

DNA damage and repair activity after broccoli intake in young healthy smokers

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Received on April 28, 2010; revised on July 9, 2010;
accepted on July 19, 2010

Cruciferous vegetables contain compounds with antioxidant properties (e.g. carotenoids, vitamin C and folates) and can alter the activity of xenobiotic metabolism (i.e. isothiocyanates). These constituents may be particularly important for subjects who are exposed to free radicals and genotoxic compounds, including smokers. The aim of the study was to evaluate the effect of broccoli intake on biomarkers of DNA damage and repair. Twenty-seven young healthy smokers consumed a portion of steamed broccoli (250 g/day) or a control diet for 10 days each within a crossover design with a washout period. Blood was collected before and after each period. The level of oxidatively damaged DNA lesions (formamidopyrimidine DNA glycosylase-sensitive sites), resistance to *ex vivo* H₂O₂ treatment and repair of oxidised DNA lesions were measured in peripheral blood mononuclear cells (PBMCs). We also measured mRNA expression levels of repair and defence enzymes: 8-oxoguanine DNA glycosylase (*OGG1*), nucleoside diphosphate linked moiety X-type motif 1 (*NUDT1*) and heme oxygenase 1 (*HO-1*). After broccoli consumption, the level of oxidised DNA lesions decreased by 41% (95% confidence interval: 10%, 72%) and the resistance to H₂O₂-induced DNA strand breaks increased by 23% (95% CI: 13%, 34%). Following broccoli intake, a higher protection was observed in subjects with glutathione S-transferase (GST) M1-null genotype. The expression level and activity of repair enzymes was unaltered. In conclusion, broccoli intake was associated with increased protection against H₂O₂-induced DNA strand breaks and lower levels of oxidised DNA bases in PBMCs from smokers. This protective effect could be related to an overall improved antioxidant status.

Introduction

Diet, together with healthy lifestyle, plays a significant protective role against the development of several chronic diseases such as cancers (1). Specific groups of vegetables are particularly rich in potentially protective phytochemicals; the Cruciferae and especially *Brassica* genus contains high concentration of constituents with antioxidant properties (e.g. carotenoids, vitamin C and folates) as well as glucosinolate precursors of isothiocyanates (ITCs) and indoles that modulate the activity of xenobiotic biotransformation (2). Indeed, many studies point to specific cancer protective effects of cruciferous vegetables towards at least lung and stomach cancer (3,4).

One protective mechanism of action of *Brassica* vegetables is thought to involve reduction of free radical-related molecular damage and this could be particularly important in subjects with high exposure. Cigarette smoke contains a large amount of reactive oxygen species (ROS) as well as other substances that generate ROS (5). ROS can cause oxidative damage to DNA such as oxidised bases and strand breaks. These DNA lesions, including the promutagenic 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) could be implicated in early steps of cancer development (6,7). Fortunately, mammalian cells have a large array of repair systems for the removal of oxidised DNA lesions; the most important pathway for oxidised bases is considered to be base excision repair (BER), whereas the nucleotide excision repair enzymes most likely functions as a backup system. The repair of 8-oxodG by the BER system is initiated by 8-oxoguanine DNA glycosylase (*OGG1*), which cleaves the *N*-glycosidic bond leaving behind a free base and an AP-site (apurinic/aprimidinic site) (8,9). In the nucleotide pool, dGTP can be oxidised to 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP), which may give rise to 8-oxodG in DNA if the oxidised nucleotide is incorporated during replication or repair. 8-Oxo-dGTP is removed from the nucleotide pool by the nucleoside diphosphate linked moiety X-type motif 1 (*NUDT1*) enzyme (10).

In a previous study (11), we observed that the intake of broccoli increased resistance to *ex vivo* H₂O₂-induced DNA strand breaks in peripheral blood mononuclear cells (PBMCs) of smokers and non-smokers, whereas oxidation of purines was reduced significantly only in smokers. It has been debated whether or not smokers *per se* have different levels of biomarkers measured by the comet assay; for instance, it has been suggested that publications from countries in southern part of Europe had a tendency to show a large effect of smoking because the brands of tobacco products were stronger than the brands in the Northern part of Europe (12). This is in keeping with a meta-analysis documenting that smokers have higher level of DNA damage measured by the comet assay, although there was large heterogeneity between publications (13).

Results obtained in our previous dietary intervention study (11) indicate that broccoli constituents may act either as free radical scavengers or may increase the endogenous defense against oxidative stress, and this could be particularly

important in subjects who smoke. For this reason, we developed a new intervention study to further investigate the effect of broccoli consumption on DNA damage (endogenous and oxidatively induced) and defense systems, including OGG1 repair activity as well as expression levels of *OGG1*, *NUDT1* and *heme oxygenase (decycling) 1 (HMOX1)* or commonly referred to as *HO-1* in PBMCs from smokers. HO-1 is the rate-limiting enzyme in the degradation of heme to equimolar biliverdin, carbon monoxide and ferrous iron and is considered an important inducible protective mechanism against oxidative stress (14). The transcription factors for expression of *HO-1* include the redox-sensitive *nrf2*, which is also involved in the induction of phase II enzymes through the antioxidant responsive element by ITCs (15). Thus, we may hypothesise that an enhanced expression of *HO-1* in lymphocytes can be considered as part of such enzyme induction and a response to oxidative stress, possibly modulated by nutritional interventions. Studies in cultured cells indicated that ITCs can enhance *HO-1* expression (16); to the best of our knowledge, such findings have not been reported or investigated in a human intervention trial.

The subjects enrolled in our study were also genotyped for the common polymorphism determining the presence of glutathione *S*-transferase (GST) M1, which is involved in the elimination of ITCs and may modulate the effects of cruciferous vegetables (3,4,17–19).

