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The Effect of Flavonoids in Soybean Products in Lymphocytes from IBD and Colon Cancer Patients After Treatment with Food Mutagens and Hydrogen Peroxide

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1. Introduction

The generation of DNA damage by environmental, medical or life style factors is considered to be an important initial event in carcinogenesis. At the cellular level, a balance between the production of oxidative radicals and the compensational action of antioxidants, which might become pro-oxidant at high concentrations (Anderson et al., 1994) is crucial for our health. Imbalance on either side, especially towards an increase in oxidative stress, might result in various detrimental effects including cell death and cancer. Despite various cellular mechanisms to counteract these adverse events the sheer number of potentially carcinogenic compounds leading to oxidative stress can negatively affect the DNA integrity of cells.

Dietary flavonoids acting as antioxidants (Rice-Evans, 2001) have been identified to be capable of counteracting these adverse oxidative effects (Ross and Kasum, 2002). They are classified as low-molecular-weight polyphenolic compounds that are ubiquitously present in fruit and vegetables and categorised according to their chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Flavonoids such as quercetin and rutin present in soybean products have potent antioxidant properties and mimic oestrogens, hence are being used to ease menopausal symptoms. Soy flavonoids are also believed to lower the blood level of triglicerydes and cholesterol preventing coronary heart disease as well as osteoporosis (Valachovicova et al., 2004). They have a wide variety of biological effects acting either as anti- or pro-oxidants depending on their concentration (Anderson et al., 1997; Duthie et al., 1997) and/or in combination with food mutagens (Anderson et al., 1997). Anderson et al. observed positive responses with flavonoids when lymphocytes were treated with them alone in the Comet assay, and in combination with food mutagens they were showing exacerbating effects at low doses and were protective at high doses (Anderson et al., 1997). The antioxidant potency of several widespread dietary

flavonoids showed a dose-dependent reduction of induced oxidative DNA damage *in vitro*, highlighting an even higher protective effect than vitamin C (Noroozi et al., 1998). It has also been shown that flavonoid intake can lower the mortality rate caused by coronary heart disease (Kaur et al., 2007). Typical flavonoids are kaempferol, quercetin and rutin (the common glycoside of quercetin), belonging to the class of flavonols. The strongest evidence for a cancer-preventive effect shows quercetin with strong antioxidant properties (Chondrogianni et al., 2010; Hollman et al., 1996). It has been reported that the average intake of flavonoids is 23 mg per day with quercetin contributing almost 70% (Hollman et al., 1996; Wach et al., 2007). As it scavenges highly reactive species such as peroxynitrite and hydroxyl radicals (Boots et al., 2008), quercetin protects not only against various diseases such as atherosclerosis, cancer, osteoporosis, pulmonary and cardiovascular diseases but also against ageing (Chondrogianni et al., 2010; Ekstrom et al., 2010; Ossola et al., 2009; Terao, 2009; Zhou et al., 2010).

Lifestyle factors like alcohol intake, physical inactivity, stress, food additives, high animal fat and/or red meat intake and also cooking-derived carcinogens such as heterocyclic amines (HCA), have been identified as having a strong impact on human health and being involved in the aetiology of cancer in general (Adamson et al., 1996; Bogen, 1994). Evidence for a positive association of colorectal cancer and adenomatous polyps with HCA exposure has been provided by several studies (Butler et al., 2003; Felton et al., 2007; Gunter et al., 2005; Knize and Felton, 2005; Murtaugh et al., 2004; Navarro et al., 2004; Nowell et al., 2002; Shin et al., 2007; Wu et al., 2006). HCA are formed by cooking proteinaceous food, mainly seen as heat-induced non-enzymatic browning that involves creatinine, free amino acids and monosaccharides (Schut and Snyderwine, 1999). More than 20 carcinogenic/mutagenic HCA have been isolated so far (Nagao et al., 1997; Wakabayashi et al., 1992). Major subclasses of HCA found in the human diet comprise of aminoimidazoazaarenes (AIA), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Schut and Snyderwine, 1999). It has been estimated that the daily intake of HCA can reach 50 μg (Knize et al., 1995; Krul et al., 2000), depending on the type of meat, temperature and the method of cooking (Messner and Murkovic, 2004; Wu et al., 2001). The heterocyclic amine PhIP is considered to be the most abundant HCA responsible for inducing various types of tumours in rats (Felton et al., 2004).

More than 600 individual compounds and complex dietary mixtures have been studied for protective effects towards HCA (Schwab et al., 2000) and numerous articles have been published regarding mammalian enzymes involved in the bioactivation and detoxification of these compounds (Eisenbrand and Tang, 1993). The genotoxicity of HCA originates from their activation by a series of reactions involving cytochrome P450 when the parent compound is converted to an electrophilic derivative such as a nitrenium ion that covalently binds to DNA resulting in DNA adducts and subsequently in nucleotide alterations and chromosomal aberrations (Goldman and Shields, 2003; Hatch et al., 2001). IQ induces unscheduled DNA synthesis in liver cells and shows strong mutagenic properties in the *Salmonella typhimurium* test system, which contribute to its classification as a potent carcinogen (Maeda et al., 1999; Murata et al., 1999; Weisburger et al., 1986) Also in addition to the formation of DNA adducts, oxidative damage to the DNA itself plays a crucial role in the carcinogenic process of food mutagens (Maeda et al., 1999; Murata et al., 1999). Heterocyclic amines like IQ are able to generate free radicals in the presence of NADPH and cytochrome b5 reductase (Maeda et al., 1999).

Colorectal tissue is constantly exposed to different chemicals and free oxygen radicals formed during metabolic activation. High intracolonic levels of free radicals may form active carcinogens or mitogenic tumour promoters through the oxidation of procarcinogens, either by hydroxyl radicals in faecal water or by secondary peroxyl radicals (Babbs, 1990). Within an inflamed bowel, disproportionate amounts of reactive oxygen species (ROS) can be additionally produced (Loguercio et al., 1996; Simmonds and Rampton, 1993). Ulcerative Colitis and Crohn's disease are inflammatory disorders of the gastrointestinal tract, associated with increased risk for colorectal cancer (Soderlund et al., 2010), which are unevenly distributed within the populations throughout the world. Although the exact cause of inflammatory bowel disease (IBD) remains unknown, the epidemiology of IBD has provided an insight into the pathogenesis of the disease by examining geographic, ethnic and other IBD risk factors (genetic, environmental, etc.) as well as their natural history (Danese and Fiocchi, 2006). Interestingly, reactive oxygen species (Seegert et al., 2001) are produced in abnormally high levels in cells from IBD patients (Rezaie et al., 2007) leading to oxidative stress and thus to DNA damage due to an imbalance between innate and exogenous antioxidants and ROS (Hemnani and Parihar, 1998; Soffler, 2007). Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Davies, 1995). Oxidative stress arising from the pathophysiology of cancer, may even serve as a biomarker (Hopkins et al., 2010), when there is an imbalance between production of ROS and their removal by intrinsic antioxidants (catalase) and antioxidant micronutrients.

