Evaluation of antioxidative and antimutagenic effects of quercetin in humans, in vitro and ex vivo

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ISBN: 978-90-5278-683-4

Production: Datawyse | Universitaire Pers Maastricht

The studies presented in this thesis were funded by The Netherlands Organisation for Health Research and Development (project number 01412012) and performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which is part of Graduate School VLAG (Food Technology Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 20 december 2007 om 16.00 uur

door

Lonneke Cécile Wilms

UNIVERSITAIRE PERS MAASTRICHT

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Chapter 1

General introduction

Food and disease

Our daily environment is a sheer endless source of challenges to life. Besides the obvious risks we consciously take, like amongst others smoking, or taking on dangerous sports, there are many threats that occur without people realising it. For instance, our body is continuously exposed to free radicals, creating a situation of oxidative stress, which may lead to several degenerative diseases. Oxidative stress is the condition where the balance between oxidising and reducing compounds is disrupted. Excess in radicals, which is the result of this disruption, may interact with biomaterials and thus induce cellular oxidative damage. This oxidative damage may cause adverse modifications of DNA, proteins and lipids [1].
Environmental carcinogens form another cause of risks to human health. By causing changes to DNA they initiate a multi-step process ultimately leading to cancer. A scheme presented in Figure 1, adapted from Singh et al. [2] visualises this multi-stage process. Fortunately, organic life has found a way to cope with these cancerous threats in order to survive. The human body possesses a solid defence mechanism to minimise carcinogenic risks. This defence mechanism is active on different levels, and in different stages of the process of carcinogenesis.

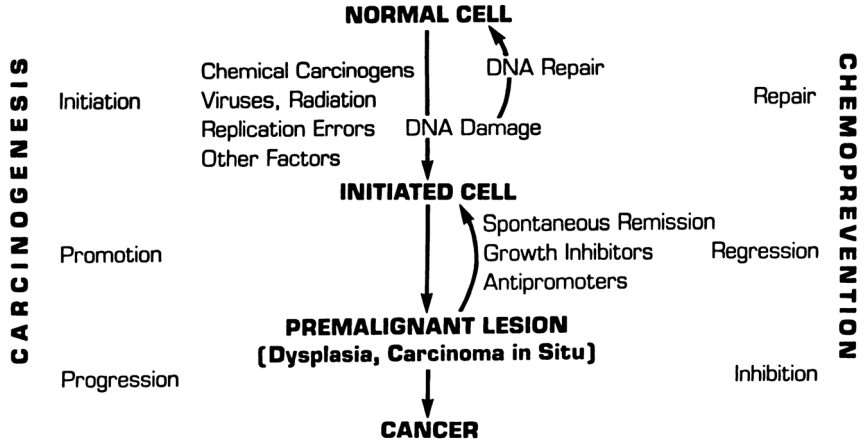

Figure 1: scheme of the multistage process of carcinogenesis. From: Singh et al. [2]

From epidemiological data we know that there is a causal relationship between diet and cancer. According to Doll diet influences as much as 30 – 70% of the cancer cases [3]. The causality depends on a variety of factors in the diet. For instance, the intake of carcinogenic contaminants of food, but also of fat, meat, or products resulting from food processing, plays an important role in the aetiology of cancer.
Food processing, in particular heating introduces new chemical structures in the food. The Maillard reaction, which is the reaction that is associated with browning of meat,

and greatly enhances flavour, is also responsible for the formation of hazardous chemical structures. During the process of cooking, several kinds carcinogens are formed e.g. acrylamide, nitrosamines, heterocyclic aromatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs). Acrylamide, a probable human carcinogen (IARC 2A) and known neurotoxicant, arises from the heat-induced reaction in cereal products, potato-based products and coffee. Nitrosamines are formed from nitrite, usually in the heating of meat (for instance heating of bacon). Heterocyclic aromatic hydrocarbons also occur from the heat treatment of meat. PAHs are formed when meat is heated over an open fire. Oxidative damage of lipids is the beginning of a chain reaction called lipid peroxidation. Lipid peroxidation consists of three stages, initiation, i.e. interaction of a radical with a polyunsaturated fatty acid, resulting in fatty acid radical, which in turn reacts with another polyunsaturated fatty acid, thus prolonging the chain reaction (peroxidation). This reaction can go continuously, most often resulting in cell death. Next to being formed in the heating of meat, nitrosamines are being formed in the human body, predominantly in the large intestine, as a reaction between nitrite and secondary amines. Since exposure to these carcinogens predominantly occurs through food, many of these have been associated in particular with gastro-intestinal cancers.

For this study on the anticarcinogenic effects of quercetin, we chose two model carcinogenic food stressors, namely PAHs and reactive oxygen. Below, modes-of-action of these two model compounds are elaborated.

Oxidative stress: Under normal conditions, the human body is constantly being exposed to free radicals, but maintains a delicate equilibrium between oxidants and anti-oxidants. The situation where this balance is disrupted is called oxidative stress and this is thought to play a role in the initiation of several degenerative diseases such as cancer [4] and coronary heart disease [5], since oxidative damage in cells can cause unfavourable modifications of DNA, proteins and lipids [1]. Anti-oxidants, like flavonoids, can avert this damage on different levels: first, by preventing the formation of radicals, second, by scavenging free radicals, and third, by endorsing their decomposition [6]. An excess of radicals can lead to disruption of the aforementioned equilibrium. One of the natural occurring reactive oxygen species is hydrogen peroxide. Hydrogen peroxide is an intermediate from the reduction of oxygen, and is formed through dismutation of the superoxide anion radical. This reaction is shown in equation 1. When hydrogen peroxide reacts with the superoxide anion radical, a hydroxyl radical is formed, via the Haber-Weiss reaction, which is shown in equation 2 below.

Equation 1: $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

Equation 2: $O_2^{\bullet-} + H_2O_2 \rightarrow OH^- + OH^{\bullet} + {}^1O_2$

The hydroxyl radical is a very powerful and reactive oxidant, which almost immediately reacts with any available biological substance, like for instance DNA [7]. This reaction

to DNA leads to single strand breaks in cells, which, after unwinding of the DNA, can be visualised and analysed in the comet assay [8-10]. Oxidative damage to DNA like strand breaks and the formation of 8-oxo-dG may lead to mutations in the DNA, that in turn may lead to cancer [11].

B(a)P exposure: Benzo(a)pyrene is a polycyclic aromatic hydrocarbon (PAH). The chemical structure of these PAHs consists of several combined benzene rings. PAHs can be found in coal tar, crude petroleum and in products of incomplete combustion for instance from cigarette smoke, stoves and incinerators, and as such are very common in our environment [7]. With regards to their carcinogenic properties, they range from non-carcinogenic (anthracene) to very potent carcinogens like benzo(a)pyrene. PAHs form a group of carcinogens that have irrefutably been involved in the induction of lung cancer in smokers [12]. Benzo(a)pyrene is not toxic itself, it needs metabolic activation before expressing its carcinogenic potency. Below, Figure 2A shows the schematic metabolism of benzo(a)pyrene into its four benzopyrene-7,8-diol-9,10-epoxides. Its carcinogenicity can be explained by the presence of the bay region; this region has strong electrophilic properties and has a preference for covalent bonding. The (+)-anti-B[a]P-7,8-diol-9,10-epoxide is the most potent ultimate carcinogen; apparently it provides the best steric conditions. Figure 2B shows opening of the bay region epoxide and covalent binding to a deoxyguanosine.

These BPDE-DNA adducts can be measured by labelling them with a radioactive phosphate (^{32}P) and separating them by means of thin layer chromatography. Reddy and Randerath [14] first described the nuclease P1 enriched ^{32}P-postlabelling method used to analyse the BPDE-DNA adducts. PAH-DNA adducts in lymphocytes have been identified as reliable biomarkers for the prediction for cancer risk in prospective case-control studies in smokers and former smokers [15, 16].

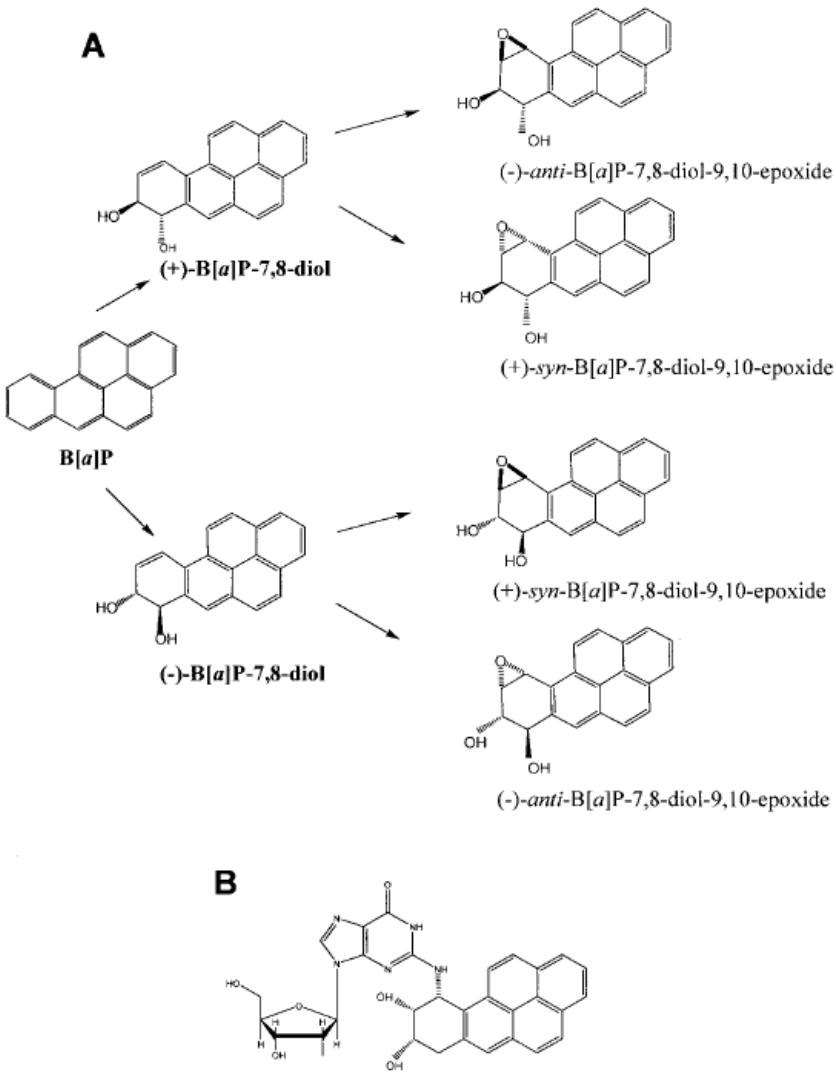

Figure 2 A: Metabolism of benzo(a)pyrene to diol epoxides. B: Opening of the bay region epoxide in a cis or trans fashion to adduct to DNA bases. From: Baird et al. [13]

Fruits and vegetables

A varied and healthy food intake is essential for human well-being. Long before science gained interest in it, intake of fruits and vegetables as well as the healing properties of certain plants has been associated with human health already in very primitive cultures. Many epidemiological studies have suggested an inverse correlation between the intake of fruits and vegetables and cancer-incidence and cancer-mortality [3, 17,

18]. Others have linked consumption of fruits and vegetables to a reduction of several other degenerative diseases like cardiovascular disease and ageing [19]. The epidemiological evidence for protection by fruit and vegetable intake seemed overwhelming, as indicated by a review by Block et al. that revealed that 128 out of 156 studies showed a significant inverse relation with fruit and vegetable consumption and relative risk for development of cancer [18]. However, recently the epidemiological evidence for this association has been weakened by some prospective studies [20-22] that lacked to find statistically significant protection by a diet rich in fruits and vegetables, and many controversies exist about the actual impact of fruit and vegetable intake on health. An elaborate meta-regression analysis by Bjelakovic et al. of 68 randomised trials of anti-oxidant intake through supplements revealed that beta-carotene, vitamin A and vitamin E significantly increased all-cause mortality [23]. Selenium and vitamin C supplementation had no significant effect on mortality, either positive or negative.

Flavonoids and other anti-oxidants like vitamins are thought to play an important role in the protection by fruits and vegetables against cancer and other degenerative diseases [24]. However, the 'magic bullet', if present, remains to be found. Despite the discovery of the so-called vitamin P by Szent-Györgyi [25] in 1937, for most of the time, flavonoids have been regarded as non-nutrients, with no special beneficial properties. Probably, the interest in flavonoids has been incited by the "French paradox" [26]. This inconsistency comprises the French diet high in saturated fatty acids and an unexpectedly low incidence of coronary heart disease in France, when compared with epidemiological data from other Western countries and corrected for risk factors [26]. This unexpected finding is supposed to be explained by the combination of a higher alcohol intake as well as a higher intake of anti-oxidants, in particular of flavonoids, both provided by the intake of predominantly red wine.

Caution is however needed when encouraging increased flavonoid intake. It is hypothesised that also adverse health effects occur from excessive flavonoid intake, since anti-oxidant effects can turn into pro-oxidant effects [27, 28]. This can be brought about by auto-oxidation of the flavonoid [27], or by its metabolism resulting in *o*-semiquinone and *o*-quinone structures. This *o*-quinone can form a quinone-methide-structure, which in turn arylate protein thiols. Excessive flavonoid intake will most likely occur by ingestion of commercially available food supplements. Manufacturers' recommended doses greatly exceed the dose that can be reached by a normal or even vegetarian diet [29].

The conflicting evidence from epidemiological studies and the need for extended knowledge about the possible beneficial role flavonoids in human health demand a benefit-risk evaluation. Given the fact that flavonoids comprise about 4000 different structures, varying in occurrence in the food chain and in chemical properties, for our studies, we have chosen quercetin, the best studied flavonoid, as a model flavonoid. Below, flavonoids in general and quercetin in particular are described in more detail.

Flavonoids

Flavonoids form a class of naturally occurring polyphenolic compounds and many of them provide attractive colours to flowers, fruit and leaves. Flavonoids can be subdivided into some of the following classes: flavanols, flavanones, flavonols, flavones, and anthocyanidins [30]. Table 1 lists these classes, their main dietary sources, and the content in various fruits and vegetables. Within a class, differences are based on the variation in number and arrangement of hydroxyl groups and on alkylation or glycosylation of these groups. The widespread variety of flavonoids and the large differences in flavonoid content of certain food items makes it difficult to estimate the daily intake of flavonoids. It was stated by Kühnau in 1976 that an average daily diet in the Western world contains approximately 1 gram of a variety of flavonoids [31]. However, according to Hollman and Katan, this number is most likely overestimated; they consider the daily intake of flavonoids to be only a few hundred milligrams, expressed as aglycons [32].

Flavonoids	Dietary source In *italic* the most important source	Content* (mg/100g edible portion)	References
Flavanol			
Epicatechin	*Green tea,* black tea, red wine	8.5	[33-39]
Catechin		2.7	
Epigallocatechin		17	
Epicatechin gallate		21	
Epigallocatechin gallate		83	
Flavanone			
Naringin	Citrus fruits, e.g. *oranges*	11	[37, 40]
Taxifolin	Peel of citrus fruits		
Flavonol			
Kaempferol	*Broccoli,* endive, leek, radish, grapefruit, black tea	6.2 20	[37, 40-43] [37, 40, 42, 44-50]
Quercetin	*Onions (red),* berries, apple skin, broccoli, cranberry, olive, tea, red wine	4.3	[40, 47, 51, 52]
Myricetin	*Cranberry,* grape, red wine		
Flavon			
Chrysin	Fruit skin		
Apigenin	*Parsley,* celery	302	[40, 42, 53]
Anthocyanidins			
Malvidin	*Red wine*, red grapes	5.7	[54-57]
Cyanidin	*Cherries*, raspberries, strawberries, grapes	111	[58]
Apigenidin	Coloured fruit and peels		

Table 1: Classes of flavonoids, examples and their dietary source (adapted from [30]and [59])

* Content of the most important source; content varies widely per season, batch and type

Quercetin

General information Quercetin (3,5,7,3',4'-pentahydroxyflavone; *Figure 3*) is a flavonoid in the class of flavonols. It is a very common flavonoid, especially present in high levels in apples, onions and blueberries; in these fruits quercetin is usually bound to a glycoside [60]. In some cases, the presence of a sugar leads to another trivial name, for instance with a rutinose-sugar as a 3-O-glycoside the compound is called rutin, whereas quercetin with a rhamnose on the same position is referred to as quercetrin. The mean flavonol intake in The Netherlands is estimated at 23 mg/day; the largest part of it being quercetin at 16 mg/day [32]. Quercetin's large contribution to the mean flavonol intake is probably the reason why it is one of the most investigated flavonoids.

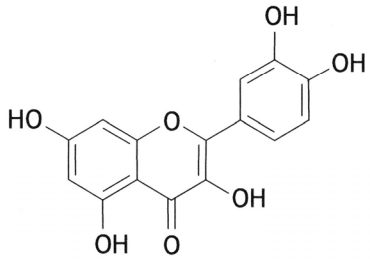

Figure 3: chemical structure of quercetin

Biological availability In order to exert beneficial effects the compound must access the human body first. For a long time there has been uncertainty about the actual biological availability of quercetin and other flavonoids. It was thought that its structure made it impossible for the compound to be absorbed intact by the human body. However, in 1995 Hollman and co-workers [61] investigated quercetin uptake by healthy ileostomy patients, and showed that a reasonable amount of quercetin was taken up, and could be recovered in ileostomy bags. They were the first to notice that absorption of quercetin was enhanced when it was conjugated to a sugar. Absorption of quercetin-glucosides from onions was 52%; whereas absorption of the aglycon (without sugar) did not exceed 24% [61]. The presence of any sugar will not suffice as such; the nature of the sugar greatly influences biological availability. A rutinose at the same chemical position (3) as in quercetin-glucosides from onions renders absorption of only 15% (as opposed to 52% from onions). When Walle et al. [62] re-investigated the absorption of quercetin glucosides in ileostomy patients they found that in the small intestine, glucosides were efficiently hydrolysed and thereupon the absorption of the aglycone may even reach 65-81%. The positioning of the sugar-group in relation to quercetin does not seem to influence the absorption. Olthof and co-workers showed

no difference in absorption between quercetin with a 3-glucose and with a 4'-glucose [63]. In plasma, quercetin is usually present in a glucuronidated form, and only a trace amount as aglycon [64], although some have reported presence of quercetin glycosides in plasma [65]. Elimination half live of quercetin is approximately 24 hours [60], therefore, repeated consumption of quercetin-rich foods will lead to an accumulation of quercetin in plasma.

Biochemical activities Quercetin, being a strong anti-oxidant, is renowned as scavenger for highly reactive species like hydroxyl radicals and peroxinitrite [66, 67] and superoxide radicals [68-70]. Consequently, it has been shown to protect against oxidative DNA damage (single strand breaks) *in vitro* in human lymphocytes [71-74] and sperm [74]. Also in cell lines Caco-2, Hep G2, and V79, quercetin is able to protect against DNA single strand breaks in a direct manner, instead of an increase of repair rate which is known from flavonoids [75]. In a human melanoma cell line (HMB-2) quercetin reduced the frequency of chromosomal aberrations induced by H_2O_2 [76]. Preventing oxidative damage is not limited to single strand breaks in DNA, quercetin is also capable of protecting against lipid peroxidation and formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [77]. Protection of ortho-phenols like quercetin against damage caused by H_2O_2 is not limited to direct scavenging of radicals. Experiments in a human neuronal cell line IMR-32 lead to the finding that ortho-phenols strongly induce glutathione S-transferase; and this induction correlated with the increased resistance against H_2O_2 measured in IMR-32 cells [78]. Furthermore, quercetin has been shown to possess anticarcinogenic properties in animal studies, which may be ascribed to either its anti-oxidative capacity or to other mechanisms of anticarcinogenicity [79]. In Hep G2 cells; incubation with up to 10 μM of quercetin inhibits B[a]P-induced DNA adducts by induction of Cyp1A1 [80]. Lautraite and co-workers [81] investigated inhibition of DNA adduct formation by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and B[a]P by several flavonoids in V79 hamster cells expressing human or rat CYP450s. In correspondence with Kang's findings, they conclude that quercetin inhibits rat CYP1A2 but not human CYP1A1. Consequently, the amount of IQ-adducts is lessened, while the quantity of B[a]P adducts remained the same [81]. Schwarz and Roots investigated *in vitro* the inhibitory effects of flavonoids on human CYP1A1 by measuring epoxidation of 7,8- dihydrodiol-benzo[a]pyrene [82]. Quercetin appeared to be a very strong inhibitor of the diolepoxide, with an IC_{50} of about 1.5 μM.

Quercetin has been linked to induction of several other genes. Valerio clearly showed an induction of gene expression and enzyme activity of human NAD(P)H: quinone oxidoreductase (NQO1) in a MCF-7 (human breast carcinoma) cell line [83]. NQO1 is a phase II detoxifying enzyme; therefore induction of this enzyme may cause protection against carcinogenic chemicals. In Caco-2 cells quercetin and chrysin induced intestinal UDP-Glucuronosyl-Transferase (UGT) another detoxifying enzyme [84].

Toxic effects In 1977, quercetin was found to be mutagenic in salmonella typhimurium (Ames test) and other short-term *in vitro* tests [85-87]. Quercetin already showed mutagenicity without microsomal activation, upon microsomal activation, its mutagenicity increased significantly [85]. Friedman found in 1984 that this

mutagenicity of quercetin could be irreversibly inactivated by for instance oxygen, oxidising enzymes and alkaline pH [88]. Like every anti-oxidant, quercetin demonstrates pro-oxidant activities as well, depending on concentration and environment. According to Metodiewa et al. this undesirable side effect takes place after metabolic oxidoreductive activation to *o*-semiquinone and *o*-quinone [28]. The *o*-semiquinone is thought to facilitate superoxide formation and GSH depletion thus leading to pro-oxidant action. The *o*-quinone is responsible for magnifying this process [28]. Kessler et al. pursue auto-oxidation of quercetin to be responsible for its pro-oxidative properties [70]. Boots et al. have concluded that, in the absence of GSH, another undesirable side effect of quercetin, is that by exerting its anti-oxidant activity toxic by-products are formed which can arylate thiol proteins [89]. Furthermore, oxidation of quercetin can result in the covalent binding of quercetin to macromolecules like protein and to a lesser extent to DNA. Walle et al. have demonstrated this phenomenon in human intestinal Caco-2 cells and Hep G2 cells [90]. Following this study, Van der Woude [91] investigated peroxidase and tyrosinase activity on the formation of covalently bound quercetin as well as its chemical stability. An elevated level of oxidative enzyme activity lead to a larger amount of covalent quercetin adducts. In NER-deficient cells, these quercetin adducts appear to be of a transient nature, possibly providing the reason for lack of carcinogenic effects of quercetin *in vivo* [91].

Inter-individual differences in quercetin-mediated effects. The toxic effects that quercetin can exert in humans depend on several circumstances, as illustrated by the possible inactivation of the mutagenicity. With respect to its anti- or pro-oxidant effect, the dose of quercetin is key, as well as its environment. Besides these variable circumstances, inter-individual differences may play a role, as for instance indicated by well-known confounding factors like gender and age but also genetic make up. NQO is an enzyme known to be involved in the metabolic activation of quercetin. As mentioned before, metabolic activation results in pro-oxidant action. There are two common genetic polymorphisms for *NQO1* gene, namely *NQO1*1* and *NQO1*2*. The variant genotype is associated with decreased enzyme activity. This implies that individuals carrying the variant genotype have less metabolic activation of quercetin, and thus would experience less pro-oxidant effects from quercetin. This concept can be expanded to a variety of genetic polymorphisms of genes involved in for instance biotransformation, DNA repair and oxidative stress. These are then expected to influence the anti-oxidative and anti-genotoxic efficacy of intervention by micronutrients. Indeed, Mooney et al [92] have shown that the association between smoking-adjusted plasma β-carotene levels and DNA damage appeared only significant in those subjects lacking the *GSTM1* detoxification gene. Moreover, Palli *et al*. concluded that *GSTM1* nulls showed strong inverse associations between DNA adduct levels and vegetable intake [93].