Materials and methods

Dietary intervention study

Thirty healthy smokers were recruited within the student population of the University of Milan and enrolled on the basis of their food habits, evaluated by means of a food frequency questionnaire, in order to have a homogenous group for lifestyle and food intake and comparable with the characteristics of the group previously studied. The subjects were included in the study if they were males <30 years and if they smoked >10 cigarettes per day.

Exclusion criteria were high (more than five portions per day) or low (less than two portions per day) intake of fruit and vegetables, regular use of medications or dietary supplements, habitual alcohol consumption (less than three drinks per week were tolerated) and adherence to specific vegetarian diets (e.g. vegan or macrobiotic). The study was approved by the Local Ethical Committee and in accordance with the Declaration of Helsinki and informed consent was signed by each participant. Characteristics of subjects recruited for the study are reported in Table I.

The investigation was designed as a crossover study. The volunteers were randomly divided into two groups of 15 subjects as follows: Group 1 was assigned to the sequence broccoli diet/washout/control diet, whereas Group 2 followed the sequence control diet/washout/broccoli diet. The period of broccoli or control diet was 10 days long and there was a 20-day washout period between dietary treatments. The study was conducted during the period of February to May 2007, which was carried out independently of our previous

intervention study on broccoli consumption (March to May 2006). Five of the subjects in our previous study were also recruited for the present study.

Broccoli and control diet

We used frozen ‘Marathon’ broccoli (*Brassica oleracea* L. var. *italica*) (Di Stasi Company, Basilicata, Italy), as in the previous study, however, this product, grown 1 year later, had a lower concentration of glucosinolates as evaluated by high-performance liquid chromatography analysis (108 versus 200 µmol ITCs per portion), thus the portion prepared for the volunteers was slightly increased. Apart from ITCs, each portion of broccoli provided 3.1 mg of lutein, 1.4 mg of β-carotene and 146 mg of vitamin C. We have used plasma concentrations of carotenoids as marker of broccoli intake and effectiveness of the intervention because these phytochemicals are not expected to be biotransformed by GST enzymes, plasma concentrations of ITCs were considered a priori to be suboptimal as marker because there could be effect-modification by GST polymorphisms. Moreover, we evaluated folate plasma concentrations, which were significantly increased in the previous broccoli intervention.

During the trial, every day broccoli was steam-cooked for 15 min and portioned (250 g) into appropriate food containers that were given to the subjects.

On Fridays, subjects were given two extra portions of broccoli to eat during the weekend. The subjects were instructed to keep their habitual diet during the entire trial. In addition, they were told to avoid the intake of cruciferous vegetables throughout the trial. We assessed the food intake during the study by a food diary, which the subjects were instructed to update daily.

Blood samples. Peripheral blood samples were collected in microtubes with heparin at the beginning and at the end of each treatment period (0, 10, 30 and 40 days). Samples were drawn early in the morning after overnight fasting.

PBMCs were isolated by density gradient centrifugation of 100 µl whole blood with Histopaque 1077 (Sigma Chemicals Co, St Louis, MO, USA). The PBMCs layer was removed from the gradient and the cells were washed with phosphate-buffered saline (PBS), centrifuged and used immediately for the determination of *ex vivo* resistance to H₂O₂-induced DNA strand breaks. A different batch of isolated PBMCs was suspended in a solution containing 50% foetal bovine serum (FBS), 40% culture medium (RPMI) and 10% dimethyl sulphoxide (DMSO) and stored at –80°C for the determination of formamidopyrimidine DNA glycosylase (FPG)-sensitive sites, DNA repair activity and expression levels of *OGG1*, *HO-1* and *NUDT1*. Moreover, serum and plasma were isolated from blood in order to evaluate carotenoids and folate concentrations.

Polymorphisms analysis GSTM1. A polymerase chain reaction (PCR) method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes in genomic DNA samples obtained from blood samples through DNeasy® Blood and Tissue kit (QIAGEN) following the manufacturer’s technical instruction.

DNA was amplified in a total reaction volume of 25 µl containing 4 µl of DNA, 1.5 mM dNTPs, 1.5 mM MgCl₂, 5 pmol of each oligonucleotide primer and 0.12 µl (5 U/µl) TaqGOLD. DNA samples were amplified using the following primers: for *GSTM1*, forward primer 5’-GGGCTCAAATA-TACGGTGGGA and reverse primer 5’-GGAGGAACCTCCCTGAAAAGC; for *GSTT1*, forward primer 5’-GGCCTTCTTACTGGTCCTC and reverse primer 5’-GAAGAGGTCCTCCCCACT; for albumin, forward primer 5’-AAAGC-CAGAGCTGGAAGTCA and reverse primer 5’-CAGCTTTGG-GAAATCTCTGG.

PCR was performed with TaqGOLD activation for 5 min at 95°C, the temperature profile repeated for the first two cycles was denaturing 30 sec at 95°C, annealing 15 sec at 60°C and extension 30 sec at 72°C; for other two cycles, denaturing 30 sec at 95°C, annealing 15 sec at 58°C and extension 30 sec at 72°C; for other 2 cycles, denaturing 30 sec at 95°C, annealing 15 sec at 56°C and extension 30 sec at 72°C and 29 cycles with denaturing 30 sec at 95°C, annealing 15 sec at 54°C and extension 30 sec at 72°C and final extension for 5 min at 72°C and storage at 4°C. The amplified products were electrophoresed through 2% agarose gel and visualised by ethidium bromide staining.

