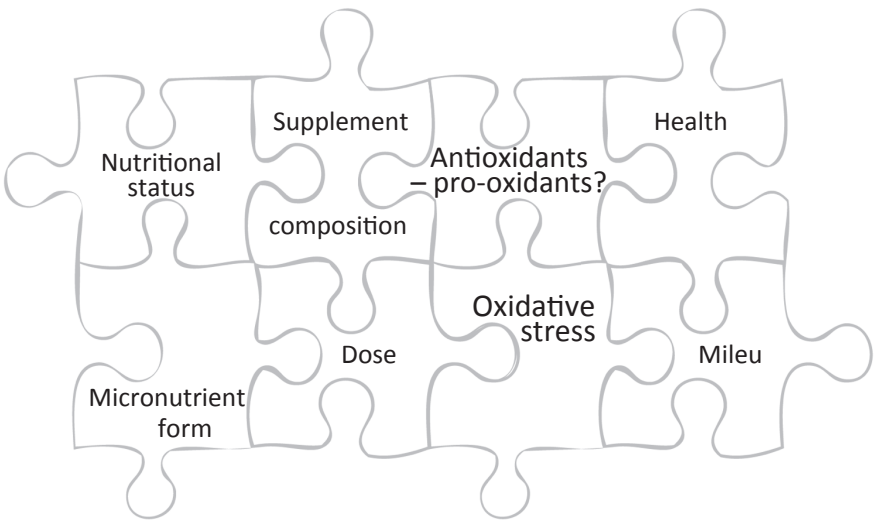


Figure 3. Factors believed to contribute to the contradicting results from micronutrient supplements. Jigsaw illustration by Cassandra Reynolds.

3 RESEARCH AIM

The overall aim of this thesis is to investigate properties and health effects of micronutrients. Particular focus is put on properties related to oxidative stress since it is believed to be involved in the varied outcomes from micronutrient supplements, while their exact role is yet not fully understood. Emphasis is put on the effect of micronutrients on DNA, as well as on the ability of antioxidants or micronutrients to act as pro-oxidants.

Specific aims have been to answer the following questions:

1. Do micronutrients/antioxidants chemically act as pro-oxidants and if so, do they do so with different strengths?
2. Do micronutrients with pro-oxidant potential act as pro-oxidants in cultured cells?
3. Do different compounds in supplements classed as either vitamin A or C differ in their abilities to act as pro-oxidants?
4. Do folate levels, intake or genes related to folate metabolism predict levels of DNA lesions?
5. Does a supplement containing a berry extract rich in antioxidants reduce DNA lesion levels, improve health or improve the oral health of chronic kidney disease patients?

4 MICRONUTRIENTS

So why do micronutrients receive so much attention? What is each of them good for? This section will briefly explain the role of individual micronutrients, giving an overview of their role in humans (also illustrated in Figure 4) as well as of potential supplementation recommendations. First, however, it is time to clarify what micronutrients, vitamins and antioxidants are and what their differences are.

4.1 MICRONUTRIENTS, ANTIOXIDANTS AND VITAMINS – WHAT IS THE DIFFERENCE?

Micronutrients, vitamins and antioxidants are often used without discrepancy in the literature. In fact, vitamins and antioxidants are often micronutrients, and many vitamins are also antioxidants, which justifies the confused use of the three terms.

Micronutrients are elements needed in minute (small) quantities. They include vitamins, antioxidants, metals (minerals) and other compounds needed for the health of human cells. **Vitamins** are *essential* in minute quantities for a human body to function properly. Humans cannot synthesise these compounds and they are therefore required through the diet. 13 known vitamins exist. **Antioxidants** are substances that, at low concentrations, delay or inhibit oxidative damage to molecules [48]. They may for example do so by reacting with and neutralising oxidants (free radicals or other reactive species), thereby preventing damage that the oxidants may otherwise have caused. Many, but not all, vitamins can act in this way and are therefore also classed as antioxidants.

The term "micronutrients" is extensively used in this thesis and refers to vitamins, antioxidants as well as other micronutrients.

4.2 VITAMIN A

In its natural form vitamin A is found in animal products, and it serves as a helper in night vision. Particularly rich sources include liver, fish oil, dairy products and eggs. Consumption of vitamin A is required for a functioning vision, gene expression, cell growth and development, immune function and embryonic development [49]. Vitamin A is a generic term, a 'family name', that refers to a group of fat-soluble compounds with the biological activity of *retinol*. It constitutes both preformed vitamin A including retinyl esters and retinol, as well as provitamin A including β-carotene, α-carotene and β-cryptoxanthin. The latter class is cleaved (i.e. cut) to vitamin A *in vivo* and serves as an important source of vitamin A [40].

When consumed as a supplement, vitamin A can be harmful at high doses. In its natural form, such high doses can only be achieved through excessive consumption of for example liver. High doses of vitamin A can affect vision, muscular coordination, liver, bones and joints and give rise to osteoporotic diseases. In Sweden, vitamin A was

withdrawn from AD-drops given to babies and infants in 2009, as much as 10 years after our neighbours in Denmark. Nevertheless, vitamin A has limited antioxidant capacity and has been shown to have preventive effects against certain cancer forms [50]. According to an extensive report conducted by the World Cancer Research Fund and American Institute for Cancer Research, there is 'limited evidence' that vitamin A protects against skin cancer [6]. Vitamin A is further described and discussed in chapter 7, and constitutes, together with vitamin C, is the theme of papers I and II.

4.3 B VITAMINS

B vitamins comprise around 30 substances, whereof eight essential:

B_1 – thiamine	B_6 – pyridoxine
B_2 – riboflavin	B_7 – biotin
B_3 – niacin	B_9 – folate (synthetic form: folic acid)
B_5 – pantothenic acid	B_{12} – cobalamins

They are required as cofactors, for DNA-synthesis, replication, conformation (folate), neurotransmitter synthesis (B_6), gene expression, development of red blood cells (B_6), and for preventing neural tube defects (folate) [40]. It is also proposed that B vitamins also have preventive properties against DNA damage [51]. Even if many of the B-vitamins do not have an antioxidant property *per se*, they cooperate in the protection against lipid peroxidation in the glutathione redox cycle (e.g. B_2). Studies have also proposed B vitamins to affect cancer [40,52,53].

Folate. Folic acid (the synthetic, manufactured, form of folate, vitamin B_9) is one of only two vitamins, the other being vitamin D, that the Swedish National Food Agency currently (2012) recommends Swedes to take supplements of. The recommendation is aimed at women who are planning to become, or are, pregnant (during the first trimester). The main reason for this recommendation is the preventive of folic acid on neural tube defects in new-borns, but evidence also suggests folates to be important in reproductive health as well as in preventing spontaneous abortions [54]. Several countries, including the US and Canada apply folic acid fortification of for example grains and cereals, while some countries, among which Sweden is one, have decided not to. Recent studies have also revealed a possible non-beneficial effect where high intake of folic acid supplements has been associated with increased cancer incidence [53,55,56]. Natural sources of folate include liver, green leafy vegetables (e.g. spinach, broccoli), citrus fruits and beans [40]. Paper III and chapter 9 are entirely devoted to folate and will discuss this in more detail.

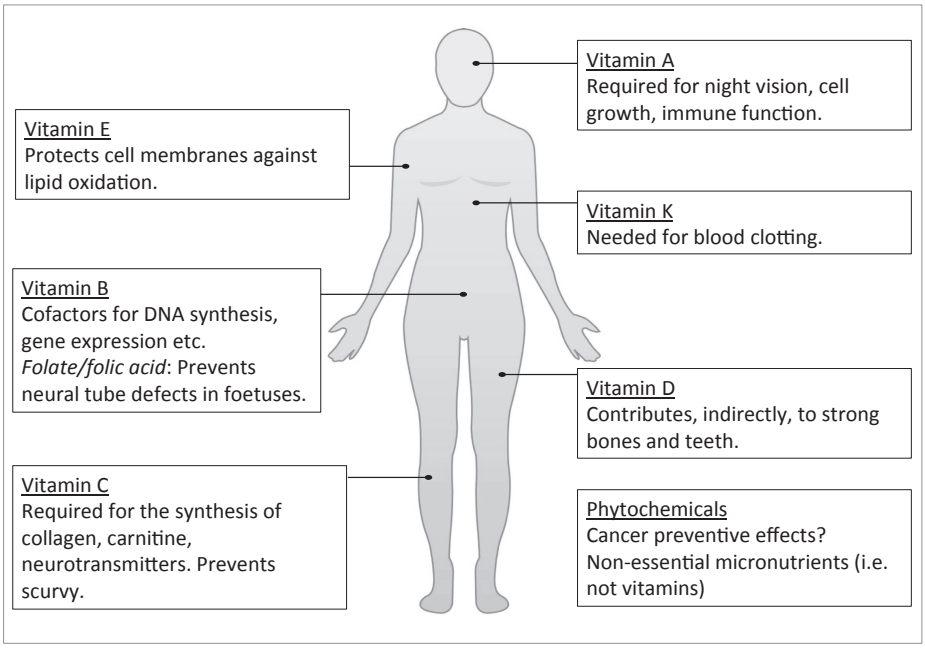


Figure 4. Selected functions of vitamins and other micronutrients. Human illustration by Cassandra Reynolds.

4.4 VITAMIN C

Vitamin C, also known as *ascorbic acid* and *ascorbate* is found in many fruits and vegetables. Citric fruits, berries and potatoes are particularly good sources. As vitamin C is a cofactor for a number of enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters, it is essential to obtain vitamin C through the diet [40]. However, the vitamin is prone to high temperature, oxidation as well as high amounts of water. Therefore, rough cooking procedures may reduce vitamin C levels in food. Deficiency of vitamin C leads to a weakening of collagenous structure, giving rise to tooth loss, joint pains, poor wound healing, bone and connective tissue disorders and eventually scurvy – a potentially fatal disease [40]. Vitamin C is a well-established antioxidant, with the ability to frequently react with free radicals and thus potentially prevent biomolecules from damage. It has been seen to lower the incidence of both cardiovascular disease and cancer [57]. Recently, vitamin C at high doses has also been suggested to have chemotherapeutic properties [58].

Side-tracking beyond the scope of this thesis, vitamin C is commonly consumed in supplemental form with the aim to prevent, or improve symptoms of, the common cold. Scientific evidence does, however, not support a reduction of colds in the general population due to vitamin C supplementation. Nonetheless, a meta-analysis concluded that the incidence of colds in marathon runners undergoing acute physical stress could be reduced by supplementation [59]. Vitamin C is further studied and discussed in papers I and II, and in chapter 8.

4.5 VITAMIN D

Vitamin D helps the body to utilise calcium and phosphorous, which are necessary to build up strong bones and teeth. Vitamin D is yet another generic term comprising a group of steroid compounds of which vitamin D_2 and D_3 are most prominent. Vitamin D_3 is produced in the skin when the precursor 7-dehydrocholesterol is hit by sunlight. Together with other hormones it helps to maintain the calcium balance. Through this action, vitamin D is important for blood clotting, nerve pulse transmission, membrane structure and muscle contraction. A deficiency of vitamin D leads to low calcium and phosphorous levels which contribute to demineralisation of bone, which can lead to softening of bones in adults (osteomalacia) and rickets, causing the spine and legs to crook in children [40].

Since the body can produce vitamin D_3 itself, vitamin D is not "essential" to consume in the same sense as other vitamins, or as the term 'vitamin' implies (see section 4.1 for definition). When the amount of sunlight is insufficient, or large amounts of air pollution, clothing or sunscreens are hindering its entry; vitamin D_3 is, however, required through the diet. Fatty fish is a good source of vitamin D, but to raise the consumption of this micronutrient, milk is fortified with vitamin D_3. Vitamin D is the second, and last, vitamin that the Swedish National Food Agency recommends supplementation of (the other being folic acid). The recommendation is aimed at infants and toddlers between one month and two years of age, and elderly who are not sufficiently exposed to sunlight. In recent years, vitamin D and its link to cancer has become of interest to researchers and discussion have been raised about a link to cancer of for example the breast, colon and pancreas, as well as mortality due to cancer [60].

4.6 VITAMIN E

E vitamins are different from other vitamins in that they are not functioning as co-factors or hormones, nor do they have a role in the metabolism. Instead vitamin E's important role is due to its antioxidant properties. They scavenge ("neutralise") radicals, interrupt chain reactions and protect cells against lipid oxidation. They protect the membrane of cells against lipid oxidation [40].

Vitamin E is found in for example vegetable oil, sunflower seeds and almonds. It is a generic name referring to eight fat-soluble, structurally similar compounds present in two groups named *tocopherols* and *tocotrienols* [40]. The structural difference between tocopherols and tocotrienols give rise to differences in the potency and efficacy of antioxidant capacity [42]. Although all E vitamins are naturally found in food items, vitamin E in supplements is commonly found only in the synthetic α-tocopherol form [40]. In addition, the synthetic form of α-tocopherol contains eight isomers whereas only one of them is believed to meet human requirements [40]. Vitamin E is present in the sea buckthorn berry that is discussed in paper IV and chapter 10.

4.7 VITAMIN K

By serving as a cofactor vitamin K promotes the function of proteins involved in the clotting, or coagulation, of blood, and a deficiency in vitamin K leads to an acute life-threatening condition as a result of excessive bleeding. This vitamin also plays a key role in maintaining bone strength, inhibiting arterial calcification and regulating cell growth [61]. Two natural forms of vitamin K exist, vitamin K_1 and K_2. Vitamin K_1 is present in green leafy vegetables such as spinach and broccoli and is the primary vitamin K source for humans. Vitamin K_2 comprises a group of compounds produced by bacteria [40].

Vitamin K deficiency is rare in adults, consequently the Swedish National Food Agency does not give recommendations concerning vitamin K intake. Neonates, new-born babies, are however more at risk of developing vitamin K deficiency [62]. Neonates have an insufficient intestinal colonisation (i.e. insufficient vitamin K_2 production) and, probably of greater importance, run the risk of not receiving sufficient amounts of vitamin K through breast milk, or through the placenta as foetuses [62]. As a consequence of this, many countries, including USA and Sweden, intravenously supplement neonates with vitamin K to prevent an increased risk of bleeding.

4.8 PHYTOCHEMICALS

Phytochemicals embrace a class of compounds to which thousands of compounds belong. The five main categories include phytoestrogens, carotenoids, phytosterols, isothiocyanates and chlorophyll. Phytochemicals are plant compounds that are non-essential to humans, i.e. they are not classed as vitamins [63]. In recent years, phytochemicals have served much attention within research and interest has been drawn to their potential cancer-preventive effects. Most of the studies suggesting cancer-preventive effects are animal or cell culture studies and a recent review from 2012 on the available epidemiological (human) studies have concluded that most phytochemicals do not affect cancer. Nonetheless, some studies have shown protective effects from for example β-carotene, β-cryptoxanthin, isothiocyanates, lignans and flavonoids on different cancer types [63].

Carotenoids. Carotenoids constitute of a group of yellow to deep-red pigments found in many bright coloured fruits and vegetables. Over 600 known compounds belong to this group, whereof one is β-carotene. As a precursor to vitamin A, β-carotene is an important source of vitamin A. As opposed to vitamin A, high concentrations of β-carotene consumed from natural sources has historically not been observed to have harmful effects [40]. This is because the absorption of β-carotene is regulated, and is irreversibly dependent on its *in vivo* concentration [40]. In reality, this means that β-carotene will only be taken up by the cells if the body is in need of it. However, environmental factors might affect its destiny and as discussed in chapter 7, β-carotene supplementation caused increased lung cancer risk in smokers in the ATBC study and CARET [12,16].

4.9 METALS/MINERALS

The human body also requires different elementary metals. Many enzymes require metal ions in order to function. In supplements, metals such as calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, selenium, zinc are also common. Many so called *multivitamins* which contain both antioxidants, vitamins and metals are available on the market. Metals are often referred to as *minerals* on dietary supplement containers. The effect of a combination of certain metals and vitamin A or C is brought up in paper I and in chapters 7 and 8.

5 OXIDATIVE STRESS, ANTIOXIDANTS AND DNA LESIONS

This chapter brings up some of the most relevant conditions and elements for the research within this thesis. It will take you through the wonders of oxidative stress, its cause and prevention as well as damage that can occur to DNA.

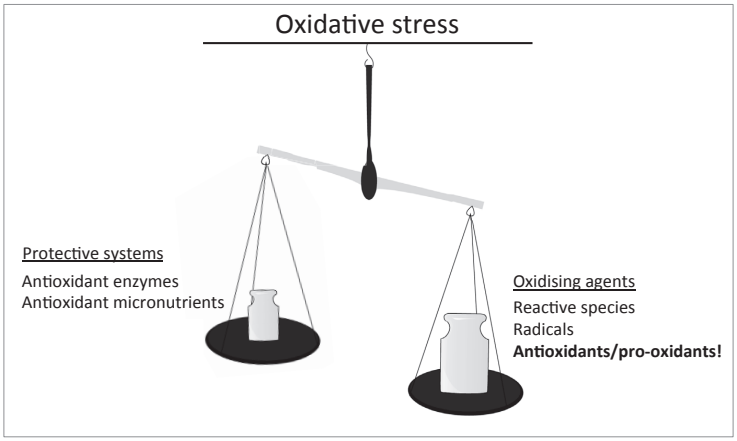


Figure 5. Oxidative stress. Scale illustration by Cassandra Reynolds.

5.1 OXIDATIVE STRESS

Oxidation is a constant threat to a cell, with the potential to cause damage to biomolecules, the compartments of the cell. Not only is it a threat though, oxidation is in fact foremost a normal and important step of many reactions taking place in cells, reactions in which electrons are transferred between molecules. Oxidising agents and other reactive species are continuously formed during normal processes such as during energy production in the mitochondrial electron transport chain and during the defence against foreign particles by phagocytes [48]. When the oxidising events overwhelm the antioxidant protective system of the cell, a condition referred to as oxidative stress evolves. Oxidative stress is an imbalance between the oxidising agents and the protective system, the term was first introduced in 1985 by Sies and defined in 1991 [64] as:

"*a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage*"

Since then, the expression has been broadened and oxidative stress was in 2007 redefined by Halliwell and Gutteridge [48] as:

"*the biomolecular damage caused by attack of reactive species upon the constituents of living organisms"*

Oxidative stress can cause damage to biomolecules including proteins, lipids and DNA. Through this updated definition it becomes clear that oxidative stress can cause

not only oxidative damage to these biomolecules, but also that the condition oxidative stress itself can give rise to other events and other types of damages. Section 5.3 below describes different types of DNA lesions that may or may not arise as a consequence of oxidative stress.

5.1.1 Causes of oxidative stress

In principal, oxidative stress can arise from either of these two scenarios:

- *Elevated levels of reactive species*. Reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals and other reactive compounds can give raise to oxidative stress. Collectively these are referred to as *reactive species*. Examples of ROS include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) while RNS include for example peroxynitrite (NO_3). The concentration of reactive species might be increased above controllable levels by 1) an excessive production of naturally occurring reactive species in the cell, 2) exposure to particularly high O_2 levels, or 3) the presence of toxins producing reactive species [48].

- *Reduced antioxidant protection*. The other option for oxidative stress to occur is by an inadequate antioxidant defence, either by insufficient antioxidant enzymes or dietary antioxidant depletion. The level of antioxidant enzymes can be hampered by, for example, specific mutations, while a lack of exogenous antioxidants might arise from an insufficient dietary intake or an incorrect administration, distribution or metabolism.