Other polymorphisms of relevance given quercetin's known modes-of-action as described above, relate to the *CYP* family, *MnSOD* and *OGG1*. Of further interest is that toxic effects of environmental stressors such as reactive oxygen and PAHs against

which protective effects of quercetin are directed, are also influenced by numerous genetic polymorphisms.

Quercetin has an ambiguous character; it ranges from anti-oxidant to pro-oxidant, depending on its environment and/or metabolic activation. Further, it ranges from mutagen upon metabolic activation to anti-mutagenic compound by inducing or inhibiting phase II or phase I enzymes respectively. Since most of the existing data on quercetin emerged from *in vitro* experiments and animal studies it is difficult to weigh health benefits and risks of quercetin, and interpret the consequences for the human situation. Dose dependency and inter-individual differences in response further complicate the interpretation. Therefore, targeted human research is required.

Aim and outline of the thesis

There is a lack of knowledge about the mechanism underlying suggested beneficial health effects instigated by consumption of fruits and vegetables, where flavonoids are thought to play an important role. As described earlier, damage to DNA can lead to several diseases including cancer; therefore, protection against formation of DNA damage can be regarded as an ultimate prevention of the disease. Intake of fruits and vegetables has been suggested as a way of defence against DNA damage and thus, against degenerative diseases. In order to attain insight in this phenomenon, a human model situation would be valuable. However, obtaining target (epithelial) tissue for chemical carcinogens is invasive, and difficult also because of ethical constraints. Therefore, in general, molecular epidemiology approaches apply biomarkers analysis to lymphocytes because of their availability and easily accessibility, rather that studying carcinogenic events in target tissues. Biomarkers are defined as cellular, biochemical, or molecular alterations that are measurable in biological media including surrogate tissues, and can be seen as parts of the continuum between exposure and disease. Consequently, biomarkers of effect of carcinogen exposure are supposed to represent predictors of carcinogenesis, rather than symptoms of already developed tumours. Validated biomarkers of environmental carcinogenesis applicable to lymphocytes from exposed humans, comprise DNA adducts [16, 94] and chromosomal damage like chromosome aberrations and micronuclei [95, 96]. Similarly, evaluating protection by hypothesized anti-carcinogens against DNA damage in lymphocytes as monitored by established biomarkers of effect, can serve as a model of chemoprevention against carcinogenesis. Also, exposing human lymphocytes in *in vitro* assays can mimic damage that poses a threat to DNA in daily life.
In this thesis I therefore undertake to provide insight about effectiveness of chemoprevention by quercetin, ultimately by investigating a direct association between increased flavonoid intake in healthy volunteers and protection at the biomarker level. In this approach we tried to prove that anti-oxidative and chemopreventive efficacy of quercetin known from literature and *in vitro* studies, can

also be demonstrated *in vivo* in humans. For this intervention study, increased quercetin intake was achieved by supplementing volunteers with a blueberry/apple juice. Blueberry juice was chosen for its naturally high content of quercetin. This natural supplementation was preferred over an intervention with food supplements because of various reasons. The level of quercetin in food supplements is very high, and exceeds the intake via natural foods by far, which thus may also lead to toxic effects by quercetin. Further, the natural matrix enhances biological availability in comparison with that of the quercetin aglycon as present in food supplements. Besides facilitating biological availability, the surrounding matrix can also counteract possibly adverse effects induced by excessive quercetin intake. Caution is however warranted in extrapolating *in vitro* data to beneficial health effects in humans because of the use of flavonoid aglycons in *in vitro* research; glycosides or conjugates are assumed to be less potent.
This thesis sets out to collect evidence for chemopreventive effects of quercetin and other flavonoids against DNA damage in humans. Furthermore, we hypothesised that certain individuals, carrying a genotype leading to deficient defence against inducible DNA damage and/or to a higher efficacy of quercetin treatment, might benefit relatively more from this chemoprevention. We furthermore suggest that it is this underlying inter-individual variability that masks epidemiological associations between fruit and vegetable intake and the incidence of cancer and CVD. The study model we applied for this thesis, was investigating the impact of increased quercetin intake by human volunteers on *ex vivo* induced DNA damage in their lymphocytes. In order to investigate DNA protective effects of quercetin, lymphocytes were exposed *ex vivo* to two DNA damage-inducing systems, H_2O_2 for induction of oxidative stress and B[a]P for formation of DNA adducts.

Therefore the research topics of the present thesis are:

- To validate our biomarker model and establish protection of lymphocytes against induced DNA damage by *in vitro* quercetin pre-treatment of human lymphocytes followed by *in vitro* exposure to different stressors.
- To determine whether protection by *in vitro* pre-treatment with quercetin can indeed be influenced by pivotal genetic polymorphisms, in particular of *GSTM1* and *GSTT1*.
- To investigate the chemopreventive effect of increased quercetin intake on *ex vivo* exposed lymphocytes, by supplementing healthy volunteers with a quercetin-rich fruit juice and consider the role of multiple genetic polymorphisms in this process.

The outline of this thesis is the following:
Chapter 2: Quercetin is known as a scavenger of reactive oxygen species, with a different affinity for various types of radicals. It is also capable of protection against oxidative DNA damage in lymphocytes. In the present study, protection against $OH^{\bullet}$-or $O_2^{\bullet -}$-induced oxidative DNA damage by quercetin was investigated in human

lymphocytes *in vitro* using Comet assay. Furthermore, the direct scavenging capacity of quercetin for again these two stressors was measured by ESR spectroscopy. The difference in protective capabilities with respect to the biological effect, i.e. oxidative DNA damage in lymphocytes, was compared to the difference in chemical effect, i.e. direct scavenging capacity by quercetin of $OH^{\bullet}$ or $O_2^{\bullet -}$ radicals.

Chapter 3: In this chapter, *in vitro* protective effects of quercetin are investigated in human peripheral lymphocytes pre-treated with quercetin and subsequently exposed to hydrogen peroxide and benzo(a)pyrene, in order to validate our biomarker model. When quercetin pre-treatment *in vitro* proved to be capable of reducing induced damage, it had to be tested whether quercetin *in vivo* could provide protection. Therefore, in a pilot study in human volunteers, it was investigated whether increased intake of quercetin via a blueberry-apple juice mixture resulted in a similar protection of *ex vivo* exposed lymphocytes.

Chapter 4: In order to identify sensitive groups based on their genetic predisposition, this *in vitro* study on human lymphocytes aims to establish the impact of genetic polymorphisms in *GSTM1* and *GSTT1* on H_2O_2-induced oxidative DNA damage and on the effectiveness of quercetin and ascorbic acid in preventing this induced damage in human peripheral lymphocytes. Polymorphisms in these genes which are involved in detoxification and anti-oxidative mechanisms, have been associated with increased cancer risk for several different cancers, as well as increased levels of biomarkers for precarcinogenic events like DNA adducts in smokers [92, 97].

Chapter 5: The pilot study described in Chapter 3, demonstrated the feasibility of a large-scale intervention study for evaluating anti-oxidative and anti-genotoxic properties of quercetin in humans. For this larger-sized study, 168 healthy volunteers were supplemented for four weeks with a quercetin-rich blueberry apple juice, produced specifically for this study by Riedel Drinks (Riedel, Ede, The Netherlands). This mixture contained about 97 mg of quercetin per litre, most of it bound to a glucoside or a galactoside at the 3-position, which makes it biologically well available. The very research biomarker model developed as described in Chapter 2, was applied here, which implies that before and after intervention, lymphocytes from healthy volunteers were exposed to H_2O_2 and benzo(a)pyrene, and the amount of induced damage was measured in an appropriate manner. Effects of the intervention on plasma anti-oxidant status, plasma quercetin content and plasma vitamin C content were recorded. Further, the effect on the level of oxidative or mutagenic DNA damage in respectively H_2O_2 – or B[a]P - exposed human lymphocytes, are described, as well as the impact of several relevant genetic polymorphisms on these parameters.

Chapter 6: The main findings of *in vitro* and *ex vivo* studies described in this thesis are discussed and summarised.

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Chapter 2

Discriminative protection against hydroxyl and superoxide anion radicals by quercetin in human lymphocytes *in vitro*

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In press: Toxicology in Vitro

Abstract

Antioxidants play a vital role in the cellular protection against oxidative damage. Quercetin is a well-investigated antioxidant and known to be able to protect against cellular oxidative DNA damage. In this study, we tried to relate the protection by quercetin pre-treatment against oxidative DNA damage in human lymphocytes *in vitro* to the interaction of quercetin in solution with hydroxyl and superoxide anion radicals as measured by ESR (Electron Spin Resonance) spectrometry, using DMPO as a spin trap. Further, scavenging capacity of quercetin-treated lymphocytes *in vitro* was evaluated by ESR spectrometry. Quercetin appears capable of protecting human lymphocytes against oxidative DNA damage caused by hydrogen peroxide in a dose-dependent manner. The protection of lymphocytes against superoxides is ambiguous. Incubation concentrations of quercetin (1, 10, and 50 μM) reduced levels of superoxide-induced oxidative DNA damage, while at 100 μM the amount of damage was increased. These results are supported by ESR-findings on quercetin in solution, also showing a prooxidant effect at 100 μM. ESR spectrometry showed rate constant values for the reaction kinetics of quercetin in lowering iron-dependent hydroxyl radical formation and NADH-dependent superoxide anion formation of respectively 3.2 x 10^{12} M^{-1} s^{-1} and 1.1 x 10^{4} M^{-1} s^{-1}. This shows that quercetin is a more potent inhibitor of hydroxyl radical formation than a scavenger of superoxide anions.

Introduction

The human body is incessantly exposed to endogenously and exogenously formed free radicals. Under normal conditions, nature maintains a delicate balance between antioxidants and oxidants. However, at excessive free radical production, this balance is disrupted, leading to a situation of oxidative stress. This is thought to play a role in the initiation of several degenerative diseases such as cancer, for instance by inducing oxidative damage in cells, and causing unfavourable modifications of DNA, proteins and lipids (Morrissey and O'Brien, 1998). Damage caused by free radicals can be prevented by antioxidants that act via different mechanisms: first, by preventing the formation of radicals; second, by scavenging of radicals; and third, by endorsing their decomposition (Li et al., 2001). Both the endogenous antioxidant defence mechanisms as well as antioxidants, for instance available through intake of fruits and vegetables, can restore the balance and prevent cellular damage. Flavonoids form an important group of antioxidants and high levels are found in fruits and vegetables like apples and onions, and in beverages like tea and red wine (Justesen et al., 1998). Total flavonoid intake from fruits, vegetables, and beverages is estimated to be several hundreds of milligrams per day (Hollman and Katan, 1999). Quercetin is the most prominent flavonol; on average, a daily Western-type diet contains about 16-18 mg of quercetin (Hertog et al., 1993; Theodoratou et al., 2007).

Pre-incubation with flavonoids proved to be capable of protecting against oxidative damage caused by hydrogen peroxide, in human lymphocytes *in vitro* (Duthie et al., 1997; Noroozi et al., 1998). Quercetin has furthermore been shown to possess anti-carcinogenic properties in animal studies, which may be attributable to either its anti-oxidative capacity (van Acker et al., 1996) or to other mechanisms of anti-carcinogenicity (Stavric, 1994). Quercetin is already well-known as scavenger for highly reactive species like hydroxyl radicals ($OH^{\bullet}$) and peroxynitrite (Bors et al., 1994; Heijnen et al., 2001). It has been shown earlier that in oxidant systems using iron-dependent $OH^{\bullet}$ formation, the antioxidant effect of quercetin is caused by metal chelation (Ferrali et al., 1997; Ozgova et al., 2003). In contrast, till date, studies investigating the interaction between superoxide anion radicals ($O_2^{\bullet-}$) and quercetin are scarce. $O_2^{\bullet-}$ can be easily converted into $OH^{\bullet}$, and with multiple cellular sources present, capable of facilitating this conversion like mitochondria and NADPH oxidase, excessive $O_2^{\bullet-}$ formation is related to several toxic effects, including DNA damage (Valko et al., 2004). Like every anti-oxidant, quercetin demonstrates pro-oxidant activities as well, depending on concentration and environment. According to Metodiewa et al. this undesirable side effect takes place after metabolic oxidoreductive activation to *o*-semiquinone and *o*-quinone (Metodiewa et al. 1999). The *o*-semiquinone is thought to facilitate $O_2^{\bullet-}$ formation and GSH depletion thus leading to pro-oxidant action. The *o*-quinone is responsible for magnifying this process

(Metodiewa et al. 1999). Kessler et al. pursue auto-oxidation of quercetin to be responsible for its pro-oxidative properties (Kessler et al, 2003).
This paper focuses on the protective capacity of quercetin pre-treatment against oxidative DNA damage caused by either $OH^{\bullet}$ or $O_2^{\bullet-}$ in human lymphocytes *in vitro*, as determined by comet assay. The kinetics of inhibition of formation of these oxygen radicals by quercetin were measured by ESR spectrometry, and the radical reducing capacity of quercetin-treated lymphocytes was also assessed by means of ESR spectrometry. We thus set out to relate the extent of protection on oxidative DNA damage in quercetin-treated human lymphocytes with reduction in radical signal by quercetin or quercetin-treated human lymphocytes *in vitro.*

Materials and methods

Superoxide dismutase (SOD) was obtained from Boehringer-Mannheim (Mannheim, Germany). Quercetin, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide (NADH, reduced form) and all other chemicals were purchased from Sigma (St Louis, MO, USA). DMPO (5,5-dimethyl-1-pyrolline N-oxide) and solutions of DMPO in nitrogen flushed Milli-Q water were purified by charcoal treatment. Stock concentrations of DMPO were determined spectrophotometrically (ε = 7700 M^{-1} cm^{-1}, 234 nm).
Collection and quercetin treatment of lymphocytes: From 3 non-smoking volunteers (2 female, 1 male, aged 27-36), 10 ml of venous blood were collected into a vacuum heparin tube (Terumo, Canada) for the isolation of lymphocytes. After dilution by adding an equal volume of phosphate-buffered saline (PBS, pH 7.4), heparin blood was layered over Lymphoprep™ (Axis-shield, Norway) in a greiner tube (Greiner Bio-one, Germany). Samples were centrifuged at 860 g for 20 minutes at room temperature for the separation of lymphocytes, which were then removed, washed, and taken up in PBS at a concentration of $1*10^6$ cells per ml PBS. The lymphocytes of the three different donors have been worked up and analysed separately. All incubations have been performed in duplicate. Lymphocytes were pre-treated for 30' at 37˚C with a concentration range of quercetin (0, 1, 10, 50, and 100 μM for ESR experiments and 0, 1, 5, 10 and 100 μM for Comet assay) in 0.5% DMSO. In earlier experiments we have shown that 0.5% DMSO does not alter the Comet response, therefore, the zero quercetin concentration is regarded as the negative control. After this pre-treatment, cells were washed once in PBS, and then resuspended in PBS. Subsequently, cells were exposed to oxygen radical generating systems, and measured directly (ESR) or after 60' incubation at 37˚C (Comet) as described below.
Comet assay: Quercetin-treated lymphocytes were incubated with either H_2O_2 for formation of $OH^{\bullet}$, or PMS and NADH for formation of $O_2^{\bullet-}$. Earlier *in vitro* studies using a dose range of H_2O_2 (Wilms et al., 2005) and PMS/NADH (data not published), a 60 minute incubation at 37°C using either 25 μM of H_2O_2, or a combination of 3.3 μM of PMS and 50 μM NADH, proved suitable for evaluating both increasing and decreasing

effects on oxidative DNA damage by quercetin pre-treatment. In order to verify that the oxidative DNA damage induced by the PMS/NADH system was actually mediated by $O_2^{\bullet-}$, control experiments were performed with SOD (1000 U/ml). All incubations were performed at non-cytotoxic levels as assessed by trypan blue exclusion and the 3-(4,5dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) toxicity test (Mosmann, 1983).

The alkaline Comet assay (Singh et al. 1988; Olive et al., 1990; Kiesewetter et al., 2000) and slightly modified as described earlier (Wilms et al., 2005), was used to evaluate the extent of radical-induced oxidative damage in lymphocytes. For visualisation of the oxidative damage, comets were stained with ethidium bromide (50 μl; 10 μg/ml). Per coded slide, a total of 50 cells were scored using fluorescence microscopy. For analysis of the damage the software program Comet assay III (Perceptive Instruments Ltd, United Kingdom) was used. The amount of oxidative damage was defined as mean tail moment (MTM), based on the definition by Olive et al. (Olive et al., 1990) tail moment is calculated as follows: tail moment = (tail intensity/total Comet intensity) x (distance from tail centre of distribution to head centre).

Electron spin resonance (ESR) measurements: In order to measure direct effect of quercetin on $OH^{\bullet}$ and $O_2^{\bullet-}$ formation, a concentration range of quercetin solutions (0, 1, 5, 10, 25, 50 and 100 μM in 0.5% DMSO) was co-incubated either with 500 μM $FeSO_4$ and 500 μM H_2O_2 to generate $OH^{\bullet}$, or with 3.3 μM PMS and 50 μM NADH to generate $O_2^{\bullet-}$. Experiments were performed in triplicate.

$OH^{\bullet}$ and $O_2^{\bullet-}$ formation were detected by ESR spectrometry using DMPO (100 mM) as a spin trap. ESR spectra were recorded at room temperature in glass capillaries (100 μl, Brand AG Wertheim, Germany) on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were: magnetic field: 3490 G; scan range: 60 G; modulation amplitude: 1 G; receiver gain: 1 x 10^5; microwave frequency: 9.85 GHz; power: 50 mW; time constant: 40.96 ms; scan time: 20.97 s; number of scans: 25. Spectra were quantified by peak surface measurements using the WIN-EPR spectrum manipulation program (Bruker, Germany). The observed reaction rate constants of quercetin with $OH^{\bullet}$ and $O_2^{\bullet-}$ formation were calculated as described previously (Aherne and O'Brien, 2000), using k_d values of 2.1 x 10^9 M^{-1} s^{-1} (Aherne and O'Brien, 2000) and 10 M^{-1} s^{-1} (Finkelstein et al., 1979) for the reaction of DMPO with $OH^{\bullet}$ and $O_2^{\bullet-}$ radicals respectively.

This same system was used in order to investigate (intra)cellular scavenging of oxygen radicals by quercetin pre-treated lymphocytes. In the presence of the spin trap DMPO (100 mM), quercetin pre-treated lymphocytes were co-incubated with 500 μM $FeSO_4$ and 500 μM H_2O_2 to generate $OH^{\bullet}$, or with 3.3 μM PMS and 50 μM NADH to generate $O_2^{\bullet-}$.

Statistical analysis: The Friedman test and t-test for paired samples were used to evaluate biological and chemical effects.

Results

Comet assay: Quercetin pre-treatment showed a clear dose-dependent decrease (p=0.02, Friedman test) in H_2O_2-induced oxidative DNA damage, reflected as the relative decrease of Mean Tail Moment in lymphocytes from three individuals (Figure 1). An incubation concentration of 5 μM of quercetin already significantly reduces the level of single strand breaks in lymphocytes by 50%. A dose of 10 μM of quercetin approaches the maximal level of protection possible, since there is no statistical significant difference (t-test, paired samples) between the levels of protection at 10 μM compared to 100 μM pre-treatment.

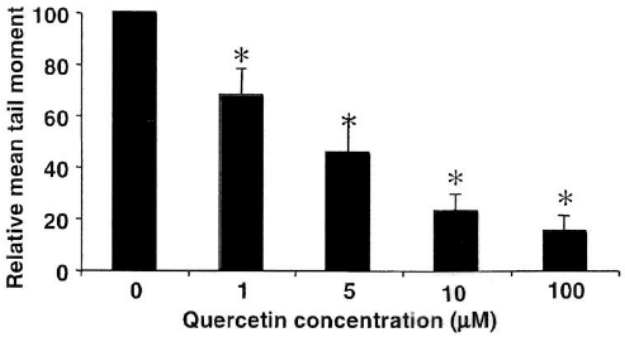

Figure 1. Dose-dependent decreases (n=3, average ratio + SEM) in H_2O_2-induced oxidative DNA damage detected by the Comet-assay in human lymphocytes after quercetin pre-incubation and related to initial level of damage. Details are described in materials and methods. Stars indicate statistical significant effects (t-test, paired samples, p<0.05)

The effect of quercetin pre-treatment on the level of oxidative DNA damage induced by superoxide anion radicals is visualised in Figure 2. The overall effect of quercetin on the level of damage induced by the PMS/NADH system is not statistically significant (p=0.1, Friedman test). In a paired samples t-test, only the lowest dose of quercetin (1 μM) leads to a statistically significant decrease, reducing the level of oxidative DNA damage by 20%. However, at 100 μM the level of oxidative DNA damage has almost doubled (p<0.05). In order to verify that the oxidative DNA damage induced by the PMS/NADH system was actually mediated by $O_2^{\bullet-}$, control experiments were performed with SOD. In the presence of this enzymatic $O_2^{\bullet-}$ scavenger, PMS/NADH-induced DNA strand break formation in lymphocytes was completely inhibited.

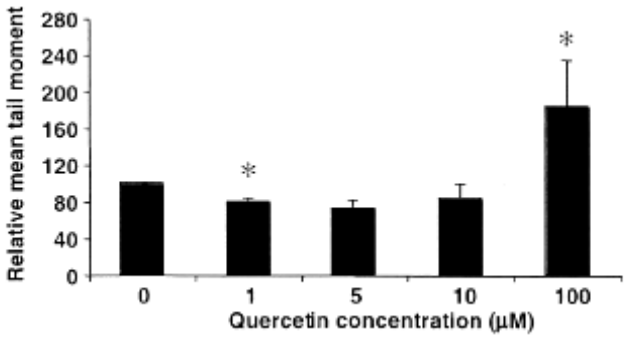

Figure 2. Dose-related changes in PMS/NADH-induced oxidative DNA damage detected by the Comet-assay in human lymphocytes by quercetin pre-incubation related to initial level of damage (n=3, average ratio + SEM). Details are described in materials and methods. Stars indicate statistical significant effects (t-test, paired samples, $p<0.05$)

Inhibition of radical formation analysed by ESR spectrometry: In order to quantify the dose-dependent lowering by quercetin of iron-dependent $OH^{\bullet}$ formation, radicals were generated in a Fe^{2+}/H_2O_2 system in the presence of 0.5% DMSO. As shown in Figure 3a, the radical spectrum consisted of a $DMPO^{\bullet}$-OH and a very small $DMPO^{\bullet}$-CH_3 signal, indicating that trapped radicals are $OH^{\bullet}$ that were not scavenged by 0.5% DMSO. Hydroxyl radical formation was lowered in a dose-dependent way, leading to a maximum inhibition of 44% at 100 μM quercetin (Figure 3b). By plotting the data according to the equation $V/v -1 = K_E[Quercetin]/K_d[DMPO]$ (Figure 3c) (Aherne and O'Brien, 2000) with V being the rate of $OH^{\bullet}$ spin trapping in the absence of quercetin, and v being the rate of $OH^{\bullet}$ formation in the presence of quercetin and using k_d values of 2.1×10^9 M^{-1} s^{-1} (Aherne and O'Brien, 2000) it was calculated that the observed rate constant of the reaction of quercetin with $OH^{\bullet}$ formation ($k_{E,\,obs}$) is 3.2×10^{12} M^{-1} s^{-1}.