Resistance to H₂O₂-induced DNA strand breaks in PBMCs. The resistance to H₂O₂-induced strand breaks in PBMCs was evaluated by means of the Comet assay as previously reported (20). The measurement of H₂O₂-induced DNA strand breaks is considered to be a marker of the cellular resistance to oxidative stress because H₂O₂ generates DNA damage by oxidative attack. Briefly, each measurement consisted of duplicate slides as follows: one slide was subjected to a H₂O₂ treatment (submerging it into a solution of H₂O₂ 500 µM in PBS for 5 min in the dark), whereas the other one acted as a control (submerging it in

Table I. Characteristics of subjects before the intervention study (mean values ± SD)

Characteristics	Number of subjects	Results
Age (years)	27	22.1 ± 2.5
BMI (kg/m ²)	27	23.0 ± 2.7
H ₂ O ₂ -induced strand breaks (% DNA in tail)	27	62.9 ± 8.8
FPG-sensitive sites (%DNA in tail)	27	14.0 ± 6.3
OGG1 activity (%DNA in tail)	11	1.9 ± 2.3
mRNA expression of NUDT1/18S (×10 ^{–6})	17	0.5 ± 1.3
mRNA expression of OGG1/18S (×10 ^{–6})	17	4.6 ± 5.8
mRNA expression of HO-1/18S (×10 ^{–5})	17	1.6 ± 3.0

BMI, body mass index.

PBS for 5 min). The slides were then immersed in lysis solution (0.1 M Na₂EDTA, 10 mM Tris, 2.5 M NaCl, 1% sarcosine, pH 10 with NaOH, 1% Triton X-100 and 1% DMSO added just before use). After lysis of the cells, slides were immersed in an electrophoresis solution (0.3 M NaOH, 1 mM Na₂EDTA; pH > 13) for 40 min, prior to an electric field being applied (25 V, 300 mA, 20 min). The slides were then neutralised to pH 7, stained with ethidium bromide (2 µg/ml) and analysed using an epifluorescence microscope (Olympus CX 41; Olympus Italia) attached to a high-sensitivity CCD video-camera (CFW 1808M; Scion Corporation, Germany) and to a computer provided with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). DNA damage was expressed as percentage DNA in tail. For each subject, the percentage DNA in tail of control cells (not treated with H₂O₂) was subtracted from the percentage DNA in tail of treated cells.

FPG-sensitive sites. Detection of endogenous oxidised DNA base was performed by means of the enzyme FPG, in order to detect the major purine oxidation product (8-oxo-7,8-dihydroguanine) as well as other altered purines. We have participated in the European Comet Assay Validation Group, demonstrating that the intra-assay variation of the comet assay is very low and we could detect dose-dependent increase in FPG sites in coded samples from monocytic THP1 standardised cells (21,22). In the present study, cryopreserved PBMCs were used, thus a higher inter-individual variability is not excluded. Moreover, cold storage can partially affect the amount of strand breaks; however, the subtraction of endonuclease buffer (EB) data from FPG data is sufficient to correct for this effect.

In brief, cryo-preserved PBMCs were thawed and washed with fresh RPMI medium and cold PBS and then embedded in agarose on fully frosted slides to perform the Comet assay as above described. After the lysis phase, slides were immersed in 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin (BSA), pH 8.0 with KOH, used also for the enzyme dilution for three times 5 min each. Then, 50 µl of a diluted solution of FPG (100 ng/ml) or buffer alone (control) was added and the slides incubated at 37°C for 45 min. The FPG enzyme was a gift from Professor Andrew Collins, University of Oslo, Norway. FPG, added to the DNA in the gel, converts altered purines into DNA breaks (23).

Alkaline treatment and electrophoresis then followed. Net enzyme-sensitive sites were calculated by subtracting the % DNA in tail in the slides incubated with buffer from the % DNA in tail obtained by incubating the slides with FPG.

DNA repair activity. The DNA repair activity was determined in cryo-preserved PBMCs. Assessment of DNA repair incisions was analysed by the Comet assay as previously described by Guarnieri *et al.* (24).

The repair activity was measured as the incision activity of substrate DNA treated with Ro19-8022/white light, which generates 8-oxo-7,8-dihydroguanine (24–26). Oxidised bases were introduced into A549 lung epithelial cells substrate nuclei by irradiating cells with white light in PBS with 1 µM Ro 19-8022 (the photosensitiser was a gift from F. Hoffmann-LaRoche, Basel, Switzerland) at 0°C. The cells were washed and resuspended in freezing medium (50% FBS, 40% RPMI and 10% DMSO) to a concentration of 3 × 10⁶ cells/ml and frozen at –80°C.

For the preparation of human PBMCs extracts, the cryo-preserved cells were centrifuged (300 g, 5 min and 4°C), and the pellet was resuspended in buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol; pH 7.8) at a volume of 20 µl per 10⁶ cells. The resuspended cells were divided in aliquots of 50 µl to which 12 µl 1% Triton X-100 was added. The lysate was centrifuged (700 g, 5 min and 4°C) and the supernatant was mixed with 200 µl buffer B (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA and 0.2 mM BSA; pH 8). Approximately 3 × 10⁴ substrate cells were embedded in agarose and applied on 85 × 100 mm GelBond® films and lysed as described for the Comet assay. After three times washing (5 min each) in buffer B, repair incisions were detected by incubation of the agarose-embedded nuclei with 60 µl PBMCs extract or buffer B for 20 min at 37°C. The subsequent alkaline treatment and electrophoresis were identical to the conditions used to determine DNA damage using the Comet assay. An assay control (a sample for FPG-sensitive sites evaluation) was included in each electrophoresis run.