The production of many ROS in the body is strongly associated with redox cycling of transition metals. One well-established such reaction is the **Fenton reaction**, where the highly reactive hydroxyl radical is produced from the conjunction of a transition metal (M) ion (e.g. Fe^{2+}, Cu^{+}) and hydrogen peroxide.

$$M^{2+} + H_2O_2 \rightarrow M^{3+} + OH^- + OH^{\bullet} \quad (1)$$

The significance of the Fenton reaction *in vivo* is, however, unclear. While it is known to occur *in vitro*, the prevalence of for example free catalytic iron within the body is small under normal conditions due to the protection of many metal-binding proteins. There are, however, conditions under which metal ion levels are increased *in vivo*, and where Fenton reactions might possess a potential source of reactive species.

5.1.2 Consequences of oxidative stress

Oxidative stress can promote cell proliferation, an up-regulation of the antioxidant defence system, cell injury or even cell death [48]. This thesis focuses on effects on DNA, and oxidative stress can induce a number of different DNA lesions, oxidative DNA lesions. Lipids, proteins and other biomolecules are also prone to damage caused by oxidative stress, but these will not be further discussed in this thesis.

Oxidative stress and an increased production of ROS has been connected to more than hundred diseases. Its impact seems to be particularly strong in cancer and neurodegenerative diseases, including Parkinson and Alzheimer diseases [65].

5.1.3 Protection against oxidative stress

The defence system against oxidative stress consists of endogenous and exogenous, dietary, antioxidants.

Endogenous antioxidants
Cells have their own defence system to fight attack from reactive species, and by such defending themselves against oxidative stress. The defence comprises several antioxidant molecules and antioxidant enzymes as well as enzyme systems that either directly reduce reactive species or reduces other biomolecules, such as proteins. Much attention has been on the enzymes superoxide dismutases (SOD), catalase, glutathione peroxidases, and thioredoxin reductases as well as the antioxidant glutathione [48].

Exogenous – dietary – antioxidants
Protection against oxidative stress is also achieved through the entry of antioxidants from the diet. These antioxidants can scavenge, i.e. react with – and thereby neutralise – reactive species including ROS and other free radicals. In this way, they are able to minimise the exposure to oxidising agents and the risk of oxidative stress to evolve.

5.2 ANTIOXIDANTS – PRO-OXIDANTS?

Antioxidants are substances that delay or inhibit oxidative damage to molecules [48]. They can scavenge free radicals or neutralise other reactive substances through electron transfers. In addition to their scavenging properties, some antioxidants can promote antioxidant enzymes or even repair existing DNA damage by e.g. reducing the guanyl radical, which is formed as an intermediate during oxidation of the DNA base guanine [66]. Alarmingly, their ability to react with electrons also enable antioxidants to act as *pro-oxidants*, *causing* oxidative stress as opposed to *preventing* it, as illustrated in Figure 5.

Environmental factors influence the antioxidant or pro-oxidant activity and a high oxygen pressure (such as that of a smokers lung) and a high antioxidant dose has, for example, been seen to promote pro-oxidant behaviour [67,68].

5.3 DNA LESIONS

The human genetic make-up our DNA is a necessity for life and is the key to the construction of all cellular components. It is also responsible for passing on properties from one generation to the next. The DNA, or deoxyribonucleic acid, is built up of four building blocks, so called nucleotides. The nucleotides consist of a pyrimidine (cytosine, C; thymine, T) or purine base (adenine, A; guanine, G), a five carbon sugar (2-deoxyribose) and one phosphate group, as seen in Figure 6. The carbon sugar

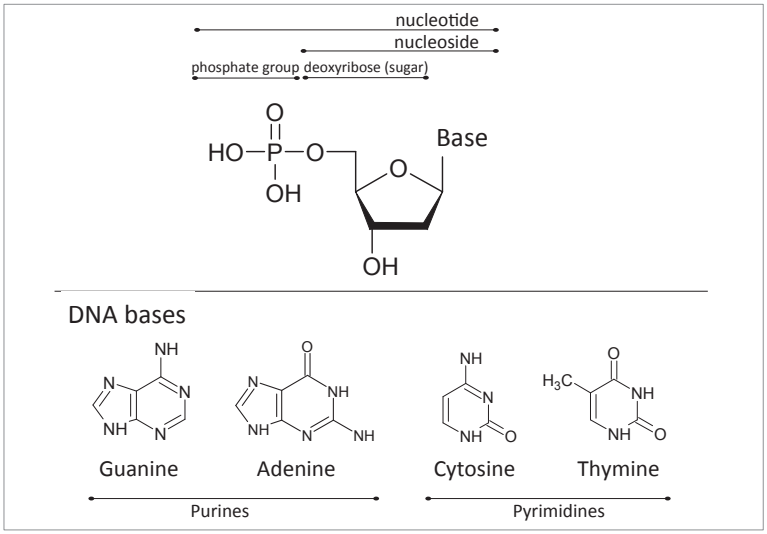


Figure 6. Chemical structures of the DNA building blocks, nucleotides and DNA bases.

(deoxyribose) and the phosphate group build the backbone of a DNA strand while the nucleobase "sticks out" allowing bonds to be formed between bases on opposite DNA strands. G and C pair together through three hydrogen bonds and A and T bind through two bonds. This base binding holds two DNA strands together, forming the double-stranded DNA helix for which DNA is recognised.

Both the sugar-phosphate backbone and the bases are vulnerable to attack, which may cause different types of damage (lesions). Many important DNA lesions are, or can be, created as a consequence of oxidative stress, including the vastly studied 8-oxoguanine. Different DNA lesions are described below.

5.3.1 Strand breaks

Damage to the DNA backbone can lead to strand breaks, either single-strand breaks in which one of the strands are broken, or double strand breaks in which both strands are broken. If incorrectly repaired, these lesions can induce mutations [69]. Strand breaks can also be formed as an intermediate step of DNA repair.

5.3.2 Oxidative DNA lesions

The four DNA bases are receptive to oxidative damage by e.g. ROS. These damages are extensively referred to as *oxidative DNA lesions/damages* but because the resulting lesions are not necessarily oxidants themselves, but merely a result of oxidation reactions, some researchers encourage the use of phrases such as *oxidatively damaged DNA* and *oxidative damage to DNA* for a more accurate description.

8-oxoguanine

The base guanine has the lowest redox potential out of all four DNA bases [70]. A low redox potential means that the agent more easily donates electrons, and thus becomes oxidised. Guanine is therefore the DNA base that is most susceptible to oxidative attack. A key site of radical attack is at the 8-position of guanine, where guanine is oxidised and 8-oxoguanine formed, see Figure 7. 8-Oxoguanine alone or its nucleoside equivalent (i.e. guanine + deoxyribose, the sugar) 8-oxoguanosine are common biomarkers for oxidative stress on DNA [71]. 8-oxoguanosine is commonly abbreviated to 8-oxoG or 8-oxoGua. Known by many names, 8-oxodeoxyguanosine is also referred to as 8-oxo-2'-deoxyguanosine and 8-oxo-7,8-dihydro-2'deoxyguanosine, and commonly abbreviated to 8-oxodG or 8-oxodGua. Undamaged G base pairs with C, both present in their *anti*-configuration. 8-oxoG can also base pair with C, but it can also adopt *syn* conformation and base pair with A instead. In this way a G:C to T:A transversion occurs during replication, resulting in a mutation [72]. 8-oxoG is thus a pro-mutagenic lesion. Whilst the clinical importance of oxidative DNA lesions remains unresolved to date, elevated levels of 8-oxoG have suggested associations with several diseases, including cancer and diabetes [73]. Due to it being relatively easily measured, in combination with its pro-mutagenic property, 8-oxoG (with or without the attached sugar unit) is the most studied lesion [74]. Under normal conditions a background level of 0.3-4.2 8-oxoguanine/10^6 guanine has been established by the European Standards Committee on Oxidative DNA Damages, ESCODD [71].

Other oxidative DNA lesions

Oxidation of guanine can also lead to other lesions including Fapy-guanine (FapyG; a ring opened form) and hyperoxidised guanine products, including for example oxazolone (Oz), spiroiminodihydantion (Sp), guanidinohydantoin (Gh) and imidazolone Iz [72]. Other DNA bases can also be oxidised at different positions, creating oxidative lesions including 8-oxoadenine, Fapy-adenine (FapyA), thymine glycols, 5-formyluracil, cytosine glycol, 5,6-dihydroxycytosine – to mention some [48].

5.3.3 DNA adducts

Alkylation

Alkylation of the DNA bases, in which carbon-hydrogen molecules bind to the oxygen atom, changes the size and shape of the DNA molecule and enables mismatched base pairing which may lead to mutations.

Crosslinking

DNA crosslinks are formed when nucleotides covalently bind to each other, either on the same strand or on opposite strands, forming intra- or inter-crosslinks, respectively. ROS can induce certain nucleotide modifications that can give raise to crosslinks. Crosslinks can block DNA replication and transcription and are believed to be mutagenic [75].

5.3.4 Misincorporation of incorrect bases into DNA

Uracil misincorporation
Another mutagenic DNA lesion is the presence of uracil (U) in DNA. Uracil is a nucleobase normally present in RNA, not in DNA. Deamination of cytosine results in the formation of uracil. Folate deficiency can also increase the presence of uracil, since a deficiency of folate prevents the conversion of dUMP to dTMP, deoxythymidine monophosphate, the thymine nucleotide required for DNA synthesis. Indeed, folate deficiency has been associated with uracil misincorporation [52], and this is further discussed in Paper III and chapter 9.

5.3.5 Consequences of DNA lesions

As is described in the following section, many DNA lesions are repaired and further damage to the cell is thus avoided. However, DNA lesions (e.g. FapyG and FapyA) might also block DNA replication and transcription, possibly resulting in mutation (i.e. an incorrectly incorporated base). Under circumstances when the repair is insufficient or even fail, mutations may also be created. Mutations occur in cells under normal conditions, without developing into cancer. It is primarily when the mutations occur in specific genes, for example in oncogenes, tumour-suppressor genes or stability genes, that cancer might follow. The process when normal cells are converted into proliferating cancer cells is referred to as carcinogenesis and is a complex multistep process [48].

5.4 REPAIR OF DNA LESIONS

Two principal repair paths take care of DNA lesions. Small alterations to bases, including oxidative DNA lesions, alkylations and single strand breaks, are mainly repaired by base excision repair (BER) whereas bulky adducts or bulky single strand breaks primarily are repaired by nuclear excision repair (NER). The cells also have repair systems for mismatches (misincorporations) as well as for double strand breaks [69].

5.4.1 Base excision repair

In principal, BER assures that the lesion is cut off, a break is formed and subsequently mended by the insertion of a correct nucleotide. BER steps in and a lesion specific glycosylase (enzyme) directly removes the lesion, i.e. the base. In the absence of a base the result is an apurinic/apyrimidinic (AP) site, which is converted to a break. During the repair process, a single strand break is thus formed. Some polymerases are bifunctional and can cut the DNA themselves while others need AP endonucleases to do so. The small gap is mended by DNA polymerase (usually polymerase β) and ligase, which removes the AP site, inserts a correct nucleotide and seal the DNA. BER steps in and removes lesions before DNA replication occurs, avoiding further consequences [48]. BER is the path most often used for the repair of oxidative lesions, but it can also repair for example alkylations and strand breaks [69]. For example, 8-oxodG is directly

repaired by BER, through the specific removal by the human 8-oxoguanine DNA glycosylase, OGG1 (hOGG1). In addition, this glycosylase also removes FapyG.

5.4.2 Nuclear excision repair

NER cuts the DNA some distance from the lesion and the oligonucleotide is removed. A DNA polymerase then fills the gap, and a ligase seals the DNA. The action of NER is down to several protein complexes. The lesion is recognised, unwound and cut (by excision) by several sets of proteins, where proteins referred to as XPC, XPB, XPD and XPF are involved. While BER gives rise to a gap the size of one nucleotide (short patch BER) or, by extension during the repair, up to eight nucleotides (long patch BER), NER contributes to a much larger gap. NER is particularly important in removing damage induced by UV exposure, but it can also help removing oxidative lesions [48].

5.4.3 Mismatch repair

Mismatch repair corrects incorrectly paired bases. While BER and NER acts before replication, mismatch repair acts on damages post replication. G:T, G:G, C:C or A:C mismatches are recognised by the Mut proteins and a nuclease removes the mispaired base. For example, incorrectly paired adenine can be removed by MUTYH. This glycosylase recognises adenine when it is incorrectly paired with C, G or 8-oxoG, and is thus also a repair mechanism for the 8-oxoG lesion. Misincorporated uracil is removed from DNA by uracil DNA glycosylase (UDG).

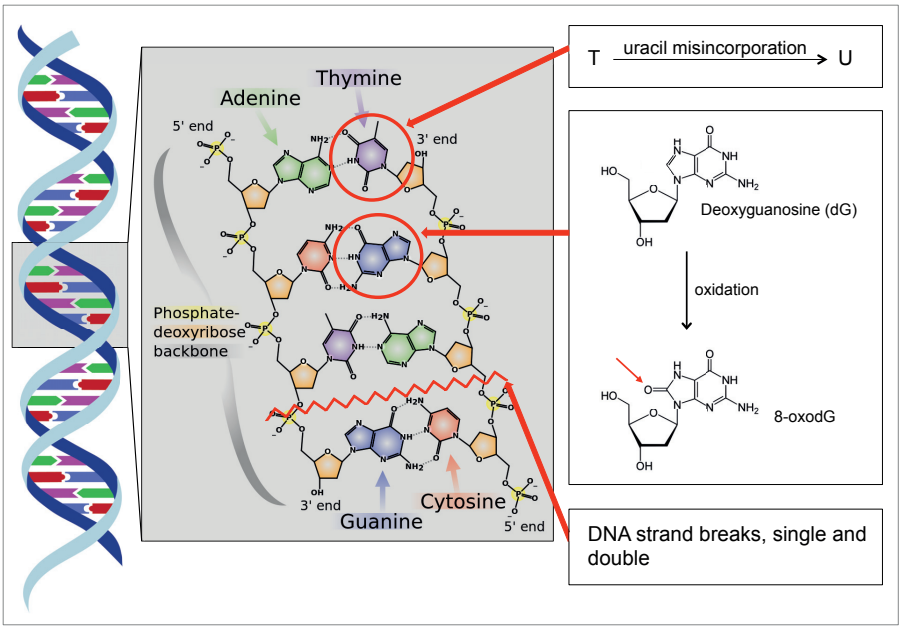


Figure 7. A schematic illustration of the DNA lesions analysed in papers I-IV. Many more oxidative lesions and misincorporations exist, as well as other types of DNA lesions. DNA illustration by Cassandra Reynolds and the zoomed in illustration in the middle is credited to Madeleine Orice Ball.

6 MONITORING THE PROPERTIES AND EFFECTS OF MICRONUTRIENTS - METHODS

6.1 HUMAN STUDIES

The ultimate goal is of course to elucidate the actual/real effects of micronutrients, vitamins, antioxidants and supplements *in humans*. Different approaches can be taken when conducting human studies. As can be seen in Table 3, the different study types are categorised depending on their design. In this thesis, one intervention study (paper IV) and one observational, yet experimental, study (paper III) have been conducted.

Table 3. Human Studies [76,77]

Observational studies	
No interference in what humans are exposed to.	
Surveys	*Simultaneous collection of data on multiple exposures and outcomes (cross-sectional), using surveys. Explore for associations.*
Case-control study	*Investigates exposure retrospectively, comparing e.g. a diseased group with a healthy control group and looking for common denominators in the past.*
Cohort study	*Investigates the effect of e.g. exposure in the future, following and comparing disease incidence in e.g. an exposed group and a control group.*
Case study	*In contrast to the other analytical approaches, this is a descriptive study in which e.g. cases of diseased patients and exposure information are reported. Case studies can be prospective or retrospective and are often small in size.*
Intervention studies	
Experimental studies in which human exposure is being interfered, e.g. through supplementation.	
Randomised, controlled trials	*The "golden standard", in which subjects are randomised to intervention or control group.*

In the case of studies with micronutrient supplements, large randomised, placebo-controlled intervention studies/trials are considered the 'golden standard'. In these studies, one or more micronutrients are given as supplements over a given time period. The study population is randomly divided into two (or more) groups in which one group receives the supplement whereas the other group (the control group) receives placebo, a tablet with the same appearance but without the micronutrients of interest. In this type of study, it is possible to compare the effect with and without the supplement, avoiding some of the confounders that hamper the interpretation of many observational studies. Nonetheless, observational studies, particularly case-control and cohort studies, also constitute a helpful tool in researching the effects of micronutrient supplements. Case-control studies enable us to investigate the impact on previous supplement usage and its association to a particular disease. This study type can be time and cost efficient and enable the study of rare diseases. One of its drawbacks is, however, the risk of recall bias. In cohort studies, this risk is avoided. Here, the effect of supplement use on disease can be studied by following people who take a certain supplement, and compare the prevalence of disease amongst them with those of a control group. However, selection bias, failure to follow-up the participants, possesses a challenge in this type of study.

In experimental human studies, the output analysed may be a disease itself, or one or more biomarkers associated with e.g. a disease. DNA lesions are one example of biomarkers used in human studies. In papers III and IV the amount of different types of DNA lesions were analysed using the comet assay, a method described in section 6.6.2. Different inflammation markers were also analysed as biomarkers of immune response.

6.2 IN VIVO STUDIES

In many research areas animal studies, i.e. *in vivo* studies, are utilised in order to come closer to a human situation, but without risking the health of human participants. When investigating micronutrients, cellular studies and human studies are more common and no attention is given to *in vivo* studies in this thesis. In addition, laboratory rats and mice seem to be responding differently to antioxidant supplements than humans do [65].

6.3 IN VITRO STUDIES

In vitro studies is a broad term that includes many different types of studies, often designed with the aim to investigate a particular issue. The common denominator is that they are not conducted in humans or animals, instead components of an organism, or cell lines are used. In studies investigating mechanisms and properties of micronutrients, the use of cell lines enables fast and simple experiments, in comparison to human studies. In papers I and II, *in vitro* methods are utilised.

6.4 CHEMICAL STUDIES

Another approach to investigating micronutrients' chemical properties is through strictly chemical studies in which no human material or cells are used. In the absence of human material as well as cell lines, it is for example possible to investigate antioxidant and pro-oxidant properties of antioxidants without the interference of other components. Through such experiments, the chemical ability of micronutrients to act as either antioxidants or pro-oxidants can be investigated. Papers I and II uses this approach.

6.5 STUDY DESIGNS USED IN PAPERS I-IV

6.5.1 In vitro and chemical studies – Papers I and II

The main aim of papers I and II was to investigate and compare whether a range of different micronutrients – of which a majority are antioxidants – have pro-oxidant properties. These antioxidants and micronutrients are by EU regulations permitted in dietary supplements. The approach used in papers I and II were of *in vitro* and chemical character, and the study designs are seen in Figure 8.

In vitro studies. In papers I and II, pro-oxidant properties of micronutrients, through their ability to cause oxidative damage to DNA, were studied using cell lines. Cells were grown in culture and exposed to micronutrient for one (paper I) or 24 (paper II) hours after which the cells were analysed for DNA lesions using the comet assay, as well as for cell death (cytotoxicity) using trypan blue staining. The analytical methods are described in section 6.6. The levels of damage were compared to those in cells that were not exposed to micronutrient (i.e. controls). Since antioxidants commonly are distributed through the blood stream, the cells used were of blood origin, the promyelocytic leukaemia cell line HL-60. In paper I, it was also investigated whether the pro-oxidant property of chosen antioxidants was affected in the presence of a metal (also found in supplements) and/or the ROS hydrogen peroxide, H_2O_2. The cells were then exposed to metal and/or H_2O_2 at the same time as micronutrient.

Chemical studies. When investigating the micronutrients' ability to chemically act as pro-oxidants, the DNA nucleoside deoxyguanosine (dG) was used as a model in order to see the chemical effects on a biologically relevant molecule. Since dG is a constituent of the DNA, potential oxidation of it by a micronutrient is directly relevant to the human situation. dG was exposed to a micronutrient for one hour and the oxidation product 8-oxodG was analysed using the HPLC-EC/UV system described below.