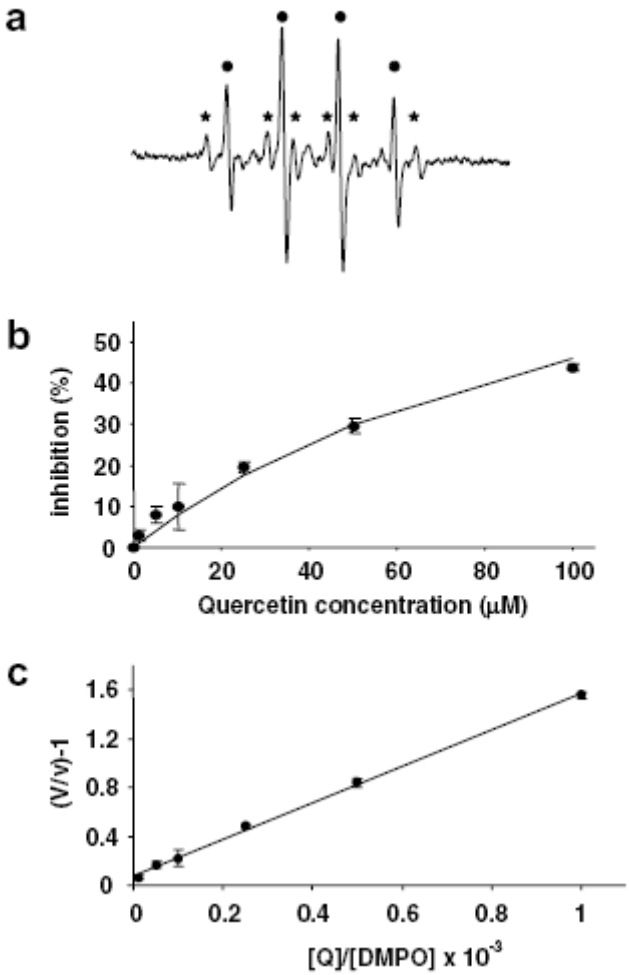

Figure 3. a) ESR spectrum in a mixture of 500 μM $FeSO_4$ and 550 μM H_2O_2 and 100 mM DMPO. Dots indicate the DMPO•-OH signal and asterisks indicate the DMPO•-CH_3 signal. b) inhibition of the DMPO•-OH signal by a dose range of quercetin. c) Plot of the data according to the equation $V/v -1 = K_E[Quercetin]/K_d[DMPO]$.

In an identical approach, the $O_2^{\bullet-}$ scavenging activity of quercetin was determined, using the non-enzymatic PMS/NADH system. A typical ESR spectrum (Figure 4a) of this system consisted of a pronounced DMPO•-OOH/DMPO•-OH signal and a small DMPO•-CH_3 signal. This DMPO•-OOH/DMPO•-OH signal arises because DMPO•-OOH decomposes into DMPO•-OH (Finkelstein et al., 1980). However, complete inhibition of the radical signal by SOD (1000 U/ml) proved that the PMS/NADH system only produced $O_2^{\bullet-}$. Utilizing this system, the quercetin concentration-dependent inhibition of the DMPO•-OOH signal was measured. Interestingly, at a concentration of 100 μM,

inhibition of $O_2^{\bullet-}$ formation was significantly ($p < 0.05$) decreased compared to concentrations of 25 or 50 μM, indicating a significant formation of oxygen radicals at this high quercetin concentration (Figure 4b). Subsequently, data points were plotted (Figure 4c) as described for OH scavenging activity of quercetin. Utilizing a k_d value of 10 M^{-1} s^{-1} (Finkelstein et al., 1979) for the reaction of DMPO with $O_2^{\bullet-}$ radicals, and excluding the values measured at a concentration of 100 μM quercetin in the calculation of the regression line because of the significantly decreased inhibition, it was calculated that the rate constant of the reaction of quercetin with $O_2^{\bullet-}$ is 1.1 x 10^4 M^{-1} s^{-1}.

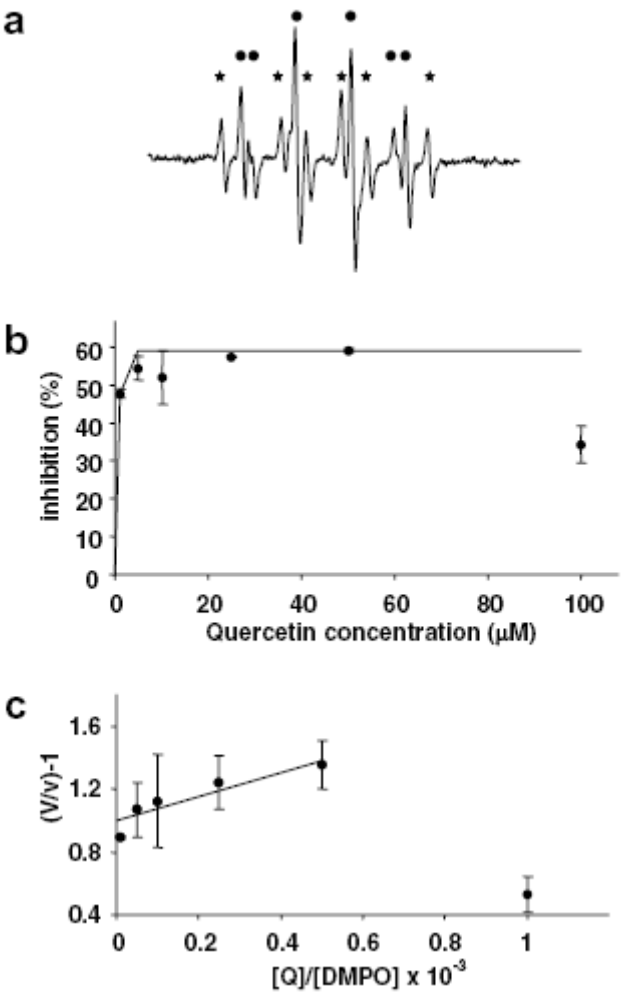

Figure 4. a) ESR spectrum in a mixture of 3.3 μM PMS and 50 μM NADH and 100 mM DMPO. Dots indicate the $DMPO^{\bullet}$-OOH signal and asterisks indicate the $DMPO^{\bullet}$-CH_3 signal. b) Inhibition of the $DMPOO^{\bullet}$-OH signal by a dose range of quercetin. c) Plot of the data according to the equation $V/v - 1 = K_E[Quercetin]/K_d[DMPO]$.

ESR measurements were also performed to detect changes in the (intra)cellular radical scavenging activity of human lymphocytes on generated hydroxyl and superoxide anion radicals in the presence of the spin trap DMPO as the result of a pre-treatment with quercetin *in vitro*. Experiments performed with lymphocytes pre-incubated with 1, 10, 50 or 100 μM quercetin showed that none of these treatments had any significant effects on the hydroxyl or superoxide radical signal intensity if compared to the signals obtained from lymphocytes not pre-treated with quercetin (figures not shown).

Discussion

Quercetin is the major flavonoid found in the Western diet and has been intensively investigated for its possible preventive properties against several degenerative diseases such as coronary heart disease, rheumatoid arthritis and cancer (Dragsted et al., 1993; Doll, 1990; Scalbert et al., 2005; Graf, et al. 2005). In the aetiology of these pathologies a common phenomenon is the increased formation of reactive oxygen species and quercetin-related health effects are partly ascribed to its antioxidant capacity. However, there are still many controversies concerning the mechanisms involved and the role that antioxidants play (Azzi al., 2004). In this study, we investigated protection by quercetin against oxygen radical ($OH^{\bullet}$ and $O_2^{\bullet -}$)-induced DNA single strand breaks as analysed by alkaline comet assay. We compared this with scavenging activity of quercetin in solution and after cellular uptake by human peripheral blood lymphocytes as detected by ESR spectrometry.

In line with earlier studies using comet assay, we confirm that quercetin appears capable of protecting human lymphocytes against oxidative damage caused by hydrogen peroxide in a dose-dependent manner (Noroozi et al., 1998; Wilms et al., 2005). In addition, we observed that the protection of lymphocytes against superoxides is ambiguous. The lowest tested incubation concentrations of quercetin (1, 10, and 50 μM) tend to reduce the level of oxidative DNA damage. At 100 μM however, the level of DNA damage is approximately doubled indicating a pro-oxidant effect of quercetin, possibly aggravated by superoxide exposure. This pro-oxidant effect was also observed in ESR measurements in the absence of cells, where the presence of PMS/NADH in combination with 100 μM quercetin results in additional production of pro-oxidative quercetin metabolites. Pro-oxidant effects of quercetin can be the consequence of conversion into *o*-semiquinone and quinodal products (Metodiewa et al., 1999) or decrease of antioxidant enzyme activity (Sahu and Gray, 1996; Boots et al., 2003). Considering the fact that quercetin in solution is a very potent inhibitor of iron-dependent hydroxyl radical formation, we conclude that the protective effects in quercetin pre-treated lymphocytes as determined by comet assay, seem to be related to direct lowering of radical formation by this antioxidant. The bioavailability of quercetin depends on its sugar moiety. Quercetin from for instance onions, has a bioavailability of 52% (Hollman et al., 1995). Recent studies with

quercetin supplementation (Hubbard et al., 2004) and infusion with a water-soluble form of quercetin (Mulholland et al., 2001) show that levels in the micromolar range up to 20 μM are detected in plasma. In plasma quercetin is mostly present in its conjugated form, which may influence antioxidant capacity or enzyme inducing abilities. Although quercetin is easily conjugated to methoxy, sulfate, and glucuronic acid groups, specific organs like lung, liver, and kidney can also deconjugate it as was found by de Boer et al. in rats (de Boer et al., 2005).

ESR spectrometry revealed rate constant values for the reaction of quercetin in lowering iron-dependent $OH^{\bullet}$ formation and NADH-dependent $O_2^{\bullet-}$ formation of respectively 3.2 x 10^{12} M^{-1} s^{-1} and 1.1 x 10^{4} M^{-1} s^{-1}. This indicates that quercetin is a very efficient inhibitor of iron-dependent $OH^{\bullet}$ formation rather than an $O_2^{\bullet-}$ scavenger. The observed reaction rate constant for H_2O_2 formation inhibition by quercetin is two orders of magnitude higher than previously found for well-established antioxidants such as ascorbate, GSH and cysteine (Aherne and O'Brien, 2000), as well as gallate esters of flavanols (Bors and Michel, 1999). The rate constant for the reaction of quercetin with superoxides which we have calculated here, is comparable with that found by Felipe and co-workers using pulse radiolysis (Filipe et al., 2002). While the lowest tested quercetin concentrations reduced the amount of superoxide radicals in our study, 100 μM of quercetin caused a significant ($p<0.05$) decrease in inhibition of superoxides indicating a pro-oxidant effect. This is in confirmation with what has been reported earlier at higher doses of quercetin (Metodiewa et al., 1999; Galati et al., 2002). An explanation of why this pro-oxidant effect at 100 μM quercetin only occurs in the superoxide system, may be the presence of NADH, which is co-oxidised by quercetin-reactive metabolites (*o*-semiquinone and quinone-methide isomere derivatives), enhancing the pro-oxidant effect (Buss et al., 2005; Galati, et al., 2002). Altogether, determining the role of quercetin in oxygen radical formation in solution showed that quercetin is a more potent inhibitor of $OH^{\bullet}$ formation than a scavenger of $O_2^{\bullet-}$.

Applying ESR spectrometry in combination with the cellular spin trap DMPO, it was observed that quercetin pre-treatment of lymphocytes did not lead to significant lowering of generated $OH^{\bullet}$ and $O_2^{\bullet-}$. Although formation of radicals by both systems sufficiently occurs in the presence of lymphocytes, apparently concentrations used for quercetin pre-treatment in this study had no added value to the natural scavenging capacity of lymphocytes.

Overall, we showed here that quercetin is a very potent inhibitor of iron-dependent $OH^{\bullet}$ formation, when compared to its $O_2^{\bullet-}$ scavenging capacity, and much stronger capable of protecting against $OH^{\bullet}$-induced DNA damage in human peripheral blood lymphocytes. Other antioxidant mechanisms like preventing the formation of radicals or endorsing their decomposition (Young and Woodside 2001,) by induction of phase II enzymes (Stavric, B. (1994) may still play an important role in the protection of lymphocytes. Our results clearly show that quercetin can protect human lymphocytes against H_2O_2-induced oxidative DNA damage. The ESR experiments revealed that this protection may well be caused by direct scavenging of hydroxyl radicals.

Acknowledgement

We would like to thank the Netherlands Organisation for Health Research and Development for financial support of this project in the context of the Nutrition: Health, Safety and Sustainability program (Grant number 01412012).

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Chapter 3

Protection by quercetin and quercetin-rich fruit juice against induced oxidative DNA damage and formation of BPDE-DNA adducts in human lymphocytes

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Abstract

Flavonoids are claimed to be able to protect against cardiovascular disease, certain forms of cancer and ageing, possibly by preventing initial DNA damage. Therefore, we investigated the protective effects of quercetin against the formation of oxidative DNA damage and formation of bulky DNA adducts in human lymphocytes both *in vitro* and *ex vivo*. First, human lymphocytes were pre-incubated with a dose range of quercetin, followed by incubation with hydrogen peroxide; protection against oxidative DNA damage was evaluated by Comet assay. Second, quercetin pre-treated human lymphocytes were challenged by benzo(a)pyrene, and BPDE-DNA adduct formation was measured by ^{32}P-postlabelling. Third, in a pilot study, lymphocytes from female volunteers who consumed a quercetin-rich blueberry apple juice mixture for four weeks, were treated *ex vivo* with an effective dose of H_2O_2 and B(a)P, respectively, at three different time points, before (t=0 weeks), during (t=2 weeks) and after (t=4 weeks) the intervention. *Results in vitro*: a significant dose-dependent protection by quercetin against both the formation of oxidative DNA damage ($p<0.01$) and of BPDE-DNA adducts ($p<0.05$) has been observed. *Results in vivo:* four weeks of juice intervention led to a significant increase in the total antioxidative capacity of plasma, as reflected by the increase of the TEAC value from 773 µM trolox equivalent at t=0 to 855 µM at t=4 ($p=0.04$) and an increase in plasma quercetin content from 5.0 nM to 10.6 nM plasma ($p=0.03$). After intervention, the level of oxidative damage upon *ex vivo* exposure to H_2O_2 was non-significantly ($p=0.07$) decreased by 41%, and *ex vivo* induced BPDE-DNA adduct level was nonsignificantly decreased by 11%. The combination of our findings *in vitro* and *ex vivo* renders evidence of the principle that quercetin is able to protect against chemically induced DNA damage in human lymphocytes, which may underlie its suggested anti-carcinogenic properties.

Introduction

Several epidemiological studies [1,2] have associated the consumption of fruits and vegetables with decreased cancer-incidence and -mortality rates. Fruits and vegetables represent the most important source of dietary anticarcinogens [3], and animal studies have consequently shown antitumorigenic effects of commonly consumed vegetables [1]. The anticarcinogenic potential of fruits and vegetables can be explained by several mechanisms of action of both nutrients and of non-nutrients [4,5]. Compounds of interest are flavonoids in general, and quercetin in particular, which are non-nutrients, present in daily food in fairly high levels, leading to a mean flavonol intake of 23 mg per day, 16 mg of which is quercetin [6]. Quercetin has been shown to possess anticarcinogenic abilities, attributable to its anti-oxidative capacity [7] or other mechanisms of anticarcinogenicity in animal studies [8]. However human data on anticarcinogenic effects of quercetin is still very limited. Analysing genotoxic events in human white blood cells can serve as a biomarker to interpret cancer risk. Godschalk et al. [9] showed in rats that BPDE-DNA adduct levels in WBC correlate with adduct levels in target tissue, upon exposure to benzo(a)pyrene (B(a)P) via various routes. Most importantly, Tang et al. [10] concluded from a prospective cohort study that PAH-DNA adducts in lymphocytes are a significant predictor of lung cancer risk in smokers. Should a flavonoid intervention be capable of reducing BPDE-DNA adduct levels upon B(a)P exposure in lymphocytes, then this might be interpreted as anticarcinogenic prevention. An indication of this protective mechanism has been provided by Kang and co-workers [11], who showed inhibition of CYP1A1 gene expression by quercetin, and a consequent reduction of B(a)P-derived adducts in B(a)P-exposed human HepG2 cells.

Oxidative damage in DNA is mostly caused by reactive oxygen species (ROS). ROS are continuously formed from both endogenous and exogenous sources, and are able to damage proteins, lipids, and DNA [12]. Even though cells possess an elaborate antioxidant system that removes these radicals, an imbalance in this system can occur and leads to oxidative stress. Oxidative stress has been proven to be involved in several degenerative diseases as cancer [13] and coronary heart disease [14]. Oxidative damage causes single strand breaks in DNA, which are measured after unwinding of the DNA, by the Comet assay. Consequently, a decrease in oxidative damage upon flavonoid intervention might be interpreted as a decrease in cancer risk. *In vitro*, quercetin has already shown its ability of protecting human lymphocyte DNA against hydrogen peroxide treatment in the Comet assay [15].

Goal of the present study is to evaluate protective effects of flavonoids in humans, using lymphocytes as a target. The biological end point is DNA damage in lymphocytes, which has been established as a biomarker for cancer risk [10].

First, we have investigated protective effects of quercetin *in vitro* in human lymphocytes by pre-treating the cells with a dose range of quercetin ranging from

physiological level to the maximal solubility (0, 1, 10, 50, and 100 μM) prior to exposure to H_2O_2, levels of oxidative DNA damage being determined using the Comet assay. Second, human lymphocytes were treated with a dose range of quercetin (0, 1, 10, 100 μM), and subsequently exposed to an effective dose of the model human carcinogen (B(a)P); formed BPDE-DNA adducts were analysed using ^{32}P-postlabelling assay. Third, since these experiments indicated that quercetin is capable of protecting human lymphocytes against both oxidative and bulky DNA damage *in vitro*, in a feasibility study, we have investigated whether a flavonoid-rich blueberry/apple juice mixture can protect against *ex vivo* induced DNA damage in lymphocytes. This blueberry-apple juice mixture has been chosen for its high quercetin content. Quercetin is thus used in this study as a model compound for the abundant variety of flavonoids. An important reason for choosing a natural supplementation is that the bio availability of flavonoids such as quercetin is strongly depending on its naturally occurring sugar moiety and the food matrix [16-18]. In this design, biological effects as differences in uptake and metabolism, and differences in susceptibility are taken into account.

Materials and methods

***In vitro* studies:** human lymphocytes of three different non-smoking donors (one male, two female, aged 35, 25, and 28) were pre-treated in duplicate (1h, 37˚C) with a dose range of quercetin (ranging from physiological to the maximal solubility: 0, 1, 10, 50 and 100 μM) and then exposed *in vitro* in duplicate to an effective dose of H_2O_2 (25 μM, 1h, 37˚C). After these treatments, lymphocytes were embedded in low melting point agarose for Comet assay (see below). Besides that, lymphocytes from three donors were pre-treated with a dose-range of quercetin (0, 1, 10, 100 μM), prior to *in vitro* exposure to an effective dose of benzo(a)pyrene (1 μM) in 0.5% DMSO, or to 0.5% DMSO as a control (18h, 37˚C). After 18 hours, cells were washed and harvested; lymphocyte pellets were stored at –20˚C until DNA isolation and ^{32}P-postlabelling (see below).

***In vivo* studies:** Eight female volunteers, aged 21 – 29 years, were recruited from personnel of the Department. All of them were considered healthy based on self-experienced health status, and all were non-smokers. Subjects did not use any medication or vitamin supplementation at the moment of the intervention (oral contraceptives were permitted). The medical ethical committee of Maastricht University and the Academic Hospital Maastricht approved of the protocol. Subjects were fully informed about the details of the study and gave their written informed consent.

Low flavonoid diet: Volunteers were given a list of foodstuffs rich in flavonoids in general, and quercetin in particular, which they had to avoid during a 5-day washout period prior to the intervention. Subjects were instructed not to consume onions, apples, tea, red wine, biological and freshly pressed fruit juices, berries (e.g.

blueberries and elderberries), broccoli, cabbage, beans, lettuce, tomato and raisins, because of their high quercetin content [19]. Volunteers were also asked to minimise their intake of spices and herbs.

Intervention: Subjects in this paired design acted as their own control. The intervention consisted of a mixture of 500 ml of a commercially available blueberry juice (Natufood, Harderwijk, The Netherlands) and 500 ml of a commercially available apple juice (Riedel, Ede, The Netherlands). Blueberry juice (34 mg quercetin/l) was mixed with apple juice (1,5 mg quercetin/l). The volunteers had to consume the mixture gradually throughout the day. Consumption of one litre of this mixture leads to an extra daily intake of about 18 mg of quercetin. Other compounds present in blueberry juice and in apple juice were not analysed. Volunteers were encouraged to stick to their normal dietary habits and lifestyles, and instructed to report their food-intake once a week. The intervention lasted for four weeks in order to reach a steady state in plasma quercetin concentration, and consequently in lymphocytes. The duration of four weeks was chosen based on literature data: Young and co-workers found no increase in plasma quercetin concentration after a juice intervention that lasted only one week [20]. Beatty and co-workers found no significant difference in level of background oxidative DNA damage after a two-week high flavonoid diet [18]. Erlund [21] proved that berries are a good source of bioavailable quercetin, and found a significant increase in plasma quercetin after four and eight weeks of supplementation with berries; consuming 100 g of berries (blackcurrants, lingonberries and bilberries) increased quercetin intake in 20 healthy subjects by 12,3 mg/day. Based on the elimination half-life of quercetin-3-glucoside of about 18.5 hours [22], steady state of quercetin in plasma should be reached well within a week. Based on these data we decided upon the longer intervention period of four weeks.

Collection of samples: Samples were collected at three different time points (t=0, t=2 and t=4 weeks), in which t=0 is the time point before the start of the quercetin intervention, directly after the 5-day washout period, t=2 is at two weeks after the beginning of the intervention, and t=4 is four weeks after the beginning. Samples were collected on each day between 9.00 and 9.30 A.M. Subjects were allowed to have breakfast before sampling but not to drink any juice. Venous blood samples were collected into vacuum heparin tubes (30 ml, venoject II) for separation of lymphocytes and into vacuum EDTA tubes (10ml, venoject II) for plasma analysis. After dilution by adding an equal volume of phosphate-buffered saline (PBS pH 7.4), heparin blood was layered over lymphoprep^{TM} (Axis-shield, Norway) in a leucosep tube (Greiner Bio-one, Germany). Centrifuging at 860 g for 20 minutes at room temperature separated lymphocytes, which were then washed and taken up in RPMI 1640 culture medium (Gibco). EDTA blood was centrifuged at 860 g for 10 minutes at room temperature for separation of plasma, and aliquots of plasma were stored at -80°C until analysis.

Analytical methods: Total plasma quercetin content was analysed by HPLC with coulometric array-detection, after enzymatic hydrolysis [23]. Antioxidative capacity of plasma was determined by means of the TEAC assay as described by Arts et al. [24].