After neutralisation with 0.4 M Tris–HCl (pH 7.5), cells were placed in 96% ethanol for 1.5 h or overnight. Nuclei were visualised as previously described for strand breaks and FPG-sensitive sites estimation after staining with 40 µl ethidium bromide (4 µg/ml) in PBS solution. The repair activity of the PBMCs extract was determined as the difference in % DNA in tail between parallel gels incubated with extract and control solution.

Quantification of OGG1, HO-1 and NUDT1 mRNA expression in PBMCs by real time reverse transcription–PCR. PBMCs samples were stored in freezing medium. Approximately 0.4 µg RNA was used for complementary DNA

(cDNA) synthesis in a reaction volume of 20 µl using the TaqMan GeneAmp RT–PCR Kit as recommended by Applied Biosystems (Nærum, Denmark). Quantitative PCR reactions were carried out in ABI PRISM 7900HT (Applied Biosystems), using primers and cDNA-specific probes purchased from Applied Biosystems. We used as the reference gene 18S rRNA, which is commercially available as a probe and primer solution (Eukaryotic 18S rRNA Endogenous Control, 4352930E; Applied Biosystems). Below are probes and primers for the genes. Sequence accession ID numbers are from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>; accessed 12 February 2007): *hHO-1*, forward primer 5'-CATGAGGAACCTTCAGAGGGC-3' and reverse primer GATGTGGTACAGGGAGGCCAT-3'; TaqMan probe: 5'-6-FAM-TGACCCGAGACGGCTTCAAGCAGCTG-TAMRA-3' (NM_002133). *hOGG1*, forward primer 5'-AAATTCGAAGGTGTGCGACTG-3' and reverse primer 5'-GCGATGTTGTTGTTGGAGGA-3'; TaqMan probe: 5'-6-FAM-CAAGACCCATCGCAATGCCTTTCTCTTTTAMRA-3' (U96710). *hNUDT1*, forward primer 5'-CATCGAGGATGGGGCTAG-3' and reverse primer CAGAAGACATGCACGTCCATGA-3'; TaqMan probe: 5'-6-FAM-TCGCCACGAACCTCAAACACGATCT-TAMRA-3' (D16581). The PCRs were performed in triplicate using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. For the PCR, the following protocol was used as follows: activation of *Taq* polymerase for 20 sec at 95°C, followed by a total of 45 temperature cycles for 0.01 sec at 95°C and 20 sec at 60°C. In each run, a standard was included and verified on the efficiency plot, and the variation coefficients of the repeated measurements were 2.98% (27).

Determination of carotenoids and folate. Lutein, β-carotene and folate were analysed in blood as previously reported (26). These dietary markers were selected as we previously have demonstrated that the plasma concentrations was increased after 10 days of broccoli consumption, whereas no effect on vitamin C level was observed in the previous study (28).

Statistical analysis. The results were analysed by analysis of variance (ANOVA) with sequence (broccoli intake versus control diet or *vice versa*) as independent factor in order to assess carry-over effects. As no carry-over effects were detected (always $P > 0.05$) data were analysed by ANOVA for repeated measure design. Only results from subjects with a complete set of data available were included in the statistical analysis. This analysis with type of treatment and time as independent factors was used to investigate the effect of broccoli consumption on cell resistance against H₂O₂-induced strand breaks, levels of endogenous oxidatively damaged DNA lesions (FPG-sensitive sites) and DNA repair activity and *OGG1*, *HO-1* and *NUDT1* mRNA expression. Moreover, ANOVA with type of treatment as independent factor was used to evaluate percentage changes [i.e. (after treatment – before treatment)/before treatment × 100] in the different end point of DNA damage considered following broccoli diet with respect to control diet. Lastly, data were also analysed by adding GSTM1 genotype as independent factor.

Differences were considered significant at $P < 0.05$; post-hoc analysis of differences between treatments or genotypes were assessed by the Fisher Least Significant Difference test with $P < 0.05$ as level of statistical significance.

Regression analysis was applied in order to verify correlations between the % changes in plasma carotenoids and folate concentrations registered in the whole experimentation (broccoli and control diet treatments) and the % changes in the end points of DNA damage considered.

Statistical analyses were performed by means of STATISTICA 5.0 software (Statsoft Inc, Tulsa, OK, USA).

Results

Twenty-seven subjects completed the entire study, whereas three subjects dropped out during the experimental period. We ended up having fewer results on oxidatively damaged DNA, repair activity and mRNA expression because of problems during the storage of the samples and we have only included data where the complete set of the four samples were available. No side effects were observed in the whole group of subjects. Figure 1 and Figure 2 show the levels of H₂O₂-induced strand breaks and FPG-sensitive sites in PMBCs following the broccoli diet and control diet treatments, for each single subject.

Despite the inter-individual variation in the response to treatments (where biological variability cannot be excluded),