In the present study, we used the Comet assay, which evaluates direct DNA breaks and is a fast and reliable method to assess DNA integrity in virtually any cell type without the requirement for cell culture (Moller, 2006). Three groups of individuals served as blood donors: healthy volunteers, IBD patients as well as patients with histopathologically confirmed, untreated colon cancer. It is known that lymphocytes from colon cancer patients exhibit higher levels of DNA damage caused by the intrinsic oxidative stress arising from colorectal cancer (Hopkins et al., 2010) and that these lymphocytes may also serve as an early predictive marker of cancer risk (Vodicka et al., 2010). Separated lymphocytes from IBD patients and healthy individuals were treated with H_2O_2 co-treated with quercetin and IQ with epicatechin. Also lymphocytes from colon cancer patients and healthy individuals were treated with IQ and PhIP with and without the supplementation of the antioxidant flavonoids, quercetin and rutin to show that these three flavonoids are able to reliably protect cells against the damaging effects of reactive oxygen species, even in the context of diseases like IBD or colorectal cancer where levels of ROS are already highly increased. Non-physiological doses were used *in vitro* to study the genotoxicological responses where higher, yet non cytotoxic doses are used as a routine procedure.

2. Materials and methods

2.1 Chemicals

The chemicals for the Comet assay were purchased from the following suppliers: RPMI-1640 medium, agarose and low melting point agarose from Invitrogen, Ltd. (Paisley, U.K.); DMSO (dimethyl sulfoxide), ethidium bromide, Trypan blue, EDTA, Trizma base, Triton X-100, quercetin, epicatechin, rutin and hydrogen peroxide H_2O_2, from Sigma Chemical Company (Dorset, U.K.); sodium chloride and sodium hydroxide from BDH Laboratory

Supplies (Poole, England); Lymphoprep cell separation gel from Nycomed Pharma Axis Shield (Oslo, Norway); FCS (foetal calf serum) from Nalgene, Rochester (New York, USA). The food mutagens, IQ (2-amino-3-methyl-3h-imidazo[4,5-f]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), were obtained from Toronto Research Chemicals, Inc. (Downsview, Ontario, Canada).

2.2 Collection of samples

After informed consent, approximately 10 ml heparinised blood were taken by venepuncture from the IBD and colon cancer patients at the Department of Gastroenterology, Bradford Royal Infirmary (BRI) and St. Luke's Hospital, Bradford, UK. Healthy volunteers were recruited within the Division of Biomedical Sciences at the University of Bradford (West Yorkshire, UK). Ethical permission was obtained from both the BRI Local Ethics Committee (Reference no.: 04/Q1202/15) and the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8). Over a period of three years, a set of samples from healthy controls, IBD patients and colon cancer patients was obtained.

2.3 Questionnaire for patients and controls

A questionnaire was administered to each donor immediately after taking the blood sample. The completed questionnaire for the patient and control groups provided essential information about lifestyle, endogenous (gender, age) and exogenous factors (intake of medicines and alcohol, smoking habits and diet). There were two different studies performed: study I involving IBD patients and study II – colorectal cancer patients. Both studies had separate treatment regimes.

2.4 Lymphocyte separation for the Comet assay

The heparinised blood was diluted with 0.9% saline in a 50:50 proportion and 6 ml of this dilution was carefully layered on top of 3 ml of Lymphoprep in 15 ml conical tubes followed by centrifugation (20 minutes at 800 g) at room temperature. The buffy coat layer of lymphocytes (above the Lymphoprep layer) was then transferred to another tube pre-filled with 10 ml of saline and centrifuged (15 minutes at 500 g). The supernatant was removed without disturbing the pellet which was then resuspended in PBS or RPMI-1640 medium and used for the *in vitro* experiments. Some of the lymphocytes were frozen in liquid nitrogen for long-term storage. In this case the pellet was resuspended in FCS containing 10% DMSO, and lodged in liquid nitrogen vapour overnight, before final storage after complete insertion in the storage Dewar.

2.5 Cell viability

Cell viability at the concentrations chosen for each experiment was checked after treatment and before performing the Comet assay. Viability was determined by Trypan blue dye exclusion indicating intact cell membranes (Phillips, 1973). 10 µl of 0.05% Trypan blue was added to 10 µl of cell suspension and the percentage of cells excluding the dye was estimated using a Neubauer Improved haemocytometer (Pool-Zobel et al., 1992). Only concentrations with viability over 80% were accepted for use in the studies to avoid artefactual results from cytotoxicity (Henderson et al., 1998).

2.6 Treatment of lymphocytes

2.6.1 Treatment for IBD group of experiments

Isolated lymphocytes (approx. 10^6 cells per ml) from IBD patients (n=10) and healthy controls (n=10) were treated without metabolic activation for 30 minutes in RPMI at 37 ^oC either with different concentrations of quercetin (0, 100, 200, 250 μM) in the presence of hydrogen peroxide (50 μM) or with different concentrations of epicatechin (0, 25, 50, 100 μM) in the presence of IQ (50 μM). Lymphocytes from healthy individuals served as the control groups. After the treatment, the cells were pelleted (5 minutes at 900 g). For DNA damage studies, the cell suspension was mixed with the same volume of 1% low melting point agarose for the Comet assay.

2.6.2 Treatment for colorectal cancer group of experiments

Lymphocyte suspensions (100 μl, 10^6 cells per ml) from colon cancer patients (n=20) and healthy controls (n=20) were exposed to defined concentrations of food mutagens and/or flavonoids in the presence of RPMI in a total volume of 1 ml. The treatment was for 30 min at 37 °C. As lymphocytes showed little or no difference in response with metabolic activation, the Comet assay was performed in the absence of metabolic activation to avoid any confounding factors (Anderson, 1997, 1998). To investigate DNA damage, the following concentrations were used 10, 25, 50 and 75 μM for PhIP and 25, 75, 100 and 150 μM for IQ based on preliminary studies (data not shown). To investigate the modulatory effect of flavonoids, the highest concentrations of IQ and PhIP were used for simultaneous combination treatment with the flavonoids, quercetin and rutin, supplemented at concentrations of 100 (50 for rutin), 250 and 500 μM.

2.7 Alkaline comet assay

The slide preparation for the Comet assay and the assay itself was carried out as previously described (Tice et al., 2000). An aliquot of 100 μl of lymphocyte suspension was mixed with 100 μl of 1% low melting point agarose (in PBS, <40 °C warm) and 100 μl of this suspension were spread onto each of the two microscope glass slides pre-coated with 1% normal melting point agarose (in water, dried overnight). After cover-slips were applied, the slides were placed on an ice-cold tray. Once the agarose set, the cover-slips were removed and a final third layer of 0.5% low melting point agarose (in PBS) was added and allowed to solidify as well on ice for 5 min. For each concentration, two replicate slides were produced. For cell lysis, the slides were immersed laterally in a container with cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and incubated at 4 ^oC overnight. Then, the slides were placed on the tray of an electrophoresis tank, filled with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH <13) and incubated for 30 minutes at 4 ^oC in the dark to allow the unwinding of DNA and expression of alkali labile sites. Electrophoresis was conducted at the same temperature for 30 minutes at 0.75 V/cm. The current was adjusted to 300 mA by raising or lowering the buffer level. After electrophoresis, the slides were removed from the tank and soaked three times for 5 minutes each with neutralizing Tris buffer (400 mM, pH 7.4). Cellular DNA was stained with 60 μl of 20 μg/ml ethidium bromide and cover-slips applied. Slides were examined using a fluorescence microscope equipped with a charge couple device (CCD) monochrome camera and a computerised image analysis system, Komet 4.0 (Kinetic Imaging, Liverpool, UK) to measure the comet parameters. All slides were coded by an independent person ensuring that scoring took place completely randomized and in a "blind" manner (Faust et

al., 2004). For each replicate slide, 25 cells were scored (50 cells in total) for each individual in each group making 500-1000 observations per experimental point, allowing a more than adequate statistical power to detect effects (Hartmann et al., 2003).