Quiescent peripheral blood lymphocytes (PBL) were exposed *ex vivo* in duplicate to an oxidative stressor (hydrogen peroxide) or a food carcinogen (benz(a)pyrene).

For the oxidative challenge, lymphocytes at a concentration of one million cells per ml of RPMI were exposed to two non-toxic concentrations of H_2O_2 (25 or 50 µM), for one hour at 37˚C, directly after the isolation. A slightly modified Comet assay [25-27] implemented according to recent guidelines [26], was used to measure the extent of oxidative damage. After the incubation, cells were centrifuged, supernatant was removed, and cells were taken up in phosphate-buffered saline. The cell suspension was mixed with low melting point agarose (Sigma), and placed on 1.5% agarose-coated slides. After setting, the slides were placed in cool lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM tris·HCl, 250 mM NaOH, 1% sodium lauryl sarcosinate, pH 10, 1% triton X-100). After lysis, slides were placed in electrophoresis buffer for 20 minutes for unwinding of DNA, after which fragments were separated by electrophoresis for 20 minutes (25 V, 300 mA, on ice). Slides were stored at 4˚C until scoring. Comets were stained with ethidium bromide for fluorescence microscopy; per blindly scored slide, a total of 50 cells were scored. The software program Comet assay III (Perceptive Instruments Ltd, United Kingdom) was used to analyse the cells.

For the exposure to B(a)P cells were taken up in RPMI 1640 at a concentration of 2 million cells per ml culture medium. Cells were incubated for 18 hours at 37˚C with 1 µM of B(a)P in 0.5% of DMSO (dimethyl-sulfoxide). This concentration of B(a)P proved to induce sufficient adduct levels in quiescent lymphocytes for analysis by ^{32}P-postlabelling assay. Both concentrations of B(a)P and of DMSO were shown to be non-toxic to lymphocytes. Control samples were incubated with 0.5% of DMSO only. After incubation, cells were resuspended carefully and centrifuged at 860g. Supernatant was removed, and cells were washed twice, using PBS. The remaining lymphocyte pellet was frozen at –20˚C until DNA isolation. DNA was isolated from the lymphocytes by phenol extraction. A modified ^{32}P-postlabelling assay [28] was used to quantify the amount of BPDE-DNA adducts. In short, DNA was digested using micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). This was followed by an enrichment step using nuclease p1, to prevent normal nucleotides from being labelled. Polynucleotide kinase transferred ^{32}P from ATP to the adducted nucleotide. Labelled adducts were separated from each other and from normal nucleotides by thin layer chromatography. TLC sheets were placed in phosphor screens, which were read by a phosphor imager, after which adduct spots were analysed using the imager software.

Viability of lymphocytes was determined by trypan blue exclusion, and turned out to be over 95% in all treatments and all subjects.

Statistical analysis: Friedman two-way ANOVA was used to assess *in vitro* efficacy of quercetin pre-treatment in B(a)P exposure; paired samples t-test was used to assess *in vitro* efficacy of quercetin pre-treatment in H_2O_2-exposure. Efficacy of blueberry/apple juice intervention was assessed by comparing *ex vivo* induced DNA damage in lymphocytes, sampled before and after the four-week intervention period. SPSS-software (advanced statistics version 6.1.1 for Macintosh) was used for the statistical

analysis of the data. The non-parametric Wilcoxon matched-pairs signed-ranks test was used to calculate p-values.

Results

***In vitro* studies:** Figure 1a shows a dose dependent decrease in H_2O_2-induced median tail moments (MTM) in lymphocytes from a single subject which were pre-treated with a dose range of quercetin. In Figure 1b MTM are shown of lymphocytes from three subjects, which were pre-treated with 0 or 10 µM of quercetin prior to H_2O_2 incubation.

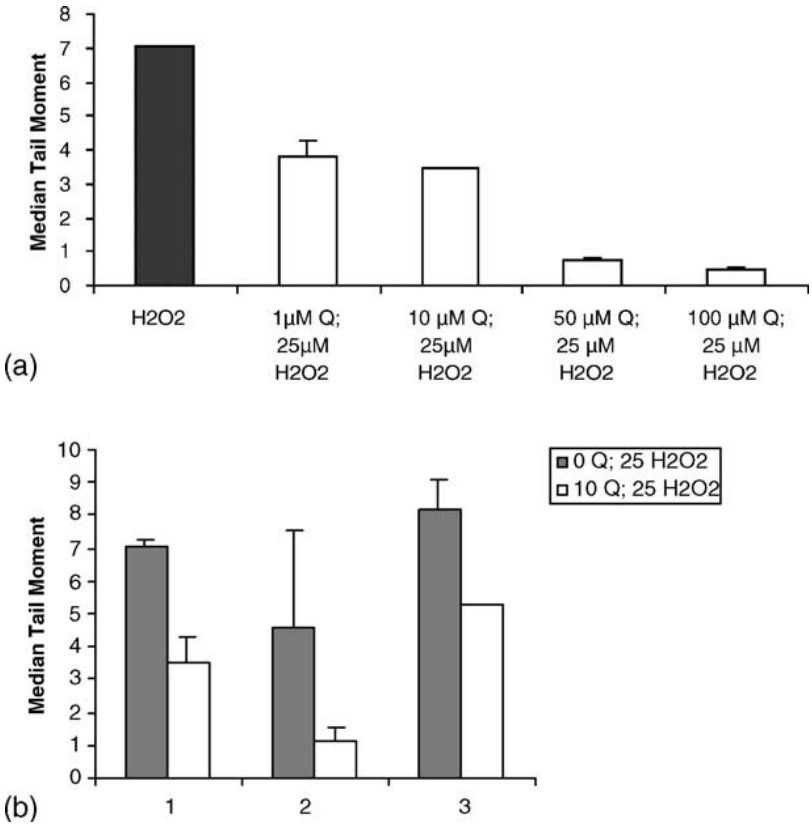

Figure 1.(a) Concentration range of quercetin pre-incubation prior to H_2O_2 incubation in human lymphocytes in one subject. (b) Level of oxidative damage in lymphocytes from three donors with and without quercetin pre-incubation prior to H_2O_2 incubation (p<0.05).

In all lymphocyte sets, quercetin pre-treatment leads to a significant (p=0.004; paired samples t-test) decrease in H_2O_2-induced oxidative damage. Figure 2 shows ^{32}P-

postlabelling results on BPDE-DNA adduct levels after the *in vitro* quercetin pre-treatment, and subsequent B(a)P exposure, in lymphocytes from one subject. A dose dependent effect is clearly observable, and significant ($p<0.05$, Friedman two-way ANOVA). In cells pre-treated with 100 µM of quercetin the BPDE-DNA adduct level has decreased below detection limit. Results on quercetin-mediated inhibition of BPDE-DNA adduct formation in lymphocytes from three different donors are shown in Table 1.

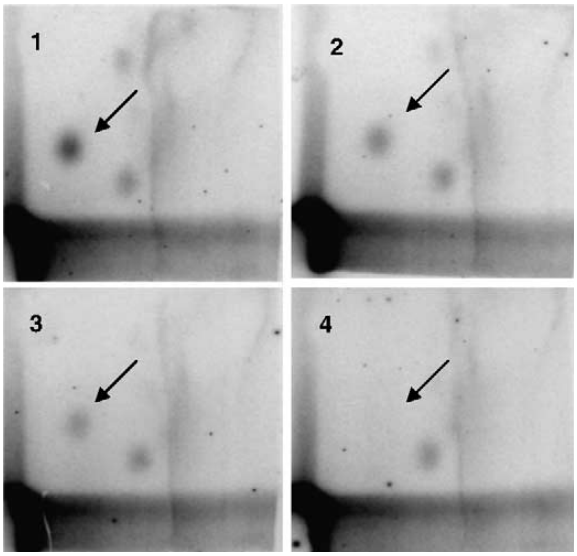

Figure 2. BPDE-DNA adduct spots (indicated by an arrow) after B(*a*)P exposure. Sheet numbers 1–4 show results of samples pre-treated with 0, 1, 10 and 100 µM of quercetin, respectively

Table 1: BPDE-DNA adduct level in lymphocytes pre-treated with quercetin, expressed as adducts per 10E8 normal nucleotides (average±sd)

	Subject 1	Subject 2	Subject 3
0 µM quercetin	8.3±4.9	4.7±1.5	2.3±0.0
1 µM quercetin	6.1±1.0	2.4±1.4	2.5±1.1
10 µM quercetin	4.0±1.1	1.3±1.8	1.0±1.5
100 µM quercetin	0	0	0
Control (no B(a)P, no quercetin)	0	0	0

***In vivo* studies:** All volunteers completed the entire intervention. Table 1 summarises the results of this feasibility study. All results are displayed as averaged group levels; individual data are not shown. Four weeks of blueberry/apple juice supplementation significantly (p= 0.03) increased plasma quercetin content (from median 5.0 nM, range 2.0-10.9 nM, at t=0 weeks, to median 10.6 nM, range 6.0-12.2 nM, at t=4 weeks). After two weeks, a non-significant increase in plasma quercetin concentration was seen (median 10.3 nM, range 8.3-21.5 nM). Plasma antioxidative capacity as measured by the TEAC assay, significantly increased by 10% (p= 0.04), from 773 (733-831) to 854 (788-915) µM trolox equivalent after 4 weeks. Two weeks of juice supplementation increased TEAC-values (non-significantly) by 3 percent. The level of *ex vivo* H_2O_2-induced oxidative DNA damage after four weeks of blueberry/apple juice intake was only 59% upon exposure to 25µM H_2O_2 (p=0.07) and 88% upon exposure to 50µM H_2O_2 (p=0.07) of the respective control levels at t=0. As for the protection against formation of DNA adducts by the food carcinogen B(a)P, the level of *ex vivo* induced BPDE-DNA adducts was nonsignificantly decreased by 11% at t=4 in comparison to t=0. BPDE-DNA adduct level in untreated lymphocytes was not detectable (<1 adduct per 10^{10} normal nucleotides), which is normal for non-smokers.

Table 2: Results of intervention study experiments with human volunteers: levels of quercetin concentration and antioxidant capacity in plasma, level of *ex vivo* induced oxidative damage and of BPDE-DNA adducts in PBL, expressed as median (range).

	t=0 weeks	t=2 weeks	t=4 weeks	P
Quercetin concentration (ng/ml plasma)	1.5 (0.6-3.3)	3.1 (2.5-6.5)	3.2 (1.8-3.7)	0.03*
TEAC (µM Trolox equivalent)	773 (733-831)	802 (752-826)	854 (788-915)	0.04*
Ratio of mean tail moment (25µM H_2O_2)	2.9 (2.1-10.6)	3.9 (1.5-4.1)	1.7 (1.4-2.1)	0.07#
Ratio of mean tail moment (50µM H_2O_2)	5.2 (4.5-17.3)	5.5 (3.2-10.7)	4.6 (3.3-5.6)	0.07#
BPDE-DNA adducts (adducts per 10^8 normal nucleotides)	12 (3.5-45.4)	8.2 (2.6-77.7)	10.7 (5.6-27.4)	0.24

P values obtained by Wilcoxon matched-pairs signed-ranks test

* Significant difference (t=4 compared to t=0), p<0.05

P value based on a group average of n=4

Discussion

In vitro pre-treatment of human lymphocytes with quercetin is very effective in preventing induced oxidative DNA damage in a dose dependent manner. Even small doses as 1 and 10 µM of quercetin already provide a large effect. This *in vitro* protection against oxidative DNA damage in human lymphocytes by quercetin pre-treatment has been described earlier [15]. Also Noroozi et al. [29] have shown in the Comet assay that a quercetin pre-treatment reduces the amount of oxidative DNA damage, in human lymphocytes, upon H_2O_2 exposure. *In vitro* pre-treatment of human lymphocytes with a dose range of quercetin prior to exposure to the food carcinogen

B(a)P also proves to be very effective in reducing bulky DNA lesions. To our knowledge, we are the first to show a quercetin pre-treatment dose-dependent reduction in the level of BPDE-DNA adducts in human lymphocytes after B(a)P exposure. De and co-workers [30] found that quercetin is capable of reducing the amount of DNA damage determined by the Comet assay *in vitro* induced by DMBA in murine lymphocytes in a dose-dependent manner; however, the Comet assay is no direct indicator for the amount of DNA adducts formed. Very importantly, these protective effects of quercetin against chemically induced DNA damage in human lymphocytes are observed at concentrations reported to be reached in plasma after a single dose of quercetin [31].

Four weeks of blueberry/apple juice intake proved to be sufficient for inducing antioxidative defence mechanisms, as is indicated by significantly increased plasma levels of quercetin and by significantly increased plasma TEAC values. Although, plasma levels did not reach the level at which quercetin exerted effects *in vitro*, protective effects were found against *ex vivo* induced oxidative DNA damage in lymphocytes. *Ex vivo* induced BPDE-DNA adduct levels showed a considerable variability, but median adduct level was lower after four weeks of intervention, than before.

In contrast to our findings, Young et al. [20] found no change in plasma quercetin concentration in a one-week study with three different doses of quercetin through blackcurrant and apple juice, after a one-week washout; the highest quercetin intake (9.6 mg/day) was obtained by consuming 1500 ml of juice. In the intervention described here, consuming one litre of the blueberry/apple juice mixture after a 5-day washout lead to an extra quercetin intake of 18 mg/day. Both the lower quercetin intake and the shorter intervention period in their study might explain the fact that Young et al. found no significant relation between plasma quercetin concentration and juice dose.

Increase of plasma quercetin level does not automatically lead to an increase in antioxidative capacity, let alone protection at the lymphocytic level.

Even though Beatty et al. [18] described a significantly increased plasma quercetin concentration after a 14-day treatment with one onion cake (150 grams, containing 89.7 mg of quercetin) a day, no significant differences in oxidative DNA damage (assayed by GC-MS) were found. Possibly, even though 14 days of treatment was sufficient to increase the plasma concentration of quercetin, it was too short a period to induce any biological effects on a cellular level (i.e. protection against oxidative damage in lymphocytes). Since in this study, lymphocytes were not *ex vivo* exposed to an oxidative stressor, the level of spontaneously generated oxidative DNA damage might have been too low to find protective effects, in contrast to our study in which we applied *ex vivo* challenging using H_2O_2.

Underlying mechanisms of chemoprevention may be several: not only does quercetin possess good radical-scavenging abilities, but it might also be capable of inhibiting or inducting phase I and phase II enzymes, respectively. Valerio and co-workers showed that quercetin is able to increase NAD(P)H: quinone oxidoreductase (NQO1) protein

levels in a human (breast carcinoma) MCF-7 cell line [32]. NQO1 is one of the major phase II detoxification systems. Lautraite and co-workers [33] showed in a Chinese hamster cell line (V79) that flavonoids are capable of reducing the amount of BPDE-DNA adducts induced by an *in vitro* challenge of the cells. However, they concluded that chrysin and apigenin were capable of reducing BPDE-DNA adducts by inhibition of CYP1A1, but quercetin was not, due to its specific inhibition of CYP1A2 only. In human HepG2 cells, however, Kang and co-workers [11] showed inhibition of CYP1A1 gene expression by quercetin, and a consequent reduction of B(a)P-derived adducts. Possibly, the protective effect observed in this study, is related to inhibition of gene expression, rather than to inhibition of the enzyme activity itself. Another mechanism for DNA adduct reduction by for instance quercetin is inactivation of benzo(a)pyrene-derived reactive metabolites, as suggested by Shah and Bhattacharya [34].

Protective effects on the level of BPDE-DNA adduct formation in our intervention study are not significant; however, a downward trend is present. *In vitro* results, showing a clear relationship between the dose of quercetin and the level of BPDE-DNA adducts, however, substantiate that quercetin is indeed capable of protection against adduct formation, at doses which can be reached *in vivo*.

Accumulation of quercetin in blood upon supplementation of flavonoid rich foods has been shown before [35], and is consistent with the findings in this study. However, it has to be borne in mind that blueberry juice also contains other flavonoids such as anthocyanins and catechins, which also possess antioxidant capacity, and these components may also attribute to the anticarcinogenic properties as observed.

Due to the relatively small size of this pilot study, not all effects were significant and clear. A power-calculation (power: 0.9, CI: 95%) based on the results of this pilot study, reveals that statistically significant effects on *ex vivo* induced DNA damage can be obtained with this blueberry/apple juice in a sample size of approximately 12 to 21 volunteers for oxidative damage and BPDE-DNA adducts respectively. We will therefore conduct a larger study for assessing protective effects of quercetin upon an *ex vivo* exposure to carcinogenic/oxidative compounds. In this upcoming study, we will take genetic predisposition of interindividual differences into account.

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Chapter 4

GSTM1 and *GSTT1* polymorphism influences protection against induced oxidative DNA damage by quercetin and ascorbic acid in human lymphocytes *in vitro*

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Food Chem Toxicol (2007)

Abstract

Antioxidants are of major importance in the protection against cellular oxidative damage caused by endogenous as well as exogenous free radicals. This study aims to establish the impact of genetic polymorphisms in *GSTM1* and *GSTT1*, which encode for enzymatic antioxidative defence, on H_2O_2-induced oxidative DNA damage and on the effectiveness of quercetin and ascorbic acid in preventing this induced damage in human lymphocytes. Lymphocytes from 12 healthy volunteers were pre-incubated either with 10 μM of quercetin or with 10 μM of ascorbic acid, and exposed to 25 μM H_2O_2 for 1h. The induction of oxidative DNA damage was quantified using the Comet assay. Genotyping of these 12 subjects showed that six individuals were *GSTM1+* and six were *GSTM1-*; eight were *GSTT1+* and four *GSTT1-*. *Results:* Baseline levels of oxidative DNA damage did not differ between *GSTM1* or *GSTT1* variants and their respective wild types. Also with respect to *ex vivo* induced levels of oxidative DNA damage, no significant difference was seen between variants and wild types of both genotypes. The protection against H_2O_2-induced oxidative DNA damage by quercetin was significantly higher in *GSTT1* wild types than in *GSTT1* variants (57% and 9% decrease, respectively; p=0.01); furthermore, *GSTT1* wild types were protected against induced oxidative DNA damage by ascorbic acid pre-incubation while *GSTT1* variants showed an increase of damage (16% decrease versus 91% increase; p=0.01). For *GSTM1* variants and wild types, observed differences in protective effects of quercetin or ascorbic acid were not statistically significant. Overall, quercetin proves to be better in protecting human lymphocytes *in vitro* against oxidative DNA damage upon H_2O_2 challenge than ascorbic acid.

Introduction

The human body is continuously being exposed to endogenous and exogenous free radicals. Excessive production of these very reactive compounds is thought to play a role in the initiation of several degenerative diseases and ageing, by inducing cellular oxidative damage, and causing adverse modifications of DNA, proteins and lipids (Morrissey and O'Brien, 1998). Antioxidants may prevent damage caused by free radicals by preventing the formation of radicals, by scavenging radicals or by endorsing their decomposition (Young and Woodside, 2001). Endogenous antioxidant defence mechanisms, as well as antioxidants present in fruits and vegetables, can prevent cellular damage caused by free radicals.

Pre-incubation with flavonoids or ascorbic acid proved to be capable of protecting against oxidative damage in human lymphocytes *in vitro* (Duthie et al., 1997, Noroozi et al., 1998). Quercetin has furthermore been shown to possess anti-carcinogenic properties in animal studies, which may be attributable to either its anti-oxidative capacity (van Acker et al., 1996) or to other mechanisms of anti-carcinogenicity (Stavric, 1994).

Human glutathione S-transferases (GST) are mainly known as phase II enzymes involved in the detoxification of endogenous and exogenous electrophilic compounds, but due to their glutathione-dependent peroxidase activity they also play an important role in protection against oxidative stress (Sram, 1998, Hayes and Strange, 1995). Four GST gene families have been identified: alpha, mu, theta and pi, and polymorphisms in these gene families which are involved in detoxification and antioxidative mechanisms, have been associated with increased cancer risk for several different cancers, as well as increased levels of biomarkers for precarcinogenic events (Strange and Fryer, 1999, Dusinska et al., 2001, Scarpato et al., 1997). Mooney et al. found an inverse association between DNA adducts and β-carotene levels in smokers lacking the *GSTM1* gene indicating that vitamin status may be critical in individuals who cannot detoxify PAHs via the GSTM1-pathway (Mooney et al., 1997). The impact of GST polymorphism combined with the effect of anti-oxidant supplementation on DNA adducts has been previously investigated in several human intervention studies (Palli et al., 2000, Pool-Zobel et al., 1998, Weiserbs et al., 2003). We now hypothesize that individuals carrying the unfavourable polymorphisms in genes encoding for antioxidative defence, are more susceptible to the induction of oxidative DNA-damage, and consequently, will benefit more from protection by dietary anti-oxidants. Hence this study aims to establish the impact of genetic polymorphisms of the GST-superfamily (GSTM1 and GSTT1) on the level of H_2O_2-induced oxidative DNA damage and on the level of protection by model antioxidants, namely quercetin and ascorbic acid, against this induced oxidative DNA damage in an *in vitro* model in human lymphocytes.

Materials and methods

Collection of samples: From each of a total of 12 non-smoking volunteers (five female, seven male, aged 23-45), 10 ml of venous blood was collected into a vacuum heparin tube (venoject II, Terumo, Canada) for the isolation of lymphocytes. After dilution by adding an equal volume of phosphate-buffered saline (PBS, pH 7.4), heparin blood was layered over lymphoprep^{TM} (Axis-shield, Norway) in a greiner tube (Greiner Bio-one, Germany). Samples were centrifuged at 860 g for 20 minutes at room temperature for the separation of lymphocytes, which were then removed, washed and taken up in PBS at a concentration of one million cells per ml of buffer.

Methods: Immediately upon isolation, lymphocytes were pre-incubated either with 10 µM of quercetin (in 0.5% THF) or 10 µM of ascorbic acid, or with their respective controls (0.5% THF or PBS) for 30 minutes at 37˚C, at a concentration of one million cells per ml PBS (pH 7.4). THF was preferred over DMSO in order to prevent false-negative results, since the latter is a well-known hydroxyl radical scavenger (Klein et al., 1980). In order to compare antioxidative effectiveness, we used equimolar concentrations of ascorbic acid and quercetin. In a pilot study, it was established that the combination of this thirty-minute pre-incubation and this particular dose of 10 µM of quercetin (Wilms et al., 2005) or vitamin C is sufficient to protect human lymphocytes against induced oxidative DNA damage. After 30 minutes, pre-incubated cells were washed and resuspended in PBS. Incubation with 25µM of H_2O_2 for 60 minutes at 37°C proved suitable for evaluation of both increasing and decreasing effects of pre-treatments. A dose range of quercetin (1, 10, 50 and 100 µM) pre-treatment was tested at this fixed dose of H_2O_2 (Wilms et al., 2005). As far as ascorbic acid is concerned, several authors have covered this topic (Anderson and Phillips, 1999, Noroozi et al., 1998, Fabiani et al., 2001). Viability of lymphocytes was determined by trypan blue exclusion, and turned out to be over 95% after all treatments (H_2O_2, quercetin and ascorbic acid) and in all subjects, implying that all incubations were performed at non-cytotoxic levels.