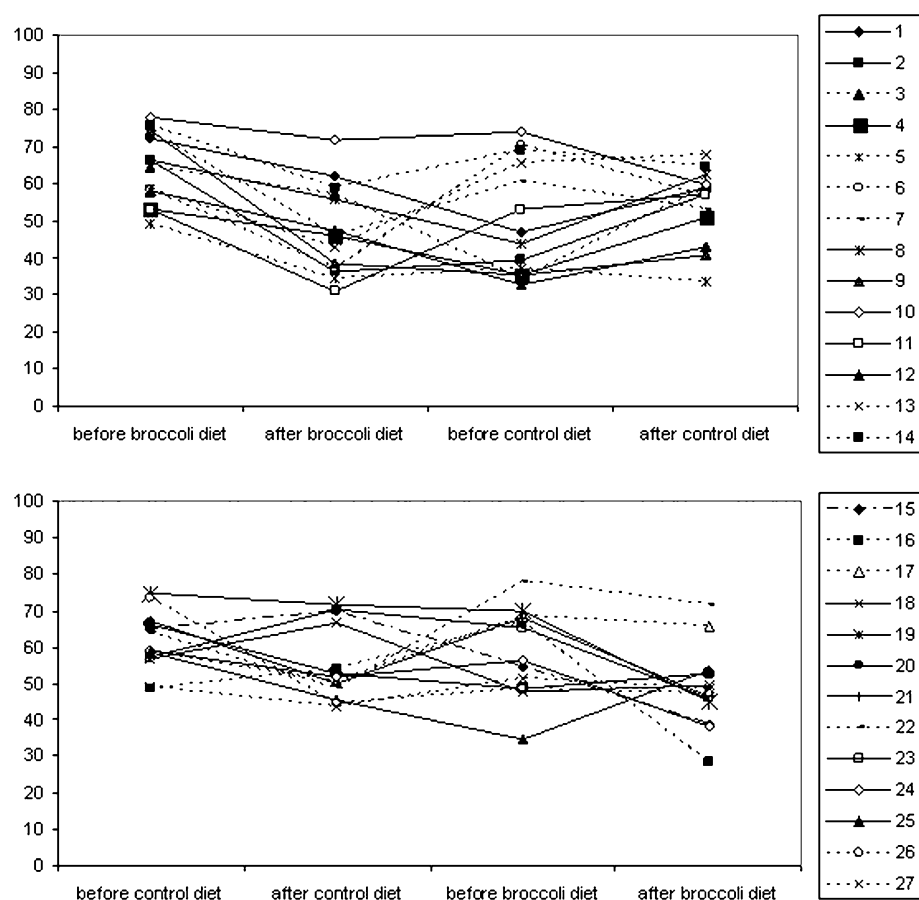


Fig. 1. Effect of broccoli diet and control diet on H_2O_2 -induced strand breaks evaluated by means of comet assay. (a) Individual values registered in Group 1 ($n = 14$) assigned to the sequence broccoli diet/washout/control diet; (b) Individual values registered in Group 2 ($n = 13$) assigned to the sequence control diet/washout/broccoli diet. The symbols (and identification numbers) represent different individuals.

there was a significant reduction in the level of DNA damage following the broccoli diet ($P < 0.01$).

The levels of H_2O_2 -induced strand breaks and FPG-sensitive sites before and after each period are reported in Table II. Background strand breaks (PBS and EB) are also reported. The mean level of H_2O_2 -induced strand breaks in PBMCs was decreased by 23% (95% CI: 13%, 34%) in the period of broccoli intake as compared to the period of control diet (Table II). This difference was driven by a reduction in the level of H_2O_2 -induced strand breaks after the broccoli intake period [−22% (95% CI: −13%, −31%)], whereas there was no difference in the resistance after the control diet [−0.4% (95% CI: −10%, + 9.4%)].

The subjects had 41% (95% CI: 10%, 72%) lower level of FPG-sensitive sites in PBMCs after 10 days of broccoli consumption compared with the control diet. This difference was driven by decreased level of FPG sites in the period of broccoli intake [−34% (95% CI: −53%, 15%)], whereas it was unaltered during the control diet period [4% (95% CI: −20%, +28%)].

Analysis of effect-modification by the GSTM1 polymorphism revealed significant single-factor effects of the genotype ($P < 0.05$) and diet ($P < 0.01$) on H_2O_2 -induced strand breaks, whereas the interaction was not statistically significant ($P > 0.05$). The subjects with GSTM1-null genotype had higher baseline levels of H_2O_2 -induced DNA strand breaks (Table II); however, at the end of the 10 days period of broccoli

consumption, there was no difference in the resistance to *ex vivo* generation of strand breaks depending on the genotypes. Consequently, the effect on resistance towards *ex vivo* generation of strand breaks following broccoli intake (% changes) was most pronounced in subjects with the GSTM1-null genotype (−27.6%, 95% CI: −37.9%, −17.4%), whereas the subjects with GSTM1-positive genotype had lower level of resistance (−13.1, 95% CI: −27.3%, 1.1%) as showed in Figure 3.

In contrast, the effect of broccoli consumption on the level of FPG-sensitive sites was not significantly related to GSTM1 polymorphism (Table II).

The OGG1 repair incision activity, gene expression levels of *OGG1*, *HO-1* and *NUDT1* are reported in Table II; there was no significant effect of broccoli intake on these biomarkers in PBMCs.

The circulating levels of folate and lutein significantly increased after broccoli consumption (+17.1 and +39.3%, respectively), whereas no significant effect on β -carotene concentration was observed (Table III).

Regression analysis showed an inverse and significant correlation between changes in serum folate concentrations and changes in H_2O_2 -induced strand breaks ($R^2 = 0.23$; $P < 0.001$).

Discussion

The present study showed that 10 days of consumption of steamed broccoli by smokers decreased the level of