Concentrations. Physiological concentrations of micronutrients were used throughout these studies, based on normal plasma levels. We also included concentrations up to ten times normal plasma levels, since both plasma and specific tissues/cells can have much higher concentrations. Intravenous administration of vitamin C, for therapeutic purposes, can, for example, produce plasma levels as high as 20 mM (400 times normal levels) [58]. The studies in papers I and II are further discussed in Chapters 7 and 8.

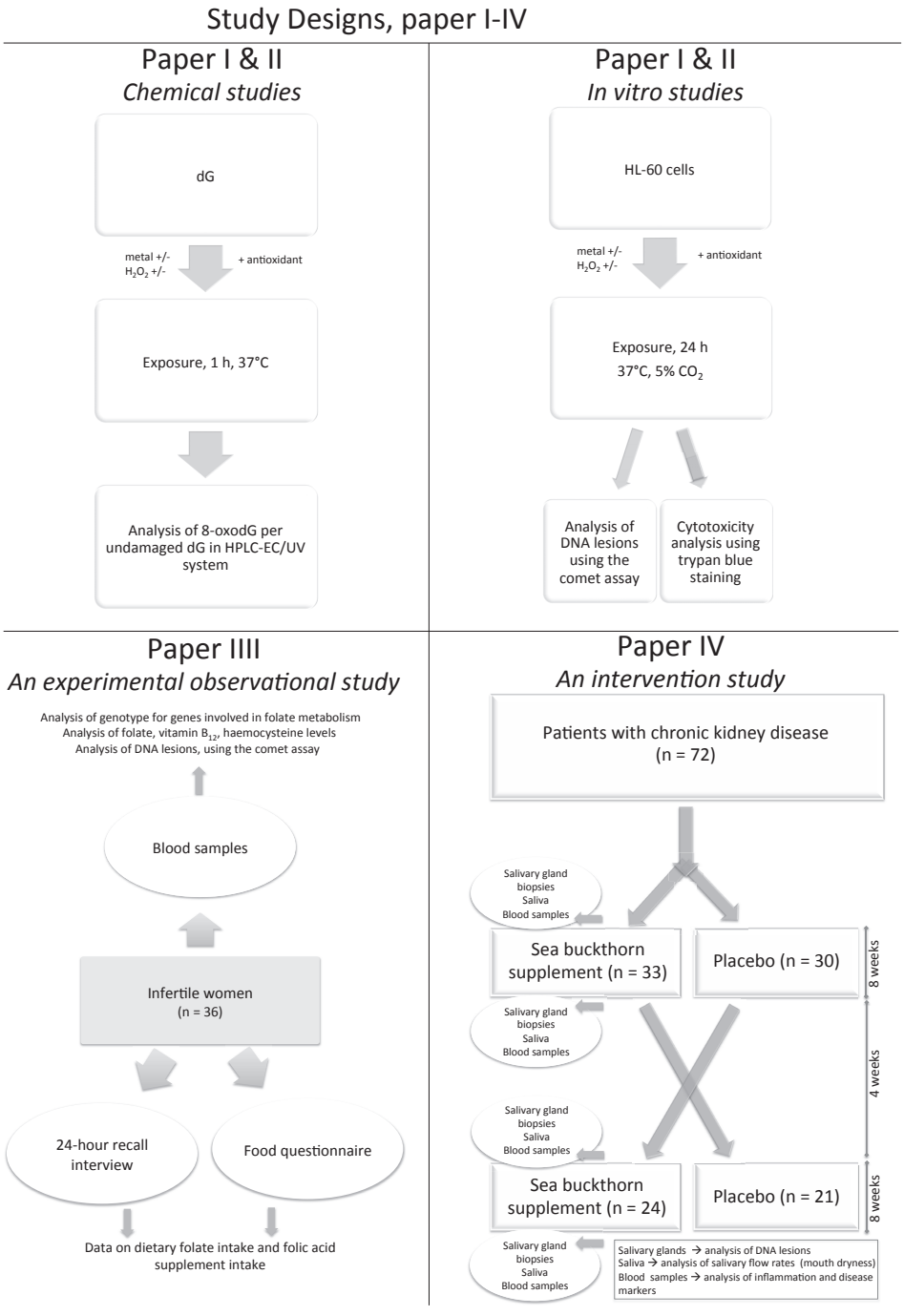


Figure 8. A chematic view over the study designs used in papers I-IV.

6.5.2 An observational, experimental human study – Paper III

Paper III investigates the impact of folate status and metabolism on different types of DNA lesions. The study group consisted of 36 women with unexplained infertility, who were attending the Department of Obstetrics and Gynecology at the Karolinska University Hospital in Huddinge, Sweden. A dietary survey and an interview were conducted wherefrom intake of dietary folate and supplement use was gained. Blood samples were also collected, and used for the analysis of e.g. folate, vitamin B_{12} and polymorphism in genes involved in folate-metabolising pathways. Using the comet assay, DNA lesions, including uracil misincorporation into DNA, strand breaks and oxidative DNA lesions (FPG-sensitive sites), were also analysed from drawn venous blood samples. Associations between folate status, intake and metabolism were then explored. This study is further discussed in Chapter 9.

6.5.3 An intervention study – Paper IV

Paper IV is an intervention study in which patients suffering from chronic kidney disease are given a supplement containing an extract from the berry sea buckthorn. This berry is rich in many antioxidants as well as fatty acids. As can be seen in Figure 8, the intervention was a crossover study in which the patients were randomly assigned into two groups. One group were given the sea buckthorn extract while the others received placebo, then they changed. The supplementation period went on for eight weeks and before given the other type of tablet, there was a four weeks wash-out period in order to limit the risk for carry-over effects, i.e. effects from period one to carry over to period two. Before and after each treatment period (i.e. four times in total), salivary glands biopsies, saliva and blood samples were collected. The main outcomes were different DNA lesions in minor accessory salivary glands, salivary flow rates and inflammation markers in blood. Paper IV is further discussed in Chapter 10.

6.6 METHODS TO DETECT THE BIOMARKERS IN PAPERS I-IV

Oxidative stress is a condition that cannot be measured *per se*. Instead the presence of damaging agents (reactive species or ROS) need to be measured, alternatively the levels of damage. ROS are short-lived, and thus challenging to measure. Another approach is therefore to measure the amount of damage that they cause. These so called *biomarkers* are often more feasible and robust to measure. A commonly measured biomarker for oxidative stress is the oxidative DNA lesion 8-oxodG. The methods used in papers I-IV to detect biomarkers and other types of damage are described below.

6.6.1 HPLC-EC/UV system

The oxidative DNA lesion 8-oxodG can be quantified using high performance liquid chromatography (HPLC) coupled to electrochemical (EC) and ultraviolet (UV) detection, as seen in Figure 9 [78]. The oxidation product 8-oxodG and the undamaged nucleoside dG are separately detected. 8-oxodG is measured using the EC detector at 350 mV and quantified via the peak area. The amount of dG is, on the other hand, measured via the UV detector using a wavelength of 290 nm. From the results, it is

Figure 9. The HPLC-EC/UV system. From the left: PC for analysis of chromatograms, EC detector on top, pump at bottom, UV detector, autosampler. The glass bottles contain the eluent, and washing solutions.

possible to calculate the amount of dG that is oxidised (i.e. the amount of 8-oxodG formed) in relation to the undamaged dG that is retained. The result is often reported as 8-oxodG/10^6 dG.

This method can be used both for chemical studies as well as for cell and human studies. The analysis of 8-oxodG levels in blood requires sample preparation that takes several hours, before analysis of 8-oxodG and dG is feasible. The long work-up procedure adds a challenge – the creation of artificial oxidation, which encumbers the interpretation of the results. In material where dG is already isolated, this problem is avoided. The HPLC-EC/UV system for quantification of 8-oxodG is specific and has previously been evaluated and validated by ESCODD [79]. In the chemical studies of papers I and II, the HPLC-EC/UV system was used for analysis of oxidative damage.

6.6.2 The comet assay

The comet assay, or single-cell gel electrophoresis, is a sensitive and established method in which different types of DNA lesions can be measured. The alkaline comet assay is the most extensively used version. In its simplest form, it detects strand breaks and alkali labile sites, which together are referred to as DNA breaks in this thesis. Alkali labile sites are apyrinic sites formed during alkaline conditions. The comet assay can also be modified by the addition of lesion-specific repair enzymes enabling other DNA lesions to be analysed [80]. Enzymes utilised include formamido pyrimidine DNA glycosylase (FPG), human 8-oxoguanine DNA glycosylase (hOGG1), endonuclease III (EndoIII), 3-methyladenine glycosylase II (AlkA), T4 endonuclease V and uracil-DNA glycosylase (UDG), detecting oxidatively damaged bases (FPG, hOOG1, EndoIII), alkylated bases (AlkA), cyclobutane pyrimidine dimers (UV-induced bulky DNA adducts; T4 endonuclease V) and misincorporated DNA (UDG) [80,81]. FPG is the bacterial equivalent to the human hOGG1 and recognises oxidised purines, primarily 8-oxoG but also e.g. fapy-guanine and fapy-adenine.

As depicted in Figure 10, the comet assay consists of a number of steps. In principal, cells to be analysed are embedded in agarose on a microscope slide where after they are lysed (i.e. broken down). To allow the DNA to unwind, treatment with an alkaline solution is performed, which is followed by electrophoresis under alkaline conditions. During this step, the presence of DNA breaks contribute to the extension of DNA

loops. Structures resembling comets are formed, for which the size and intensity of the "tail" indicate the amount of DNA breaks. The "head" contains the supercoiled DNA attached to a matrix or scaffold [82]. After electrophoresis, the cells are washed and fixated. Following staining, the comets can be analysed using a microscope and computerised image analysis. Different units can be used when reporting the level of DNA, where the percentage of DNA in tail ('% DNA in tail') is widely recommended as the most accurate to use [83]. The software calculates the percentage of intensity in tail (i.e. damage) compared to intensity of head (i.e. intact DNA).

When other DNA lesions are analysed with the addition of an enzyme, additional steps are added between the lysis and alkaline treatment. Directly after lysis, the cells are then equilibrated in enzyme buffer and subsequently incubated with enzyme. The enzyme locates and cuts the lesion, and an apurinic/apyrimidinic site is formed. In the subsequent alkaline treatment, a break is formed. These samples include both 'general' DNA breaks and enzyme specific lesions. Therefore, samples that are not treated with enzyme need to be included. The amount of enzyme specific DNA lesions is then calculated by

% DNA in tail (enzyme treated) - % DNA in tail (without enzyme) (2)

The comet assay has been extensively validated, both in ESCODD and the European Comet Assay Validation Group, ECVAG (within the European Network of Excellence ECNIS) [71,84]. The method requires only a small number of cells, is sensitive in detecting low levels of DNA damage and can be applied on most eukaryotic cell types [83]. The comet assay is used in all constituent papers of this thesis, both without enzymes (papers I-IV), with FPG (papers I-IV) and with UDG (paper III). As far as I am aware, only a handful studies have previously used the UDG in the comet assay in human biomonitoring samples [85-87].

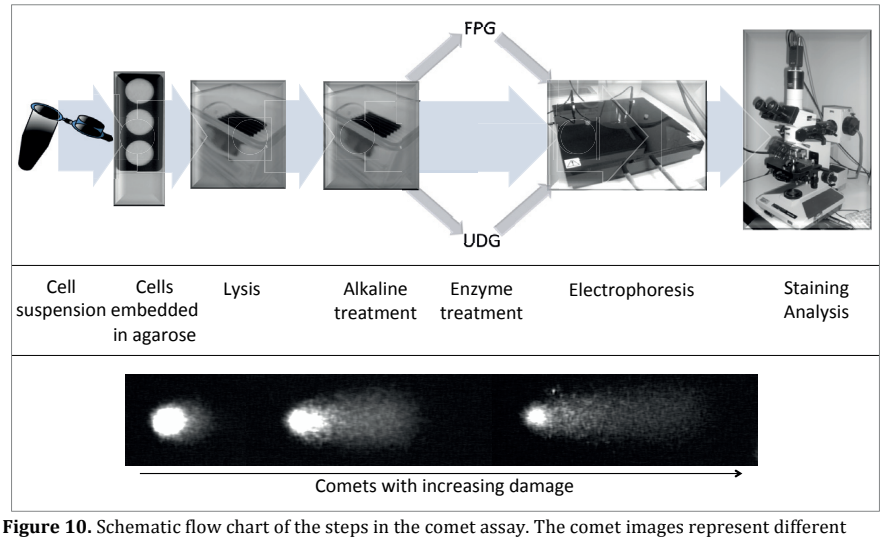


Figure 10. Schematic flow chart of the steps in the comet assay. The comet images represent different degrees of damage detected by the comet assay.

6.6.3 Trypan blue staining

Different viability assays can be used to monitor the viability of cells. Also referred to as cytotoxicity tests, these assays are readily used in toxicity testing, in order to detect whether an agent is cytotoxic, i.e. causes the death of cells. Some assays require cells to be viable in which case the utilization of viability assays are crucial prior to analysis. In this thesis, the viability of cells has been analysed both as a toxicity test of the micronutrients (in the *in vitro* cell studies) as well as in order to assure a high percentage of viable cells prior to running the comet assay.

Trypan blue staining, or trypan blue exclusion test, is a 'simple' method for the analysis of cell viability. The trypan blue stain penetrated damaged cell membranes allowing discrimination between damaged (unviable) cells and intact (viable) cells. Cells are incubated with the stain and the cells are then observed in a microscope and the number of stained (unviable) and unstained cells (viable) counted, from which a percentage of unviable cells can be computed. Strictly speaking, the trypan blue exclusion test detects cells with disrupted cell membranes; these cells are not necessarily dead *per se* [83]. Nonetheless, the output from trypan blue staining is often loosely referred to as cytotoxicity or unviable cells.

6.6.4 Other methods

Micronutrient intake. Surveys or interviews can be used to obtain information on the intake of dietary and supplemental micronutrients. In paper III, 24-hour recalls was used, an interview method where the subjects report all food and beverage consumed during the last 24 hours. In addition, questionnaires were used from which supplement intake was obtained. The supplement content was approximated using the labels on supplement containers.

Micronutrient status. The plasma or blood level of a micronutrient can be measured using for example immunoassays, fluorometric or HPLC methods. In paper III folate, homocysteine and vitamin B_{12} levels were measured using a solid-phase time resolved fluoroimmunoassay, a flourescence polarization immunoassay and a fluorometric method, respectively.

6.7 OTHER METHODS OF INTEREST

6.7.1 Detection of DNA lesions

Oxidative DNA lesions. While the comet assay has been recommended by ESCODD for detecting oxidative DNA lesions due to minimal risk of 'artificial' oxidation, chromatographic methods might be necessary when the detection of the damage needs to be more precise than what the enzymes used in the comet assay can offer. The HPLC-EC/UV system for the detection of 8-oxodG is one such chromatographic method. Others include gas chromatography-mass spectroscopy (GC-MS) and HPLC-MS/MS. Capillary electrophoresis (CE)-EC or enzyme-linked immunosorbent assay (ELISA) can also be utilised to measure oxidation products of DNA [71]. ELISA is, however, not explicit for 8-oxodG since antibodies are not specific to a particular oxidised base [88].

Bulky DNA adducts. Bulky DNA adducts can be analysed using for example ^{32}P-postlabelling and HPLC, immunoassays and GC-MS.

6.7.2 Antioxidant assays

This thesis does not focus on the antioxidant capacity of micronutrients/antioxidants. However, there are of course a number of assays in which the strength of the antioxidant property can be measured/analysed. Even though all antioxidants protect against oxidation, they have different preferences regarding what reactive species they quench, for example singlet oxygen ($^1O_2$, an electronically excited state of O_2; e.g. carotenoids), other ROS (e.g. vitamin C) or peroxyl radicals (e.g. phenolics). Since antioxidant capacity assays measure the quenching of specific reactive species, the choice of assay might reflect the results. Common total antioxidant capacity (TAC) assays include FRAP, ORAC, TRAP and TEAC [89].

When we are interested in the antioxidant effect on DNA, detection of DNA lesions through the methods mentioned above is another appropriate approach. The major difference compared to the pro-oxidant assay in papers I and II, described in section 6.5.1, is that *damaged* cells are used. Cells are first exposed to a damaging agent and then incubated with an antioxidant. Using the comet assay, the level of DNA lesions can subsequently be analysed. Lower levels of DNA lesions compared to control (i.e. cells not incubated with antioxidant) indicate a direct antioxidant effect. Lower levels can, however, also indicate an upregulation of repair enzymes instead, an indirect antioxidant effect by the antioxidant.

6.8 METHODOLOGICAL ISSUES

The ultimate goal with any *in vitro* assay would be to imitate an *in vivo* situation so well that the effects would be identical to a human situation *in vivo*. Unfortunately, no assay is able to imitate the human situation with such preciseness, although immense effort is dedicated to getting as close as possible. Nevertheless, every assay has its drawbacks and issues that need to be taken into consideration when interpreting the results. Even human studies have challenges that need to be overcome.

6.8.1 Chemical studies

Chemical studies enable us to dig deep and investigate properties of micronutrients in more detail. It provides us with the opportunity to study individual properties with no or few interfering factors. The benefits from this type of study can, however, also pose challenges. Extrapolation to a human situation should be done with great care, if at all, particularly in studies with antioxidants. Antioxidants are highly dependent on the surrounding and factors such as temperature, buffer content, oxygen pressure and antioxidant concentration can affect how an antioxidant acts. Pro-oxidant properties of β-carotene have, for example, been seen to be promoted by high oxygen pressure and high β-carotene dose [41,90]. As seen in Figure 11, the choice of buffer highly affected the pro-oxidant property of different vitamin C compounds in paper II. Chemical

studies provide us with insight of the chemical properties of antioxidants, of how they are capable of acting, but only under the specific experimental conditions used.

dG. The use of dG in the chemical studies of papers I and II enables a direct connection to the *in vivo* situation, since dG is a biological component of DNA. However, dG was dissolved and present in solution when exposed to antioxidant in these studies. *In vivo* dG is present in the nucleotide pool and incorporated in DNA, clearly less accessible to attack. Nonetheless, (d)G is susceptible to oxidative attack even when present in DNA. Hence, exposure of pure dG in chemical experiments provide us with insight of an antioxidant's ability to oxidise the base, but it is important to keep in mind the different circumstances that characterise an *in vivo* situation.

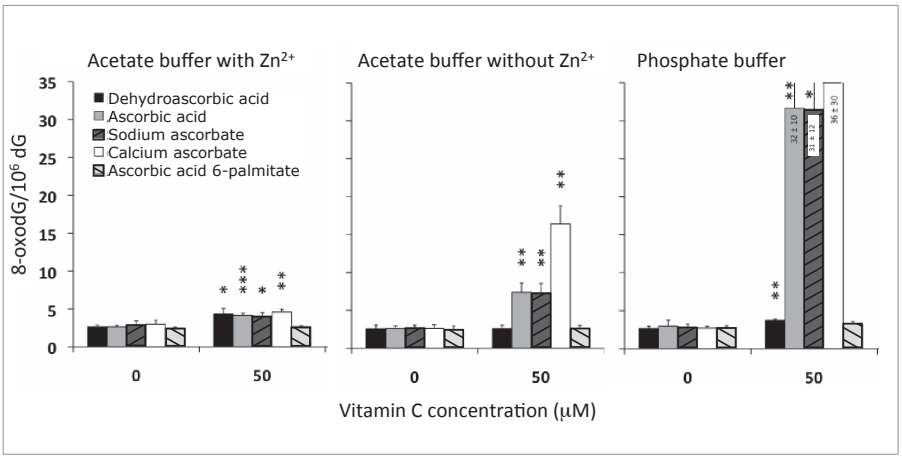


Figure 11. Oxidation of the DNA nucleoside dG by different vitamin C compounds present in different buffers. The results show that the immediate surrounding affects the strength of pro-oxidative activity by vitamin C.