The Comet assay (Singh et al., 1988, Tice et al., 2000, Olive et al., 1990) implemented according to recent guidelines (Tice et al., 2000) and slightly modified (Wilms et al., 2005), was used to evaluate the extent of oxidative damage. All incubations were carried out in duplicate. Per coded slide, a total of 50 cells were scored using fluorescence microscopy. For analysis of the damage the software program Comet assay III (Perceptive Instruments Ltd, United Kingdom) was used. The amount of oxidative damage is defined as median tail moment (MTM). Based on the definition by Olive et al. (Olive et al., 1990) tail moment is calculated as follows: tail moment = (tail intensity/total comet intensity) x (distance from tail centre of distribution to head centre).

Leftover lymphocyte pellets were stored at -20˚C until DNA isolation for genotyping. DNA was isolated from the lymphocytes by phenol extraction.

Genotyping: All subjects were genotyped for *Glutathione-S-Transferase (GST) M1* and *GSTT1* genetic polymorphisms. Both polymorphisms of *GSTM1* and *GSTT1* consist of

genomic deletions. Genotyping is performed as described by Zhong et al. for *GSTM1* (Zhong et al., 1993) and Wiencke et al. for *GSTT1* (Wiencke et al., 1995). In short, the technique consists of PCR amplification of genomic DNA. Exons 4 and 5 of the *GSTM1* gene, and the 3' non-coding region of the *GSTT1* gene are amplified. The absence of an amplification product combined with the presence of a positive control band indicates the null (variant) type for both polymorphisms. This PCR method is not suited for distinguishing between homozygous (+/+, wild type) and heterozygous (+/-); therefore, these two groups were combined and compared with the variant group (-/-).

Statistical analysis: The Mann-Whitney U-Test was used to evaluate differences between levels of oxidative DNA damage in *GSTT1* and *GSTM1* variants and wild types. A power-calculation (power 0.80; CI 95%) based on earlier *in vitro* studies (Wilms et al., 2005) indicates that in a paired design a group size of n=4 will suffice for establishing protective effects by quercetin, as influenced by *GSTT1* polymorphism, assuming that the polymorphism accounts for 50% of the protective effects.

Results

Of 12 subjects, six were *GSTM1* wild type (homozygous and heterozygous) and six were *GSTM1* variant (-/-); this corresponds with the standard distribution in a Caucasian population (48,2%) (Wormhoudt et al., 1999). Of these same 12 subjects, eight were *GSTT1* positive (+/+ or +/-), and four were *GSTT1* -/-; standard percentage in a Caucasian population is 10% for *GSTT1* null (Wormhoudt et al., 1999). Five out of twelve subjects were *GSTT1*+/*GSTM1*+; three out of 12 were variant for both genotypes.

Figure 1 shows a box plot of median (n=12) levels of H_2O_2-induced oxidative DNA damage in human lymphocytes with and without quercetin or ascorbic acid pre-incubation. The level of oxidative DNA damage caused by H_2O_2 incubation is significantly reduced by quercetin pre-incubation (median tail moment 8.6 (H_2O_2) vs. 5.8 (quercetin and H_2O_2) (n=12, $p<0.01$)). Pre-treatment with ascorbic acid does not change the level of H_2O_2 -induced oxidative DNA damage.

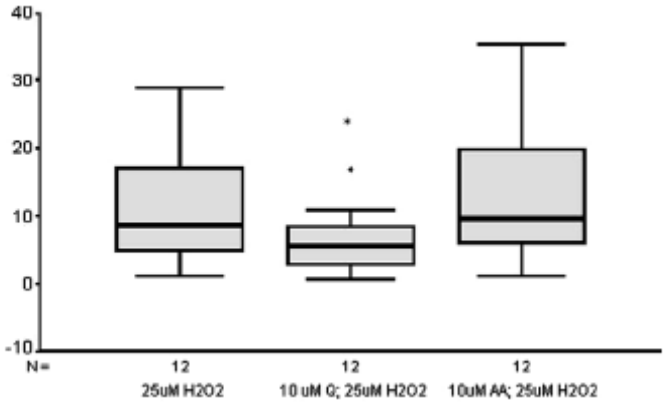

Figure 1: Median ([range], n=12) levels of oxidative DNA damage in Tail Moment with and without quercetin or ascorbic acid pre-treatment, respectively. ♦ Denotes outlying value, and * Indicates significant decrease ($p<0.05$)

Table 1 summarises levels of oxidative damage upon different co-incubations (median [range]) in *GSTM1* and *GSTT1* subpopulations. No differences were observed in baseline or H_2O_2-induced levels of DNA damage between *GSTM1* variants and *GSTM1* wild types. Also there appeared to be no difference in baseline and H_2O_2-induced damage levels between *GSTT1* variants and *GSTT1* wild types. Both quercetin and ascorbic acid slightly increased baseline levels of oxidative DNA damage in lymphocytes, an effect that was stronger for ascorbic acid than for quercetin. In both *GSTM1* variants and *GSTT1* variants, the increasing effects of quercetin and ascorbic acid on baseline levels of oxidative levels are more pronounced than in their wild type counterparts. However, none of these effects reached statistical significance. The last two columns of Table 1 are concerning the effects of anti-oxidant pre-treatment. In order to overcome the interindividual differences and describe the data to the fullest, we calculated the relative effect of the anti-oxidant pre-treatment per subject. This ratio comprised the relative increase or decrease in level of oxidative damage by dividing the amount of damage upon anti-oxidant pre-treatment and subsequent oxidative challenge by the level of damage induced by H_2O_2. These ratios can be found in Table 2.

Table 1: Level of oxidative damage, reflected as Tail Moment (median [range]) for the four different polymorphic subgroups.

	Control	H_2O_2	Quercetin	Ascorbic acid	Q* and H_2O_2	AA* and H_2O_2
GSTM1+ (n=6)	1.4	8.0	2.4	3.8	6.3	8.8
	[0.2;6.5]	[1.2;28.8]	[0.3;4.5]	[0.4;11.6]	[0.7;10.8]	[1.1;21.7]
GSTM1- (n=6)	2.2	11.2	2.7	4.5	5.6	14.1
	[0.7;3.8]	[4.6;21.6]	[1.3;14.2]	[2.1;25.4]	[1.1;16.9]	[3.8;35.3]
GSTT1+ (n=8)	1.9	10.1	2.7	3.9	5.4	9.1
	[0.2;6.5]	[1.2;28.8]	[0.3;4.5]	[0.4;11.6]	[0.7;10.8]	[1.1;22.3]
GSTT1- (n=4)	1.8	7.6	2.1	4.5	7.0	13.6
	[0.7;3.7]	[7.0;19.8]	[1.5;14.2]	[4.1;25.4]	[4.6;16.9]	[8.6;35.3]
n=12	1.8	8.6	2.4	4.1	5.6	9.5
	[0.2;6.5]	[1.2;28.8]	[0.3;14.2]	[0.4;25.4]	[0.7;16.9]	[1.1;35.3]

* Q= quercetin; AA = ascorbic acid

Table 2 reflects the change in H_2O_2-induced oxidative DNA damage caused by antioxidant pre-treatment, for *GSTM1* and *GSTT1* variants and wild types. In *GSTT1* wild types, quercetin pre-incubation prior to H_2O_2 incubation results in a level of damage of only 43% of the level of damage without antioxidant present, thus a decrease in damage of 57%. This is significantly different (p=0.01) from the effect of quercetin in *GSTT1* variants, in which quercetin causes a decrease of only 9%. Ascorbic acid pre-treatment causes a relatively minor decrease in damage of 16% in *GSTT1* wild types, but leads to an increase of 91% in *GSTT1* variants (p=0.01). There is no difference in effect of quercetin pre-treatment between *GSTM1* wild types and variants (40% decrease vs. 42% decrease), while a non-significant difference caused by ascorbic acid pre-treatment (10% decrease vs. 49% increase) was observed.

Table 2: Mean relative increase or decrease in level of oxidative damage calculated as the ratio between with and without pre-incubation, in subgroups classified for GSTT1, GSTM1, or the combination of these two.

	n	Quercetin		Ascorbic acid	
GSTT1+	n=8	0.43	*	0.84	*
GSTT1-	n=4	0.91		1.91	
GSTM1+	n=6	0.60		0.90	
GSTM1-	n=6	0.58		1.49	
GSTT1+ GSTM1+	n=5	0.47	*	0.82	*
GSTT1- GSTM1-	n=3	0.79		2.11	
GSTT1+ GSTM1-	n=3	0.36	*	0.86	*
GSTT1- GSTM1+	n=1	1.26		1.32	

* Statistically significant difference, p<0.05

Table 2 also shows the effect of pre-treatment with quercetin and ascorbic acid, when the combined impact of both polymorphisms is taken into account. Lymphocytes from *GSTT1*-/- *GSTM1*-/- subjects show a smaller decrease of induced oxidative DNA damage by quercetin pre-treatment than those from *GSTT1*+ *GSTM1*-/- (21% vs. 64%; p=0.05). Pre-incubation with ascorbic acid even leads to an increase of damage in *GSTT1*-/- *GSTM1*-/- lymphocytes; whereas *GSTT1*+ *GSTM1*-/- still benefit from some protection (111% increase vs. 14% decrease; p=0.05). Double wild types (*GSTT1*+ *GSTM1*+) are better protected by quercetin pre-treatment than double variants (*GSTT1*-/- *GSTM1*-/-), namely 53% vs 21% ($p<0.05$). Double wild types seem to be protected against oxidative DNA damage by ascorbic acid; as mentioned earlier double variants show an increase of damage upon ascorbic acid pre-treatment (18% decrease, 111% increase, respectively; $p<0.05$). Since only one out of twelve subjects is *GSTT1*-*GSTM1*+ (26% increase by quercetin pre-treatment, 32% by ascorbic acid pre-treatment), no further comparisons have been made.

Discussion

Antioxidants like quercetin and ascorbic acid have the ability of protecting human lymphocytes against oxidative DNA damage. This efficacy of flavonoids and vitamin C *in vitro* has been described earlier. In the present study, we investigated the protecting effects of equimolar pre-treatment with quercetin and ascorbic acid prior to H_2O_2 incubation in human lymphocytes *in vitro*. Also, we studied the influence of glutathione S-transferase polymorphisms theta and mu. We hypothesised that individuals at risk of oxidative DNA damage because of an unfavourable genetic predisposition benefit more from anti-oxidant treatment.

In our study, we found no significant differences in background levels of oxidative damage in lymphocytes originating from subjects with *GSTM1*+ and null genotypes or from *GSTT1*+ and null genotypes. This corresponds with what Dusinska and co-workers (Dusinska et al., 2001) concluded upon testing effects of H_2O_2 challenge *ex vivo* in lymphocytes from 155 men in relation with polymorphisms of three different GSTs, namely *GSTM*, *GSTT* and *GSTP*. In their study, background levels of oxidative damage did not differ according to genetic polymorphisms of the three classes. In the comet assay, these authors found no difference in H_2O_2 sensitivity as percentage of DNA in tail between *GSTM1*+ and *GSTM1* null, but the *GSTT1* wild types (*GSTT1*+) had a significantly higher percentage of DNA in the tail as compared to the GSTT1 null genotypes. Onaran and co-workers found no differences in susceptibility to oxidative stress as an effect of *GSTM1* null, in comparison with *GSTM1*+ in human lymphocytes exposed to CumOOH (Onaran et al., 2001). We found no statistical significant differences between *GSTM1* and *GSTT1* wild types and variants in induced level of oxidative damage upon H_2O_2 treatment.

Quercetin pre-treatment provided substantial protection against oxidative DNA damage in human lymphocytes. Effects of ascorbic acid pre-treatment were

contradictory; protection against oxidative DNA damage by this compound was far less then protection by quercetin, and in some subjects, ascorbic acid pre-treatment led to an increase of oxidative damage upon H_2O_2 incubation. Noroozi and co-workers found that the protective effect of vitamin C was far less than that of flavonoids at equimolar concentrations as measured by single strand breaks in the Comet assay, upon H_2O_2 treatment (Noroozi et al., 1998). Anderson and co-workers have described a dose-dependent effect of vitamin C in cultured human lymphocytes. Low doses of ascorbic acid tended to have a protective effect, whereas higher (>200μM vitamin C) doses exacerbate oxidative DNA damage (Anderson and Phillips, 1999). In the present study, we found exacerbating effects of ascorbic acid in some individuals, whereas in other subjects, ascorbic acid demonstrated a protective role. Considering the antioxidative capacity as reflected by the TEAC value (1.05 for ascorbic acid and 3.0 for quercetin, respectively (Huang et al., 2005)), better protection against oxidative DNA damage can be expected from quercetin. Another underlying explanation for difference in effect may be differences in the bioavailability of respective antioxidants. A lipophilic compound such as quercetin is capable of diffusing across the cell membrane in particular. These contradictive effects of ascorbic acid could not be related to *GSTT1* or *GSTM1* polymorphism, as can be seen in Table 1.

A possible explanation for the observed lack of preventive effects in cross sectional studies on antioxidant supplementation (Heart Protection Study Collaborative Group, 2002) might be that anti-oxidant treatment is only protective in some subgroups, and not in others. Genetic polymorphisms might be just the key to set different subgroups, since genetic polymorphisms are known to play an important role in for instance cancer predisposition. The *GSTM1* null and *GSTT1* null genotype have, non-conclusively, been associated with an increased risk for several cancers (Strange and Fryer, 1999, Mohr et al., 2003), and impaired anti-oxidant defence has been suggested as one of the possible explanatory mechanisms.

Our data indicate that a GST mutation does not imply that an individual is less protected against oxidative DNA damage; this is in agreement with Dusinska et al. who showed that GST activity does not differ between *GSTM1* variants and wild types, or between *GSTT1* variants and wild types (Dusinska et al., 2001). Dusinska et al. also found that vitamin C status was significantly higher in *GSTM1* nulls than in *GSTM1+* subjects, whereas it was significantly lower in *GSTT1* nulls than in *GSTT1+* individuals (Dusinska et al., 2001).

We hypothesised subjects having an unfavourable polymorphism would be more susceptible to oxidative damage and possibly benefit more from antioxidant treatment. However, in this study, we showed that specifically *GSTT1+* indeed benefit from antioxidant treatment. Quercetin pre-incubation protects lymphocytes from *GSTT1* wild types far better than *GSTT1* nulls. A possible explanation for this benefit for *GSTT1* wild types could be the induction of the enzyme by quercetin. Quercetin has previously been shown as an inducer for other phase II enzymes as UGT and NQO1 (Galijatovic et al., 2000, Valerio et al., 2001). Since GSTs play an important role in oxidative stress by detoxifying electrophilic compounds an induction of GST decreases

the amount of oxidative damage. After ascorbic acid pre-treatment, *GSTT1* wild types are protected, whereas in *GSTT1* nulls pre-treatment leads to an increase of damage, which could possibly be explained by additive effects of ascorbic acid and hydrogen peroxide. In *GSTT1*+, expected additive effects are apparently replaced by an antagonistic mode of action in which the antioxidant does not induce damage and on top of that, decreases the amount of damage caused by H_2O_2. With respect to quercetin pre-treatment, *GSTM1* + and – do not differ, however, after ascorbic acid pre-treatment, *GSTM1+* show a slight decrease in damage (10%), whereas the level of damage in *GSTM1* -/- is increased by 50%.

One has to bear in mind that in plasma quercetin is mostly present in its conjugated form, which might influence antioxidant capacity or enzyme inducing abilities. Although quercetin is easily conjugated to methoxy, sulfate, and glucuronic acid groups, specific organs like lung, liver, and kidney can also deconjugate it as was found by De Boer et al. (de Boer et al., 2005) in rats.

Implications: Genetic polymorphisms allow us to identify susceptible groups for certain kinds of damage. Along the same line, once susceptible groups have been identified, one may find that some genetic polymorphisms are associated with increased protection against genotoxic damage, for instance by anti-oxidant treatment.

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Chapter 5

Impact of multiple genetic polymorphisms on effects of a four-week blueberry juice intervention on *ex vivo* induced lymphocyte DNA damage in human volunteers

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Abstract

Consumption of fruits and vegetables has been associated with a decrease in cancer incidence and cardiovascular disease, presumably caused by antioxidants. We designed a human intervention study to assess antioxidative and possible anti-genotoxic properties of fruit-borne antioxidants. We hypothesized that individuals bearing genetic polymorphisms for genes related to quercetin metabolism, B[a]P metabolism, oxidative stress, and DNA repair, differ in their response to DNA protective effects of increased antioxidant intake. In the present study, 168 healthy volunteers consumed a blueberry/apple juice that provided 97 mg quercetin and 16 mg ascorbic acid a day. After a four-week intervention period, plasma concentrations of quercetin and ascorbic acid, and total plasma antioxidant capacity (TEAC) were significantly increased. Further, we found 20% protection ($p<0.01$) against *ex vivo* H_2O_2-provoked oxidative DNA damage, measured by comet assay. However, the level of *ex vivo* induced BPDE-DNA adducts was 28% increased upon intervention ($P<0.01$). Statistical analysis of 34 biologically relevant genetic polymorphisms revealed that 6 significantly influenced the outcome of the intervention. Lymphocytes from individuals bearing variant genotype for *Cyp1B1*5* seemed to benefit more than wildtypes from DNA-damage protecting effects upon intervention. Variants for *COMT* tended to benefit less, or even experienced detrimental effects from intervention. With respect to *GSTT1* the effect is ambiguous: variants respond better in terms of intervention-related increase in TEAC, but wildtypes benefit more from its protecting effects against oxidative DNA damage. We conclude that genotyping for relevant polymorphisms enables selecting subgroups among the general population that benefit more of DNA damage-modulating effects of micronutrients.

Introduction

Numerous epidemiological studies have associated consumption of fruits and vegetables with decreased cancer incidence and cancer mortality rates [1,2], for instance between the intake of flavonoids via fruits and vegetables and several other degenerative diseases like cardiovascular disease (CVD) and ageing [3,4]. Fruits and vegetables represent the most important source of dietary anticarcinogens [5], and animal studies have consistently shown antitumorigenic effects of commonly consumed vegetables [1]. However, epidemiological evidence for this association has been weakened by some recent prospective studies [6-8].

Scavenging of reactive oxygen species and reactive carcinogen metabolites, as well as induction of phase II detoxification enzymes [9] may explain the suggested anticarcinogenic potential of fruits and vegetables. [10,11].

Oxidative stress leads to the induction of cellular oxidative damage, which may cause adverse modifications of DNA, proteins and lipids [12]. *In vitro*, a flavonoid such as quercetin has already shown its ability of protecting human lymphocyte DNA against hydrogen peroxide treatment [13]. Consequently, a decrease in oxidative damage upon flavonoid intervention may be interpreted as a plausible explanation for the protective effects of fruits and vegetables on cancer risk. Similarly, dietary antioxidants may modulate other biomarkers for cancer risk, such as aromatic DNA adducts formed by polycyclic aromatic hydrocarbons, which have been established as predictors of lung cancer risk [21]. In earlier studies, we tested the efficacy of protecting lymphocyte DNA *in vitro* by quercetin and ascorbic acid before adding H_2O_2 or benzo[a]pyrene (B[a]P). Subsequently, in a pilot study on supplementation of healthy volunteers by administering a quercetin-rich blueberry/apple juice, we demonstrated that a four-week intervention period is suitable for enhancing antioxidant defence, and that a quercetin plasma level was reached that reduced *ex vivo* induced DNA damage [14].

Genetic polymorphisms of genes involved in for instance biotransformation, DNA repair and oxidative stress, are expected to influence the antioxidative and anti-genotoxic efficacy of intervention by micronutrients. Indeed, Mooney et al [15] have shown that the association between smoking-adjusted plasma β-carotene levels and DNA damage appeared only significant in those subjects lacking the *GSTM1* detoxification gene. Moreover, Palli *et al*. concluded that *GSTM1* nulls showed strong inverse associations between DNA adduct levels and vegetable intake [16].

We therefore hypothesized that within the general population, subgroups exist that based on genetic predisposition, benefit relatively more from chemoprevention by an antioxidant-rich diet. Correspondingly, the present large-scale intervention study has been carried out in order to assess prevention of *ex vivo* induced DNA damage by increased intake of fruit-borne antioxidants. Healthy volunteers consumed one litre of blueberry/apple juice per day, leading to a dose of 16 mg ascorbic acid and 97 mg of quercetin bound to a sugar moiety that ensures biological availability. Quercetin

concentration, ascorbic acid concentration and Trolox Equivalent Antioxidant Capacity (TEAC) were monitored. Lymphocytes were treated *ex vivo* with H_2O_2 or B[a]P before and after the intervention period, and differences in induced single strand breaks in DNA and BPDE-DNA adducts assessed. Subjects were genotyped for 34 relevant polymorphisms selected on the basis of their hypothesized interaction with applied biomarkers in blood.

Materials and methods

Study population: The study population consisted of 168 healthy volunteers, 114 female and 54 male, aged 18 - 45 years. Volunteers were recruited through advertisements in local newspapers. Volunteers were considered healthy based on self-experienced health status and were included if they met with the following basic requirements: non-smokers, no use of medication (except for oral contraceptives), no use of vitamin supplementation at the moment of intervention. Further, if it was expected to interfere with the study, subjects were excluded from the study based on dietary habits, profession or other life style factors, for instance excessive PAH-exposure at work, or high antioxidant intake due to personal dietary habits. All this information was gathered through questionnaires. Subjects were fully informed about the details of the study and gave their written informed consent. The Medical Ethical Committee of Maastricht University and the Academic Hospital Maastricht approved the protocol. Demographic information of the study population is listed in Table 1.

Sex	N	Age (mean [min;max])	Lifestyle
Male	54	29 [18;45]	Healthy, non-smoking, no use of medication
Female	114	28 [18;45]	Healthy, non-smoking, no use of medication

Table 1: *Description of study population: number of subjects per sex, their mean age, and lifestyle.*

Wash-out period: As we were particularly interested in raising plasma flavonoid levels, prior to the intervention period, volunteers were given a list of food ingredients rich in flavonoids in general and quercetin in particular, which they had to avoid during a five-day washout-period. Items on this list were onions, apples, red wine, tea, biological and freshly pressed fruit juices, berries (e.g. blueberries and elderberries), grapes, cherries, raisins, parsley, broccoli, cabbage, beans and tomatoes [17]. Next to avoiding food items on the list, subjects were asked to minimise the use of spices and herbs [18,19]. The design of this intervention as well as the efficacy of the washout period was based on a pilot study, described earlier [14].

Intervention: In a paired design, each subject acted as his or her own control. As mentioned before, based on the results from our pilot study, it was concluded that best results were obtained after 4 weeks of intervention. The five-day flavonoid wash-out period was followed by a four-week intervention period with a custom-made blueberry/apple juice mixture, produced specifically for this study by Riedel Drinks (Riedel, Ede, The Netherlands). This mixture of which subjects consumed one litre per day, contained about 97 mg of quercetin per litre, most of it bound to a glucoside or a galactoside at the 3-position, which is known to facilitate its biological availability [20]. The ascorbic acid content of the juice was 16 mg per litre. The impact of seasonal variation in dietary habits or increased sensitivity was overcome by year-round random sampling.