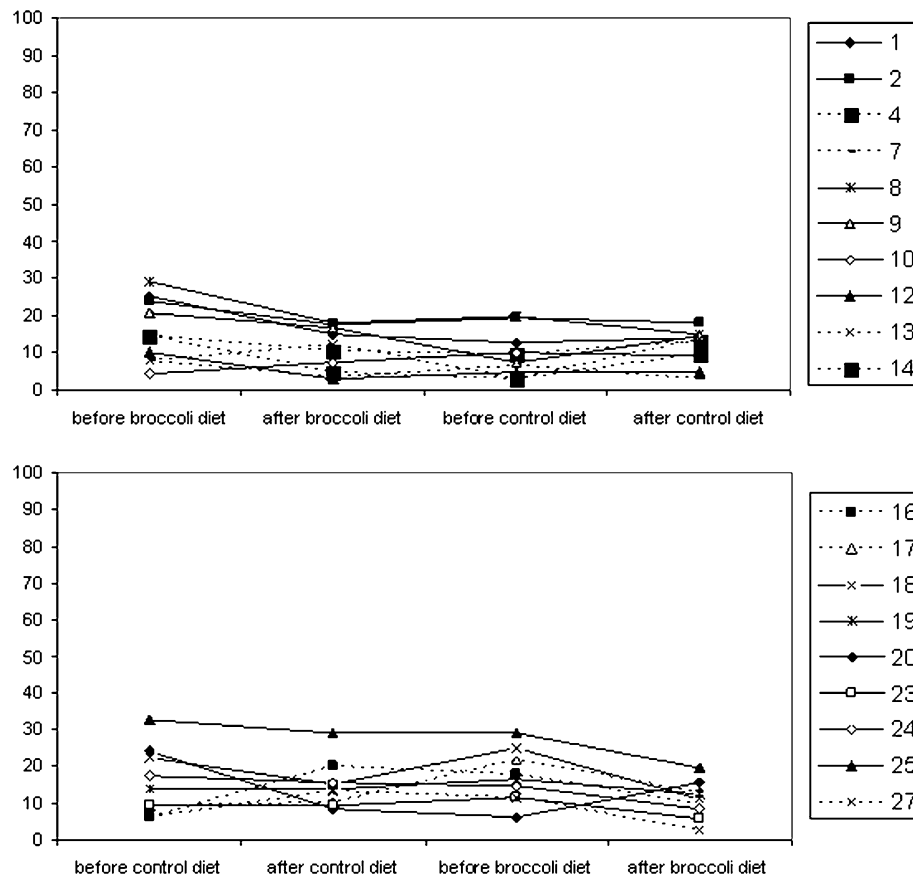


Fig. 2. Effect of broccoli diet and control diet on FPG-sensitive sites evaluated by means of comet assay. (a) Individual values registered in Group 1 ($n = 10$) assigned to the sequence broccoli diet/washout/control diet; (b) Individual values registered in Group 2 ($n = 9$) assigned to the sequence control diet/washout/broccoli diet. The symbols (and identification numbers) represent different individuals.

Table II. H_2O_2 -induced strand breaks, FPG-sensitive sites, DNA repair activity and mRNA expression of *OGG1*, *NUDT1* and *HO-1* before and after broccoli and control diet in smokers

	Before broccoli diet, mean (SD)	After broccoli diet, mean (SD)	Before control diet, mean (SD)	After control diet, mean (SD)
Background SBs (% DNA in tail, PBS)				
All subjects ($n = 27$)	7.5 (2.6)	7.7 (2.3)	7.4 (2.8)	7.4 (2.6)
H_2O_2 -induced SBs (% DNA in tail)				
All subjects ($n = 27$)	61.4 (11.1)	47.9 (11.4)*	55.4 (13.3)	55.2 (9.6)
GSTM1+ ($n = 14$)	57.6 (11.8)	48.8 (12.1)***	52.4 (12.7)	54.0 (9.4)
GSTM1- ($n = 13$)	65.4 (9.1)	46.9 (11.1)*	58.7 (13.7)	56.5 (10.1)
Background SBs (% DNA in tail, EB)				
All subjects ($n = 19$)	23.0 (8.0)	21.3 (6.5)	21.8 (8.8)	19.8 (8.0)
FPG-sensitive sites (% DNA in tail)				
All subjects ($n = 19$)	16.5 (7.7)	11.0 (5.4)**	13.2 (9.8)	13.8 (7.5)
GSTM1+ ($n = 9$)	17.3 (7.2)	11.1 (5.1)***	17.1 (12.0)	16.1 (9.4)
GSTM1- ($n = 10$)	15.9 (8.4)	10.9 (5.9)***	9.8 (6.0)	11.6 (4.8)
DNA repair activity (% DNA in tail)				
All subjects ($n = 11$)	3.7 (3.0)	3.6 (2.5)	3.0 (3.0)	4.4 (3.1)
OGG1 mRNA ($\times 10^{-6}$) ^a				
All subjects ($n = 17$)	6.4 (12.4)	5.4 (6.3)	4.2 (3.9)	6.2 (5.5)
HO-1 mRNA ($\times 10^{-5}$) ^a				
All subjects ($n = 17$)	3.3 (9.0)	2.2 (4.5)	1.5 (2.7)	1.8 (1.6)
NUDT1 mRNA ($\times 10^{-6}$) ^a				
All subjects ($n = 17$)	1.6 (5.5)	0.8 (2.0)	0.6 (1.3)	0.5 (0.7)

SB, strand break.

^aThe mRNA levels are reported as the fold compared to 18S.

*Significant difference between 'before' and 'after' each treatment, $P < 0.001$.