6.8.2 Cellular *in vitro* studies

In vitro studies using cell lines takes us one step closer towards an *in vivo* situation. Again, great care needs, however, to be given when attempting to extrapolate to an *in vivo* situation.

Cells

What is the ultimate cell model for micronutrient studies? A straight answer is far from easy to give. The kinetics of micronutrients *in vivo* enables them to be in contact with a huge variety of cell types. The ADME (administration, distribution, metabolism and excretion) route also differs between micronutrients – contributing further to the difficulty of using one universal cell type. Thereby, cell lines used in micronutrient studies in the literature vary and range from blood cells, cervical cells and colon cells to lung cells and include cell lines such as HL-60 [47], HeLa [91], Caco-2 [91] and A549 [92], respectively. Cell lines have been adapted to a cell culture environment and are able to grow for more passages (i.e. a longer tine) than primary cells. In an article from 2003 [93], Halliwell proposes that cells in culture may be exposed to a much higher oxidative stress level. During the adaptation, the cells have attained certain properties

that will differ from the original cells. It has even been argued that a considerable number of cell lines have changed to such an extent that they no longer are the cells they are supposed to be [94]. The interpretation of studies using cell lines should therefore bear in mind that this, too, is a model system.

In papers I and II, the promyelocytic leukeaemia cell line HL-60 was used. After ingestion, many micronutrients are absorbed in the gastrointestinal tract in humans and distributed via the blood stream. Blood cells are therefore exposed to many micronutrients, and are because of that one type of cells used in micronutrient studies. HL-60 cells are grown in suspension in cell medium, i.e. the cells' 'food'.

Cell Medium

In cell studies, the choice of cell medium and medium components has been widely suggested to affect the outcome of studies involving antioxidants [65]. The presence or absence of serum [95], antioxidants and metals may affect the behaviour of micronutrients. Cell media are frequently deficient in antioxidants. In addition, commonly used cell media also contain iron. For example, the DMEM (Dulbecco's Modified Eagle Medium) is used for many cell types and contains iron(III) nitrate ($Fe(NO_3)_3$). Calf (foetal bovine) serum, which contains some transferrin with bound iron in, is also often added to media. Since some antioxidants can take part in Fenton reactions, the presence of transition metals is important to consider when interpreting the results [93]. The medium RPMI (Roswell Park Memorial Institute) used in papers I and II does not contain *added* iron salts, nor copper [96]. Halliwell [65] even argues that effects of antioxidants on cultured cells may be artefacts, created by reactions with medium components or by antioxidant metabolites.

In order to investigate the impact of medium on the outcome of our results, we conducted a small pilot study comparing the effects of RPMI (with serum) and PBS. PBS, or phosphate buffered saline, is a common buffer solution used in biological research. We investigated the vitamin compounds that had revealed to be most influenced by buffer type in the chemical dG experiments of paper II. We exposed HL-60 cells to these vitamins present either in medium or PBS. No great influence of the medium (medium or PBS) beyond normal variations was observed in this pilot study (n=1), where we analysed for DNA breaks and FGP-sensitive sites.

Comet assay and cell viability

Although subjected to debate, the use of a high proportion of viable cells when running the comet assay simplifies the interpretation of the results. If unviable cells are analysed and high DNA lesion levels detected, we are faced with the chicken and egg causality dilemma of what caused what? Did the cell death cause the DNA lesions or were the DNA lesions present in the cell anyway, possibly causing the cell death? If we analyse cells that are viable instead, this confusion is avoided. Within this thesis, a cell viability of above 90% (paper I) or 94% (paper II) was accepted for the comet assay runs.

6.8.3 Human studies

Even human studies face challenges and possess many requirements in order to be considered reliable.

Intervention studies
Randomised placebo-controlled studies (randomised control trials, RCT) are considered the most rigorous method in evaluating the effect of a substance. Nonetheless, certain factors need to be considered. A high compliance and a low dropout is, for example, desirable. Dropouts refer to participants who, for whatever reason, drop out of the study, while compliance refers to how well the subjects comply to the supplementation. It is important to collect data on both compliance and the number of dropouts. There are different approaches for how to deal with dropouts during the interpretation of results. The exclusion of participants who have not complied to the intervention (drop-outs or poor compliance) from the analysis enables analysis of the efficiency of the actual treatment, and is referred to as a *per-protocol* analysis. Another approach is to include all subjects who were included in the study, regardless of how well they complied to the intervention. This is called an *intention-to-treat* analysis and shows the *practical* value of the supplementation. Furthermore, blinding of the supplements (i.e. not revealing if it is a supplement or placebo) further enhances the quality of an RCT. Blinding can be done for the study participant only or for the participant and study conductor, the latter being *double*-blinding. Ethical considerations, the sample-size, the often high costs and long time that RCTs take also need to be considered when designing an RCT [97]. In crossover study designs, as used in paper IV, a wash-out period between treatments is essential in most cases, in order to avoid the effect from treatment one to influence treatment period two. Statistical analysis for *carry-over effects* can be conducted in order to verify that the washout period was sufficient.

Observational studies
In all observational studies, there is a risk of misinterpreting results since an effect might be caused by an unknown factor, a *confounder*. Statistical methods are used in order to account for known confounders, but the risk of unknown confounders remains. Since observational studies by nature cannot use randomisation, there is also a risk of *selection bias* where the exposed and control groups differ in factors of importance. If surveys or interviews are utilised, *recall bias* can also influence the data, meaning that the memory or “exaggeration of the truth” interfere with reality.

7 DIGGING DEEPER – VITAMIN A

In papers I and II, a large focus is on vitamin A and its pro-oxidant and DNA-damaging properties. This chapter will discuss vitamin A in more detail, unravelling its action in humans. It will also discuss its properties, in context of papers I and II and the current scientific literature. The focus is on basic research, understanding the basic properties of vitamin A compounds.

7.1 WHAT IS VITAMIN A?

Vitamin A is required for a functioning vision, reproduction, membrane integrity, cell growth and cell development [49]. Vitamin A is a generic term referring to compounds with the same biological effect as retinol. Vitamin A needs to be attained from the diet, either as preformed vitamin A or as provitamin A. Preformed vitamin A include retinol and retinyl esters (including e.g. retinyl acetate and retinyl palmitate) while common provitamin A's include β-carotene, α-carotene and β-cryptoxanthin which are cleaved to vitamin A *in vivo*. β-Carotene exhibits the greatest retinol activity of the provitamin A's. The chemical structure of vitamin A compounds, or retinoids, some of which can be seen in Figure 12, consist of three parts: a cyclohexane ring with three methyl groups, a conjugated tetraene side chain and a structural sidegroup. Retinoids can display many isomeric forms due to the presence of double bonds that can attain both *trans*- and *cis*- form, but the all-*trans* configuration tends to be the most stable [40].

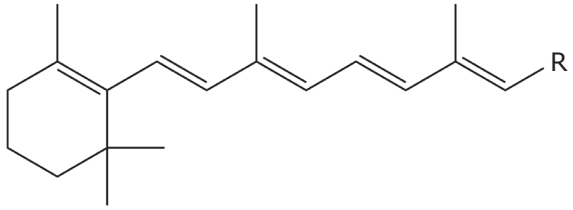
Vitamin A deficiency affects the eye, and can lead to dryness of the conjunctiva of the eye, decreased night vision or in severe cases to blindness. A deficiency can also result in growth retardation and decreased resistance to infections. The three latter conditions still occur in large numbers of children in less developed countries [98].

7.2 THE FATE OF VITAMIN A IN HUMANS

Most human tissue require vitamin A in the form of retinoic acid, while the eye needs retinal to retain vision. The vitamin A metabolism has two main functions: 1) to assure that tissues receive the correct retinoid in order for them to locally produce retinoic acid, and 2) to assure that retinol reaches the retina of the eye, in order for it to produce 11-*cis*-retinal [40].

Vitamin A compounds do not freely wonder around the blood system, instead different proteins lead their way. Lipoproteins transport retinyl esters and carotenoids (provitamin A) while retinoid binding proteins transport retinol, retinal and retinoic acid. The proteins assure that the retinoids do not damage membranes to any significant degree. In plasma, retinol is transported by retinol-binding protein (RBP). RBP is synthesised in the liver. Still present in the liver, one RBP ('apo-RBP') binds to one retinol, forming retinol-RBP ('holo-RBP') which is let out to the plasma. Transthyretin (TTR) non-covalently binds to retinol-RBP, stabilising the interaction between retinol and RBP. Moreover, RBP protects retinol from oxidation. Retinoic acids circulating in plasma are bound to albumin [40].

Vitamin A compounds



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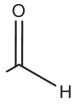
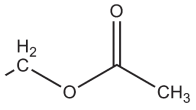
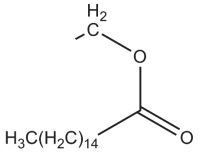
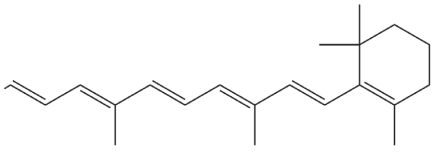
Retinal	
Retinyl acetate	
Retinyl palmitate	
β-carotene	

Figure 12. Chemical structures of vitamin A compounds analysed in paper II, and partly in paper I.

Intracellularly, retinoid binding proteins including CRBP-1, CRBP-II, CRBP-III, CRBP-IV, CRABP-I and CRABP-II protect the retinoids from degradation, protect membranes from vitamin A accumulation, facilitate the "solubility" in water, and transports the retinoids to enzymes involved in the metabolism. Some retinoids (e.g. 9-*cis*-retinoic acid) can also bind to, and thereby activate, nuclear retinoid receptors, which then act as transcription factors [40].

Regardless of the retinoid consumed, retinol is often formed during the process of absorption and thus the common form transported to tissues. The common route goes from the gastrointestinal tract, to the intestines where absorption occurs, via the blood/plasma and into the liver for storage or to extrahepatic tissues. 80% of vitamin A is stored in the liver (as retinyl esters), in stellate cells, also referred to as Ito cells, vitamin A storing cells or fat-storing cells [99]. Most tissues also store retinyl esters, but in lower concentrations. When there is a need for retinol in tissues, retinyl esters in stellate cells are hydrolysed to retinol in the liver, and then transported (with RBP) to plasma and distributed to where required. At low vitamin A concentrations, RBP is accumulated in the liver, ready to transport retinol and increase its plasma concentration. Vitamin A consumed as retinyl esters are hydrolysed to retinol in the intestines, absorbed and re-esterified in enterocytes (cells in the intestines). Retinyl esters are then transported via the plasma to the liver where the retinyl esters are hydrolysed back to retinol. The retinol enters stellate cells where it is stored as retinyl esters. In humans, the absorption of β-carotene differs from the preformed A vitamins in that it is absorbed through the intestines as intact β-carotene. β-Carotene then either passes unmetabolised across the intestine, or is cleaved to two molecules of all-*trans*-retinal. The conversion is regulated: the more there is the less is taken up. Excess intake of β-carotene from natural sources is therefore not linked to toxicity [40,49].

In tissues, the major vitamin A form is esterified retinol (retinyl esters), where the fatty acid group can be palmitate (most common), stearate or oleate [40]. The ester form protects the hydroxyl group from oxidation. Retinyl esters can be hydrolysed to retinol in tissue cells and retinol oxidised to all-*trans*-retinoic acid, via the intermediate all-*trans*-retinal. All-*trans*-retinoic acid is the most bioactive form of vitamin A. It has been seen to be capable of promoting almost all functions caused by vitamin A in humans, with the exception of vision. Retinol-RBP can also be transported to the retina in the eye, where formed retinyl ester is hydrolysed and isomerised to 11-*cis*-retinol, which in turn is oxidised to 11-*cis*-retinal. This retinal isomer is a constituent of dark adaptation (visual cycle or retinoid cycle) and is required for a properly functioning night vision [40]. Retinoic acid cannot be reduced to retinal *in vivo*, which might explain why retinoic acid does not affect vision.

Physiological plasma levels of vitamin A are normally stable, with low inter- and intra variation. Normal retinol concentrations (majority bound to RBP) are reported to be 2-2.2 μM [40] or 1-3 μM [99]. Other retinoids detected in plasma, at much lower levels, include all-*trans*-retinoic acid, 9-*cis*-retinoic acid, 13-*cis*-retinoic acid and di-*cis*-retinoic acid [40]. Retinyl esters in serum are usually below 0.2 μM [99].

7.3 VITAMIN A SUPPLEMENTS – COMPOSITION

Supplements often contain synthesised vitamin A, where the retinol is stabilised by forming acetate, propionate or palmitate esters [40]. A supplement of vitamin A can contain any of four different vitamin A compounds, without the specification of which is present. According to the European Parliament and Council's directive (2002/46/EC), any of the compounds listed in Table 4 are permitted. Consequently, the exact form of vitamin A consumed from a dietary supplement is not known to the consumer. The chemical structures of the compounds are seen in Figure 12, with the addition of retinal found *in vivo*.

Table 4. Vitamin A compounds permitted in supplements[1]

Retinol
Retinyl acetate
Retinyl palmitate
β-Carotene

[1] According to the European Parliament and Council's directive 2002/46/EC

7.4 TOXICITY OF VITAMIN A

At high concentration of vitamin A, when the RBP transport proteins capacity is exceeded and non-bound vitamin A is presented to membranes, toxicity may result. Also referred to as hypervitaminosis A, vitamin A toxicity can be acute or chronic. In acute hypervitaminosis A, excess vitamin A binds to lipoproteins, producing for example changes in biological membranes, headaches, nausea and fever. Chronic vitamin A toxicity is more common than acute and can arise after months or years of high intakes of preformed vitamin A from e.g. liver or supplements [49,99]. Chronic toxicity manifests as dry skin, hair loss, intracranial pressure or anorexia, for example. For acute toxicity the RDI needs to be exceeded by hundredfold (adults) over a few days, while chronic toxicity in adults occurs after a consumption of 25 000 IU/day (approximately eight times the RDI in Sweden) for more than six years, or over 100 000 IU (approximately 110 times the RDI) for over six months [99].

7.5 VITAMIN A – THE THEME OF PAPERS I AND II

Papers I and II aimed to investigate vitamin A properties, properties related to pro-oxidation and DNA lesions. The papers describe basic research, with the objective to understand fundamental principles, yet not assured, of vitamin A. An outline of the study designs of papers I and II can be seen in chapter 6, Figure 8. Cultured leukaemia cells (HL-60) were exposed to vitamin A for one (paper I) or 24 (paper II) hours and its potential to affect DNA and cell viability was studied. The influence of metals and/or H_2O_2 was also investigated (paper I), and in addition the different vitamin A compounds permitted in supplements were also researched and compared (paper II). Below follows a discussion on the findings from these and other related studies.

7.6 VITAMIN A AND ITS EFFECT ON DNA LESIONS

In cell studies, vitamin A has shown various effects on DNA. It has been seen to enhance the production of oxidised purines by PMA (a tumour promoter)-activated neutrophils [100], enhance strand breaks induced by the cancer drug CPT-11 [101], and contradictory, to decrease DNA adducts (induced by benzo(a)pyrene) [102]. The DNA damage promoting effects in these studies were enhancements of already occurring damage. In our cell studies, we did not induce damage using other agents, on the contrary the aim was to investigate if vitamin A has properties that enable it to cause damage to DNA alone (but in the presence of cell medium etc.), without promoting damage by other agents. In our studies, retinol did not show any capability to cause DNA strand breaks or oxidative DNA lesions (FPG-sensitive sites) to HL-60 cells, nor did any of the other preformed vitamin A compounds analysed. Contradictory to our results, a study from 2000 revealed retinol to induce strand breaks, 8-oxodG and DNA fragments at concentrations of 1-5 μM, also in HL-60 cells [47]. The oxidation in that study was increased with concentration. We used concentrations four times higher than in the 2000 study and yet did not observe DNA lesions by retinol. While copper and iron have been suggested to promote pro-oxidant behaviour of retinol [47,103], the addition of copper or iron ions did not affect the results on DNA damage in our study. In HP-100 cells, derived from HL-60 cells but more resistant to H_2O_2, retinol did not cause oxidative damage in the study by Murata and colleagues [47].

It is notable that HL-60 cells can be induced to differentiate into melocytes, metamyelocytes and neutrophils by different agents, including DMSO, retinoids and ascorbic acid (vitamin C) [104]. When dissolving the retinoids in papers I and II, DMSO was initially used. During cell exposure with A vitamins, ≤ 0.5% DMSO was present. This is 1.5-3 times lower than what was used when differentiation of HL-60 cells was previously achieved [104]. Retinoic acid at 1 M, has been shown to differentiate over 90% of HL-60 cells in six days and close to 20% in one day [105]), consequently some of the cells exposed to retinoids in paper II (although not retinoic acid *per se*) are likely to have differentiated after the 24 hour exposure. Differentiation by retinol in paper I is assumed to be much less pronounced (if even present), due to the shorter exposure time of one hour. Differentiation can also lead to a difference in the oxidative defence, primarily in regard to the oxidative burst, the release of ROS when for example fighting bacteria [106]. It is unknown whether the cells in our studies and the study by Murata *et al*. were in the same stage or not when it comes to differentiation and related properties. In addition, the presence or absence of serum can affect the action by certain antioxidants [95], a possible contributing factor to differences in results. In papers I and II, 10% FBS (foetal bovine serum) was used when culturing the cells, while 6% was used by Murata *et al.* [47].

Retinal, the vitamin A metabolite formed from retinol, β-carotene and retinyl esters, has previously been seen to have pro-oxidant properties [47]. In paper II, retinal – as the only vitamin A compound – caused cytotoxicity to cultured cells. The cell death was almost complete, leaving next to no cells alive, or with intact cell membranes (as detected with trypan blue staining). Due to the high cytotoxicity by retinal, DNA damage had to be analysed using a lower concentration compared to the other vitamin

A compounds. 2 μM did not induce any DNA breaks or oxidative DNA lesions in our study. Retinal has, however, previously been reported to cause strand breaks, 8-oxodG and DNA fragments [47]. In that study, a stronger DNA damaging effect was seen from retinal than from retinol. As will be revealed further in the coming section, retinal clearly has the ability to induce oxidation to biomolecules, such as oxidising dG to 8-oxodG, something which together with results from the other authors, suggests an underlying oxidative mechanism not detectable in the cell-based experiments in paper II to be behind the cytotoxicity by retinal.

When comparing the effects on DNA damage by the different vitamin A compounds permitted in supplements, the differences were minimal. None of the preformed A vitamins induced either DNA breaks or oxidative DNA lesions. The provitamin A β-carotene stood out, inducing a small but statistically significant increase in oxidative DNA lesions, as will be discussed below. None of the preformed A vitamins caused cytotoxicity to the cells. In this aspect the compounds found in supplements seem to act similarly to each other. Their chemical properties, discussed in the next chapter, reveal another story.