Collection of samples: After the five-day wash-out period, the first blood sample, before intervention, was drawn between 8 and 9 AM. Volunteers were allowed to have breakfast before sampling, but were not allowed to drink any fruit juice. After the four-week intervention period, the second blood sample was drawn between 8 and 9 AM, and treated the following way. Again, breakfast but not any juice was allowed. Venous blood samples were drawn into one 10 ml EDTA vacuum tube for plasma analyses and into two 10 ml vacuum lithium heparin tubes (venoject II, Terumo, USA) for isolation of lymphocytes. The EDTA tubes were kept at 4°C; subsequently, tubes were centrifuged for 10 minutes at 265 x *g* at 4°C to separate plasma. For analysis of ascorbic acid, plasma was de-proteinised and stabilised using 10% of TCA (final concentration 5%). For TEAC and quercetin determination, aliquots were frozen without any treatment. All plasma samples were kept at -80°C until analysis. For isolation of lymphocytes, heparinized blood was diluted by adding phosphate buffered saline (PBS, pH 7.4), and by layering this mixture over lymphoprep™ (Axis-shield, Norway) in a leucosep tube (Greiner Bio-one, Germany). Centrifuging for 20 minutes at 860 x *g* at room temperature separated lymphocytes, which were thereupon washed and resuspended in PBS.

Analytical methods: Total quercetin concentration in plasma, defined as the sum of quercetin glucuronides and sulfates, was analysed by means of HPLC with coulometric array-detection after enzymatic hydrolysis, as described by De Boer et al. [21]. The concentration of ascorbic acid was analysed by means of HPLC as described earlier [22]. Calibrators containing the same amount of TCA as the samples were prepared freshly. TEAC assay was used to determine the total antioxidant capacity in plasma of all volunteers, according to procedures as described by Fischer et al. [23].

Oxidative treatment of samples: Before and after intervention, quiescent peripheral blood lymphocytes were exposed *ex vivo* and in duplicate to oxidative stress. For this, immediately upon isolation, lymphocytes at a concentration of $1*10^6$/ml PBS were exposed to 25 μM of H_2O_2 for 1h at 37°C. Dose and duration had previously been established in a dose range-finding experiment (unpublished data). In all subjects, viability of lymphocytes turned out to be over 95% after treatment (assessed by trypan blue exclusion). We used a slightly modified alkaline (pH>12) comet assay [24-26], implemented according to recent guidelines [25], to assess the level of single strand

breaks in DNA. After the incubation, cells were washed once in PBS (pH 7.4) and then taken up in PBS, again at a cell concentration of $1*10^6$/ml. The cell suspension was mixed with low melting point agarose (Sigma), and positioned on 1.5% agarose-coated slides. Further procedures were as described earlier [14]. Comets were stained with ethidium bromide (50µl, 1mg/ml) for fluorescence microscopy; per blindly scored slide, a total of 50 cells was scored using the software program Comet assay III (Perceptive Instruments Ltd, United Kingdom), background levels of single strand breaks being corrected by subtracting the levels in non-exposed lymphocytes of each individual from that in H_2O_2-exposed lymphocytes of the same subject.

B[a]P treatment of samples: Before and after intervention, quiescent peripheral blood lymphocytes were exposed *ex vivo* to the food carcinogen benzo[a]pyrene (B[a]P) in duplicate. Immediately after isolation, lymphocytes were taken up in RPMI 1640 at a concentration of 2 million cells per ml culture medium. Cells were exposed to 1 µM B[a]P in 0.5% DMSO (dimethyl sulfoxide) for 18h at 37°C. As was investigated before [14], this concentration of B[a]P was sufficient to produce well-detectable levels of adducts in quiescent lymphocytes for analysis by ^{32}P-postlabelling. Control samples were treated with 0.5% DMSO. Both B[a]P and DMSO were applied at non-toxic concentrations; viability of lymphocytes turned out to be over 95% after treatment in all subjects (trypan blue exclusion). After incubation, cells were resuspended carefully and centrifuged for 20 minutes at 860 g. Cells were washed twice using PBS. The remaining lymphocyte pellet was kept at -20°C until DNA isolation. Phenol extraction was used to isolate DNA from lymphocytes. BPDE-DNA adducts were assessed by ^{32}P-postlabeling following the nuclease P1 enrichment technique as described by Reddy and Randerath [27] with some modifications [28]. In all experiments two BPDE-DNA standards with known adduct levels (1 adduct/10^7 and 1 adduct/10^8 nucleotides) were analyzed in parallel for quantitation purposes. Quantitation was performed using Phosphor-Imaging technology (Fujifilm FLA-3000). BPDE-DNA adduct levels were corrected for the amount of DNA in the reaction, which was analysed by HPLC-UV analysis of an aliquot of the digested DNA.

Genotyping: DNA for genotyping was isolated from lymphocytes by standard phenol extraction procedures. All 168 volunteers were genotyped for a total of 34 single nucleotide polymorphisms (SNPs). The SNPs were selected based on a known or an expected association with oxidative stress, biotransformation of quercetin and B[a]P, as well as DNA repair. The Cancer SNP 500 database was used to obtain DNA sequences and allele frequencies (http://snp500cancer.nci.nih.gov). SNPs were analysed using a multiplex PCR method developed by Knaapen et al. [29] and further developed by Ketelslegers et al. who describes 17 out of 34 SNPs analysed here [30]. All analysed SNPs are located in the encoding region, which implies they are all functional.

Required group size: The experimental setup described here was tested before in a pilot study supplementing 8 healthy volunteers with a blueberry apple juice. Information from this study, namely a reduction of oxidative DNA damage of 59% and a reduction in BPDE-DNA damage of 11%, in combination with the observed variation

between subjects was used as input for a power calculation. The power calculation (power 0.9; CI 95%) performed on data obtained in a pilot study revealed that a group size of 12 to 21 subjects would suffice in order to obtain statistically significant results [14].

Statistical analyses: Paired samples t-test was used to assess the efficacy of the intervention on the concentration of quercetin and ascorbic acid in plasma, on plasma TEAC, on the level of *ex vivo* induced oxidative DNA damage, and on the level of *ex vivo* induced BPDE-DNA adducts. Multivariate, stepwise linear regression was used to assess the impact of sex, age, and the different relevant polymorphisms on the efficacy of the intervention. Parameters rendering a statistically significant outcome of the stepwise linear regression were further analysed by paired samples t-test. Pearson's correlations were calculated between increase in ascorbic acid, quercetin, and TEAC, respectively versus *ex vivo* induction of oxidative DNA damage and B[a]P adduct level, respectively, corrected for the polymorphism involved. SPSS software (12.0.1 for Windows) was used for all statistical analyses of the data.

Table 2: List of SNPs, their position, and the amino acid change that is related to the polymorphism.

Single Nucleotide Polymorphism	Amino acid change	dbSNP ID	Function	Effect on enzymatic function	Frequencies wt / hz / v*		
*CYP1A2*1F*		rs762551	Phase I bioactivation	Higher inducibility	86	69	13
*GSTM1*0*	Deletion		Phase II detoxification	No enzyme activity	90	-	78
*GSTP1*2*	I105V	rs947894	Phase II detoxification	Decreased enzyme activity	61	85	22
*GSTP1*3*	A114V	rs1799811	Phase II detoxification	Decreased enzyme activity	140	28	0
*GSTT1*0*	Deletion		Phase II detoxification	No enzyme activity	139	-	29
*NAT2*5*	I114T	rs1801280	Phase II detoxification	Decreased enzyme activity	66	76	26
*NAT2*6*	R197Q	rs1799930	Phase II detoxification	Decreased enzyme activity	84	67	17
*NAT2*7*	G286E	rs1799931	Phase II detoxification	Decreased enzyme activity	161	7	0
*XRCC1*2*	R194W	rs1799782	DNA repair	Increased enzyme activity	147	20	1
*XRCC1*3*	R280H	rs25489	DNA repair	Decreased enzyme activity	142	25	1
*XRCC1*4*	Q399R	rs25487	DNA repair	Decreased enzyme activity	66	77	25
*XRCC3*1*	T241M	rs861539	DNA repair	Decreased enzyme activity	71	71	26
*XPD*5*	K751Q	rs1052559	DNA repair	Decreased enzyme activity	27	85	56
*XPD*6*	R156	rs238406	DNA repair	Decreased enzyme activity	35	85	48
*OGG1*2*	S326C	rs1052133	DNA repair	Decreased enzyme activity	100	60	8
*BrCA2*1*		rs1799943	DNA repair	Decreased enzyme activity	102	55	11
*BrCA2*3*	N372H	rs144848	DNA repair	Decreased enzyme activity	90	57	21
*GPX1*1*	P198L	rs1050450	Oxidative stress	Decreased enzyme activity	71	80	17
*APEX1*1*	D148E	rs3136820	Oxidative stress	Decreased enzyme activity	48	87	33
*mEH*2*	Y113H	rs1051740	Phase II detoxification	Decreased enzyme activity	79	77	12
*mEH*3*	H139R	rs2234922	Phase I bioactivation	Increased enzyme activity	105	55	8
*CAT*1*		rs1001179	Oxidative stress	Decreased enzyme activity	104	48	16
*MnSOD2*1*	V16A	rs1799725	Oxidative stress	Decreased enzyme activity	46	81	41
*NQO1*2*	R139W	rs4986998	Phase II detoxification	Decreased enzyme activity	153	15	0
*NQO1*1*	P187S	rs1800566	Phase II detoxification	Decreased enzyme activity	95	67	6
*CYP1A1*2A*		rs5030838	Phase I bioactivation	Decreased enzyme activity	145	22	1
*CYP1A1*2C*	I462V	rs1048943	Phase I bioactivation	Decreased enzyme activity	159	8	1
*CYP1A1*4*	T461N	rs1799814	Phase I bioactivation	Decreased enzyme activity	160	8	0
*CYP1B1*5*	V432L	rs1056836	Phase I bioactivation	Decreased enzyme activity	55	83	30
*CYP1B1*7*	N453S	rs1800440	Phase I bioactivation	Decreased enzyme activity	115	46	7
*CYP2E1*5*		rs6413420	Phase I bioactivation	Decreased enzyme activity	151	16	1
*CYP3A4*1B*		rs2740574	Phase I bioactivation	Decreased enzyme activity	154	12	2
MPO		rs2333227	Oxidative stress	Decreased enzyme activity	94	64	10
*COMT*1*	V158M	rs4680	Phase II detoxification	Decreased enzyme activity	41	84	43

*: wt = homozygous wildtype, hz = heterozygous, v = homozygous variant. The numbers reflect the numbers of subjects carrying that genotype; a hyphen indicates that the method was not able to distinguish between heterozygous or homozygous wild type (in case of a deletion), therefore both of these polymorphisms are gathered under homozygous wild type.

Results

Single nucleotide polymorphisms: Table 2 lists all analysed SNPs, universal ID codes, the amino acid change related to the polymorphism, enzyme function as well as the expected effect of the polymorphism. The last three columns of this table represent the frequency of wild types, heterozygous and variants of these polymorphisms in the current intervention population.

Effects on quercetin concentration: 4-week intervention with a blueberry/apple juice significantly increased mean (±SE) plasma quercetin levels from 28.8 nM (± 1.05) to 79.2 nM (± 5.14). Regression analysis showed that the level of increase significantly depended on *NQO1*2* (p=0.000). Sex and age did not have any impact. As can be seen in Figure 1, *NQO1*2* heterozygous subjects showed a significantly larger increase in plasma quercetin concentration than their wild type counterparts; homozygous subjects were however not present in this particular population.

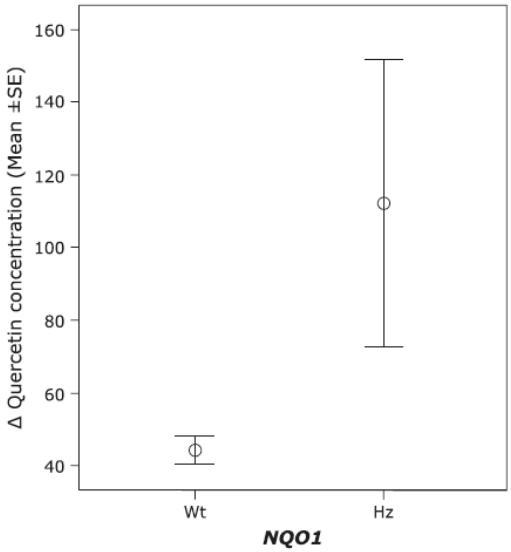

Figure 1: effect of *NQO1*2* polymorphism on the increase of plasma quercetin concentration upon intervention.

Effects on ascorbic acid concentration: The mean (±SE) plasma concentration of ascorbic acid was significantly increased by the intervention, from 58μM (± 1.19) to 61μM (± 1.07) (p=0.001). Sex and age were no confounding factors for this parameter. The change in plasma concentration of ascorbic acid was strongly influenced by *Cat1* (p=0.006; Figure 2). Carriers of the *Cat1* wild type showed hardly any increase in ascorbic acid concentration (1.27 ±1.13 μM), whereas heterozygous and variants show a large (6.80 ± 2.25 μM, and 6.68 ± 2.26 μM, respectively) increase in ascorbic acid concentration.

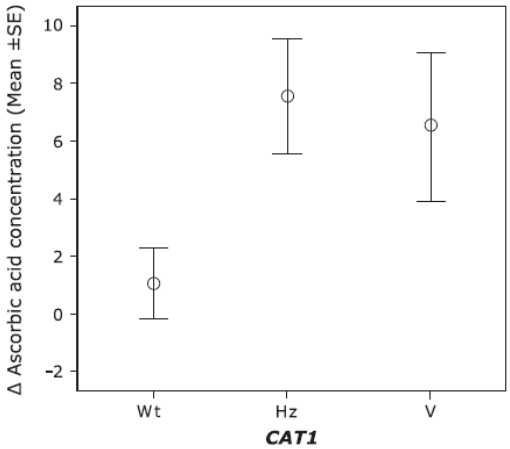

Figure 2: effect of *CAT1* on the increase in plasma ascorbic acid concentration

Effects on antioxidant capacity: The mean (± SE) TEAC value was significantly (p=0.000) increased by the intervention from 781 μM (± 3.95) to 800 μM (± 4.02) trolox equivalent. Sex and age had no effect on the TEAC outcome. Increases of plasma TEAC levels appeared to be partly associated with observed increases in plasma quercetin levels. Stepwise regression indicates that only *GSTT1* (p=0.045) was significantly involved in the effect of a 4 week intake of blueberry/apply juice on plasma TEAC: persons with the *GSTT1* deletion showed the largest increase in plasma anti-oxidative capacity (Figure 3).

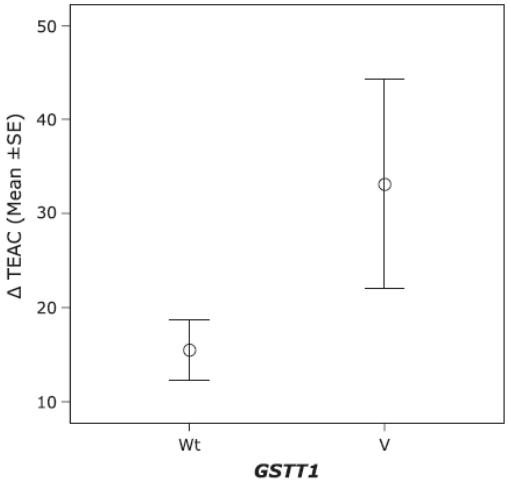

Figure 3: effect of *GSTT1* on the increase of plasma TEAC upon intervention.

Effects on oxidative DNA damage: Overall, paired analysis of background corrected samples revealed a significant (p=0.006) protection of 20% against *ex vivo* induced

oxidative DNA damage upon intervention by blueberry/apple juice intake. Before intervention, the mean Tail Moment was 9.9 (± 0.51 (mean ± SE)), after intervention, the mean Tail Moment was reduced to 8.0 (±0.52 (mean ± SE)). The impact of all genetic polymorphisms, as well as sex and age, was assessed by stepwise regression analysis. *GSTT1* (p=0.004), *XRCC1*4* (p=0.006) and sex (p=0.018) were significant predictors of the intervention effect on Comet assay. Lymphocytes obtained from persons with *GSTT1* wild type showed a larger intervention related decrease in single strand breaks than lymphocytes from those carrying the *GSTT1* deletion (Figure 4a). However, this was not associated with intervention-related changes in plasma levels of quercetin and ascorbic acid, nor of plasma TEAC levels. Finally, men showed a larger protective effect upon blueberry/apple juice intervention than women (Figure 4b).

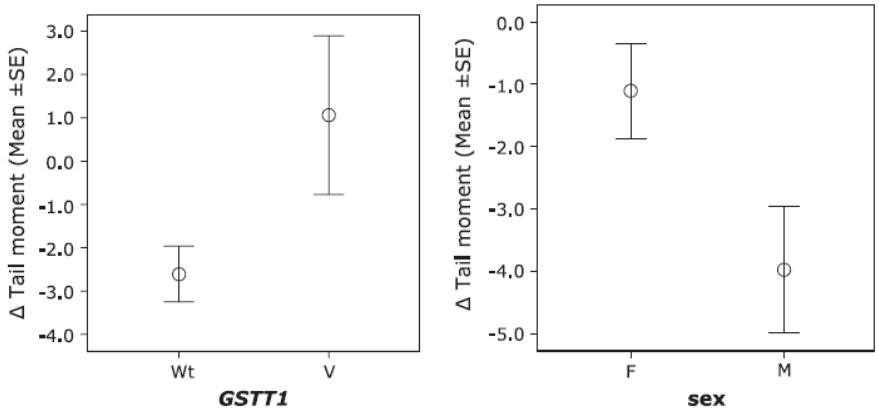

Figure 4: Effect of *GSTT1* polymorphism (a) and sex (b) on the level of induced oxidative DNA damage in human lymphocytes.

Effects on BPDE-DNA adduct level: Upon intervention, in the whole study population, levels of *ex vivo* induced BPDE-DNA were significantly increased from 1.48 (±0.07; mean ± SE) adducts per 10^7 normal nucleotides to 1.90 (±0.12; mean ± SE) adducts per 10^7 nucleotides. Sex and age did not influence the level of BPDE-DNA adducts. Stepwise regression revealed that *CYP1B1*5* (p=0.015) and *COMT1* (p=0.038) significantly predicted these increases in *ex vivo* induced adduct levels (Figure 5a and 5b, respectively). *Cyp1B1*5* wild types showed the largest increase in B[a]P-induced DNA adducts while by contrast, *Cyp1B1*5* variants showed intervention-related absence of *ex vivo* increased BPDE-DNA adduct level, however, without association with increases in plasma quercetin, ascorbic acid and TEAC levels. As for *COMT1,* levels of adducts after the intervention increased with the number of polymorphic alleles, meaning that *COMT1* variants show the largest increase in *ex vivo* induced BPDE-DNA adducts upon intervention. The difference in induced BPDE-DNA adduct levels and the increase in plasma TEAC appear inversely correlated in *COMT1* wildtype subjects (R=-0.317; p=0.043).

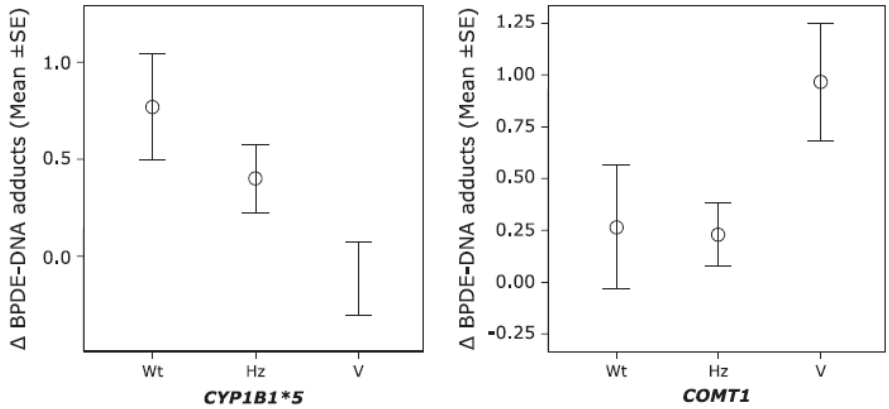

Figure 5: Effect of *Cyp1B1*5* (a) and *COMT1* (b) on the intervention related change in BPDE-DNA adduct level.

Discussion

Several *in vitro* studies have shown that quercetin and other flavonoids, as well as ascorbic acid, possess a strong antioxidant activity related to a strong scavenging capacity [31-38]. Further, quercetin has been investigated *in vitro* for its anti-genotoxic effects [39-41], partly related to its antioxidative capacity. Although biologically relevant, *in vitro* models have limitations with respect to extrapolating results to the *in vivo* situation. In earlier studies, we have found that quercetin or ascorbic acid pre-treatment of human lymphocytes *in vitro* before adding a stressor like H_2O_2 or B[a]P significantly reduced the level of single strand breaks and BPDE-DNA adduct levels, respectively. Subsequently, we have assessed whether supplementation of healthy volunteers with a quercetin-rich fruit juice also results in prevention of *ex vivo* provoked DNA damage [22]. From this pilot study, we concluded that a four-week intervention period is suitable for enhancing antioxidant defence, as was supported by the increase in quercetin plasma concentrations, an increase in TEAC value and a decrease in H_2O_2-provoked DNA damage *ex vivo*. However, observed large inter-individual variations in this pilot study required a more in-depth investigation in a larger study population.

Aim of the present intervention study therefore was to assess effectiveness of chemoprevention of an increased intake of fruit-borne antioxidants, by evaluating effects on biomarkers of oxidative or genotoxic risk. For that purpose, lymphocytes were treated *ex vivo* with an oxidative stressor or a carcinogenic compound before and after a 4-week intervention period with a daily intake of quercetin-rich blueberry/apply juice. Since hypothetically, genetic variation may underlie the observed inter-individual variations in the DNA-protecting effect of an antioxidant intervention, a large set of

genetic polymorphisms of genes involved in metabolism, DNA repair and oxidative stress was taken into account.

This blueberry/apple juice intervention significantly increased plasma quercetin concentration, ascorbic acid concentration and concomitantly, plasma antioxidant capacity. Neither increases in plasma ascorbic acid nor increases in plasma quercetin concentration appeared exclusively responsible for the increase of plasma TEAC. By contrast, in a blueberry and cranberry juice intervention study by Pedersen [42] an increase of plasma antioxidant capacity was caused solely by the increase of plasma ascorbic acid concentration and not by increase of phenols. Obviously, the specific blueberry/apple juice mixture we applied may contain other, hitherto unidentified, antioxidants that contribute to the increase in TEAC.

With respect to protection by 4 week dietary intake of this fruit juice against *ex vivo* H_2O_2- or B[a]P-induced DNA damage, within the whole study population effects were modest and strongly biased by large inter-individual differences. Despite this, we did find a significant protection against H_2O_2-induced oxidative DNA damage. However, we also observed a significant increase in BPDE-DNA adducts induced *ex vivo* upon intervention. Upon classification of subjects according to genetic polymorphisms, of the 34 polymorphisms investigated in the present study, 6 appeared to influence the outcome of the intervention.

*NQO1*2* heterozygous subjects showed a larger increase in plasma quercetin levels by the intake of blueberry/apple juice than their wild type counterparts. This may well be explained by the fact the *NQO1*2* wild types have a higher enzyme activity, and therefore probably higher metabolism of quercetin [43], which results in a lower end concentration of free quercetin in plasma. Further, quercetin has been associated with an induction of *NQO1* gene expression [44], resulting in an even more enhanced metabolism of quercetin.

As for *Cat1,* the increase of plasma ascorbic acid concentration increases with the number of polymorphic alleles. Wild types show no increase, while heterozygous and homozygous variants show a rather large increase in plasma ascorbic acid concentration. Catalase is an endogenous antioxidant enzyme and together with superoxide dismutase and glutathione peroxidase, it serves as primary defence against oxidative stress in the antioxidant network. It can be suggested that the lack of endogenous antioxidant defence is compensated by a more efficient uptake of exogenous ascorbic acid.