2.8 Statistical analysis

Data were tested for normality prior to statistical analysis. Normal distributions were checked through the Kolmogorov-Smirnoff and Shapiro-Wilk's Test to assess whether parametric statistics could be used. Study I: Gaussian normality was violated for many of the scale variables as endorsed by the Kolmogorov-Smirnov test. Therefore, non-parametric test procedures were adopted wherever necessary, such as the Kruskal-Wallis (K-W) and the Mann-Whitney (M-W) tests for independent samples. When testing intra-subject differences in DNA damage, the Wilcoxon Signed Rank (WSR) test was applied. For the binary response variables, Fisher's Exact (FE) test was applied. Throughout the analyses, a significance level of 5% was used and unilateral alternative hypotheses preferred to bidirectional tests (wherever appropriate). Study II: differences in measured parameters between healthy and colon cancer subjects were assessed by the parametric One Way ANOVA Test, since data were normally distributed. The relationship between DNA damage and various parameters characterising colon cancer status and unexposed control was analyzed using Post-hoc analysis (Dunnett test). The mean of each set of data was used in the statistical analysis. A probability level at $p<0.05$ was regarded as statistically significant. Differences between two experimental groups were tested by the unpaired Student *t*-test when comparing confounding factors due to gender, diet, smoking and drinking habits. The SPSS package version 16 was used to compare patient and control groups at different doses of H_2O_2, food mutagens and flavonoids.

Statistical analyses were performed on mean values for each cluster group (healthy individuals, IBD and colon cancer patients) for each possible combination of chemicals. The experimental unit was the individual. The Comet data parameters used to measure DNA damage were Olive tail moment (OTM; arbitrary unit, the fraction of DNA in the tail multiplied by the tail length) and % tail DNA (the percentage of DNA in the tail) recommended to be the most reliable comet measurements with OTM being the most statistically significant (Kumaravel and Jha, 2006). Of these two parameters, OTM is one of the most commonly reported measures of DNA damage but is recommended to be provided together with % tail DNA (Tice et al., 2000). Together they clearly define the comets indicating a linear relationship to the DNA break frequency over a wide range of levels of damage and both can be applied for scientific purposes (Hartmann et al., 2003; Kumaravel et al., 2009).

3. Results

Mutagenic effects of food mutagens and H_2O_2 in lymphocytes of all groups, healthy individuals and IBD/cancer patients, were examined *in vitro* by comparing the untreated controls to the different treatment doses. Supplementing the treatment with various concentrations of the flavonoids, quercetin, epicatechin and rutin, the reduction of the genotoxic impact of chemicals was also evaluated. The different combined treatments of H_2O_2/food mutagens and flavonoids were then compared to a positive control being a non-

supplemented high dose of H_2O_2/food mutagens. Subsequently, an intergroup comparison between healthy individuals and IBD/cancer patients was carried out evaluating the baseline DNA damage as well as the difference in sensitivity of lymphocytes of all groups. Also, the contribution of confounding factors was evaluated for all experiments.

3.1 Study I: IBD patients

3.1.1 Patient versus control groups

As shown in Table 1 and Figure 1, there was a significant difference in baseline DNA damage before *in vitro* treatment in lymphocytes of IBD patients when comparing them with healthy individual controls ($p < 0.001$). The study groups as well as the control groups after treatment showed significant increases in DNA damage induced by H_2O_2 ($p < 0.001$) and IQ ($p < 0.001$). The induced damage caused by the *in vitro* treatment with H_2O_2 or IQ decreased significantly in both groups when co-treated with the flavonoids quercetin or epicatechin, respectively.
Flavonoid supplementation at the highest concentration (250 µM quercetin or 100 µM epicatechin) caused an overall significant reduction of the induced DNA damage within the patient group and the control groups. This resulted in a 48.6% ($p < 0.001$) reduction of H_2O_2 induced DNA damage and a 43% ($p < 0.001$) reduction of IQ induced DNA damage within the patient groups. For both control groups, reductions in DNA damage of 35.2% and 57.1%, respectively, were observed (both, $p < 0.001$) (Table 1 and Figure 1). As expected, the two different control groups showed similar baseline DNA damage (M-W, $p = 0.174$).

Different concentrations	H_2O_2 + Quercetin (group one)				Different concentrations	IQ + Epicatechin (group two)			
	Olive tail moment		% tail DNA			Olive tail moment		% tail DNA	
	Study group	Control group	Study group	Control group		Study group	Control group	Study group	Control group
A1	10.9 ± 2.1	4.1 ± 1.3	19.9 ± 1.5	9.2 ± 1.2	A2	17.5 ± 2.2	4.6 ± 2.4	26.8 ± 1.1	9.2 ± 0.8
B1	14.4 ± 3.2	12.8 ± 2.4	24.8 ± 0.2	18.5 ± 0.6	B2	22.2 ± 3.1	15.9 ± 1.7	30.8 ± 1.2	21.4 ± 0.4
C1	5.3 ± 1.8	5.7 ± 1.7	21.1 ± 1.9	9.0 ± 0.6	C2	15.2 ± 1.9	7.8 ± 2.2	23.1 ± 0.5	13.0 ± 0.8
D1	7.1 ± 1.4	6.9 ± 1.2	22.6 ± 1.4	11.8 ± 0.7	D2	16.5 ± 1.8	5.6 ± 2.7	25.6 ± 1.4	9.5 ± 2.1
E1	8.8 ± 3.1	6.2 ± 1.1	21.6 ± 2.1	11.5 ± 1.2	E2	12.7 ± 3.9	6.4 ± 2.6	21.6 ± 0.2	11.5 ± 1.3

Table 1. IBD patient/study and control groups, Olive tail moment (OTM) and % tail DNA after *in vitro* treatment with H_2O_2 and IQ and supplementation with the flavonoids at different concentration levels. A1, A2**:** No treatment; B1: H_2O_2 50 µM + Quercetin 0 µM ; B2: IQ 50 µM + Epicatechin 0 µM; C1: H_2O_2 50 µM + Quercetin 100 µM; C2: IQ 50 µM + Epicatechin 25 µM; D1: H_2O_2 50 µM + Quercetin 200 µM; D2: IQ 50 µM + Epicatechin 50 µM; E1: H_2O_2 50 µM + Quercetin 250 µM; E2: IQ 50 µM + Epicatechin 100 µM;