Human and animal studies. A report by the European Network of Excellence ECNIS (Environmental Cancer risk, Nutrition and Individual Susceptibility) concluded in 2007 that vitamin A has a limited capacity to act as an antioxidant *in vivo*, and that there is a lack of intervention studies using increased doses of vitamin A [98]. Since then, some evidence further suggesting antioxidant behaviour, but also pro-oxidant behaviour has emerged. In a study from 2012, supplementation of a multivitamin containing vitamin A (as retinol and β-carotene) along with other antioxidants (selenium, vitamin C and vitamin E) to young, healthy, non-smoking adults reduced the levels of DNA strand breaks in those with the highest damage levels before supplementation, as analysed with the comet assay [107]. This indicates a potential to reduce high levels of DNA damage. Other studies have shown both positive [108] and negative [109,110] associations between vitamin A and 8-oxodG levels. In animal studies, protection against induced DNA damage detected as 8-oxodG in leukocytes [111] or strand breaks [112] has been reported in rats by retinyl acetate and retinoic acid, respectively. On the other hand, oxidation of lipids and proteins in rats have also been reported [113], emphasising that vitamin A has potential to protect as well as promote damage to biomolecules *in vivo*.

7.7 VITAMIN A AND PRO-OXIDANT PROPERTIES?

Out of thirteen micronutrients, vitamin A stood out in paper I as one of three showing chemical pro-oxidant properties. It also showed to be one of only two that acted as a pro-oxidant at singular doses as well as caused a dose-response, increasing oxidation with an increased concentration of vitamin A – in the form of retinol. Vitamin A even showed a stronger pro-oxidant effect than the well-known antioxidant/pro-oxidant vitamin C. Under the conditions of the study in paper I, retinol caused oxidation to the DNA nucleoside dG. Normal plasma levels were not sufficient to induce oxidation. When present at three or ten times plasma levels, however, retinol acted as a pro-oxidant, causing oxidation to dG. From this, it is clear that vitamin A is yet another

micronutrient whose chemical pro-oxidant action is dependent on its concentration, a phenomenon that has been proposed for other antioxidants [45-47,90]. Suggestions have been made that retinol-induced oxidative damage to DNA is mediated by transition metals, Murata *et al.* [47] identified copper (Cu(II)) and Dal-Pizzol *et al.* [103] identified iron to be required for the DNA to be oxidatively damaged by retinol. In addition, superoxide and the generation of H_2O_2 was discussed to be behind copper(II)-mediated damage to DNA by retinol [47]. In our cellular experiments, the presence of copper or iron did not affect the outcome on DNA damage suggesting the involvement of these metals to be lower than anticipated. The chemical pro-oxidant effect by retinol was, however, significantly enhanced by both copper and iron, iron by far showing the strongest effect. Contrary to the results from the cellular exposure, this is in line with a role of copper and iron in retinol's pro-oxidative potential, as suggested by authors of the studies mentioned above. Moreover, the complete mechanisms of how retinol causes its pro-oxidant properties is to my knowledge not clear. Vitamin C can form complexes with iron and copper, while vitamin A is, to my knowledge not known to do so. In paper I, we speculate whether vitamin A can form weak complexes or not. Retinol might also induce the production of ROS in cells [114].

From paper II and Figure 13, it is very clear that the vitamin A compounds permitted in supplements differ in their chemical properties with rather great variety, specifically in their potential to act as pro-oxidants. Two out of the four vitamin A compounds permitted in supplements showed chemical oxidation potency (retinol and retinyl acetate), while two (retinyl palmitate and β-carotene) did not. Retinal also revealed pro-oxidant properties. These results support the studies mention in the previous section discussing DNA lesions, where vitamin A has been seen to induce oxidation to DNA in cells and rodents [47,109,113].

Vitamin A is a fat-soluble vitamin. With the intention to produce a supplement with less risk of toxic effects, a water-soluble vitamin A has been manufactured. Ironically, the water-soluble vitamin A was in 2003 shown to be more toxic than the fat-soluble [115], certainly emphasising the difference between different vitamin A compounds and more importantly the significance of knowing their properties when choosing compound for supplement use.

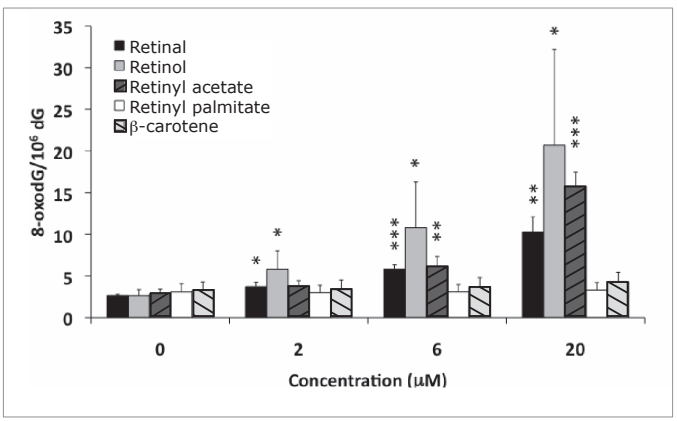


Figure 13. Oxidation to the DNA nucleoside dG in acetate buffer by different vitamin A compounds permitted in supplements. 2 μM represents a normal plasma level of retinol.

7.8 VITAMIN A AND CANCER

Vitamin A has limited antioxidant capacity and has been shown to have preventive effects against certain cancer forms [50]. The World Cancer Research Fund and American Institute for Cancer Research concluded in 2007 that there is limited evidence that vitamin A protects against skin cancer [6]. In addition, as reviewed in [116] preventive effects on breast and cancer have also been observed. Studies are, however, not completely unified and disappointing results from vitamin A supplementation on cancer are also evident. For example, no effect on head, neck or lung cancer was observed after two years of supplementation [117]. During therapeutic use it is also important to avoid hypervitaminosis A and one approach taken to improve the effectiveness of vitamin A in preventing and treatment of cancer is to synthesise new retinoids with more specific actions and less toxic effects [116].

7.9 PROVITAMIN A – β-CAROTENE

Following the ATBC trial and CARET in the 1990s, β-carotene has received much attention. Partly in line with our results, β-carotene has previously been concluded to exhibit pro-oxidant properties. The effect is promoted by high concentrations and an oxidised environment (such as that of a smokers lung) [41,90]. We observed β-carotene to cause an increase in FPG-sensitive sites in HL-60 cells, but not strand breaks or cytotoxicity at a concentration 40 times that of normal plasma levels, and ten times that of normal vitamin A levels. Neither did β-carotene cause oxidation to dG in our chemical set-up. The increase in FPG-sites was statistically significant, nonetheless the increase was small. The specific environment of a smoker's lung, characterised by 1) a high oxygen pressure, 2) low antioxidant levels (especially vitamin C and E, normally stabilising β-carotene) and 3) elevated levels of free radicals, can promote oxidation of β-carotene. In the literature, the metabolites formed have been suggested to play an important role in the lung cancer promoting effect in smokers [43,44]. Considering that we only observed a damaging effect on one out of four parameters and that the observed effect was small, our results are in line with that hypothesis.

7.10 CONCLUSION

A PubMed search on the Mesh term 'vitamin A' reveals 35 745 publications on the topic. Although vitamin A is well studied, many question marks concerning its properties, mechanisms and effects on cancer or other human health aspects remain.

While vitamin A is essential for humans, it has chemical pro-oxidant properties in addition to its antioxidant properties. Thus it has the potential to cause oxidative damage to biomolecules. Studies have shown damaging effects by retinoids to for example DNA, as well as no effect. The exact mechanisms to how and when vitamin A causes oxidation is still not completely clear, but might be linked to the presence of transition metals, the formation of ROS and resulting pro-oxidant action, as well as the vitamin A concentration. In addition it became clear from our studies that the different vitamin A compounds permitted in supplements greatly vary in their chemical potential to act as pro-oxidants, a potential concern during manufacturing of supplements.

8 DIGGING DEEPER – VITAMIN C

This chapter will guide you through basic research on vitamin C, unravelling its properties on its potential to act as a pro-oxidant.

8.1 VITAMIN C – A HISTORICAL ANTIOXIDANT

Vitamin C is a well-documented antioxidant. It has an active hydroxyl group and is therefore a very efficient free radical scavenger. Under this process, a radical form of vitamin C is formed, as seen in Figure 14. This vitamin C radical is significantly more stable than other radicals due to resonance stabilisation, and therefore the ascorbyl radical does not possess a threat to the cell. Vitamin C is probably the most known vitamin of them all. It was on voyages with Vasco da Gama and other explorers in the 15th and 16th centuries that the symptoms of scurvy were observed and the consequences of feeding the crew a diet without e.g. fruit, vegetables or herbs were learnt the harsh way [118]. Subsequent research led to the identification of vitamin C in the 1920s [119]. Humans lack a key enzyme ($_L$-gulonolactone) needed for the synthesis of ascorbic acid and vitamin C intake is therefore required through our diet. During evolution the enzyme has been retained in the kidney of many fish, reptiles and birds, and in the liver of many mammals [40].

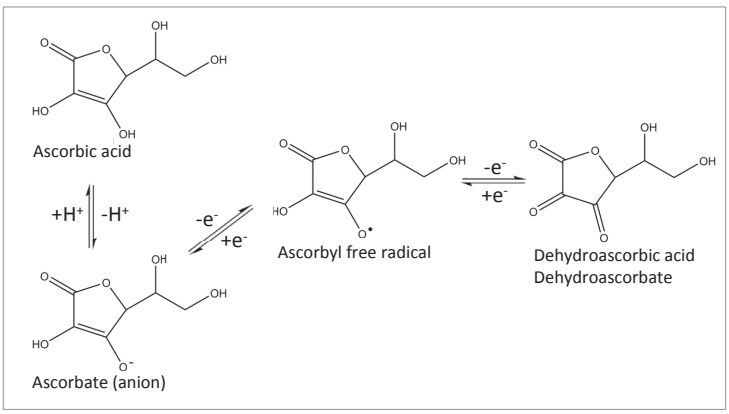


Figure 14. Antioxidant action by vitamin C. Scavenging of e.g. a free radical by ascorbate, the anionic form of vitamin C commonly found *in vivo*, produces the ascorbyl free radical, a much less reactive radical.

8.2 THE FATE OF VITAMIN C IN HUMANS

Vitamin C is water-soluble and is therefore able to circulate fluids without transport proteins. Vitamin C ingested through food or supplements is absorbed in the small intestine, transported in to the blood stream and distributed to key tissues where it is absorbed and utilised as co-factor for enzymes or as an antioxidant [120]. Moreover, the kidneys play an important role in reabsorbing ascorbic acid from the renal filtration. Vitamin C can adopt two main forms, the reduced form ascorbic acid (AA), and the oxidised form dehydroascorbic acid (DHAA). See Figure 14 or 15 for their structural

differences. At physiological pH, ascorbic acid is negatively charged and present as ascorbate (an anion), while DHAA remains uncharged.

Ascorbate and DHAA require special transport systems aiding them across cell membranes. Absorption in the intestine occurs via sodium dependent transporters or via glucose transporters. Ascorbate is transported across membranes via the sodium dependent transporters SVCT1 or SVCT2, one or both which are found in most tissues [121], while the glucose transporters GLUT1, GLUT3 and GLUT4 (the latter in insulin sensitive cells) transports DHAA. Once absorbed, DHAA is quickly reduced to ascorbate. After absorption, vitamin C is transported in portal blood and distributed to tissues. When normal physiological concentrations, 20-150 μM of ascorbic acid is present, high-affinity transporters help to transport ascorbic acid into tissues. At unphysiological vitamin C concentrations, transportation into tissue occurs via low-affinity transporters. Certain tissues have particularly high concentration of vitamin C. These tissues exhibit special transporters that facilitate the intake of vitamin C despite an already high intracellular concentration [122].

In recent years, it has become evident that vitamin C efflux also occurs, i.e. vitamin C is transported out of cells through activated transport systems. DHAA can thereby be absorbed, reduced to ascorbate and ascorbate then transported to the extracellular fluid. Under physiological pH DHAA is unstable and it is rather rapidly, and irreversibly, hydrolysed to 2,3-diketo-$_L$-gulonate. The purpose with vitamin C efflux could therefore be to convert unstable vitamin C to a more stable form, maintaining vitamin C concentrations, or simply to transfer electrons across membranes [123].

Vitamin C plasma concentrations from food sources are strictly controlled around 70-85 μM, where excess amounts are excreted with urine. Oral supplementation has, however, been predicted to achieve levels of up to 220 μM and intravenous administration can lead to a vitamin C plasma concentration of 1500 μM [124].

8.3 VITAMIN C SUPPLEMENTS – COMPOSITION

A vitamin C supplement can contain any of the five compounds presented in Table 5, in accordance with the directive by the European Parliament and Council (2002/46/EC). Their chemical structures are seen in Figure 15, with the addition of DHAA, the oxidised form. As with vitamin A and other micronutrients, the exact form of vitamin C present in a supplement is not known to the consumer. In addition, the matrix and formula of the supplement might influence to what extent oxidation occurs in and from supplements. For example, it has been reported that metal-catalysed oxidation of vitamin C can be slowed down by altering the matrix [125].

Table 5. Vitamin C compounds permitted in supplements[1]

L-Ascorbic acid
Sodium L-ascorbate
Calcium L-ascorbate
Potassium L-ascorbate
L-Ascorbyl-6-palmitate

[1] According to the European Parliament and Council's directive 2002/46/EC

8.4 VITAMIN C – THE THEME OF PAPERS I AND II

Similarly as for vitamin A, papers I and II also aimed to investigate vitamin C properties, properties related to pro-oxidation and DNA lesions. The papers describe basic research, with the objective to understand fundamental principles of vitamin C. The study design is outlined in Figure 8, chapter 6. In the chemical set up, the DNA nucleoside dG was exposed to vitamin C for one hour and the amount of oxidised dG was analysed. Cultured leukaemia cells (HL-60) were exposed to vitamin C for one (paper I) or 24 (paper II) hours and its potential to affect DNA and cell viability was studied. The influence of metals and/or H_2O_2 was also investigated (paper I). Furthermore, the different vitamin C compounds permitted in supplements were investigated and compared (paper II). It is noteworthy that the vitamin C concentrations used for exposure were of physiological concentrations, equivalent to plasma concentrations and up to ten times plasma levels, representing intracellular levels [126]. Below follows a discussion on the findings from these and other related studies.

8.5 VITAMIN C AND ITS EFFECT ON DNA LESIONS

Vitamin C is an ambiguous compound, which has the ability to act in a range of different ways. It is, as mentioned, known to act as a pro-oxidant when involved in Fenton reactions. Reports on the effect of vitamin C on DNA have been varied and cell culture studies (of cell lines or *ex vivo* cells) have concluded vitamin C to protect against DNA lesions [127-129], induce DNA lesions [130-132], or leave the DNA unaffected [130,131,133]. The dose has been pinpointed to affect the outcome, where a low dose has a greater tendency to give protection [127,129] and higher doses to induce DNA damage [131]. This does, however, not always hold true. For example, exposure to HepG2 cells (a hepatocellular carcinoma cell line) with 70 μM AA (a low dose) for 24 and 48 hours induced DNA damage in a study from 2011 [132].

In papers I and II, we exposed cultured cells to concentrations up to ten times the normal plasma level, i.e. 500 μM, and saw no effect on the DNA lesions measured using the comet assay. In experimental studies, concentrations are often much above physiological levels, and the dose used in papers I and II is by such standards considered low, adding to the evidence of non-pro-oxidant behaviour of vitamin C in cells at low concentrations. We also investigated the effect of ascorbic acid together with metals that are also found in supplements, commonly together with vitamin C in multivitamins. A different scenario was seen in the presence of copper. Ascorbic acid and copper stood out as the only combination that caused DNA damage in the cellular studies – both DNA breaks and oxidative DNA lesions (FPG-sensitive sites). Co-supplementation of ferrous (Fe^{2+}) salts and vitamin C has been reported to increase oxidative stress in the gastrointestinal tract [134], suggesting that the effects observed in the present study *might* also affect an *in vivo* situation. The effect of vitamin C and metals will be further discussed in section 8.7.

The different vitamin C compounds permitted in supplements did not differ in their potencies to induce DNA lesions to cells under this study design, none of them caused damage to DNA. Contrary, ascorbic acid, sodium ascorbate and calcium ascorbate,

Vitamin C compounds

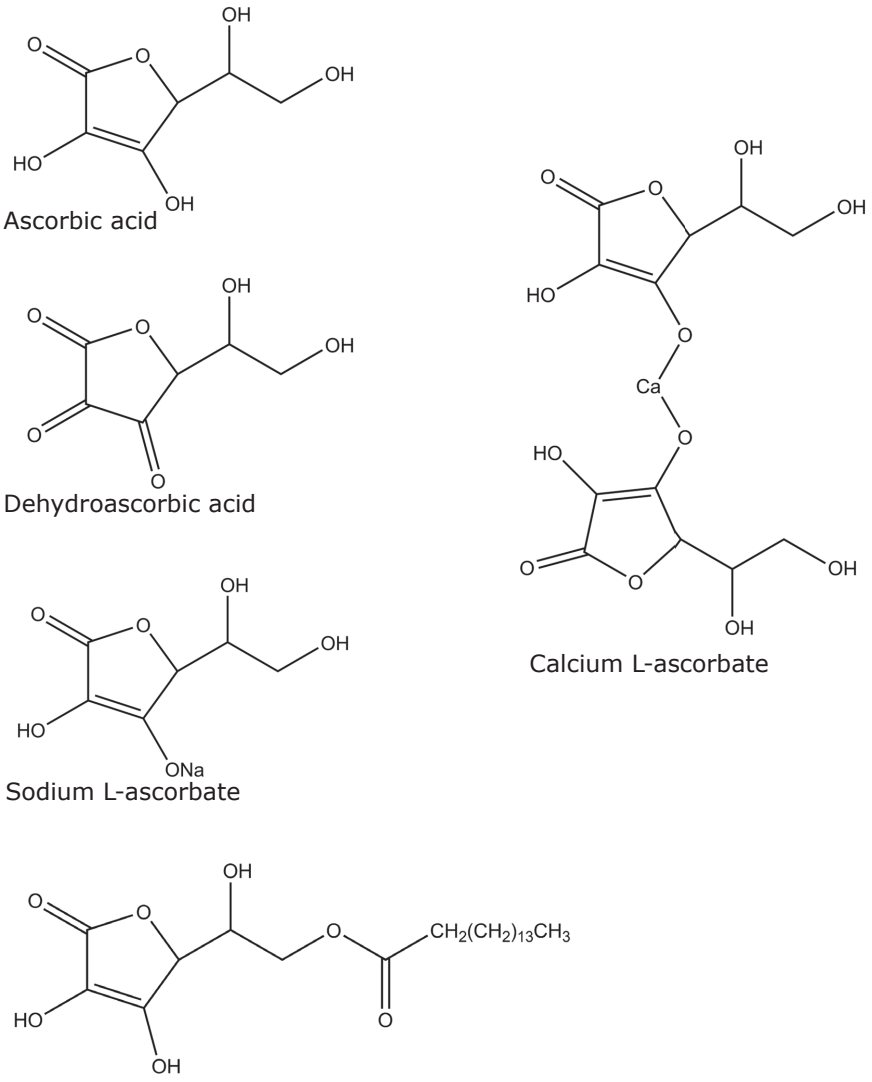


Figure 15. Chemical structures of the vitamin C compounds analysed in paper II, and partly in paper I.

three out of the four compounds, caused cytotoxicity to cells to different extents, confirming what others have previously reported concerning ascorbic acid and sodium ascorbate [135-137]. DNA lesions were not affected by these compounds indicating that the cytotoxicity was initiated by other factor(s)/mechanisms. However, because of the cytotoxicity caused at 500 μM, the concentrations used for analysis of DNA damage had to be lower for ascorbic acid, sodium ascorbate (250 μM) and calcium ascorbate (150 μM). At 500 mM, ascorbic acid did in fact induce FPG-sensitive sites, but since the cytotoxicity limit set was exceeded it cannot be concluded if these oxidative lesions underlie the cytotoxicity or not, or if the cytotoxicity caused the DNA lesions. Moreover, ascorbic acid has been reported to protect against apoptosis (cell death) induced by depriving the cells of serum [138,139]. DHAA is not permitted in supplements, but is the oxidised form of ascorbic acid, also present *in vivo*. While ascorbic acid promoted cytotoxicity of cells, DHAA did not. This was somewhat surprising since HL-60 cells express GLUT transporters that normally transport DHAA into the cells [140]. However, DHAA forms a bicyclic structure in aqueous solution and predominantly exists as the bicyclic hemiketal hydrate, while AA remains in the monocyclic form, contributing to physiochemical differences [125]. Moreover, HL-60 cells have the ability to stabilise extracellular ascorbate, increasing its presence [141].