GSTT1 polymorphism had a significant impact on the effect of this fruit juice intervention on plasma TEAC. Human *GSTT1* encodes for a phase II enzyme involved in detoxification of various electrophilic compounds, and plays an important role in anti-oxidative defence. In our study, *GSTT1* variants showed the largest increase in plasma TEAC (P=0.045). Since *GSTT1* deletion is associated with a reduced enzyme activity, it is expected to cause lower plasma TEAC as compared to *GSTT1* wild type. Presumably, by taking in relatively larger amounts of exogenous antioxidants, *GSTT1* variants may possess a certain very effective mechanism to compensate for this loss of activity.

Another explanation for this finding is that antioxidants contributing to TEAC-value are more efficiently metabolised and excreted in *GSTT1* wild type.

In contrast, it appeared to be *GSTT1* wild type subjects who benefited most from antioxidant treatment with regard to protection against *ex vivo* induced oxidative DNA damage in their lymphocytes. It is suggested that absence of functional *GSTT1* shifts the oxidant/antioxidant balance towards more prooxidative conditions that may lead to oxidation of polyphenols and possibly other antioxidants taken in through the blueberry/apple juice. In this respect, it is of interest that *GSTT1* null individuals are not more susceptible to oxidative damage in a non-challenged situation [45].

Our study showed no decrease but rather an increase in BPDE-DNA levels *ex vivo* induced in lymphocytes upon intervention in the whole study population. This corresponds to findings by Anderson and coworkers [46] who investigated several flavonoids for protective properties against food mutagens like PhIP (2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine) and IQ (2-amino-3-methylimidazo (4,5-f) quinoline). At high doses, quercetin reduced genotoxic damage by these compounds in both lymphocytes and sperm while at low doses, these flavonoids tended to have exacerbating effects, the mechanism responsible for this effect, however, is unknown. Plasma quercetin levels as achieved by our blueberry/apple juice intervention, were within the nanomolar range, and therefore may have resulted in an increase in BPDE-DNA adducts. Effects on the amount of BPDE-DNA adducts can be explained by the inhibition of phase II enzymes, like CYP1A1, CYP1B1 and other CYPs, by quercetin [40,47,48] In our study, the effect of *CYP1B1*5* polymorphism on *ex vivo* formation of DNA adducts is obviously intervention-related, since this polymorphism does not influence levels of BPDE-DNA adducts induced before intervention (data not shown). Variants, who may be associated with a relatively lower CYP1B1*5 activity show lower levels of BPDE-DNA adduct formation *ex vivo* upon intervention, possibly due to inhibition by quercetin of the remaining enzyme activity.

Furthermore, we have found that *COMT1* variants demonstrated the most profound increase in *ex vivo* induced BPDE-DNA adduct levels. This is probably due to the lower activity of Catechol O-Methyltransferase that is inherent to the variant genotype [49]. COMT is a phase II enzyme that is involved in eliminating B[a]P metabolites [50], and has been shown to be inhibited by tea catechins [51]. None of the *COMT1* genotypes are associated with intervention-related protection against adduct formation, but in *COMT1* wild type subjects, there only is a mild increase in induced DNA adducts in lymphocytes which is inversely correlated with increases in plasma TEAC. This seems to imply that the increase in antioxidant status lowers the intervention-related increase in BPDE-DNA adducts. A remarkable finding is that an increase in plasma TEAC, clearly indicating an antioxidant effect, is not correlated with a decrease in oxidative DNA damage in either genotype. Taking several genoptypes into account by grouping individuals by calculating the sum of relevant risk alleles did not further establish intervention effects.

In line with previous findings [52-56] we confirmed absence of antioxidative effects by micronutrients on base-line DNA damage; quercetin appeared to only exert its

protective effects upon oxidative challenge *ex vivo*. Moller and Loft [57] also argued that dietary interventions with antioxidants showed higher beneficial outcomes in oxidatively stressed subjects like diabetics or HIV-infected patients.

Supplementation with ascorbic acid turned out to be effective in oxidatively challenged lymphocytes. Duthie et al, [58] have investigated oxidative DNA damage in smokers and non-smokers by means of comet assay. A 20-week supplementation with vitamin C (100 mg/day), vitamin E (280 mg/day), and beta-carotene (25 mg/day), resulted in a significant decrease in endogenous oxidative damage. Further, lymphocytes of these treated subjects showed an increased resistance *in vitro* to H_2O_2-provoked oxidative DNA damage. Mooney et al. [59] investigated the impact of vitamin C and vitamin E supplementation for over 12 months in smokers. Their study revealed that smoking women, not men, showed a significant 31% decrease in BPDE-DNA adducts in leukocytes upon vitamin C/vitamin E intervention. An interesting finding was that this decrease was even more pronounced (43%) in women carrying the *GSTM1*-null genotype.

Altogether, in this intervention study, we demonstrated that four-week intake of a quercetin-rich blueberry/apple juice, was sufficient to increase plasma concentrations of quercetin, ascorbic acid and other unidentified antioxidants. Furthermore, the antioxidative capacity of plasma increased significantly, as reflected by the increased TEAC value. These results show that the blueberry/apple intervention induced antioxidative defence mechanisms. Despite the fact that plasma quercetin concentrations did not reach the level at which quercetin is known to exert protective effects *in vitro* [14], protective effects were clearly observed in H_2O_2-exposed lymphocytes following intervention. By contrast, *ex vivo* induced levels of BPDE-DNA adducts in B[a]P-exposed lymphocytes increased upon intervention suggesting increased risk of blueberry/apple juice intake. Overall, out of 168 subjects, 139 *GSTT1* wild types (83%) and 30 *CYP1B1*5* variants (18%) may benefit most from increased fruit-borne antioxidant intake with respect to reducing risks of DNA damage, while for 43 carriers of the *COMT1* variants (26%), risks may be elevated. Evaluation of the role of genetic polymorphisms thus appears to provide a helpful tool in assessing susceptible groups and groups that benefit from specific dietary interventions. However, more integrated approaches are required for definitely assessing health costs and benefits of micronutrients.

Acknowledgements

We would like to thank the Netherlands Organisation for Health Research and Development for financial support of this project in the context of the Nutrition: Health, Safety and Sustainability program. Further, we would like to thank Riedel Drinks for providing the fruit drink.

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Chapter 6

Summary and general discussion

A healthy lifestyle is associated with decreased risk of cancer and of other degenerative diseases like cardiovascular diseases (CVD) and ageing. Epidemiology has rendered a fair amount of evidence that fruit and vegetables support a healthy lifestyle, and just may be the cause of preventing such diseases. Antioxidants as present in fruits and vegetables are suggested to play a role in the protection against degenerative diseases. However, recently epidemiological evidence for this has weakened, first, by the MRC/BHF where vitamin supplementation in over 20,000 high-risk individuals did not result in a decrease in mortality (Lancet, 2002. 360(9326): 23-33), and then even further by a recent meta-analysis of randomised controlled trials revealed that supplementation with vitamin A, E, or beta-carotene have resulted in an increased mortality (Bjelakovic, Jama, 2007. 297(8): p. 842-57).
In the studies described in this thesis, we hypothesised that not all human individuals respond equally to the suggested anticarcinogenic effects of fruits and vegetables, and that it is this underlying interindividual variability which masks epidemiological associations between fruit and vegetable intake and the incidence of cancer and CVD. More specifically, we hypothesised that if study populations were subdivided in certain groups differing in susceptibility, as genetically determined by polymorphisms in genes that play a pivotal role in carcinogenesis, protective effect by fruits and vegetables will become apparent. In order to test this hypothesis, a human intervention study was required.
For evaluating this hypothesis, we needed to identify a model anti-carcinogen. However, there seems to be no 'magic bullet', no single vitamin or specific antioxidant that appears to be able to claim sole responsibility for the protective effects by vegetables and fruits. From the many options, we chose to use quercetin as a model compound for the group of flavonoids, which are abundantly present in fruits and vegetables. Quercetin possesses good antioxidative properties, and has already been shown *in vitro* to be able to protect against DNA damage (Duthie, Mutat Res, 1997. 393(3): p. 223-31). We decided to dose quercetin through food e.g. through a specific fruit juice mixture; because in this juice, quercetin occurs in its natural form, bound to sugar moieties, through which we aimed to achieve high biological availability. In order to test our hypothesis, we aimed to discover whether certain subgroups within the study population are more susceptible to these DNA damage-preventing features of quercetine, based on their specific genotype. For this purpose, subjects were analysed for single nucleotide polymorphisms (SNPs) in the coding regions of 34 genes selected because of their known or suspected involvement in oxidative stress, biotransformation of quercetin and B[a]P, as well as DNA repair.

Chapter 2 focuses on the protective effect of quercetin on oxidative DNA damage *ex vivo* induced by two different reactive oxygen species involved in oxidative stress, superoxide anion radicals ($O_2^{\bullet -}$, PMS/NADH-mediated) and hydroxyl radicals ($OH^{\bullet}$ (Fenton reaction, iron-induced)). Further, we tried to relate the difference in protective effect on oxidative DNA damage in human lymphocytes to quercetin's scavenging capacity of the two reactive oxygen species, i.e. we were trying to relate its

effectiveness as a scavenger to its effectiveness as a preventive measure against oxidative DNA damage. Human lymphocytes were chosen since they represent the only readily available source of DNA for assessing systemic effects in human intervention studies. Protection by quercetin against *ex vivo* induced oxidative DNA damage in the Comet assay has been compared with its direct scavenging capacity of quercetin measured by ESR spectroscopy. Quercetin appears capable of protecting human lymphocytes against H_2O_2-induced DNA damage *in vitro* in a dose-dependent manner. However, the protection of lymphocytes against $O_2^{\bullet-}$ is ambiguous. Low pre-incubation concentrations of quercetin (1 μM) tend to reduce the level of superoxide-induced oxidative DNA damage, while at 100 μM DNA damage is induced. The observed rate constant for the inhibition reaction of quercetin with $OH^{\bullet}$ formation (3.2 x 10^{12} M^{-1} s^{-1}) appears to be much higher as compared to the reaction with $O_2^{\bullet-}$ (1.1 x 10^4 M^{-1} s^{-1}). Hence we conclude that quercetin is very potent in protecting against oxidative DNA damage caused by hydroxyl radicals and is a more potent inhibitor of hydroxyl radical formation than a scavenger of superoxide radicals. Based on the clear effect of quercetin both as a direct scavenger of $OH^{\bullet}$ measured by ESR as well as the protection against single strand breaks in lymphocytic DNA as measured by Comet assay, it was decided that H_2O_2 is the preferred oxidative stressor for our test system.

To further optimise our study model, in Chapter 3 we started out to investigate the ability of quercetin to prevent DNA against induced damage in *in vitro* exposed human peripheral blood lymphocytes. First, we pre-incubated lymphocytes *in vitro* with a range of quercetin dosages, followed by a DNA-oxidizing challenge with hydrogen peroxide. The level of H_2O_2-induced oxidative damage was determined by use of the single-cell gel electrophoresis (Comet) assay. Second, similarly quercetin-pretreated human lymphocytes were challenged by treatment with benzo(a)pyrene (B(a)P) which induces bulky DNA lesions, and this BPDE-DNA adduct formation was measured by ^{32}P-postlabelling. Quercetin showed a clear and significant dose-dependent protection *in vitro* against both the formation of oxidative DNA damage ($p < 0.01$) and of BPDE-DNA adducts ($p < 0.05$). Since these studies demonstrated that quercetin pre-treatment of lymphocytes *in vitro* was very effective in significantly reducing the amount of induced DNA damage, in the third part of this Chapter we performed a pilot study *in vivo* where lymphocytes from eight female volunteers who consumed a quercetin-rich blueberry/apple juice mixture for four weeks, were treated *ex vivo* with an effective dose of H_2O_2 and benzo(a)pyrene, respectively, at three different time points, i.e. before (t = 0 weeks), during (t = 2 weeks) and after (t = 4 weeks) the intervention. Results of the small scale *in vivo* study substantiated the idea of internally charging human lymphocytes by fruit antioxidants. Daily intake of blueberry juice during a four week period was capable of boosting the total antioxidant capacity of plasma, as reflected by the increase of the TEAC value from 773 μM trolox equivalent at t = 0 to 855 μM at t = 4 weeks ($p = 0.04$) and by an increase in plasma quercetin content from 5.0 to 10.6 nM ($p = 0.03$). After intervention, levels of oxidative damage upon *ex vivo* exposure to H_2O_2 were non-significantly ($p = 0.07$) decreased by an average of 41%,

while the mean BPDE-DNA adduct level induced *ex vivo,* was non-significantly decreased by 11%. This combination of our findings *in vitro* and *ex vivo* provides evidence that quercetin is able to protect against chemically induced DNA damage in human lymphocytes, which may underlie its suggested anticarcinogenic properties. However, large inter-individual differences in prevention against *ex vivo* induced DNA damage were observed suggesting inter-individual differences in response to fruit antioxidants that may be explained by differences in genetic predisposition.

Our hypothesis that genetic polymorphisms could be at the basis of susceptibility of groups of people and might enable the prediction of the extent of an intervention effect was assessed in an adjusted *in vitro* model as described in Chapter 4. We started to challenge this hypothesis by investigating the impact of two quite common genetic polymorphisms and their effect on *in vitro* induced damage in human lymphocytes and the extent of protection by pre-treatment using an antioxidant. Two anti-oxidants, quercetin and ascorbic acid, were used at equimolar concentrations. Ascorbic acid was included in this test system as a positive control since it is a model dietary antioxidant. We aimed to establish the impact of genetic polymorphisms in *GSTM1* and *GSTT1* on H_2O_2-induced oxidative DNA damage and on the effectiveness of quercetin and ascorbic acid in preventing this induced damage. *GSTM1* and *GSTT1* encode for enzymatic antioxidative defence. Lymphocytes of 12 healthy volunteers were pre-incubated either with 10 μM of quercetin or with 10 μM of ascorbic acid, and subsequently exposed to 25 μM H_2O_2 for 1h. Induction of oxidative DNA damage was quantified using the Comet assay. Overall, quercetin proves to have increased protective capability in human lymphocytes *in vitro* against oxidative DNA damage upon H_2O_2 challenge when compared to ascorbic acid. Genotyping of these 12 subjects showed that 6 individuals were *GSTM1+ (homozygous wildtype and heterozygous)* and 6 were *GSTM1- (variant)*; 8 were *GSTT1+ (homozygous wildtype and heterozygous)* and 4 *GSTT1- (variant)*. Baseline levels of oxidative DNA damage did not differ between *GSTM1* or *GSTT1* variants and their respective wild types. Also with respect to *ex vivo* induced levels of oxidative DNA damage, no significant difference was observed between variants and wild types of both genotypes. Strikingly, the protection against H_2O_2-induced oxidative DNA damage by quercetin was significantly higher in *GSTT1* wild types than in *GSTT1* variants (57% and 9% decrease, respectively; p=0.01). Furthermore, *GSTT1* wild types were protected against induced oxidative DNA damage by ascorbic acid pre-incubation while *GSTT1* variants showed an increase of damage (16% decrease versus 91% increase; p=0.01). For *GSTM1* variants and wild types, observed differences in protective effects of quercetin or ascorbic acid were not statistically significant. Overall, it is concluded that genetic polymorphisms appear to influence the protective effect against induced DNA damage of *in vitro* pre-treatment by quercetin and ascorbic acid; however, interpretation of results, although obtained in this relatively simple model, is complex.

The ultimate part of this thesis comprises the large-scale human dietary intervention study, which is described in Chapter 5. We designed this study to assess antioxidative and possible anti-genotoxic properties of fruit-borne antioxidants, to verify the hypothesis that individuals bearing polymorphisms in genes related to quercetin metabolism, B[a]P metabolism, defence against oxidative stress, and DNA repair, differ in their response to DNA protective effects of increased antioxidant intake. This was inspired by a study by Mooney et al. (Carcinogenesis, 1997. 18(3): p. 503-9) who found that DNA damage in smokers was affected by genetic polymorphisms and nutritional status: The association between smoking-adjusted plasma β-carotene levels and DNA damage appeared only significant in those subjects lacking the *GSTM1* detoxification gene. Further, Palli et al. (Carcinogenesis, 2004. 25(4): p. 577-84) concluded from data from the EPIC Italy study that *GSTM1* nulls, not *GSTM1* wildtypes showed strong inverse associations between DNA adduct levels and vegetable intake. In the present study 168 healthy volunteers consumed a blueberry/apple juice that provided 97 mg quercetin and 16 mg ascorbic acid per day. After a four-week intervention period, plasma concentrations of quercetin and ascorbic acid, and total plasma antioxidant capacity (TEAC) indeed were significantly increased. Overall, the approach to use a situation of elevated stress in order to more reliably monitor protection by quercetin proved to be very successful, since at baseline levels, there were hardly any effects measurable. We found 20% protection ($p<0.01$) against *ex vivo* H_2O_2-provoked oxidative DNA damage, as assessed by Comet assay. However, the averaged level of *ex vivo* induced BPDE-DNA adducts was increased by 28% upon intervention ($P<0.01$). Statistical analysis of 34 biologically relevant genetic polymorphisms revealed that 6 polymorphisms significantly influenced the outcome of the intervention. Lymphocytes of individuals bearing variant genotype for *Cyp1B1*5* seemed to benefit more than wildtypes from DNA-damage protecting effects upon intervention by blueberry/apple juice. Variants for *COMT* tended to benefit less, or even experienced detrimental effects from intervention. With respect to *GSTT1,* the effect is ambiguous: variants respond better in terms of intervention-related increase in TEAC, but wild types benefit more from its protecting effects against oxidative DNA damage.

Summarising results as described in this thesis, it is concluded that both *in vitro* pre-treatment as well as *in vivo* administration of quercetin result in protective effects in *ex vivo* oxidatively challenged human lymphocytes. We proved that a four-week dietary intervention, in which healthy volunteers consumed a quercetin-rich fruit juice, is capable of boosting antioxidative defence mechanism. With respect to oxidative stress, internal charging of lymphocytes by quercetin and probably other fruit-borne antioxidants appeared effective in preventing *ex vivo* induced DNA damage. These effects were even more pronounced in different subpopulations. For example, *GSTT1* variants showed the largest increase in plasma antioxidant capacity, whereas *GSTT1* wild types showed the largest decrease in single strand breaks. Interestingly, in the *in vitro* study described in Chapter 4, *GSTT1* wild types also showed the largest protection from *in vitro* quercetin pre-treatment. With respect to oxidative DNA damage, there

was a close match between *in vitro* experiments and the intervention study. As in *in vitro* experiments, the amount of *ex vivo* induced oxidative DNA damage was decreased upon intervention. Further, *GSTT1* wildtypes, that showed the largest decrease in *in vitro* induced oxidative DNA damage, also formed the group that benefited most from the intervention. However, when it comes to B[a]P treatment, the *in vitro* pre-treatment was very effective in reducing the amount of B[a]P-induced BPDE-DNA adducts. Unfortunately, upon intervention, the amount of B[a]P-induced BPDE-DNA adducts was increased instead of decreased. This means that care has to be taken in translating *in vitro* acquired information to the *in vivo* situation.
Grouping subjects according to their genetic polymorphism revealed interesting aspects. For instance, for a parameter like induced oxidative DNA damage, an average population-wide intervention-related decrease in damage was measured, whereas subjects carrying the *GSTT1* homozygous wild type showed a more pronounced beneficial effect of the intervention. Based on the finding that 6 polymorphisms significantly influenced the outcome of the intervention we conclude that genotyping of relevant polymorphisms enables selecting subgroups among the general population that benefit more of DNA damage-modulating effects of micronutrients. This implies that our proposed hypothesis was proven to be correct. This outcome may be taken into account in giving dietary recommendations on the intake of vitamins and antioxidants to the general public.

Implications for further research:
Epidemiological evidence for the role of fruits and vegetables in disease prevention is not unambiguous or entirely clear, nor is the underlying biological or biochemical mechanism. *In vitro* studies may be very helpful in discovering biochemical responses in human lymphocytes thereby providing crucial mechanistic insights. However, although rendering useful information, *in vitro* studies represent a model, which cannot take all biological factors of a human body into account. Since also human intervention studies cannot give all the answers, due to their complexity, *in vitro* and *in vivo* studies will have to remain to be synergistically performed in order to complement each other. However, through the research presented in this thesis, we showed that an alternative approach applying biomarkers for (anti-)carcinogenesis, and classifying different susceptible groups of people based on their genotype, may reveal patterns of prevention of disease that in previous epidemiological evaluations were possibly masked by inter-individual differences in susceptibility. It might be useful to re-evaluate some of the larger epidemiological trials by using this novel research approach. The present study should thus be seen as a starting point for further dedicated research, in which a stricter dietary intervention or an elaboration of the amount of stress posed on lymphocytes may help to further elucidate mechanisms within the black box that is called healthy lifestyle.

Samenvatting en algemene discussie

Een gezonde levensstijl wordt in verband gebracht met een verlaagd risico op verschillende degeneratieve ziekten als kanker, hart- en vaatziekten en veroudering. De epidemiologie heeft door de jaren heen redelijk veel bewijs geleverd dat fruit en groenten bijdragen aan een gezonde levensstijl, en dat groenten en fruit een belangrijke rol kunnen spelen bij het voorkomen van dergelijke ziekten. Van antioxidanten die voorkomen in fruit en groenten wordt gedacht dat ze een rol spelen in de bescherming tegen degeneratieve ziekten. Onlangs is het epidemiologische bewijs hiervoor verminderd, in eerste instantie door de MRC/BHF studie waarin extra vitamine supplementen in meer dan 20.000 'high-risk' individuen niet leidden tot een verlaging van de sterfte (Lancet, 2002. 360(9326): 23-33). Het epidemiologische bewijs werd verder verzwakt door een recente meta-analyse van gerandomiseerd gecontroleerde experimenten die aantoonden dat aanvullende vitamine A, E of beta-caroteen inname zelfs leidden tot een verhoogde sterfte (Bjelakovic, Jama, 2007. 297(8): p. 842-57).

Bij de experimenten zoals beschreven in dit proefschrift, hebben we de hypothese gesteld dat niet alle mensen in dezelfde mate en op dezelfde manier reageren op de anticarcinogene effecten van fruit en groenten, en dat het juist deze verschillen zijn die mogelijk de epidemiologische verbanden tussen fruit en groente inname en het vóórkomen van kanker en hart- en vaatziekten maskeren. Preciezer gezegd hebben we verondersteld dat de beschermende werking van fruit en groenten duidelijker naar voren zal komen, wanneer studiepopulaties worden onderverdeeld in specifieke groepen, die verschillen in gevoeligheid op basis van onderscheid in genen.

Om deze hypothese te testen hebben we een interventie studie in mensen nodig en bovendien een model anti-carcinogeen. Helaas lijkt een wondermiddel niet te bestaan. Er is geen vitamine of specifieke antioxidant die als enige verantwoordelijk kan worden gehouden voor de beschermende werking van groenten en fruit. Uit de vele opties die voorhanden waren, hebben we ervoor gekozen om quercetine als modelstof te gebruiken voor de groep van flavonoïden die in grote mate voorkomen in groenten en fruit. Quercetine beschikt over goede antioxidatieve eigenschappen en het is al aangetoond in *in vitro* onderzoek dat het kan beschermen tegen DNA schade (Duthie, Mutat Res, 1997. 393(3): p. 223-31). We hebben ervoor gekozen om quercetine toe te dienen via een fruitdrank. In dit sap komt quercetine voor in zijn natuurlijke vorm, namelijk aan een suikergroep gebonden. Hierdoor is de quercetine beter biologisch beschikbaar. Ons doel was om specifieke subpopulaties te ontdekken in de onderzoekspopulatie die gevoeliger zijn voor de DNA beschermende werking van quercetine op basis van hun specifiek genotype. Proefpersonen werden getest op single nucleotide polymorphisms (SNPs) in het coderend deel van 34 genen, die geselecteerd zijn op basis van hun bekende of veronderstelde betrokkenheid bij oxidatieve stress, biotransformatie van quercetine en B[a]P en DNA herstel.