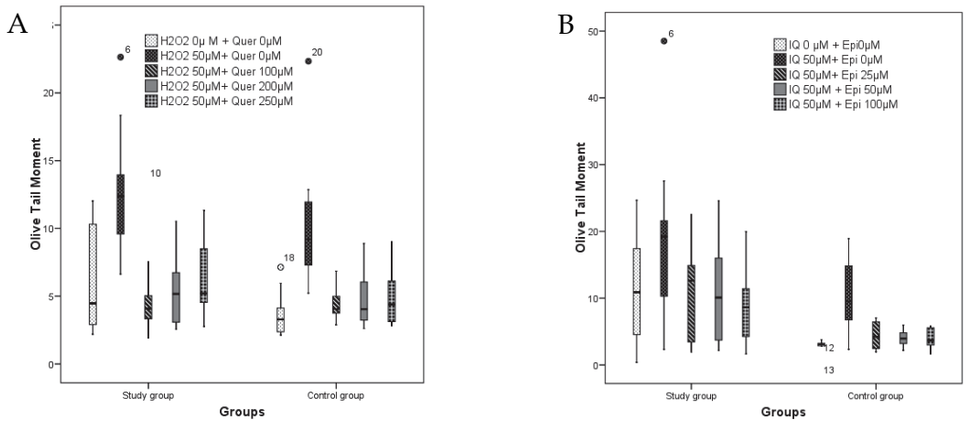

Fig. 1. IBD patient group and control group after *in vitro* treatment with A) H_2O_2 (50 µM) and supplementation with the flavonoid quercetin (Quer) at different concentration levels and B) IQ (50 µM) and supplementation with the flavonoid epicatechin (Epi) at different concentration levels. Pooling the data for each group and comparing median levels of DNA damage and 75% quartiles showed them to be significantly different (Kolmogorov-Smirnov test, $p < 0.001$). The ° symbols followed by a number indicate individual outliers with the respective patient number.

3.1.2 Differences in IBD sub-groups

As shown in Table 2 and Figure 2 in both series of experiments there was less DNA damage in the UC patient group (n = 4) than in the CD group (n = 4) each being significantly different (Kruskal-Wallis (K-W) test, $p < 0.001$) when compared with the combined patient groups, which also included the indeterminate group where it was difficult to differentiate into UC or CD (n = 2). Also there was less induced DNA damage in the study group treated with H_2O_2 and quercetin compared with the study group treated with IQ and epicatechin although the patients were selected randomly.

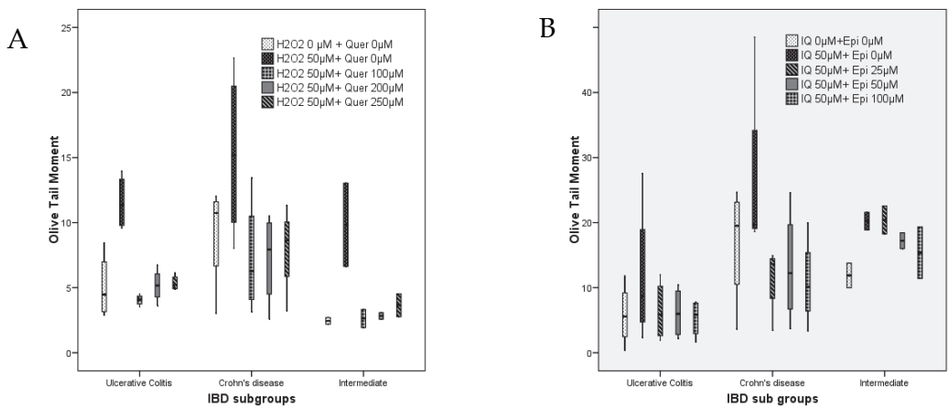

Fig. 2. DNA damage within three IBD subgroups after *in vitro* treatment with A) H_2O_2 (50 µM) and supplementation with the flavonoid quercetin (Quer) at different concentration levels and B) IQ (50 µM) and supplementation with the flavonoid epicatechin (Epi) at different concentration levels. The first two groups were diagnosed with Crohn's disease and Ulcerative Colitis. The third group was an indeterminate subgroup.

3.1.3 Confounding factors

3.1.3.1 Ethnicity, age, gender, smoking and drinking habits

There were small differences of median levels of DNA damage in Caucasians (n = 13) and Asians (n = 7) after treatment with H_2O_2 and quercetin as well as in males and females. A similar effect was observed within groups treated with IQ and epicatechin. However, these differences were not found to be statistically significant. There were also no statistically significant differences in DNA damage in the age distributions between patients as well as between control individuals (H_2O_2 with quercetin experiment: patients' mean age = 42.4 years ± 11.6, control individuals' mean age = 28.9 years ± 9.0; IQ with epicatechin experiment: patients' mean age = 39.2 years ± 10.3, control individuals' mean age = 22.6 years ± 9.2). No major differences were seen due to smoking and/or drinking habits (Table 2).

Confounding factors	H_2O_2 (0 µM) + Quercetin (0 µM)				IQ (0 µM) + Epicatechin (0 µM)			
	Defining factors	**N**	**OTM**	**p-value**	**Defining factors**	**N**	**OTM**	**p-value**
Type of IBD[#]	Ulcerative colitis	4	5.3 ± 0.5		Ulcerative colitis	4	3.5 ± 0.1	
	Crohn's disease	4	9.8 ± 0.6	p < 0.001	Crohn's disease	4	7.3 ± 0.7	p < 0.001
	Indeterminate	2	7.3 ± 0.9		Indeterminate	2	7.0 ± 2.2	
Age[Δ]	0-25	7	7.9 ± 2.3		0-25	7	8.2 ± 0.5	
	26-45	8	11.3 ± 02	p = 0.298	26-45	9	12.4 ± 2.5	p = 0.395
	More than 46	5	13.0 ± 3.3		More than 46	4	10.7 ± 0.1	
Smoking[Δ]	Active smokers	9	12.4 ± 0.6		Active smokers	9	7.4 ± 3.2	
	Ex-smokers	3	7.3 ± 1.2	p = 0.357	Ex-smokers	2	15.5 ± 0.3	p = 0.090
	Non-smokers	8	9.5 ± 2.1		Non-smokers	9	12.4 ± 1.1	
Drinking habit[Δ]	Non-alcoholic	7	10.6 ± 1.1		Non-alcoholic	10	12.7 ± 1.0	
	Moderate	8	9.3 ± 0.8	p = 0.704	Moderate	9	7.6 ± 4.2	p = 0.123
	Severe	5	12.2 ± 0.4		Severe	1	15.0 ± 0.7	
Ethnic origin[Δ]	Caucasian	13	10.6 ± 1.4	p = 0.905	Caucasian	12	9.8 ± 0.9	p = 0.487
	Asian	7	10.3 ± 0.3		Asian	8	11.6 ± 2.7	
Gender[Δ]	Female	13	10.1 ± 2.2	p = 0.700	Female	10	10.3 ± 3.0	p = 0.838
	Male	7	11.1 ± 0.9		Male	10	9.7 ± 2.3	

Table 2. Details of patient and control groups relating to confounding factors and their significant differences. OTM – Olive tail moment; # - only patient values included, values for healthy individuals shown in Table 1; Δ - patient and control group values combined

3.1.3.2 Previous medication in the IBD group as a confounding factor

Patients had been treated with a range of drugs for IBD, namely, azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study. Azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 6); asacol (n = 1); pentasa & prednisolone, prednisolone & mesalazine (n = 3). Within the treatment groups, there appeared to be differences but they were not significant.

3.2 Study II: colon cancer patients

3.2.1 Patient versus control groups

Treating lymphocytes from healthy individuals and cancer patients with food mutagens IQ and PhIP *in vitro* resulted in a dose-dependent statistically significant induction of DNA

damage for both parameters measured, Olive tail moment (OTM) and % tail DNA (Table 3). For the 25µM IQ treatment of lymphocytes from colon cancer patients, the induced DNA damage measured in % tail DNA reached significance while the evaluated OTM did not.