**Significant difference between before and after each treatment, $P < 0.005$.

***Significant difference between before and after each treatment, $P < 0.05$.

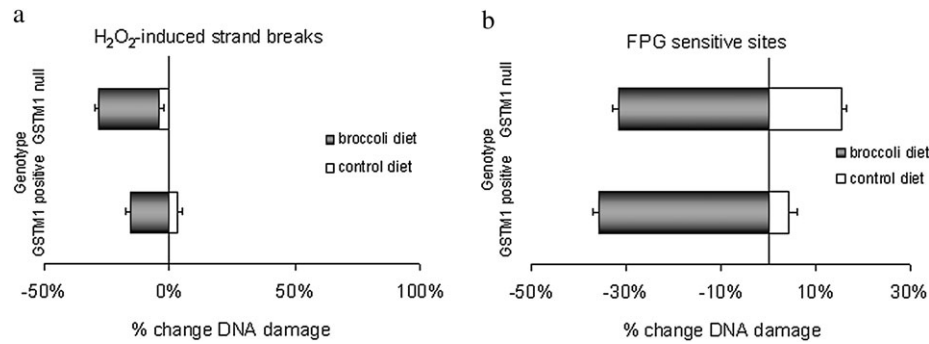


Fig. 3. Effect of broccoli diet and control diet on end points of DNA damage (FPG-sensitive sites and H₂O₂-induced strand breaks) evaluated by means of comet assay. Data reported are mean % change DNA damage [(after treatment – before treatment)/before treatment × 100] and standard error. **(a)** % change DNA damage (H₂O₂-induced strand breaks) in GSTM1-null (*n* = 13) and GSTM1-positive (*n* = 14) subjects. **(b)** % change DNA damage (FPG-sensitive sites) in GSTM1-null (*n* = 13) and GSTM1-positive (*n* = 14) subjects.

Table III. Concentrations of folate (in serum), lutein and β-carotene (in plasma) before and after broccoli and control diet

	Before broccoli diet, mean (SD)	After broccoli diet, mean (SD)	Before control diet, mean (SD)	After control diet, mean (SD)
Folate (nmol/l) (<i>n</i> = 27)	11.3 (3.9)	13.2 (3.3)**	12.0 (5.9)	12.1 (4.7)
Lutein (μmol/l) (<i>n</i> = 27)	0.31 (0.13)	0.43 (0.21)	0.30 (0.14)	0.29 (0.13)
β-carotene (μmol/l) (<i>n</i> = 27)	0.28 (0.25)	0.31 (0.22)*	0.28 (0.24)	0.29 (0.29)

*Significant difference between before and after each treatment, *P* < 0.001.
**Significant difference between before and after each treatment, *P* < 0.01.

endogenous oxidised DNA bases and H₂O₂-induced DNA strand breaks. Whereas, OGG1 repair activity and gene expression levels of *OGG1*, *NUDT1* and *HO-1* were unaltered, at least in the sub-sample of data analysed.

Our data are in accordance with the rather consistently shown protective effects of cruciferous vegetables, including Brussels sprouts, broccoli and watercress, on biomarkers of oxidative damage to DNA in human intervention studies (11,29–34). Such effects may be particularly important in subjects exposed to high levels of ROS, e.g. from cigarette smoke. Indeed, Gill *et al.* (33) showed that smokers had larger reduction in basal and H₂O₂-induced strand breaks in PBMCs than non-smokers following an intervention with 85 g of raw watercress daily for 8 weeks. Similarly, we have found that FPG sites in PBMCs decreased only in smokers, whereas the level of H₂O₂-induced DNA strand breaks decreased significantly both in smokers (*n* = 10) and non-smokers (*n* = 10) consuming 200 g of broccoli daily for 10 days (11).

A lowered level of FPG sites in PBMCs can in principle be caused by a decreased rate of guanine oxidation in DNA or in the nucleotide pool or an increased rate of repair (10). It has previously been observed that consumption of kiwi fruits increased the DNA repair activity and lowered the level of FPG sites in lymphocytes (35). In addition, Guarnieri *et al.* (24) investigated the repair activity towards oxidised DNA in human mononuclear blood cells in two placebo-controlled antioxidant intervention studies, one with well-nourished subjects who consumed 600 g/day of fruits and vegetables or tablets containing the same amount of vitamins and minerals for 24 days and another one with poorly nourished male smokers who consumed 500 mg/day vitamin C as slow- or plain-release formulations together with 182 mg/day vitamin E for 4 weeks. Only male smokers supplemented with slow-release vitamin C tablets had increased DNA repair activity by ~27%, whereas there was no significant change in

subjects supplemented with tablets with plain-release formulation of vitamin C and in subjects who consumed fruits and vegetables. In the present study, with a reduced level of FPG sites after broccoli intake, we found no change in repair activity towards oxidatively damaged guanine in DNA in extracts from the same pool of PBMCs. Similarly, there was no change in the gene expression of *OGG1* and *NUDT1*, the protein of which is responsible for repair of oxidised guanine in DNA and the nucleotide pool, respectively. A possible limitation of the present study is the number of missing data (due to samples storage problems) in the analysis of DNA repair activity and the mRNA expression. Still, it should be emphasised that neither the *OGG1* expression nor the repair activity pointed in the direction of increased level after broccoli intake, indicating that we would not find beneficial effects with even a substantially larger number of subjects in the study.

We found increased resistance towards H₂O₂-induced strand breaks in PBMCs. Thus, our data point mainly to increased resistance to oxidative stress in PBMCs after a broccoli rich diet. Increased resistance to oxidative stress induced by cruciferous vegetables could be related to upregulation of antioxidant, phase II and other defence enzymes and/or increased levels of scavengers of ROS. We found no change in the expression of *HO-1*, which is highly susceptible to oxidative stress and also a part of the *nrf2*-dependent gene battery of phase II enzymes frequently ascribed protective effects of cruciferous vegetables (2). Moreover, in our previous study with a similar broccoli intervention, we did not find modulation of GST activity in plasma (28). Similarly, in the watercress consumption study described above (36), no change in mRNA levels of catalase, glutathione peroxidase 1, glutathione S-transferase A4, glutathione S-transferase P1, superoxide dismutase 2 or UDP glucuronosyl transferase A1 was found in lymphocytes despite considerable decreases in basal and oxidatively induced DNA damage. In the same study,

the activity of SOD and GPX in red cells was not significantly changed overall, although there appeared to be an increase among subjects with the *GSTM1*-null genotype (36). In another study, the activity of SOD and GPX were unchanged after Brussels sprouts intake despite reduction of oxidatively induced DNA damage in lymphocytes (34). Moreover, the intake of regular broccoli changed the expression of only one gene tested in a very large battery of xenobiotic metabolism genes through microarray analysis. On the contrary, high sulphoraphane broccoli changed the expression of more and different genes in the gastric mucosa (37).