Hoofdstuk 2 richt zich op de beschermende rol van quercetine op oxidatieve DNA schade die *ex vivo* (buiten het lichaam) in lymfocyten veroorzaakt werd door twee reactieve zuurstof species die betrokken zijn bij oxidatieve stress. De ene zuurstof species zijn superoxide anion radicalen ($O_2^{\bullet -}$, PMS/NADH-gemedieerd) en de andere

zijn hydroxyl radicalen ($OH^{\bullet}$ (Fenton reactie)). Verder hebben we geprobeerd om het verschil in beschermend effect op het niveau van oxidatieve DNA schade in lymfocyten in verband te brengen met de radicaal-vangend vermogen van quercetine voor beide zuurstof species. Dat wil zeggen, we hebben geprobeerd om de relatie tussen zijn effectiviteit als radicalenvanger en de effectiviteit als beschermer tegen oxidatieve DNA schade in kaart te brengen. Humane lymfocyten werden gekozen omdat ze de enige goed beschikbare bron voor DNA vormen waarmee systemische effecten in interventie studies met gezonde vrijwilligers onderzocht kunnen worden. We hebben bescherming door quercetine tegen *ex vivo* geïnduceerde oxidatieve DNA schade gemeten met de zogenaamde Comet assay. Vervolgens zijn deze resultaten vergeleken met het directe radicalenvangend vermogen van quercetine gemeten met ESR spectrometrie. Quercetine blijkt goed in staat om humane lymfocyten te beschermen tegen H_2O_2-geïnduceerde DNA schade *in vitro* op een dosisafhankelijke manier. Daarentegen is de bescherming tegen schade geïnduceerd door $O_2^{\bullet-}$ tweeledig. Relatief lage concentraties van quercetine lijken het schadeniveau te verlagen, terwijl hogere concentraties (100 µM) het niveau van oxidatieve DNA schade verhoogt. De reactiesnelheid van quercetine met $OH^{\bullet}$ (3.2×10^{12} M^{-1} s^{-1}) blijkt veel hoger dan die van quercetine met $O_2^{\bullet-}$ (1.1×10^{4} M^{-1} s^{-1}). Op basis hiervan concluderen we dat quercetine heel goed in staat is te beschermen tegen oxidatieve DNA schade veroorzaakt door hydroxyl radicalen. Verder kan geconcludeerd worden dat het sterker hydroxyl radicalen kan remmen, dan dat het superoxide radicalen kan wegvangen. Op basis van dit duidelijke effect van quercetine, is besloten dat H_2O_2 de voorkeur heeft als oxidatieve stressor voor onze proefopzet.

Om ons onderzoeksmodel te optimaliseren, hebben we in Hoofdstuk 3 onderzocht of quercetine in staat was om het DNA te beschermen tegen geïnduceerde schade in *in vitro* blootgestelde humane lymfocyten. In het eerste deel zijn de lymfocyten behandeld met verschillende hoeveelheden quercetine, waarna er een hoeveelheid waterstofperoxide (H_2O_2) aan werd toegevoegd dat het DNA beschadigt. Het schadeniveau dat veroorzaakt werd door de behandeling met H_2O_2, is gemeten met behulp van de Comet assay. In het tweede deel zijn op dezelfde manier met quercetine behandelde lymfocyten blootgesteld aan benzo(a)pyreen (B[a]P) dat aan het DNA bindt en zogenoemde 'bulky adducts' veroorzaakt. Deze BPDE-DNA adducten zijn gemeten met behulp van de ^{32}P-postlabelling-techniek. Quercetine bleek duidelijk in staat om dosisafhankelijk het DNA te beschermen tegen zowel de oxidatieve DNA schade ($p < 0.01$) als tegen de vorming van BPDE-DNA adducten ($p < 0.05$). Aangezien quercetine *in vitro* goed in staat is om te beschermen tegen geïnduceerde DNA schade, hebben we in het derde deel van dit hoofdstuk een onderzoek opgezet waarbij 8 vrouwelijke vrijwilligers 4 weken lang een quercetine-rijke bosbessen/appelsap hebben gedronken. De lymfocyten van deze vrijwilligers zijn voor aanvang van de studie, na 2 weken, en na 4 weken blootgesteld aan dezelfde schadelijke stoffen als in de eerdere delen, respectievelijk H_2O_2 en B(a)P. De uitkomst van deze kleinschalige studie ondersteunde het idee dat lymfocyten in het lichaam konden worden

opgeladen met antioxidanten uit fruit. De dagelijkse inname van het bosbessen/appelsap gedurende vier weken was voldoende om de antioxidant-capaciteit van het plasma te verhogen. Het DNA-schadeniveau veroorzaakt door zowel H_2O_2 als B(a)P blootstelling was (niet significant) verlaagd na de interventie. De combinatie van deze gegevens leverde het bewijs dat quercetine in staat is om witte bloedcellen te beschermen tegen chemisch geïnduceerde DNA-schade. In deze groep vrijwilligers waren grote verschillen te zien in de bescherming tegen geïnduceerde DNA-schade. Deze kunnen mogelijk verklaard worden door verschillen in genetische gevoeligheid, bepaald door genetische polymorfismen.

De hypothese dat genetische polymorfismen de gevoeligheid van groepen mensen bepalen, en dat zo het effect van een interventie voorspeld kan worden, hebben we getest in een *in vitro* model dat beschreven wordt in Hoofdstuk 4. Om deze hypothese te testen hebben we de invloed van twee veel voorkomende genetische polymorfismen op de *in vitro* geïnduceerde schade in lymfocyten getest, en hun effect op bescherming door een antioxidant tegen deze schade bekeken. Twee verschillende antioxidanten, quercetine en vitamine C, zijn gebruikt in dezelfde concentratie. Vitamine C is meegenomen als model-antioxidant die voorkomt in voeding. Het doel was om de invloed van genetische polymorfismen in *GSTM1* and *GSTT1* op zowel H_2O_2-geinduceerde oxidatieve DNA schade als op de effectiviteit van quercetine en vitamine C in het beschermen van deze schade te onderzoeken. De keuze voor *GSTM1* en *GSTT1* is gemaakt omdat beide coderen voor enzymatische antioxidatieve afweer. Lymfocyten van 12 gezonde vrijwilligers werden behandeld met ofwel quercetine ofwel vitamine C, en vervolgens blootgesteld aan H_2O_2. De hoeveelheid oxidatieve DNA schade werd wederom gekwantificeerd met behulp van de Comet assay. Het blijkt dat quercetine in dit model in humane lymfocyten een hogere mate van bescherming biedt dan vitamine C. Uit genotyperings-analyse van deze 12 vrijwilligers bleek dat 6 van hen *GSTM1+ (homozygoot wildtype en heterozygoot)* en 6 *GSTM1- (variant)* waren; verder bleken 8 individuen *GSTT1+ (homozygoot wildtype en heterozygoot)* en 4 *GSTT1- (variant)*. De achtergrond niveaus van oxidatieve DNA schade verschilden niet tussen de verschillende groepen. Evenmin waren er verschillen in *ex vivo* geïnduceerde niveaus van oxidatieve DNA schade. Opvallend genoeg was de bescherming tegen H_2O_2-geïnduceerde oxidatieve DNA schade door quercetine significant hoger in *GSTT1* wild types dan in de *GSTT1* varianten (57% and 9% afname, respectievlijk; p=0.01). Daarnaast bleken *GSTT1* wild types beschermd door een pre-incubatie met vitamine C tegen geïnduceerde oxidatieve DNA schade terwijl *GSTT1* varianten juist een verhoging van de schade lieten zien (16% afname versus 91% toename; p=0.01). Voor wat betreft *GSTM1* varianten en wild types, waren de geobserveerde verschillen in beschermend effect van quercetine of vitamine C niet statistisch significant. In het algemeen kunnen we concluderen dat genetische polymorfismen het beschermend effect tegen geïnduceerde DNA schade door een *in vitro* pre-incubatie met quercetine of vitamine C lijken te beïnvloeden. De interpretatie van de resultaten, hoewel verkregen met een relatief eenvoudige studie opzet, is gecompliceerd.

Het laatste deel van dit proefschrift omvat de grootschalige voedingsinterventie studie in gezonde vrijwilligers, die beschreven staat in Hoofdstuk 5. Deze studie hebben we zo opgezet om de anti-oxidatieve en mogelijk anti-genotoxische eigenschappen van antioxidanten uit fruit te onderzoeken en om de hypothese te testen dat individuen met polymorfismen in genen die betrokken zijn bij quercetine metabolisme, B[a]P omzetting, afweer tegen oxidatieve stress en DNA herstel, verschillen in hun respons ten aanzien van DNA beschermende effecten van verhoogde antioxidant inname. Dit onderzoek was geïnspireerd op een studie door Mooney et al. (Carcinogenesis, 1997. 18(3): p. 503-9) die aantoonde dat DNA schade in rokers beinvloed werd door genetische polymorfismen en voedingsstatus. Het verband tussen roken en voor roken gecorrigeerde β-caroteen concentraties in plasma en DNA schade bleek alleen significant in proefpersonen zonder het *GSTM1* detoxificatie gen. Verder heeft Palli et al. (Carcinogenesis, 2004. 25(4): p. 577-84) aangetoond met gegevens uit de EPIC Italy study dat alleen *GSTM1* varianten, en niet de *GSTM1* wild types een sterk omgekeerd verband lieten zien tussen DNA adduct niveaus en de inname van groenten. In de huidige studie hebben 168 gezonde vrijwilligers een bosbessen/appelsap gedronken die voorziet in 97 mg quercetin en 16 mg vitamine C per dag. Na een vierweekse interventie periode waren zowel de plasma concentraties van quercetine en vitamine C als de totale antioxidant capaciteit van het plasma (TEAC) verhoogd. De aanpak om een toestand van verhoogde stress om betrouwbaarder de bescherming door quercetine te kunnen bepalen bleek erg succesvol, aangezien er in achtergrondniveaus nauwelijks effecten meetbaar waren. In deze studie hebben we een 20% bescherming ($p<0.01$) tegen *ex vivo* H_2O_2-veroorzaakte oxidatieve DNA schade, getest met de Comet assay. Daarentegen was het gemiddelde niveau van *ex vivo* veroorzaakte BPDE-DNA adducten verhoogd met 28% na interventie ($P<0.01$). Statistische analyse van 34 biologisch relevante genetische polymorfismen heeft aangetoond dat 6 ervan significant de uitkomst van de interventie beïnvloeden. Lymfocyten van individuen met het variant genotype voor *Cyp1B1*5* lijken meer baat te hebben bij bescherming tegen DNA-schade door de bosbessen/appelsap interventie dan individuen met het wild type. Varianten voor *COMT* lijken juist minder baat te hebben, of zelfs schadelijke effecten te ondervinden van de interventie. Wat *GSTT1* betreft is het effect dubbel: varianten hebben een betere respons als gekeken wordt naar de toename in TEAC door de interventie, maar wild types hebben meer baat bij de beschermende effecten tegen oxidatieve DNA schade.

Als we de resultaten zoals beschreven in dit proefschrift samenvatten, kunnen we concluderen dat zowel *in vitro* pre-incubatie als *in vivo* toediening van quercetine resulteert in beschermende effecten in *ex vivo* aan oxidatieve stress blootgestelde humane lymfocyten. We hebben bewezen dat een vierweekse voedingsinterventie, waarin gezonde vrijwilligers een quercetine-rijke fruitdrank drinken, in staat is om de antioxidatieve afweer te verhogen. Met betrekking tot oxidatieve stress bleek het in het lichaam opladen van lymfocyten met quercetine en andere antioxidanten uit fruit effectief in het voorkomen van *ex vivo* geïnduceerde DNA schade. Deze effecten waren

zelfs meer uitgesproken in verschillende subpopulaties. Bijvoorbeeld *GSTT1* varianten lieten de grootste toename in plasma antioxidant capaciteit zien, terwijl *GSTT1* wild types de grootste afname in enkelstrengs DNA breuken. In de *in vitro* studie beschreven in Hoofdstuk 4, lieten *GSTT1* wild types ook de grootste bescherming zien als gevolg van de *in vitro* quercetine pre-incubatie. Met betrekking tot oxidatieve DNA schade was er een grote overeenkomst tussen de *in vitro* experimenten en de interventie studie. Net als in *in vitro* experimenten was de hoeveelheid van *ex vivo* geïnduceerde oxidatieve DNA schade verlaagd na interventie. Bovendien bleek dat *GSTT1* wild types, die de grootste afname in *in vitro* geïnduceerde oxidatieve DNA schade lieten zien, ook de groep vormden die de meeste baat had bij de interventie. Bij blootstelling aan B[a]P bleek de *in vitro* voorbehandeling met quercetine erg effectief in het verminderen van de hoeveelheid B[a]P-geïnduceerde BPDE-DNA adducten. Helaas bleek na interventie de hoeveelheid B[a]P geïnduceerde BPDE-DNA adducten verhoogd in plaats van verlaagd. Dit houdt in dat men in het geval van B[a]P voorzichtig moet zijn met het vertalen van *in vitro* verworven informatie naar de *in vivo* situatie.

Het onderverdelen van proefpersonen naar hun genetisch polymorfisme heeft interessante aspecten opgeleverd. Bijvoorbeeld, voor een parameter als geïnduceerde oxidatieve DNA schade werd in de gemiddelde populatie een interventie-gerelateerde afname in schade gemeten, terwijl individuen met het GSTT1 homozygoot wild type nog een meer uitgesproken beschermend effect van de interventie lieten zien. Gebaseerd op de bevinding dat 6 polymorfismen de uitkomst van de interventie significant beïnvloeden, kunnen we concluderen dat genotypering van relevante polymorfismen het mogelijk maakt om subgroepen in de algemene bevolking te selecteren die meer baat hebben bij DNA-schade modulerende effecten van micronutriënten. Dit houdt in dat onze voorgestelde hypothese correct is. Met deze uitkomst kan rekening worden gehouden bij het geven van voedingsadviezen aan de algemene bevolking voor wat betreft de inname van vitaminen en antioxidanten.

Implicaties voor verder onderzoek:

Epidemiologisch bewijs voor de rol van groenten en fruit in de preventie van ziekten is in zekere mate dubbelzinnig of niet helemaal duidelijk, bovendien is het onderliggend biologisch of biochemisch mechanisme vaak onduidelijk. *In vitro* onderzoeken kunnen helpen in het ontdekken van biochemische reacties in humane lymfocyten en zo leiden tot cruciale mechanistische inzichten. Hoewel ze nuttige informatie leveren, blijven *in vitro* studies een modelsituatie, waarin niet alle biologische factoren van een menselijk lichaam kunnen worden meegenomen. Aangezien ook humane interventiestudies door hun complexiteit niet alle antwoorden kunnen geven, zullen *in vitro* studies en *in vivo* studies beide moeten worden uitgevoerd, zodat ze elkaar aanvullen. Met het onderzoek dat gepresenteerd werd in dit proefschrift, hebben we laten zien dat een alternatieve aanpak waarin biomarkers voor (anti)carcinogenese en het classificeren van verschillende gevoelige groepen van proefpersonen op basis van hun genotype worden toegepast, patronen kan laten zien in de bescherming tegen ziekte, die in

eerdere epidemiologische studies wellicht gemaskeerd werden door interindividuele verschillen in gevoeligheid. Het zou nuttig kunnen zijn om enkele grotere epidemiologische studies opnieuw te bestuderen met behulp van deze nieuwe onderzoeksaanpak. De huidige studie moet daarom gezien worden als een uitgangspunt voor verder gericht onderzoek, waarin een striktere voedingsinterventie of een uitbreiding van de hoeveelheid stress die aan de lymfocyten wordt toegediend kan helpen om mechanismen in de 'black box' die gezonde levensstijl heet, verder te verhelderen.

Dankwoord

Eindelijk is het dan de beurt aan dat laatste stukje tekst in een proefschrift dat vaak als eerste gelezen wordt, het dankwoord. Promoveren doe je niet alleen, dat is algemeen bekend. Er zijn zoveel mensen om me heen die me op verschillende manieren aan dit eindproduct hebben geholpen, allemaal hartelijk dank hiervoor! Een aantal van hen wil ik wat uitgebreider noemen.

Als eerste wil ik mijn promotor, Prof. Jos Kleinjans bedanken. Jos, zonder jouw kritische blik, en de snelheid van manuscripten becommentariëren (vele malen sneller dan ik ze schreef!) had dit boekje hier nu niet gelegen.

Vervolgens wil ik al mijn (oud)GRAT-collega's bedanken voor hun steun en gezelligheid. In het bijzonder diegenen die mij hebben bijgestaan tijdens de interventiestudie: Daniëlle voor het scoren van ontelbare Comet-glaasjes, Edwin als 'brute kracht' om die duizenden liters sap in de koude kamer te krijgen, Lou (heer is un good wief…) voor de DNA isolaties en het postlabel-werk, en Ralph voor de genotyperingen. Zonder jullie had ik dit niet kunnen doen! Anneloes en Marie-Claire, dank jullie wel voor de secretariële ondersteuning. Pascal, dankjewel voor je hulp met computers, op het B-lab en als 'luisterend oor'. Marcel, je was niet direct bij mijn onderzoek betrokken, maar ik kon altijd bij je terecht voor een of andere wijze raad, of om ideeën te spuien. En dan was er nog die mountainbike-tocht waarbij je een letterlijk steuntje in de rug was! Dankjewel dat je mijn paranimf wilt zijn.

Harald, dankjewel voor je hulp bij het prikken van mijn proefpersonen, en voor je enorme rust en relativeringsvermogen, je hebt me geleerd tot tien te tellen!

Jan D. en Theo, zonder jullie was ik waarschijnlijk nooit bij GRAT begonnen, maar zonder jullie had ik het zeker niet afgemaakt. Dankjewel voor jullie troostende, opbeurende, en relativerende woorden als het weer eens tegen zat.

Anne en Patries, jullie weten wat het is om naast een nieuwe baan nog een proefschrift af te ronden, dankjewel voor jullie steun en motivatie.

Alle quercetine-aio's, Vincent de Boer, Agnes Boots, Ashwin Dihal, en Hester van der Woude, en natuurlijk hun begeleiders: Gerrit Alink, Aalt Bast, Guido Haenen, Peter Hollman, Ivonne Rietjens, Rob Stierum, en Ruud Woutersen, dank jullie wel voor de nuttige en motiverende halfjaarlijkse bijeenkomsten. Agnes, naast je gezelschap en hulp op een aantal interventie-ochtenden, ben je zeker in de laatste maanden een enorme steun en toeverlaat geweest! Op naar nog meer avondjes met wijn en blauwe schimmelkaas. Erg bijzonder dat ik jouw promotiedatum mag delen, zij het een jaartje later…

Petra Dekker en Piet Haasen van Riedel drinks (nu: Frieslandfoods), hartelijk dank voor jullie bijdrage in de zoektocht naar het ideale sap, en jullie interesse voor mijn onderzoek door de jaren heen.

Leden van de beoordelingscommissie: Prof. dr. A.M. Schols, Dr. G.R.M.M. Haenen, Prof. dr. R.P. Mensink, Prof. dr. Ir. I.M.C.M. Rietjens, en Prof. dr. F.J. Van Schooten, hartelijk dank voor de snelle beoordeling van het manuscript.

Alle proefpersonen en iedereen die voor mijn onderzoek bloed heeft gegeven hartelijk bedankt voor jullie zeer essentiële bijdrage.

Mijn DSM-REG-collega's, Henk, Piet, Dick, Mariëlla, Yolanda, Sandra, Monique, Jack, Daniëlle, Jeanine, André, Bart, Wim en Hans, dankjewel voor jullie steun, interesse en vertrouwen in het afgelopen anderhalf jaar. Dankzij jullie is dit laatste stukje van het promotie-traject gemakkelijker geworden.
Al mijn lieve vrienden, dankjewel voor alle afleiding tijdens gezellige lunches, etentjes of andere activiteiten. Cindy en Nikkels, lieve Cin, urenlang 'eventjes bijkletsen', laten we dat vooral blijven doen! Sandra, onze onvergetelijke trip naar Australië en het tripje naar Berlijn vragen om herhaling! Tamara en Dave, Cindy en Lars, Riech, François, Marloes, Kim Z., Ellen, Lorena, en Luc. Tot gauw!

Als laatste wil ik mijn familie bedanken. Allerliefste Dimphy en Rogier, waar moet ik beginnen om jullie te bedanken. Vooral het laatste jaar hebben jullie mijn promotie van zeer dichtbij meegemaakt. Dankjewel voor jullie geduld en bemoedigende woorden, voor het luisteren, voor jullie hulp bij weerbarstige figuren en voor het doorploeteren van lappen tekst. Dankjewel ook voor alle gezellige avonden, samen eten, en naar het theater of de bioscoop. Heerlijk om zo dicht bij jullie te wonen. Dimphy, ik vind het heel bijzonder dat jij mijn paranimf wilt zijn. Jij hebt het zonder meer verdiend om mee te promoveren, en ik ben heel blij dat jij op de dag van de verdediging achter me staat! Lieve mama, dankjewel voor je onvoorwaardelijke liefde en steun, en voor je ongekend vertrouwen in mij. Dankjewel dat je me steeds gestimuleerd hebt om het beste uit mezelf te halen, en me steeds hebt weten op te beuren als ik het even niet zag zitten. Lieve papa, jij hebt me enorm geholpen bij mijn studiekeuze, en bij mijn verdere loopbaan, door me erop te wijzen dat ik niet naar het HLO hoefde (of mocht!) als ik op een lab wilde staan. Ik mis je, maar ik weet dat je erbij bent…
Ik hou van jullie.

Lonneke

Curriculum Vitae and publications

CURRICULUM VITAE

Lonneke Cécile Wilms was born in Maastricht, The Netherlands, on October 10th 1977. She attended high school (athenaeum) at the Sint Maartenscollege in Maastricht from 1990 until 1997. After graduation, she studied Environmental Health Sciences at Maastricht University from 1997 until 2001. The research described in her Master's thesis was performed at the Karolinska Institute in Stockholm, Sweden, in 2001 under supervision of Prof. Kari Hemminki. After obtaining the MSc degree in 2001, she started the PhD project described in this thesis at the Department of Health Risk Analysis and Toxicology, Maastricht University, under supervision of Prof. Jos Kleinjans. This project was part of a collaboration entitled "Benefit-risk evaluation of flavonoids in foods and their use as functional food ingredients", and was funded by the Netherlands Organisation for Health Research and Development in the Nutrition: Health, Safety and Sustainability program. Further, she participated in several courses as part of the Postdoctoral Education in Toxicology, like Food Toxicology and Food Safety, and Laboratory Animal Science (art. 9 of the Dutch Law on Animal Experiments), and she received a diploma in Radiation Hygiene (level 5B).
In May 2006 she joined DSM Nutritional Products, department of Regulatory Affairs, in Delft, as a toxicologist.

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