	Healthy individuals	Colon cancer patients	Healthy individuals	Colon cancer patients
	Mean OTM ± SE		% tail DNA ± SE	
Negative control	2.10 ± 0.30	4.10 ± 0.42 §§§	10.95 ± 1.30	18.18 ± 1.25 §§§
25 µM IQ	3.23 ± 0.31 **	4.84 ± 0.47 §§	14.69 ± 1.01 **	20.12 ± 1.26 * §§§
75 µM IQ	5.50 ± 0.38 ***	6.18 ± 0.40 ***	21.82 ± 1.27 ***	24.76 ± 1.23 *** §
100 µM IQ	6.03 ± 0.43 ***	6.97 ± 0.49 ***	23.98 ± 1.28 ***	25.90 ± 1.54 ***
150 µM IQ	8.47 ± 0.58 ***	9.75 ± 0.53 *** §	33.80 ± 1.22 ***	35.52 ± 1.94 ***
150 µM IQ and 100 µM Q	7.67 ± 0.51	7.24 ± 0.37 †††	29.06 ± 1.05 ††	30.11 ± 1.48 †
150 µM IQ and 250 µM Q	5.90 ± 0.46 †††	7.32 ± 0.54 †††	22.53 ± 1.62 †††	28.86 ± 1.52 †† §§
150 µM IQ and 500 µM Q	3.44 ± 0.26 †††	5.81 ± 0.71 † §§	16.13 ± 1.16 †††	24.57 ± 2.39 ††† §§
150 µM IQ and 50 µM R	7.76 ± 0.59	7.21 ± 0.37 †††	27.42 ± 1.59 †	28.95 ± 1.13 ††
150 µM IQ and 250 µM R	7.27 ± 0.47 †	6.63 ± 0.37 †††	26.21 ± 1.32 ††	26.74 ± 1.20 †††
150 µM IQ and 500 µM R	4.12 ± 0.59 †††	5.18 ± 0.50 †††	15.98 ± 1.78 †††	21.82 ± 1.38 ††† §§
Negative control	2.90 ± 0.36	3.55 ± 0.30	13.53 ± 1.30	16.51 ± 0.96 §
10 µM PhIP	3.75 ± 0.41	4.38 ± 0.33 **	16.92 ± 1.42 *	19.95 ± 1.20 ** §
25 µM PhIP	5.57 ± 0.49 ***	5.41 ± 0.33 ***	23.31 ± 1.52 ***	24.15 ± 1.03 ***
50 µM PhIP	5.70 ± 0.54 ***	7.07 ± 0.49 *** §	24.01 ± 1.58 ***	27.79 ± 1.36 *** §
75 µM PhIP	8.58 ± 0.85 ***	8.27 ± 0.71 ***	31.05 ± 2.52 ***	33.79 ± 1.64 ***
75 µM PhIP and 100 µM Q	6.80 ± 0.58 †	6.75 ± 0.53 †	27.16 ± 1.46 †††	27.68 ± 1.36 ††
75 µM PhIP and 250 µM Q	5.99 ± 0.58 ††	5.71 ± 0.49 ††	23.99 ± 1.68 †††	24.35 ± 1.41 ††
75 µM PhIP and 500 µM Q	3.72 ± 0.43 †††	4.17 ± 0.45 †††	16.73 ± 1.41 †††	18.81 ± 1.50 †††
75 µM PhIP and 50 µM R	7.63 ± 0.56	7.25 ± 0.47 †	30.07 ± 1.79	29.28 ± 1.52 †
75 µM PhIP and 250 µM R	6.39 ± 0.41 ††	6.39 ± 0.39 ††	25.38 ± 1.03 ††	27.13 ± 1.02 ††
75 µM PhIP and 500 µM R	4.34 ± 0.30 †††	5.85 ± 0.56 †† §	18.94 ± 0.72 †††	24.36 ± 1.54 ††† §§

Table 3**.** DNA damage induced *in vitro* in lymphocytes from healthy individuals and colon cancer patients by the food mutagens IQ and PhIP and its reduction by flavonoid supplementation with various concentrations of quercetin (Q) and rutin (R).

Significantly different from the negative control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$
Significantly different from highest dose of food mutagen IQ: † $p<0.05$; †† $p<0.01$; ††† $p<0.001$
Significantly different from healthy individuals: § $p<0.05$; §§ $p<0.01$; §§§ $p<0.001$

Different concentrations of the flavonoids quercetin (100, 250 and 500 µM) and rutin (50, 250 and 500 µM) showed modulating effects on human lymphocytes of both donor groups in the presence of high doses of food mutagens, 150 µM IQ or 75 µM PhIP (Table 3). In the majority of the experiments supplementation with flavonoids resulted in a significant dose-dependent reduction of the induced DNA damage ranging from 1.4 to 2.5 times. For lymphocytes from healthy individuals, only the lowest quercetin dose together with IQ and the lowest dose for rutin together with IQ and PhIP measured in OTM, as well as the lowest dose of rutin together with PhIP when evaluating % tail DNA did not reach significant levels. At the highest supplemented flavonoid dose, the DNA damage from a high dose of food mutagen was significantly reduced to levels of damage in lymphocytes (from both donor groups) which was comparable to a treatment with a six times lower dose of the food mutagen IQ and a 7.5 times lower dose of PhIP, respectively.
Intergroup comparisons showed lower basic DNA damage in lymphocytes from healthy individuals (negative control) when compared to those from colon cancer patients (Figure 3 and Table 3). This difference was highly significant ($p < 0.001$ for parameters, OTM and %

tail DNA) for the negative control of the IQ experiment and significant for the PhIP experiment ($p < 0.05$ for % tail DNA; the OTM parameter did not reach significance: $p = 0.085$). Also, after treatment with food mutagens IQ and PhIP this higher baseline damage led to a significantly higher induction of DNA damage in lymphocytes from cancer patients for IQ concentrations of 25 µM ($p < 0.01$ for OTM; $p < 0.001$ for % tail DNA), 75 µM ($p < 0.05$ for % tail DNA) and 150 µM ($p < 0.05$ for OTM) as well as PhIP concentrations of 10 µM ($p < 0.05$ for % tail DNA) and 50 µM ($p < 0.05$ for OTM & % tail DNA).