Human studies. A combination of vitamin C and E supplementation lowered DNA damage levels in breast cancer patients simultaneously undergoing chemotherapy [142]. A two to four hour supplementation of vitamin C has provided protection to oxidative damage induced *ex vivo* [143]. Oxidative DNA damage was reduced in male smokers following four weeks of vitamin C and E supplements [144]. These studies indicate that vitamin C supplementation might, despite the somewhat unclear pro-oxidant property, yield beneficial effects on DNA lesions in humans.

8.6 VITAMIN C AND PRO-OXIDANT PROPERTIES

In paper I, ascorbic acid stood out as the only micronutrient out of thirteen to induce oxidation to the nucleoside dG at *normal plasma concentrations*. The chemical pro-oxidant property was dose-dependent and oxidation increased with dose. Vitamin C has documented pro-oxidant properties [46], and the results from the chemical study in paper I adds to that evidence. More importantly, it points out ascorbic acid to chemically act as a pro-oxidant already at physiological concentrations. When, in paper II, comparing the chemical pro-oxidant properties of the different vitamin C compounds permitted in supplements, all (the same that caused cytotoxicity to cells) but one induced oxidation to dG. The exception, ascorbic acid 6-palmitate was subjected to solubility difficulties under the experimental conditions and was therefore only analysed in the lowest concentration. Therefore it cannot be excluded that it might have pro-oxidant potential at higher doses. Nonetheless, ascorbic acid 6-palmitate is more stable than Ascorbic acid and the lack of reactivity by it in the assays was therefore not unexpected [145]. Just as with DNA lesions, cell studies have shown both antioxidant [146] and pro-oxidant [147] behaviour from vitamin C, reducing or increasing ROS production.

The action by all vitamin C compounds found in supplements were highly dependent on the buffer type used in the experiments. It is clear, as observed by others [130], that the surrounding environment influences the pro-oxidant property of vitamin C. When present in phosphate buffer, ascorbic acid, sodium ascorbate and calcium ascorbate produced a several-fold higher increase in the oxidation of dG when compared to acetate buffer, as can be seen in Figure 11, chapter 6. The spread of oxidation level caused by calcium ascorbate contributed to a non-significant difference in phosphate buffer versus acetate buffer, although the visual increase was big. The presence of zinc in the acetate buffer reduced the oxidation by the same compounds, emphasising that both buffer type and metal content (other than the transition metals iron and copper) influence the pro-oxidation by ascorbic acid.

8.7 VITAMIN C AND METALS

Together with ascorbic acid, both iron and copper are well documented to catalyse oxidation reactions [134,148]. In Fenton reactions a transition metal is oxidised by H_2O_2 producing a reduced transition metal, a hydroxide ion and the reactive hydroxyl radical [48]. Ascorbic acid can interact with metals through redox reactions and reduce Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) and Cu^{2+} (cupric ion) to Cu^{+} (cuprous ion), thereby facilitating Fenton type reactions. Moreover, the combination of vitamin C and iron is referred to as the Udenfriend's system and is frequently used to promote oxidation. In this system, vitamin C acts as a two-electron donor complexed to a transition metal, inducing oxidation of aromatic moieties [148]. The results from paper I imply, however, that it might be even more efficient to use copper for such purposes. Copper really stood out when in combination with vitamin C, causing both strictly chemical oxidation to dG (synergistically) and – as the only combination – DNA breaks as well as oxidative DNA lesions to cultured cells. Vitamin C is currently being investigated as a potential future chemotherapeutic agent due to its pro-oxidant properties, and copper ions, to a greater extent than iron ions, have been suggested to be involved in the process, causing oxidative DNA lesions [134,149].

The combination of vitamin C, transition metal ions and H_2O_2 *in vitro* is well-known to cause pro-oxidation [48,148], an effect that was confirmed in the present study. The pro-oxidant effect by vitamin C has repeatedly been attributed to the presence of transition metals, in large or small quantities [150,151]. Although some evidence has pointed towards a damaging effect by vitamin C independent of metals [137], or a protective effect even in the presence of iron-overload [152]. A recent study suggests that ascorbic acid exposure in air, or ascorbic acid and H_2O_2, can produce the hydroxyl radical (and DHAA), which then promotes damage to DNA [153]. From the results of paper I, two main mechanisms were proposed to underlie the chemical pro-oxidation: 1) Fenton reactions in the presence of H_2O_2 (where such was present) and transition metal ions (e.g. Fe^{2+}, Cu^{2+}); (2) vitamin C-metal complexes are formed, promoting oxygen transfer and thereby oxidation of dG and DNA. Such chemical structures of vitamin C-metal complexes have previously been proposed [154-156]. The suggested mechanisms are schematically outlined in Figure 16. According to Halliwell *et al.*, all laboratory solutions and cell media are contaminated with transition metals unless

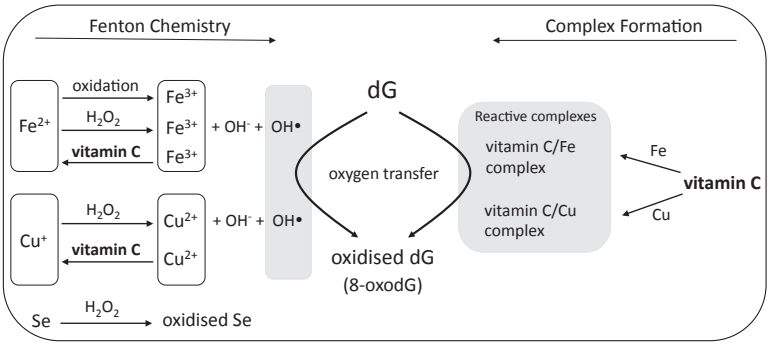


Figure 16. A schematic (non-stoichiometric) summary of the mechanisms behind the ability of vitamin C and meatls and/or H_2O_2 to oxidise dG. Abbrevitation: Cu, copper; dG, deoxyguanosine; Fe, iron; Se, selenium.

specifically purified [93]. It is therefore likely that the mechanisms mentioned above can hold even for studies where metals have not been added.

8.8 CELL EXPOSURE WITH VITAMIN C – CHALLENGES

The nature of cell cultures give rise to some factors to consider when investigating vitamin C. First, cell medium may contain metals and as discussed above, their presence might affect the action of vitamin C. For example, the very common DMEM (not used in this thesis) contains iron(III) nitrate. Serum from calves (used in this thesis) also contains transferrin, proteins with bound iron. Iron in transferrin will, however, normally not catalyse free radical reactions. In addition, laboratory equipment contain trace amounts of metals, unless specifically purified [93]. RPMI medium used within this thesis does, however, not contain added iron salts, nor copper [96].

In addition, different cell types express certain transporters to different extents. Hence, the choice of cells might affect the uptake of the vitamin. HL-60 cells has, for example, the ability to stabilise extracellular ascorbate [141], while they also express GLUT transporters [140]. Moreover, exposure using DHAA has been argued to demonstrate an artificial uptake, via GLUT [157]. It was, to me, surprising that DHAA did not affect the HL-60 in paper I, considering that ascorbic acid did (inducing cytotoxicity) and that DHAA was expected to be taken up via GLUT, and then reduced to ascorbic acid. This emphasises the challenge in foreseeing the most appropriate circumstances in *in vitro* studies. Furthermore, due to its instability, most cell media do not contain added vitamin C, nor vitamin E [93,158]. Exposure of these antioxidants have been speculated to be artefacts due to the cells lack of these antioxidants or due to oxidation in the medium [93].

8.9 VITAMIN C AND CANCER

Cancer cells display special properties, some which might intervene with the action of vitamin C. As a consequence, vitamin C has become a hot topic within cancer research and is discussed and speculated as a potential future chemopreventive agent.

Cancer cells often have an overexpression of GLUT transporters, enabling a high uptake of DHAA and accumulation of ascorbate [116]. Cancer cells also have low levels of antioxidant enzymes and high levels of ROS, and it has been speculated whether vitamin C is able to enhance this effect in such a way that it can promote death of these cells [116]. Moreover, progress has been made in achieving pharmacological concentrations of vitamin C by intravenous administration, enabling higher *in vivo* concentrations than was previously achieved by oral administration [159]. As reviewed by Verrax and Calderon, and Mamede *et al.* [58,116], vitamin C has been reported to increase the efficiency of cancer drugs *in vitro* and *in vivo*, but the opposite effect has also been observed *in vitro*, with a resulting decreased effect of cancer drugs. Importantly, cancer treatment in the form of radiotherapy generates ROS in irradiated tissues, and chemotherapies might also affect oxidative stress through its different modes of action [58]. The use of antioxidants/pro-oxidants might therefore interfere with cancer chemo- and radiotherapy. Consequently, the potential use of vitamin C as a chemopreventive agent is at an early stage and needs immense considerations and further research.

8.10 VITAMIN C VERSUS VITAMIN A

Out of the thirteen micronutrients investigated in paper I, vitamin A and C stood out in particular. Vitamin C has well-documented pro-oxidant properties while the pro-oxidant potential of vitamin A has been reported to a lesser degree. Somewhat surprising, we observed that vitamin A caused oxidation to dG with an even stronger pro-oxidant potential than vitamin C. Retinol concentrations in plasma are stable and do not fluctuate much. Three times normal plasma levels of retinol is therefore not normally achievable in human plasma, but more likely to occur in tissues utilising or storing vitamin A. Plasma levels of vitamin C are also kept stable but are able to fluctuate more than for vitamin A, for example after vitamin C supplementation [124]. Moreover, since even normal plasma levels were sufficient to cause oxidation, the relevance *in vivo* might be suspected to be higher for vitamin C. On the other hand, the properties of vitamin C is, as discussed in previous sections, highly dependent on environmental factors, and extrapolation to an *in vivo* situation is for that reason premature. The results from paper I do, nonetheless, give important insight into pro-oxidant properties of both vitamin A and C, enabling a direct comparison between the two as well as eleven other micronutrients.

8.11 CONCLUSION

The pro-oxidant potential previously documented from vitamin C is confirmed in this thesis. Vitamin C stood out among the thirteen micronutrients as the only one capable of pro-oxidant activity at normal plasma concentrations. The presence of iron or copper further enhanced the effects, and the combination of vitamin C and copper stood out the most, causing oxidation both in the chemical and cellular analyses. This is a potential concern since vitamin C and copper are both commonly found in supplements. The forms of vitamin C present in supplements also differ in their chemical properties, emphasising the importance of making an aware decision during the synthesis of antioxidant supplements. To compound the situation, supplements are also easily accessible to the public, enabling individuals to be exposed to very high doses. On the other hand, the pro-oxidant potential of vitamin C along with characteristic properties of cancer cells opens up for a potential use for vitamin C for cancer prevention and treatment.

9 DIGGING DEEPER – FOLATE

In this chapter, you will be guided through the effects of folate in humans, with an emphasis on the effect of folate status, folic acid status and the link to DNA lesions. The chapter revolves around the observational study conducted in paper III.

9.1 FOLATE OR FOLIC ACID?

Folate, vitamin B_9, is a water soluble vitamin involved in amino acid metabolism, correct DNA synthesis and in a range of transfers of methyl-groups [54]. While naturally occurring folate is found in food sources, folic acid is the synthetic form present in supplements. Folate is a generic term referring to compounds with similar chemical structures, containing three moieties, 1) a pterin ring, 2) *para*-aminobenzoic acid (joint by a methylene bridge) and 3) a glutamic residue (joint by a peptide bond) [160]. Figure 17 shows the chemical structure. The different folate compounds arise since the pterin ring can be reduced or oxidised to different extents and the glutamic acid can be extended to include a glutamate chain. A majority of the naturally occurring folates include five to eight glutamate units in its side chain [40]. The fully reduced form of folate is referred to as tetrahydrofolate and frequently abbreviated to THF. In supplements, a synthetic form of folate is commonly present – folic acid. This form is specific, containing one glutamate unit only, and a fully oxidised pterin ring [40].

9.2 THE FATE OF FOLATE IN HUMANS

In vivo, folates from oral consumption are absorbed in the intestine, transported out to the blood system and guided to tissues where it is absorbed and stored and/or metabolised. The kidney also serves as a key site for the re-absorption of folate [40]. Before absorption can occur in the intestine, folates in polyglutamate form (folate PG) are enzymatically hydrolysed to monoglutamate form (folate MG). Subsequently, several transport systems facilitate the uptake of the folate MG. The intestinal transport is believed to be mediated mainly by proton-coupled folate transporter (PCFT), reduced folate carrier (RFC) is also present as well as a mechanism by which the folates are taken up through endocytosis, folate receptor (FR)-mediated endocytosis. PCFT also takes part in the latter [54]. Once absorbed, both folate and folic acid are metabolised to 5-methyl THF, which is taken up by the peripheral blood stream via RFC and folate receptors [40]. In plasma the form most predominantly present is 5-methyl THF, found bound to albumin or to a high-affinity folate binder [40].

To be retained and stored in cells, folate MG is converted to folate PG, intracellularly folate PG is stored bound to enzymes. To be released to the extracellular milieu, the folate PG is hydrolysed back to folate MG. The uptake of folate is concentration dependent and the expression of folate transporters adapts to the extracellular concentration [40]. In addition, in most tissues, although not liver tissue, the RFC has a higher affinity for folate compared to folic acid. The presence of high levels of folic acid also affects the ADME in that absorption of folic acid also occurs through a

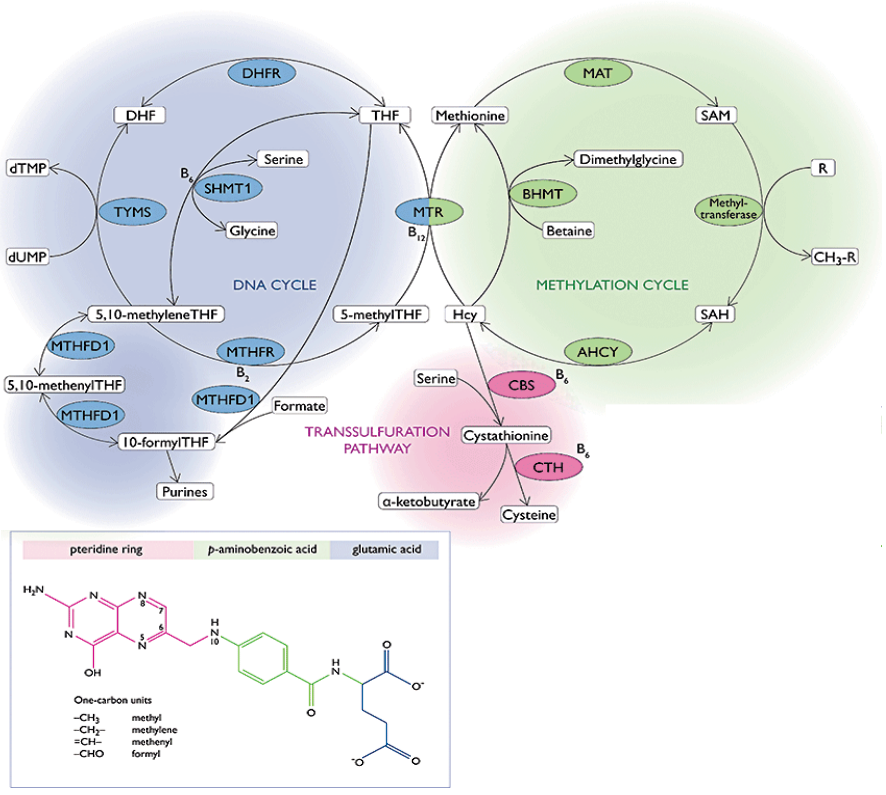


Figure 17. Folate mediated one-carbon metabolism consisting of DNA biosynthesis (DNA cycle, in blue) and methylation (in green). Below is the chemical structure of folates. Abbreviations: AHCY, s-adenosylhomocysteine hydrolase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CTH, cystathionase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; Hcy, homocysteine; MAT, methionine, adenosyltransferase; MTHFD, methylenetetrahydrofolate; MTHFR, methylenetetra-hydrofolate reductase; MTR, methionine synthase; R, methyl acceptor e.g. DNA or histones; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; SHMT1, serine hydroxymethyltransferase; . THF, tetrahydrofolate; TYMS, thymidylate synthetase. B_x denotes the respective B vitamins. The figure is reproduced from Laanpere *et al.* [54] with permission from Wiley/Nutritional Reviews.

diffusion-like process in the intestines at high folic acid concentrations. As opposed to folate, unmetabolised folic acid can be found both in plasma and excreted in urine [40].

In mammals, folate is required for the activation and transfer of one-carbon units, known as folate-mediated one-carbon metabolism. As portrayed in Figure 17, it can be seen to include two intervening pathways: 1) a DNA cycle, and 2) a methylation cycle. In the DNA cycle, adenine, guanine and thymidine (dTMP) precursors are produced, as well as the amino acids serine, glycine and methionine. In the methylation cycle, the primary methylation agent s-adenosylmethionine, SAM, is formed, as well as the amino acids homocysteine, cysteine and methionine. The two cycles compete for the use of 5,10-methylene THF, especially at low folate levels [54]. The folate-mediated one-carbon metabolism also requires other micronutrients, such as vitamin B_2, B_6 and B_{12} and low levels of these might also affect the metabolism – something which is referred to as 'functional' folate deficiency.

9.3 FOLATE – THE THEME OF PAPER III

The main aim of paper III was to investigate associations between intake or status of folate, folic acid or folate metabolising enzymes with DNA lesions. A particular interest was dedicated to the lesion of uracil misincorporated into DNA since folate depletion will prevent the conversion of dUMP to dTMP in the DNA cycle, see Figure 17. Consequently, the synthesis of thymine is inhibited and instead uracil becomes available for incorrect incorporation [54]. We aimed to investigate if folate parameters could predict levels of uracil misincorporation, oxidative DNA lesions or DNA strand breaks. Folate is extensively studied in relation to fertility and folate parameters are therefore comprehensively analysed in infertile women. For that reason, infertile but otherwise healthy women were chosen as the study population in paper III. The study design is shown in Figure 8, chapter 6, and described in detail in paper III. In short, 36 infertile women were studied. The women answered food questionnaires and interviews, from which folate/folic acid intake could be estimated. Blood samples were also drawn from the participants, from which DNA lesions (DNA breaks, oxidative DNA lesions and uracil misincorporation into DNA), levels of folate, vitamin B_{12} and homocysteine, and polymorphisms for genes involved in folate metabolism were analysed.

9.4 FOLATE, FERTILITY AND FOETUS HEALTH – IS THERE A LINK?

Folate status has, to different extents, been connected to neural tube defects, spontaneous abortions and infertility. Within 28 days of conception, the nervous system is being developed through the closure of the neural tube, which subsequently develops into the spinal cord and surroundings of the brain. In between 0.4 to 2‰ (i.e. per thousand) of all pregnancies, the neural tube is not closed properly, leading to neural tube defects [53]. Survivors of neural tube defects suffer from neurological motor deficits. They vary in severity depending on the site and size of the defect but can, for example, include water in the brain, cognitive dysfunction, learning disabilities or walking difficulties [53]. Folate is a crucial component of many one-carbon reactions, and a sufficient folate status is required for a functioning DNA synthesis, cell division, correct SAH/SAM ratio and homocysteine levels, all needed for a functioning development of the nervous system [40]. Folic acid supplementation has proven highly successful in preventing neural tube defects [161].