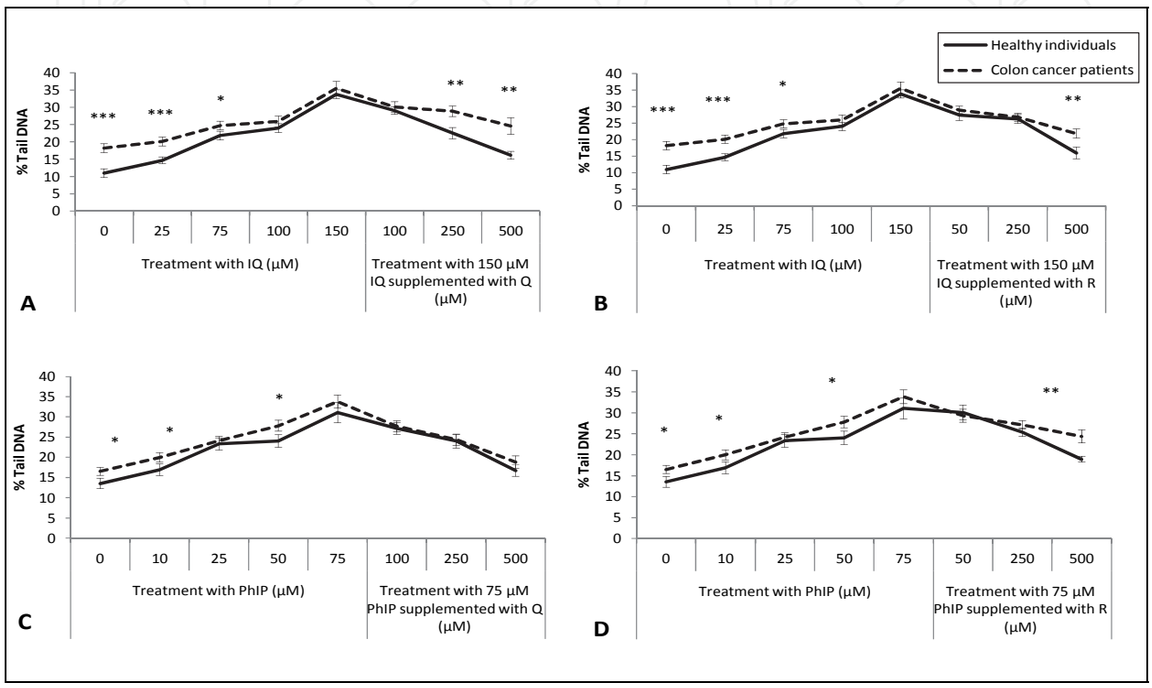

Fig. 3. The Comet parameter % tail DNA is shown indicating DNA damage in lymphocytes after *in vitro* treatment with IQ (25, 75, 100 and 150 µM) and then supplementation of the highest dose with either the flavonoid quercetin (Q) [Panel A] or rutin (R) [Panel B] as well as treatment with PhIP (10, 25, 50 and 75 µM) and then supplementation of the highest dose with either the flavonoid quercetin (Q) [Panel C] or rutin (R) [Panel D]. The flavonoids were used at different concentrations from 50 µM up to 500 µM. Intergroup comparisons of healthy individuals and colon cancer patients revealed significantly increased induction of DNA damage, when treating with heterocyclic amines alone and together with flavonoids, between healthy individuals and the colon cancer patient group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). All other types of data comparisons are shown in Table 3.

When supplementing a single high-dose treatment of either IQ (150 µM) or PhIP (75 µM) with flavonoids (quercetin or rutin), the intergroup comparison showed only at the highest levels of flavonoid supplementation significant differences in the reduction of DNA damage caused by the food mutagen (Figure 3 and Table 3). Except for the supplementation of PhIP with 500 µM of quercetin (Figure 3C), lymphocytes from colon cancer patients showed significantly higher amounts of DNA damage at higher flavonoid concentrations in comparison to healthy volunteers (Figures 3A, 3B & 3D), i.e. less reduction of induced damage by the flavonoid (IQ + 500 µM quercetin, $p < 0.01$ for OTM and % tail DNA; IQ +

250 μM quercetin, $p < 0.01$ for % tail DNA; IQ + 500 μM rutin, $p < 0.01$ for % tail DNA; PhIP + 500 μM rutin, $p < 0.05$ for OTM and $p < 0.01$ for % tail DNA). The parameter % tail DNA for genetic damage was more sensitive compared to OTM.

3.2.2 Confounding factors

Confounding factors such as age, gender, diet, smoking habits and alcohol intake were also investigated (Table 4). A significant higher baseline DNA damage ($p < 0.001$) in lymphocytes from colon cancer patients was observed for parameters OTM and % tail DNA compared to those from healthy individuals. There was also a significant difference between subjects of >50 years of age when compared to those under 50 years of age ($p < 0.01$) showing a 1.80-fold and 1.54-fold increased baseline DNA damage for OTM and % tail DNA, respectively. No statistically significant differences were found when focusing on smoking habits, alcohol intake and diet, although, when comparing Western to Asian/vegetarian type diet the OTM parameter almost reached significance ($p = 0.061$). DNA damage in male lymphocytes was significantly ($p < 0.05$) higher than in lymphocytes from females for the Comet assay parameter % tail DNA but not for the OTM parameter ($p = 0.450$).

Confounding factor	Sub-groups	N	Mean OTM ± SE	% tail DNA ± SE	Description
Diagnosis	Healthy	20	2.10 ± 0.30	10.95 ± 1.30	Colon cancer patients vs. healthy individuals
	Colon cancer	20	4.10 ± 0.42 ***	18.18 ± 1.25 ***	
Age$^{\Delta}$	<50 years	18	2.02 ± 0.32	9.99 ± 1.38	>50 vs. <50 years of age
	>50 years	22	3.64 ± 0.38 **	15.42 ± 1.16 **	
Smoking$^{\Delta}$	Active smokers	7	3.16 ± 0.85	12.12 ± 2.58	Active / ex-smokers vs. non-smokers
	Ex-smokers	16	2.69 ± 0.40	12.27 ± 1.52	
	Non-smokers	17	2.99 ± 0.44	13.80 ± 1.54	
Drinking habit$^{\Delta}$	Severe	15	3.20 ± 0.48	13.69 ± 1.60	Severe / moderate drinking vs. no alcohol
	Moderate	14	2.85 ± 0.47	12.97 ± 1.79	
	No alcohol	11	2.48 ± 0.54	11.66 ± 1.86	
Diet$^{\Delta}$	Western	25	3.22 ± 0.39	13.66 ± 1.38	Western vs. Asian/vegetarian diet
	Asian/vegetarian	15	2.30 ± 0.32	11.58 ± 1.23	
Gender$^{\Delta}$	Female	17	2.50 ± 0.35	10.79 ± 1.07	Female vs. male individuals
	Male	23	3.16 ± 2.97	14.39 ± 1.45 *	

Table 4. Confounding factors for healthy individuals and colon cancer patients and their influence on the baseline DNA damage using the Comet assay. Significantly different from the negative control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. OTM - Olive tail moment; % tail DNA- fraction of DNA in the tail; Δ - patient and control group values combined

4. Discussion

Crohn's disease (CD) and Ulcerative Colitis (UC), known as inflammatory bowel disease (IBD), are fairly common chronic inflammatory conditions of the gastrointestinal tract. Although the exact aetiology of IBD remains uncertain, dysfunctional immunoregulation of the gut is believed to be the main cause. Amongst the immunoregulatory factors, reactive oxygen species (Seegert et al., 2001) are produced in abnormally high levels in IBD (Rezaie et al., 2007). An imbalance between antioxidants and ROS results in oxidative stress, leading to cellular damage (Rezaie et al., 2007) and subsequently cell death or cancer. Colorectal cancer is a heterogeneous neoplasm consisting of cancer cells with various proliferation rates and the potential to metastasise (Ozdemirler Erata et al., 2005). Genetic alterations caused by cellular overproduction of ROS are required for neoplastic progression

(Soderlund et al., 2010). Such changes are based on DNA damage triggered by endogenous but also by environmental and lifestyle genotoxins (Bartsch et al., 2002; Ozdemirler Erata et al., 2005). As the onset of cancer is a prolonged multi-stage process where successive mutations are accumulated, continuous erosion of the genome and defects in repair contribute to this process (Hoeijmakers, 2001; Jiricny and Marra, 2003). Several factors, depending on the socio-economical status, such as large amounts of salted, cured and smoked foods, dietary mutagens, alcohol and obesity may play an important role in increasing mutagenicity leading to an inappropriate stimulation of the immune response or release of ROS subsequently generating further DNA damage (Hursting et al., 2003).

Cooking fish and beef inevitably generate HCA especially at high temperatures (Schut et al., 1999), which are carcinogenic in mice, rats and monkeys producing hepatic, intestinal and mammary tumours (Schoeffner and Thorgeirsson, 2000) and posing a potential risk to humans. HCA have been widely investigated and all of them have so far been described as mutagenic and carcinogenic (Gooderham et al., 2007). Food-derived heterocyclic amines (HCA) like IQ have been shown to be mutagenic in the Ames test inducing gene mutations and tumours *in vivo* (Adamson and Thorgeirsson, 1995; Knize et al., 1995). Food mutagens may cause different types of DNA damage from chromosomal aberrations to subtle nucleotide alterations. Most food mutagens like HCA are able to form reactive DNA adducts by covalently binding to nucleotides. However the effect of food mutagens in carcinogenesis can be modified by heritable traits, namely, low-penetrant genes that affect exposure of the mutagen to DNA through metabolic activation and detoxification or other cellular responses to DNA damage. There is strong evidence that endogenous liver and kidney enzymes or those from commensal bacteria in the intestine are able to metabolically activate HCA which in turn generate DNA damage (El-Zein et al., 2006; Krul et al., 2000). Able to activate and detoxify heterocyclic amines may be enzymes like CYP1A2, *N*-acetyltransferase, sulfotransferase, prolyl tRNA synthetase, phosphorylase and COX isomers (Wolz et al., 2000). In a recent case-control study, no associations were found between colorectal cancer (CRC) risk and polymorphisms within the genes of those enzymes (Sachse et al., 2002). This comprehensive analysis, however, failed to consider commensal bacteria and their potential impact on HCA activation, an effect independent of the host genotype. The pro-carcinogen IQ is predominantly produced through the pyrolysis of creatinine with sugars and becomes significantly mutagenic in the presence of hepatic microsomes (Sugimura and Sato, 1983). Anaerobic colonic bacteria can convert IQ to 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline-7-one (HOIQ), a direct-acting mutagen (Bashir et al., 1987). These commensal bacteria can strongly influence IQ-induced DNA damage in colonic cells and also in hepatocytes as measured by the alkaline comet assay (Knasmuller et al., 2001).

As mentioned before DNA damage seems to be also triggered by oxidative stress. When considering the human diet, it should be recognized that food contains both, mutagens and components that decrease cancer risk such as antioxidants (Goldman et al., 2003; Maeda et al., 1999). Flavonoids are known to have antioxidative properties *in vivo* (Rice-Evans, 2001) and modulate effects of food mutagens *in vitro* in human lymphocytes and sperm (Anderson et al., 1998). Green tea and, to a lesser extent, black tea are a rich source of still another group of flavonoids called catechins. The activity of quercetin is believed to be due to its antioxidative properties, however, it has been suggested that quercetin may also have pro-oxidative activities, which might then directly affect genotoxicity (Lee et al., 2003). Conclusively, quercetin acts as a strong antioxidant and scavenger of free radicals while it

might simultaneously undergo an oxidation process giving rise to the formation of the semiquinone radical (Papiez et al., 2008).

The present studies demonstrate that H_2O_2, PhIP and IQ are capable of inducing significant DNA damage as a result of oxidative stress (Figures 1, 2 and 3, Table 1 and 3). There was a significant increase of DNA damage after treating lymphocytes from healthy controls, IBD and colon cancer patients with H_2O_2, PhIP and IQ, while a significant protective effect was found in the presence of the flavonoids quercetin, rutin and epicatechin (Figures 1, 2 and 3, Table 1 and 3).

In the study I, the protective *in vitro* effect of quercetin and epicatechin against oxidative stress in lymphocytes from IBD patients and healthy individuals (Figure 1) was observed. We were able to show that untreated lymphocytes from IBD patients had significantly increased DNA damage when compared to healthy individuals as shown previously with other IBD patients (Najafzadeh et al., 2007). *In vitro* treatment with H_2O_2 and IQ significantly induced DNA damage by oxidative stress in both groups. Flavonoids reduced the baseline DNA damage in lymphocytes from IBD patients treated with H_2O_2 and IQ. Similar effects were achieved with Chaga mushroom extracts (Najafzadeh et al., 2007). When co-treated with flavonoids, a significant protective effect was shown against free radical damage to the DNA generated by H_2O_2 or IQ (Figure 1). There was a very high level of damage in the patient group without any treatment because of their background inflammation and IBD therapeutic drugs which they had taken, but both patients and controls showed a parallel and gradual reduction in DNA damage after treating with flavonoids (Figure 1 and Table 1).

Lymphocytes from CD patients in two series of study groups appeared to have a greater level of baseline DNA damage than those from UC patients when compared to the whole patient group ($p < 0.001$) , suggesting that lymphocytes from CD patients are more exposed to oxidative stress than other IBD subgroups (Figure 2). It becomes obvious that an excessive production of ROS and radical nitrogen metabolites occur during the inflammation of the intestine in IBD patients (Kruidenier et al., 2003). It seems that a misbalanced production of pro-inflammatory and anti-inflammatory cytokines is characteristic of IBD and severely affects the immune homeostasis in peripheral blood cells, even more in CD than in UC patients (Sventoraityte et al., 2008). However, all subgroups react in the same way towards exogenous oxidative stressors as well as towards the inhibition of oxidative stress by flavonoids.

The detrimental effects of two common food mutagens, IQ and PhIP, on the DNA were investigated in study II by treating *in vitro* lymphocytes from healthy individuals and from patients diagnosed with colon cancer. Pool-Zobel *et al.* found lymphocytes responses to be very similar to responses in rectal cells (Pool-Zobel et al., 2004), thus, supporting their role as surrogate cells for biomonitoring and *in-vitro* treatments. Both HCA caused in a dose-dependent manner similar levels of DNA damage in lymphocytes of both groups for the Comet assay parameters OTM and % tail DNA (Table 3), supporting the classification for IQ as "probably carcinogenic to humans" and for PhIP as "possibly carcinogenic to humans" (IARC, 1993). Higher doses significantly increased the induced DNA damage. IQ and PhIP were shown to be potent genotoxins and carcinogens (Adamson et al., 1995; Durling and Abramsson-Zetterberg, 2005; Duthie et al., 1997). Even very low doses (10^{-3} to 10^{-4} μM PhIP) induce expression of the DNA damage response proteins like p53 and increase proliferation in oestrogen receptor (ER)-negative MCF10A cells (Gooderham et al., 2007). Hence, PhIP may induce/enhance carcinogenicity via DNA damage and/or oestrogen receptors (Bennion et al., 2005; Felton et al., 2004). IQ on the other hand can form DNA adducts like N-

(deoxyguanosin-8-yl)-IQ in the presence of nitric oxide constituting a possible cancer risk for individuals with colon inflammation (Lakshmi et al., 2008).

In our study the DNA damage induced in lymphocytes of both donor groups by food mutagens IQ and PhIP was effectively and dose-dependently reduced by supplementation with the flavonoids quercetin and rutin (Table 3). The level of DNA damage from the highest HCA dose reduced by the highest dose (500 µM) of flavonoids was comparable to that of a six times (for IQ) and 7.5 times (for PhIP) lower non-supplemented dose of food mutagen.