While the influence of folate in neural tube defects is clear, its role in fertility is more uncertain and is currently being subject to research. In fact, studies have observed that folate insufficiency, high homocysteine levels (hyperhomocysteinemia) and certain polymorphisms of genes involved in folate metabolism negatively affect oocyte and embryo quality, oocyte production, ovulation, implantation and pregnancy viability. Whether folic acid supplementation can improve female fertility and foetus viability remains to be seen [54].

9.5 FOLATE AND DNA LESIONS

9.5.1 Uracil misincorporations

As can be seen from Figure 17, a low folate level will produce low 5,10-methylene THF levels, which in turn will hamper the conversion of dUMP to dTMP. The result is elevated uracil concentrations accompanied by reduced thymidine levels. Uracil and thymidine differ only by one methyl group, and their resemblance makes it possible for uracil to be misincorporated into DNA, in place of thymine. Uracil is normally present in RNA only and therefore becomes subjected to DNA repair mechanisms. During repair, uracil is removed, producing a single strand break. Unless thymidine synthesis is resumed (i.e. by raised folate status), the correct base might not be inserted and DNA double strand breaks, chromosomal aberrations and malignant transformation (i.e. initiation of cancer) may follow [52]. In human studies, the evidence is inconsistent but DNA damages including uracil misincorporation and chromosomal breakage have been observed [51,85,162-164]. In subjects with a sufficient folate status, the amount of uracil misincorporated into DNA has been seen to be either unaffected by the folate level in red blood cells, or to be negatively correlated [85]. Folic acid supplementation for twelve weeks reduced the level of uracil misincorporation among the healthy participants in the latter study [85]. In another study conducted on healthy women with sufficient folate status, the level of uracil misincorporation was not altered when folic acid supplementation was given for four weeks. In splenectomised individuals (i.e. with the spleen partially or completely removed), sufficient folate levels have been associated with normal levels of uracil misincorporated into DNA, while folate deficiency has been correlated to higher levels of uracil misincorporation [162].

In paper III, we studied infertile, but otherwise healthy, women. Since these women were aiming to become pregnant they fall under the recommended population groups who, by the Swedish National Food Agency, are advised to consume folic acid supplement. Approximately 67% in our study reported taking supplements. However, 41% consumed less than recommended despite supplement use. On average, approximately 520 μg folate and folic acid were consumed from food and supplements, and a serum level of 20 nM was achieved, which is well within normal and recommended levels. In these subjects with adequate folate levels, we did not find any association between folate status and uracil misincorporation into DNA, nor between folate status and vitamin B_{12} or homocysteine levels. These results, in combination with the results from the studies described above, indicate that even though folate status has been associated with uracil misincorporation, the effect seems to be more pronounced in subjects with a poor folate status.

9.5.2 Other DNA lesions

Folate status and its association to other DNA lesions have been reported in studies. Kapisewska *et al*. reported no correlation between folate status and DNA strand breaks in healthy women with sufficient folate status, after a four week folic acid supplementation period [86]. In another study, young healthy individuals with adequate folate levels were studied and no correlation between red cell folate and micronuclei formation in lymphocytes were observed [51]. Micronuclei formation is characteristic

of cells with DNA lesions. Despite the lack of association, the authors experienced a reduction of micronuclei formation following 24 weeks of folic acid supplementation, a reduction associated with vitamin B_{12} and homocysteine levels, but not with red cell folate [51]. In splenectomised individuals, adequate folate levels have been associated with normal micronucleated reticulocytes (immature red blood cells), while folate deficiency was associated with higher levels of micronucleated reticulocytes [162]. In another study in splenectomised subjects, those with the highest micronucleated reticulocytes were folate or vitamin B_{12} deficient [163].

As with uracil misincorporated into DNA, the women studied in paper III, who had adequate folate levels revealed no correlation between folate status and DNA breaks or oxidative DNA lesions. As with the uracil lesions, these results together with results from the other studies discussed, imply that, even though folate status has been associated with different DNA lesions, the effect seems to be more prominent in participants with a poor folate status.

Enzyme polymorphisms. The impact of different polymorphisms in key enzymes in the folate-dependent one-carbon metabolism on fertility as well as DNA lesions have been implicated in previous studies [54,86]. In paper III, we observed a small, yet statistically significant, effect by the *MTHFR* 677 polymorphism on the level of DNA strand breaks. The *MTHFR* 677 enzyme is involved in the conversion of 5,10-methyl THF to 5-methyl THF as seen in Figure 17. In paper III, the *MTHFR* 677 CT genotype was associated with lower levels of DNA strand breaks, as compared to the CC wild-type genotype. The actual difference was, however, only 0.5%, and would not have been significant if Bonferroni correction for multiple tests had been conducted. In our study, the clinical relevance of this decrease can be questioned. In addition, the power to detect effects of DNA damage by different polymorphisms was low, due to the relatively few participants. The extent of uracil misincorporation into DNA has previously been linked to different genotypes of the *MTHFR* 677 enzyme. Contrary to what was indicated in our results, CT and TT polymorphisms have instead been associated with lower levels of uracil misincorporation into DNA, as well as with lower plasma levels of folate, as compared with the CC wild-type [86]. The effect of polymorphisms of different enzymes involved in folate metabolism on DNA lesions and fertility remains to be further researched.

It cannot be excluded that a larger study population and participants with lower folate levels might have revealed a different association between DNA lesions, folate status and gene polymorphisms.

9.6 FOLATE AND CANCER

Evidence has indicated that folate deficiency might contribute to an increased risk of cancer of the colorectum, breast, ovary, pancreas, brain, lung and cervix [52]. However, recent studies and meta-analysis raise concern that high doses of folic acid supplementation might also contribute to an increased cancer risk [53,55,56].

9.7 CONCLUSION

Folate is essential for one-carbon reactions involved in amino acid metabolism, correct DNA synthesis and a range of transfers of methyl-groups. Maternal folate deficiency is associated with neural tube defects in foetuses, and has been implicated in oocyte viability, pregnancy outcome and in infertility. Folate deficiency has also been correlated with different types of DNA lesions, where the misincorporation of uracil into DNA is particularly dependent on folate status. In paper III, we investigated the association between folate and folic acid intake with uracil misincorporation into DNA, DNA strand breaks or oxidative DNA lesions. Our results add to the carefully accumulating evidence indicating that, even though folate status has been associated with DNA damage, the effect seems to be more evident in subjects with folate deficiency.

10 DIGGING DEEPER – SEA BUCKTHORN

Since many large intervention studies administering micronutrients in rather large doses have failed to show health beneficial effects, interest has been drawn to micronutrient supplements that are imitating the content of fruits and vegetables. One such approach is using extracts from fruit and berries. This chapter revolves around the effects of extracts from the sea buckthorn berry, a berry rich in antioxidants and fatty acids. The sea buckthorn will be discussed in the light of the intervention study conducted among chronic kidney disease patients in paper IV.

10.1 THE SEA BUCKTHORN BERRY

The sea buckthorn berry, as seen in Figure 18, has historically been used as a medicinal plant in China, Turkey and Russia for hundreds of years. Its Latin name is *Hippophae rhamnoides* and it is one of six species and twelve subspecies belonging to the genus *Hippophae* L. Sea buckthorn is the only one of these species that grows outside of Central Asia and can also be found in, for example, Europe, Russia, China and India. More than 80% of the land used to grow sea buckthorn, for either wild or cultivated plants, is found in China. Although to a much lesser extent, sea buckthorn is also found in the Nordic countries. It is well adapted to extreme conditions and can grow in nutritionally deficit soils, drought, salinity (an environment rich in salt), wind and at temperatures ranging from -43°C to +40°C [165].

Sea buckthorn is rich in nutrients, such as unsaturated fatty acids, B, C and E vitamins, carotenoids and polyphenols [165,166]. Oil from this berry is particularly rich in the fatty acids linoic (18:2 n-6), α-linoic (18:3 n-3), oleic (18:1 n-9), palmitoleic (16:1 n-7) and palmitic (16:0) acids, as well as vitamin E, tocopherols and tocotrienols, flavonoids and carotenoids [167]. The precise content of a sea buckthorn berry is dependent on several factors. The subspecies, the growth place and the maturity level all contribute to the levels of fatty acids and micronutrients present [167-170]. The content of sea buckthorn oil is also dependent on whether the oil has been extracted from seed or pulp, resulting in different fatty acid components and ratios. Juice and oil from sea buckthorn berries also differ in their contents [171].

Figure 18. The sea buckthorn berry. Photo by MyHavtorn, reproduced with permission from MyHavtorn.

Figure 19. A haemodialysis session for a chronic kidney disease patient. Photo by Staffan Larsson.

While the sea buckthorn plant has been used as a medicinal plant in certain parts of Asia for many centuries, it is only during recent years that an increasing interest in the plant has arisen in the Western world. Today, sea buckthorn is found on the food market, present in a wide range of products including jam, curd, juice, wine, liquors, tea, pie, cake, honey, sweets and ice cream. Sea buckthorn can also be purchased as berry oil and as a dietary supplements, and is also used in cosmetics [165].

10.2 A SEA BUCKTHORN EXTRACT – THE THEME OF PAPER IV

The aim of paper IV was to research the effect of a supplement containing sea buckthorn oil extract on oral health, inflammation markers and DNA lesions in patients suffering from chronic kidney disease (CKD). 45 patients were studied, all undergoing haemodialysis treatment. DNA lesions including DNA breaks and oxidative DNA damage (FPG-sensitive sites) were measured using the comet assay. Analyses of blood markers from routine samples of haemodialysis patients were also included. This intervention study had a randomised, double blind crossover design. Patients were given supplement or placebo to start with, following which, after a washout period, they consumed the other type of capsule. The supplementation periods lasted for eight weeks and the washout period stretched over a four-week period. The study design can be seen in Figure 6, chapter 8. A more detailed description of the study is found in paper IV.

Supplement content. One capsule contained 500 mg of sea buckthorn extract, and the participants consumed four capsules daily. The content of the sea buckthorn capsules are listed in Table 6. The placebo capsules contained coconut oil and were similar in physical appearance.

Table 6. Content (mass and weight %) of the sea buckthorn extract capsule

	Amount in one capsule (500 mg)	Daily dose (four capsules, 2 g)
Oleic acid (C18:1 n-9)	124 mg (24.8%)	496 mg
Palmitoleic acid (C16:1 n-7)	97 mg (19.5%)	388 mg
Linoleic acid (C18:2 n-6)	92 mg (18.4%)	368 mg
α-Linolenic acid (C18:3 n-3)	63 mg (12.6%)	252 mg
Vitamin E	931 μg	3.7 mg
Vitamin A	88 μg	352 μg

10.3 CHRONIC KIDNEY DISEASE AND DIALYSIS PATIENTS

CKD is a progressive loss of renal function accompanied with uremic symptoms. Patients with CKD suffer from numerous discomforts. Damage to the kidneys induces uremia, a state of intoxification that leads to alterations of many biomolecular processes. CKD can affect all tissues and organs in the human body, including the heart, blood vessels, nerves and muscles [172]. Therapy to avoid these complications consists of medication at a first stage, and ultimately of dialysis treatment or a transplant. During dialysis the blood is cleaned through a membrane enabling toxins to

be eliminated or decreased. Two forms of dialysis exists, peritoneal and haemodialysis, the latter being the most commonly used, with an estimated one million users worldwide [173]. The experience of a haemodialysis session can be seen in Figure 19.

In recent years an increasing attention has been brought to the oral health problems that CKD patients also suffer from as a consequence of both the disease and therapy. Problems with the bone as well as soft tissue structure have been reported. Many patients experience, for example, xerostomia (mouth dryness), eating-, swallowing- and speaking difficulties, tooth and gingival sensitivity, bad appetite, painful mouths, gingival bleedings and an increased risk of infections [172-175]. CKD is also associated with malnutrition, high levels of oxidative stress and inflammation, thus contributing to a higher risk of developing cardiovascular diseases. Patients undergoing haemodialysis have been revealed to have an increased production of free radicals, impaired antioxidant mechanisms and an oxidant/antioxidant imbalance, all contributing to oxidative stress [176].

Patients undergoing haemodialysis often have low nutrient levels due to the loss of water soluble vitamins during the dialysis treatment, but also due to their restricted diet as well as lost ability to, for example, activate vitamin D [177]. Because of a loss in kidney activity, certain micronutrients are prevented from being excreted and are on the contrary to others found in high levels, for example vitamin A [177]. The level of micronutrients can also fluctuate depending on the time left until dialysis treatment [178]. Treatment normally occurs at a medical centre three times per week, and requires several hours per session. Vitamin E-coated dialysis membranes have been introduced with encouraging results, reducing lipid peroxidation [179]. Supplementation with a few micronutrients has also indicated promising results. Folic acid contribute to a reduction in cardiovascular disease [180], omega-3 fatty acid to a pronounced antioxidant defence [181], a fish oil supplement to a reduction in the inflammation marker C-reactive protein (CRP) [182] and vitamin E to a reduction of DNA breaks [176], for example.

10.4 SEA BUCKTHORN AND HEALTH EFFECTS

As will be unravelled in this and the following section, the sea buckthorn berry has been attributed antioxidant properties as well as immune response regulatory effects, anti-inflammatory effects and anticancer effects in a mix of *in vitro*, animal and human intervention studies.

Inflammation is induced by the body during the course of many different diseases, and is well-known to be elevated in CKD disease patients. In paper IV, inflammation markers measured in the CKD patients undergoing dialysis treatment were not affected by sea buckthorn oil consumption. This was despite elevated inflammation in the patients group, as seen in a previous study among the same patients [183]. The inflammation markers measured were hsCRP (high sensitive C-reactive protein), antitrypsin, orosomucoid and leukocytes – some of which sea buckthorn has previously been reported to affect. For example, Larmo *et al.* [184] observed that consumption of sea buckthorn puree reduced the CRP level, an effect that was not correlated with the

flavonol content as hypothesised [185]. Anti-inflammatory activity has also been documented in rats [186]. Two obvious differences between our study and the one by Larmo and colleagues [184] are the participants and the sample size. While Larmo *et al.* included 254 healthy participants, we studied 45 chronically ill patients. In addition, the form of sea buckthorn consumed differed between the two studies. Larmo *et al.* administered sea buckthorn puree while the CKD patients in paper IV received capsules containing oil extracts. Considering that the antioxidant and fatty acid content of sea buckthorn differ between different parts of the plant and is dependent on maturity as discussed above, the content of the sea buckthorn is excepted to differ in the two studies, and it is possible that the oil extract used in paper IV has a lower ratio of less lipophilic compounds.

Certain parameters related to the development of cardiovascular disease, in addition to inflammatory markers, have also been affected by sea buckthorn. In an *in vivo* study in rabbits, sea buckthorn oil displayed anti-artherogenic and cardioprotective activity, as determined by a reduction in, for example, plasma cholesterol [187]. In a human intervention in which 229 healthy adults consumed puree from sea buckthorn the results differed, and neither LDL or HDL cholesterol, or triacylglycerol, were affected by a three months intervention [185]. Platelet aggregation has, however, been inhibited in adult men after four weeks of sea buckthorn oil consumption [188]. In paper IV, the focus was on the specific health effects related to CKD, and cholesterol values were not measured. Another health aspect for which sea buckthorn has been observed to be positively related to and which paper IV did not investigate, is the linkage to metabolic disease. Lehtonen *et al.* [189] reported improvements of different parameters related to metabolic disease in overweight and obese women after a 33-35 day intervention with sea buckthorn extract.

In paper IV, we did not expect disease parameters to significantly change during the intervention, CKD is an irreversible process and it would be premature to expect a dietary intervention to interfere with the disease progression. The hypothesis was rather to improve other health aspects such as DNA lesions or mouth dryness. As expected, creatinine and urea were not affected by sea buckthorn supplementation. A few parameters were, however, significantly affected, but their changes were small and their clinical relevance therefore questioned.

The dryness of eyes has been improved by consumption of oil from sea buckthorn [166]. Adults using contact lenses reported fewer days experiencing dry eye symptoms in comparison to placebo consumers. Analytical values that improved, not only in contact lens users, include tear film osmolarity, redness and burning. The improvement of dry eye symptoms were not caused by a change in fatty acid content of the tear film as speculated [190]. CKD disease patients commonly experience mouth dryness. Based on the improvement in dry eye symptoms, we investigated if a sea buckthorn oil extract could improve mouth dryness symptoms in CKD patients. In paper IV, the mouth dryness was analysed using salivary flow (excretion) rates, which often, but not always, correlate with mouth dryness. The CKD patients did, however, not display any improvement in oral health after supplementation.

Since the sea buckthorn berry is rich in vitamin C and other micronutrients, it comes as no surprise that its potential effect on the common cold has been investigated. Larmo *et al.* did, however, not observe any change in frequency or duration of the common cold or urinary tract infection after 90 days of consumption of sea buckthorn puree [184].

10.5 SEA BUCKTHORN AND DNA LESIONS

The sea buckthorn berry has attributed antioxidants properties as described by several *in vitro* studies, while the number of published articles with reference to sea buckthorn and human studies measuring DNA damage is low. A high antioxidant activity has been seen from extracts, using different antioxidant assays (e.g. FRAP) [191]. A reduced free radical production [192,193], apoptosis and DNA fragmentation have also been observed from sea buckthorn [192], along with an increased antioxidant status [192]. Mice have been protected against induced oxidative damage by sea buckthorn [194,195]. In a cell-free system, an oil extract has revealed potential to be metal chelating, metal (iron) reducing, and radical scavenging [196]. A study conducted in 2010 revealed antioxidant properties from a sea buckthorn extract – when at a low dose, but not at a high dose [197]. Higher doses provided a pro-oxidant effect – as with many other antioxidants in this thesis. The berry has in fact also been observed to induce DNA-protein cross-links [198]. Moreover, a protective effect on DNA breaks in lymphocytes measured using the comet assay has also been detected [199].

In paper IV we investigated the effect of supplementation with a sea buckthorn oil extract on DNA lesions in CKD patients. Considering the discomforts that CKD patients experience in the oral cavity (e.g. mouth dryness), we focused on effects in local tissue with the aim to investigate if local damage could be improved by the intervention. Our results, however, did not reveal any beneficial effect on DNA lesions, neither DNA breaks or oxidative DNA lesions, in minor salivary glands among the participants. CKD patients have elevated inflammatory levels as well as oxidative stress levels [176]. In addition, the two are intervened since ROS are produced during inflammatory response. Despite this, and along with antioxidant potential of sea buckthorn documented in *in vitro* studies, we did not observe an antioxidant effect. However – and importantly – CKD has repeatedly been reported to be associated with elevated levels of oxidative stress and DNA damage in *leukocytes* [200,201]. Whether oxidative stress levels in blood cells reflect the level in tissues is, however, unclear and it was recently shown that the CKD patients in our study had lower oxidative stress levels (as determined by oxidative DNA lesions using the comet assay) in salivary glands compared to healthy controls [183]. A lack of protective effect against DNA damage could be due to the already relatively low levels of DNA lesions present in the salivary glands, and it cannot be excluded that a different result may be achievable in tissues with initial elevated levels of DNA lesions.

Thus, in paper IV we did not reveal any protective effect from sea buckthorn on DNA lesions. Whether sea buckthorn supplementation can protect against or improve DNA damage in tissues containing higher levels of initial damage, alternatively in blood cells, needs to be further investigated.