Strong antioxidative effects of flavonoids to protect against DNA damage have been known for some time (Anderson et al., 2003; Collins, 2005; Perez-Vizcaino et al., 2009; Rice-Evans, 2001) and *in-vitro* experiments on human colonocytes suggested that especially quercetin plays a crucial role in the defence against oxidative insults (Duthie and Dobson, 1999). In human lymphocytes quercetin and rutin already showed a dose-dependently protective effect against DNA damage caused by the mutagenic anticancer drug mitomycin C (Undeger et al., 2004). However, neither myricetin, quercetin nor rutin increased the rate of DNA strand break repair in various cell types such as Caco-2, Hep G2 and V79 (Aherne and O'Brien, 2000).

We found that the number of individuals in each group was sufficient to establish statistically significant responses ($p < 0.001$) shown in our study for the food mutagens. Our results indicate that the baseline DNA damage was higher for all experiments in lymphocytes from colon cancer patients when compared to healthy individuals (Table 3 and Figure 3). Disease states which involve an overproduction of ROS may therefore inflict significantly higher DNA damage in peripheral lymphocytes from patients when compared to the baseline level of damage in healthy individuals. This has been shown for diseases like Irritable Bowel Syndrome and diabetes (Collins et al., 1998b; Najafzadeh et al., 2009; Wyatt, 2006). It also confirms findings of Vodicka *et al.* who observed increased chromosomal damage in lymphocytes of newly diagnosed cancer patients compared with healthy controls (Vodicka et al., 2010). Similar observations of a higher baseline DNA damage were made for head and neck squamous cell carcinoma patients (Palyvoda et al., 2003) and breast cancer patients (Rajeswari et al., 2000; Smith et al., 2003) in addition to higher levels of cytogenetic damage (Palyvoda et al., 2003). Even the modulating effect of flavonoids in a co-treatment with a high dose of food mutagen (Table 3) seem to be affected by the higher baseline damage as the induced DNA damage in lymphocytes from colon cancer patients was not reduced to the levels of healthy individuals. Except for the supplementation of PhIP with 500 µM of quercetin (Figure 3C), lymphocytes from colon cancer patients showed significantly higher amounts of DNA damage at higher flavonoid concentrations (Figures 3A, 3B & 3D), i.e. less reduction of induced damage, that may suggest that higher concentrations of flavonoids would be required to achieve a protective effect. A possible reason for this finding could be a reduced repair capacity which was found for breast cancer patients after *in-vitro* treatment of lymphocytes with N-methyl-N-nitro-N-nitrosoguanidine or ionising radiation (Smith et al., 2003). The repair capacity of first degree relatives to these patients was also decreased (Rajeswari et al., 2000; Smith et al., 2003). These differences in repair capacity may either be an effect of cancer *per se* due to a changed lymphocyte population alongside the oncogenic process, or a higher DNA damage and slower repair among some individuals who may be more predisposed to develop cancer (Palyvoda et al., 2003).

An analysis of confounding factors such as age, gender, diet, smoking habits and alcohol intake (Table 4) on the baseline level of DNA damage in lymphocytes in Study II showed a

significant higher damage for subjects of >50 years of age ($p < 0.01$) as previously reported (King et al., 1994; Mendoza-Nunez et al., 2001). When examining the confounding effect aspects the numbers of participants were reduced yet we still found statistically significant effects for age (>50. $p < 0.01$) for both OTM and % tail DNA and for gender (males, $p < 0.05$) for % tail DNA only. An increase in DNA damage in lymphocytes has been observed among elderly individuals but was not significant (Betti et al., 1994; Mendoza-Nunez et al., 2001). We are aware our groups are not best matched for age however these were the only individuals available at the time. Despite this fact we were able to detect an age effect in a >50 age group. Although smoking and alcohol consumption is associated with an increased risk for colorectal cancer (Bardou et al., 2002; Emmons et al., 2005) probably due to DNA damage via increased levels of oxidative stress (Lodovici and Bigagli, 2009; Obe and Anderson, 1987; Pool-Zobel et al., 2004), no statistically significant differences were found in our study when focussing on smoking habits, alcohol intake or diet. This was also the case in the IBD study (Study I). DNA damage in male lymphocytes, however, was significantly higher than in lymphocytes from females for the Comet assay parameter % tail DNA but not for OTM. According to the literature, male gender constituted a risk factor for DNA damage (Collins et al., 1998a), where elderly males had more than twice the probability of having DNA damage than females (Mendoza-Nunez et al., 2001). After *in vitro* treatment with IQ and PhIP an increase in DNA damage in lymphocytes was observed in our study but confounding factors such as diet, smoking and drinking habits did not seem to influence this response, even though numerous environmental and lifestyle compounds can have an impact on the exposure, metabolism and cell proliferation response of HCA (Felton et al., 2004). Nevertheless, in most of the cases the damage was similarly distributed and the general response to treatment may still be dependent on possible dietary interactions after exposure to food mutagens and individual factors like individual susceptibility, cellular antioxidant / micronutrient levels or disease states (Airoldi et al., 2004; El-Zein et al., 2006; Ferguson and Philpott, 2008; Han et al., 2008; Pool-Zobel et al., 2004). It has been estimated that up to 90% of the cases of colorectal cancer could be prevented with life style modifications such as balanced diet, avoidance of smoking and alcohol and physical activity (Boursi and Arber, 2007).

5. Conclusion

In conclusion, a significant protective effect of the flavonoids quercetin, epicatechin and rutin against oxidative DNA damage has been demonstrated after oxidative stress has been induced *in vitro* by H_2O_2, heterocyclic amines IQ and PhIP in lymphocytes obtained from IBD and colon cancer patients and healthy individuals. Lymphocytes from IBD, cancer patients and controls had increased DNA damage possibly due to an overproduction of ROS and there was a significant difference in response between all donor groups to treatment with HCA alone and together with flavonoids in both studies. It is believed that flavonoids operate through their antioxidant properties and responses of H_2O_2 and HCA to quercetin, epicatechin and rutin observed throughout the study, support the hypothesis that food mutagens target DNA by generating ROS. Concepts such as chemoprevention specifically focus on the long-term use of protective agents like vitamins (Boursi et al., 2007; Das et al., 2007). As shown, flavonoids have impressive antioxidant properties and are able to effectively protect the integrity of lymphocytes' DNA from ROS induced by HCA, which would make them the ideal supplemental compounds to prevent the onset of cancer

(Dashwood, 1999; Ross et al., 2002). Although the possible health benefits of consuming flavonoids seem to be obvious, there are so many biological activities attributed to them, that further study may be justified.

The flavonoids significantly reduce DNA damage *in vitro* in lymphocytes of IBD and colorectal cancer patients as well as healthy individuals. Thus, a diet containing flavonoids could be very effective in reducing baseline and exogenously induced oxidative DNA damage of IBD and colorectal cancer patients.

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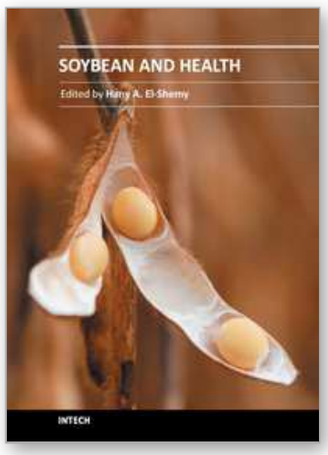

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