10.6 SEA BUCKTHORN AND CANCER

In addition to its antioxidant activity, and other health promoting properties discussed, the sea buckthorn has also been suggested to have antimutagenic potential. A study published in 2009 showed 'remarkable antitumor activity' by a sea buckthorn extract towards a two-stage carcinogenesis induced by a tumour promoter (TPA) in mice [202]. In mice, a decrease in carcinogen-induced forestomach and skin tumourigenesis have also been seen from sea buckthorn, with an indication of an up-regulation of antioxidant and phase II enzymes, and DNA-binding activity of the transcription factor interferon regulatory-1 [203]. Olsson *et al.* observed that sea buckthorn inhibit proliferation of cancer cells in a cell study, an effect that was correlated with carotenoid and vitamin C content of the berry [204]. The absence of effect on DNA lesions in CKD patients in paper IV does not add evidence to a cancer protective effect by the berry. Nevertheless, studies published on sea buckthorn and cancer effect provides promising evidence to elaborate on in future research.

10.7 CONCLUSION

In paper IV, the CKD patients undergoing dialysis treatment were not affected by sea buckthorn supplement consumption. Published clinical trials are not consistent in showing effects on inflammatory markers form sea buckthorn berry and our study adds to the evidence of no effect on inflammation. While some intervention studies have shown health beneficial effects on DNA lesions in CKD patients, sea buckthorn did not affect DNA breaks or oxidative DNA lesions in our study. We did, however, measure the damage in local tissue – minor salivary glands – while it is more common to analyse DNA lesions in blood cells since blood is simple to collect from participants. It cannot be excluded that sea buckthorn supplementation could affect the DNA of blood cells more, or that the effect of sea buckthorn could be different in another patient group. The results from paper IV add to the ambiguous evidence in the literature concerning supplementation of micronutrients or berry extracts.

11 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

11.1 CONCLUDING REMARKS

What are the contributions of this thesis to the fields of nutrition, micronutrients and toxicology? The thesis adds to the evidence that micronutrients can act differently depending on external factors – that many of them can behave both as antioxidants and pro-oxidants. It also adds to the confusion of micronutrient supplements, implying that supplements do not always have significant contributions to our DNA, specifically on the levels of DNA breaks and FPG-sensitive sites (oxidative DNA lesions). The results within the thesis also reveal that berry extracts are not automatically health beneficial even if the berries have been described as having health-promoting effects. Synthetic forms of a micronutrient, as often found in supplements, may have different qualities and take different actions compared to its natural form. An interest has therefore been drawn to the use of natural ingredients in supplements, such as extract from berries. However, the results herein show that health beneficial effects do not automatically follow simply because the micronutrients are of natural origin. The health effects from micronutrient supplements may vary – independent of if the supplement contains micronutrients of synthetic origin or extracts from natural sources. The thesis has answered the following questions, as outlined in chapter 3:

1. Do micronutrients/antioxidants chemically act as pro-oxidants and if so, do they do so with different strengths?
Some do. Out of thirteen micronutrients vitamin A and vitamin C were found to particularly stand out with strong pro-oxidant activity in a strictly chemical set-up. The pro-oxidant activity was further enhanced, synergistically, in the presence of iron or copper, also found in supplements. Environmental factors (i.e. buffer type, dose) also significantly affected the pro-oxidant capacity of the vitamins.

2. Do micronutrients with pro-oxidant potential act as pro-oxidants in cultured cells?
Mostly not, but in some combinations they can. In this thesis, vitamin A or C alone did not cause oxidative damage to the DNA of cells in culture in this thesis. Nor did they cause DNA strand breaks. Noteworthy, however, the combination of vitamin C and copper *did* cause oxidative DNA damage as well as DNA breaks – a combination standing out with particular pro-oxidant activity in this thesis.

3. Do different compounds in supplements classed as either vitamin A or C differ in their abilities to act as pro-oxidants?
Yes. It is clear that the different vitamin A compounds permitted in supplements have different chemical properties and different abilities to act as pro-oxidants. The same was concluded about vitamin C compounds present in supplements. This poses a potential concern during for example manufacturing of supplements. When it came to the effect on the DNA of cultured cells the compounds did, however, not differ between each other to the same extent and most compounds did not cause DNA lesions.

4. Do folate levels, intake or genes related to folate metabolism predict the level of DNA lesions?
The results herein imply that such predictions should not be made at this point. In our observational study, folate/folic acid intake or folate status was not associated with the level of DNA lesions in 36 infertile women. Most polymorphisms of genes involved in folate-mediated one-carbon metabolism were not associated with DNA breaks, oxidative DNA lesions or uracil misincorporation into DNA either. The folate status and intake in these participants were within a desired range, and it could be that an association between folate and DNA lesions is stronger in participants with a poor folate status – a hypothesis that needs further investigation.

5. Does a supplement containing a berry extract rich in antioxidants reduce DNA lesion levels, improve health or improve the oral health of chronic kidney disease patients?
Unfortunately not in our study. An eight week long intervention with a sea buckthorn extract did not change the level of DNA lesions in chronic kidney disease patients undergoing haemodialysis treatment. Neither did it affect mouth dryness as determined by salivary secretion, nor did it affect inflammation or disease specific parameters to any level that would be anticipated to be of clinical relevance. The initial level of DNA lesions in minor salivary glands among the patients were, however, not elevated and a different effect on DNA lesions might be achievable in patients with higher initial damage, or in a different cell type.

This thesis emphasises that the properties of micronutrients and antioxidants are in fact not straightforward, further adding to their mystery. An antioxidant is not always an antioxidant – with its ability to take part in electron transfers it can also act as a pro-oxidant. The ambiguousness is further enhanced due to that many external factors can have an effect on the micronutrients' actions. The chemical effects of micronutrients can thus vary. Neither is it clear and obvious how they act when ingested as a supplement. Will the micronutrient supplement help us in our strive to live a long and healthy life, or will they be nothing but a burden added to our economical concern? Or, if worse comes to worse, will they even cause harm? Previous studies in smokers and workers exposed to asbestos have shown just that: that antioxidants in fact are capable of causing harm. However, in many studies antioxidant supplements have simply not had any effect at all on studied parameters, and the study on patients with kidney failure presented within this thesis adds to this non-effective result by micronutrients. It is important to mention that this thesis focuses on the effects of micronutrients in supplements on DNA and cancer, and the micronutrients discussed may very well have other health effects, beneficial or not, which have not been covered in this thesis. There are also certain groups of the population that might benefit from consumption of specific dietary supplements in a different way compared to the general population. Clinically defined nutrient deficient individuals might be one such group.

11.2 FUTURE PERSPECTIVES

Most studies investigating health effects related to DNA and cancer in the general healthy population have failed to reveal beneficial effects from micronutrient supplements. Thus, future research that instead concentrates on specific high-risk groups might be more relevant at this point. Another challenge lies in identifying health effects from individual micronutrients, and a greater focus on the cocktail effect of several micronutrients might be desirable, whether as a multivitamin or a berry or fruit extract.

While the use of micronutrient supplements by the general population is being questioned, more and more interest is shifted towards the micronutrients' potential benefit within epigenetic and cancer preventing research. Epigenetics is a blooming field and refers to modifications in gene expression caused by changes in DNA methylation and chromatin structure. Epigenetic changes do not alter the DNA sequence but are – importantly – inheritable. Folate, retinoic acid and selenium have all been implied in epigenetic processes and comprise potential benefits to be investigated in future cancer preventive research [52,205].

In an article published in 2011, Halliwell [65] proposes an interesting view that might come to challenge the field of antioxidants. He argues that *reactive species* might play a more important role in the antioxidant defence in humans than dietary antioxidants, by triggering increased levels of endogenous antioxidants. From this follows that pro-oxidant action by antioxidants may play an role in enhancing the body's own antioxidant defence, while the scavenging potential by antioxidants might be of less importance – a hypothesis opening up for interesting research in the future.

12 POPULAR SCIENTIFIC SUMMARY

A strive to find a simple cure against diseases such as cancer has long been evident. The hope that it could be true arouse when it was observed that fruit and vegetables could prevent and reduce the appearance of several diseases, including cancer. Was it possible to extract the active substances and consume these in tablet form and achieve the same protective effect against cancer and other diseases? Fruit and vegetables contain small essential compounds called micronutrients. Many of these micronutrients are also so called vitamins and/or antioxidants. These small nutrients have been attributed responsible for the disease protection seen from fruit and vegetables. Keeping that in mind, vitamin supplements were manufactured with the hope that they would be just as health promoting.

Over a third of the population consume vitamin supplements on a regular basis. While many people take supplements with the belief that their health will benefit from it, scientific studies have not been able to verify that effect. Contrary, many large studies have shown that vitamin supplements often have no effect at all. In fact, micronutrient supplements have also been seen to have harmful health effects, increasing cancer and death incidence among smokers. However, studies also exist where vitamin supplements have indeed protected against cancer, primarily in deficient participants. Much research has been conducted on micronutrients, yet a lot of question marks remain concerning their properties and health effects. Several factors are believed to affect their disputed health effects, including:

- Initial health and nutrition status of the person.
- Supplement composition – are micronutrients that normally cooperate present?
- Dose – the dose is often higher in supplements than in fruit and vegetables.
- Micronutrient form – naturally occurring or manufactured?
- The surrounding environment – can influence the action of micronutrients.
- Antioxidant or pro-oxidant?
- Oxidative stress

In this thesis, my colleagues and myself have investigated some of the factors that are believed to underlie the different results obtained form vitamin supplements. I have focused on how micronutrients interfere with our genetic make-up, the DNA. Damage to the genetic make-up can sometimes give rise to cancer. The thesis describes both experimental studies on a chemical and cellular level as well as studies on humans.

Our genetic make-up contains instructions for how the components of the cell, our building blocks, are built. Imagine that the genetic make-up contains words (DNA bases) that together create instructions of how the different components should be built. If the instruction is damaged – for example if the instruction paper is ripped, letters removed or entire words removed or replaced – it severely complicates the building process. Depending on what letters that have been changed or gone missing, one scenario that may evolve is that the components or cells are built over and over again, in a quantity and speed that was not intended – this is how tumours and cancer can arise. One type of damage that can occur on our genetic make-up is through oxidation –

just as iron rusts. The body has a well functioning antioxidant system that protects against oxidation and damage that it causes (oxidative damage/lesion), but sometimes the protective system is overwhelmed, resulting in oxidative stress. Many micro-nutrients are also so called antioxidants, with the ability to protect against oxidation. There is, however, a paradox in that the property that makes them able to protect against oxidative damage (as antioxidants), also enables them to change behaviour and start to act as pro-oxidants, causing damage opposed to protecting against it.

In this thesis, we have used different model systems to compare the ability of thirteen micronutrients (whereof many antioxidants) to act as pro-oxidants, and cause oxidation to one of the 'words' in our genetic make-up (the DNA base guanine). Two micronutrients stood out – vitamin A and C. These oxidised, i.e. damaged, the DNA base and the effect increased with an increased dose. Directives at EU level regulate which compounds that are permitted in supplements. We saw that several of the compounds that are classed as either 'vitamin A' or 'vitamin C' differed in their ability to act as pro-oxidants. The pro-oxidant potential was, in line with what others have observed, affected by the immediate surrounding. The environment (probably also in the body) is thus essential for how an antioxidant/pro-oxidant behaves. We also observed that adding metals, such as copper or iron, enhanced the pro-oxidative activity. The combination of vitamin C and copper particularly stood out, causing oxidation not only to the DNA base, but also to the genetic make-up of cells in a system one step closer to a human situation (although still very far from it). Vitamin C and copper are both found together in multivitamin supplements. The different abilities by micronutrients to act as antioxidants or pro-oxidants should be taken into account when, for example, manufacturing supplements.

This thesis also includes two human studies, where the effect of supplements was investigated. In one of these studies, we investigated if folate, or folic acid, affects the level of damages on the genetic make-up. Supplementation with folic acid is recommended by the Swedish National Food Agency to women who are planning to become pregnant or who are in the beginning of a pregnancy for a normal development of the foetus. In our study of 36 infertile women, we found no associations between folate/folic acid and the level of damage on the genetic make-up. The women had adequate levels of folate and it could be that the damage levels had been affected by folate status if the participants had had low folate levels. The lack of positive health effects in many studies with vitamin supplements has led to an increasing interest for supplements that imitate the content of fruit and vegetables to a greater extent – can they give more promising results? We conducted a so called intervention study where 45 patients suffering from chronic kidney disease consumed a supplement containing oil from sea buckthorn, a berry rich in antioxidants and fatty acids. Unfortunately, the sea buckthorn supplement did not have any effect on the damage levels in the genetic make-up, neither did it improve mouth dryness or other disease related parameters.

The results in this thesis add to the growing evidence that vitamin supplementation do not always have a health protective effect. The thesis emphasises that each micronutrient has unique properties, dependent on factors in their immediate surrounding. It might be that supplementation with micronutrients provides best benefit in those with initially low nutrient levels.

13 POPULÄRVETENSKAPLIG SAMMANFATTNING

Länge har väl önskan om att finna ett enkelt botemedel mot sjukdomar så som cancer funnits. Hoppet om att detta kunde bli verkligen väcktes när man såg att frukt och grönsaker kunde motverka och minska uppkomsten av en mängd olika sjukdomar, däribland cancer. Kanske kunde man utvinna de aktiva ämnena och konsumera de i tablettform och få samma skydd mot cancer och andra sjukdomar? Frukt och grönsaker innehåller små livsviktiga ämnen som kallas för mikronutrienter. Många av dessa mikronutrienter är också så kallade vitaminer och/eller antioxidanter. Skyddet mot sjukdomar från frukt och grönt har trotts komma från dessa små näringsämnen. Med det i bakhuvudet har vitamintabletter (vitamintillskott) tillverkats med förhoppningen om att de skulle vara lika fördelaktiga för vår hälsa.

Över en tredjedel av befolkningen äter vitamintillskott regelbundet. Medan många i allmänheten äter tillskotten i tron om att hälsan ska förbättras så har studier gällande vitamintillskott inte kunnat bevisa att det faktiskt är så. Istället har många stora studier visat att vitamintillskott ofta varken gör till eller från hos ”vanliga” friska personer. Dessutom har vitamintillskott till och med visat sig kunna ha hälsofarliga effekter, genom att öka risken för cancer och dödlighet hos rökare. Dock finns även studier, främst hos folk med näringsbrist, där vitamintillskott faktiskt har visat en skyddande effekt mot cancer. Det har forskats mycket på mikronutrienter men trots det kvarstår många frågetecken vad gäller deras egenskaper och hälsoeffekter. Flera faktorer tros påverka deras omstridda hälsoeffekter, så som:

- Personens hälsa och näringsstatus från början.
- Tillskottets sammansättning – finns de mikronutrienter som brukar samarbeta?
- Dosen – ofta är mängden större i tillskott än i frukt och grönt.
- Mikronutrientens form – naturlig eller kemiskt framställd?
- Miljön omkring – vissa miljöfaktorer kan ändra hur mikronutrienter beter sig.
- Antioxidanter eller pro-oxidanter?
- Oxidativ stress.

I denna avhandling har jag och mina kollegor undersökt några av de faktorer som tros ligga bakom att vitamintillskott kan ge så skilda resultat hos olika individer. Jag har fokuserat på hur mikronutrienter påverkar vår arvsmassa, vårt DNA. Skador på vår arvsmassa kan i vissa fall ge upphov till cancer. Avhandlingen beskriver både experimentella studier på en kemisk och cellulär nivå och studier på människan.

Vår arvsmassa innehåller instruktioner för hur cellernas komponenter ska byggas. Man kan se det som att arvsmassan är uppbyggs av ord (DNA-baser) som tillsammans bildar instruktioner för hur olika komponenter ska byggas upp. Skadas instruktionen – till exempel genom att instruktionslappen rivs itu, bokstäver i orden försvinner eller att hela ord försvinner eller byts ut – så försvåras uppbyggnaden av komponenterna avsevärt. Beroende på vilka ord som försvunnit eller ändrats så kan ett scenario bli att byggstenarna eller cellerna byggs om och om igen i en mängd och fart som inte var tänkt – det är på så vis tumörer och cancer kan uppstå. En typ av skador som kan ske på vår arvsmassa är genom oxidation – precis som när järn rostar. Kroppen har ett väl

uppbyggt antioxidantsystem som skyddar mot oxidation och skador som de orsakar (oxidativa skador), men ibland överbelastas skyddssystemen, då oxidativ stress uppkommer. Många mikronutrienter är även så kallade antioxidanter och dessa kan också skydda mot att oxidation uppstår. Det paradoxala är att samma egenskap som gör att de skyddar mot oxidativa skador (som antioxidanter) också gör att de har förmågan att ändra deras sätt att verka och agera som pro-oxidanter och orsaka oxidation istället för att skydda mot det.

I denna avhandling har vi använt olika modellsystem för att jämföra förmågan av tretton olika mikronutrienter (varav många antioxidanter) att agera som pro-oxidanter och orsaka oxidation till ett av ”orden” i vår arvsmassa (DNA-basen guanin). Två mikronutrienter stack ut – vitamin A och C. Dessa oxiderade, alltså skadade, DNA-basen och effekten ökade när dosen ökades. Direktiv på EU-nivå reglerar vilka föreningar som får finnas i tillskott och vi såg att flera av de föreningar som räknas som ”vitamin A” eller ”vitamin C” skilde sig åt i sin förmåga att agera som pro-oxidanter. I linje med vad andra har sett så påverkade omgivningsmiljön deras förmåga att agera som pro-oxidanter. Omgivningsmiljön (förmodligen även kroppens) är således väsentlig för hur en antioxidant/pro-oxidant agerar. Vi såg också att tillsatsen av metallerna koppar eller järn förstärkte pro-oxidativa aktiviteten från både vitamin A och C. Kombinationen av vitamin C och koppar utmärkte sig genom att inte bara oxidera DNA-basen utan även arvsmassan i celler i ett system som efterliknar människosituationen lite mer (men som ändå är långt ifrån). Vitamin C och koppar förekommer dessutom tillsammans i multivitamintillskott. Vitaminernas olika förmågor att agera som antioxidanter eller pro-oxidanter bör tas i bejakande vid exempelvis framställningen av tillskott.

Denna avhandling innehåller också två stycken humanstudier, alltså studier på människor där effekten av tillskott i människor undersökts. I den ena studien undersökte vi om nivån eller intag av folat, eller folsyra, påverkade nivån av skador på arvsmassan. Tillskott med folsyra rekommenderas av Livsmedelsverket till kvinnor som planerar att bli gravida eller som är i början av en graviditet, för en normal utveckling av fostret. I vår studie bland 36 infertila kvinnor fann vi inga samband mellan folat/folsyra-status eller intag och skadenivåer på arvsmassan. Kvinnorna hade tillfredsställande nivåer av folat i kroppen och det kan vara så att skadenivån på arvsmassan hade påverkats mer av folsyranivån om de hade haft låga folatnivåer. Avsaknaden av positiva hälsoeffekter i många studier med vitamintillskott har bidragit till ett ökat intresse för tillskott som efterliknar innehållet i frukt och grönt i större utsträckning – kan de ge mer utlovande resultat? Vi utförde en så kallad interventionsstudie där 45 njursjuka patienter åt ett tillskott innehållande olja från havtorn, ett bär innehållande bl.a. antioxidanter och fettsyror. Dessvärre hade havtornstillskottet ingen effekt på skadenivån på arvsmassan i salivkörtlar, inte heller hade den någon effekt på muntorrhet eller andra sjukdomsrelaterade mätningar.

Resultaten i denna avhandling bidrar till den växande bevisbördan för att vitamintillskott inte har en skyddande hälsoeffekt i alla lägen. Avhandlingen betonar att varje mikronutrient har unika egenskaper, vilka också kan påverkas av dess omgivning. Det kan vara så att vitamintillskott har bäst verkan hos de med näringsbrist.

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