We did not detect differences in the level of DNA strand breaks after the broccoli supplementation. This is in keeping with the conclusion from a literature review showing that only very few publications have reported effect on DNA strand breaks in leukocytes (or subsets of leukocytes such as lymphocytes or mononuclear blood cells), whereas there is a larger proportion of publications that have reported an effect of antioxidant supplementation in terms of FPG-sensitive sites or 8-oxodG in leukocytes (38). The null effect in terms of DNA strand breaks in our study is further strengthened by the fact that it was determined in both the analysis of H₂O₂ sensitivity and FPG-sensitive sites. We detected a higher level of DNA strand breaks in the assessment of FPG-sensitive sites than the analysis of H₂O₂ sensitivity. We attribute the difference in the level of DNA strand breaks between the two measurements to methodological differences because the determination of H₂O₂-sensitive sites was carried out on fresh samples, whereas the level of FPG-sensitive sites was determined on cryopreserved samples. It should be noted that the higher level of strand breaks in the determination of FPG-sensitive sites does not affect the validity of the analysis of oxidatively damaged DNA. We have measured a level of FPG-sensitive sites that is similar to the levels reported in other studies (39). However, we did observe inter-individual and intra-individual variation in the level of FPG-sensitive sites and sensitivity to H₂O₂-induced DNA strand breaks. In addition, there is large effect of broccoli intake in some subjects, whereas other subjects appear to have no benefit. We cannot determine whether this heterogeneity is because some subjects are non-responders because this trait would only be revealed if the person participated in several independent intervention trials. However, it should be emphasized that our crossover study was not completely controlled in regard to habits of the subjects. Environmental and occupational exposures could affect the level of DNA damage and sensitivity to H₂O₂-induced DNA strand breaks such as sunlight, air pollution, exhaustive exercise or therapeutics (12). In addition, it is also possible that some subjects entering a trial on beneficial effects of dietary products change toward a healthier lifestyle. In our study this might be displayed as decreased levels of FPG sites and increased resistance toward H₂O₂-induced DNA strand breaks. Still, we believe that the reduction of DNA damage, as found in the present study, may be due to direct or indirect antioxidant scavenger functions of compounds in cruciferous vegetables, including vitamin C, carotenoids, polyphenols, folates and/or some ICTs. Indeed, we have previously shown that the broccoli consumption regime used in the present study increased the plasma concentrations of lutein and β -carotene in smokers, respectively, by ~45 and 33% as well as folate (~16%) and non-indolyl ITCs (~86%) (28). The present study confirms an increase of folate and lutein concentrations after broccoli

consumption, whereas, differently from the previous study, β -carotene levels were not significantly increased after the intervention (probably because of the higher individual variability). We also found a significant inverse correlation between the % changes in folate levels and those of H₂O₂-induced DNA damage possibly providing support for the contribution of the vitamin to the DNA protection against *ex vivo* induced damage and/or repair. Despite the lack of correlation for single carotenoids, we do not exclude a role of carotenoids in the DNA protection because the effect of these antioxidants might depend on the synergy of multiple bioactive substances rather than single compounds.

The broccoli used in the present study had lower concentration of ITCs compared to the broccoli used in our previous study (~110 versus 200 μ mol ITCs per portion provided), whereas there was the same level of protection against *ex vivo* generation of DNA strand breaks and endogenous levels of FPG sites in PMBCs from smokers (11). Thus the mechanisms for reduction of oxidative damage to DNA do not seem to involve specifically the ITCs with enzyme inducing properties. This further supports the notion that whole foods can exert a protective effect in virtue of the numerous compounds present, able to act in synergy.

To our knowledge, this study is the first reporting the possible effect-modification by *GSTM1* polymorphism in relation to modulation of DNA damage following broccoli consumption. In particular, we found an increased resistance to H₂O₂-induced strand breaks after broccoli intake in the group of subjects with the *GSTM1*-null genotype with respect to the positive genotype. Although large-scale systematic studies on the effect of the *GSTM1* polymorphism have not been published, some cross sectional studies on air pollution exposure and diet do suggest that subjects with the *GSTM1*-null and -positive genotype differ in respect to levels and response of FPG sites and H₂O₂-induced strand breaks in leukocytes (40–42). Individuals with *GSTM1*-null genotype could benefit more than subjects with *GSTM1*-positive genotype from *Brassica* vegetables intake because altered metabolism leads to a higher bioavailability of sulphoraphane or total ITCs as shown in studies with administration of a single broccoli meal, although differences were small and the interpretation is debated (17,19). The impact of *GSTM1* on possible cancer protective effects of cruciferous vegetables is also debated with effect mainly shown with the *GSTM1*- and *GSTT1*-double-null genotype in Chinese and European populations (4), whereas the *GSTM1*-positive genotype might convey beneficial effects in subjects from North America (17). Intake of cruciferous vegetables, including broccoli, is most popular in the latter region, whereas other *Brassica* vegetables in the former region as well as multiple other dietary, lifestyle and genetic differences can play roles in these apparent discrepancies.

In conclusion, in the present study, the intake of broccoli seems protective, as far as DNA damage is concerned, in smokers who are exposed to oxidative stress. This protective effect may be due to improved antioxidant status.

Funding

Ministry of Education, University and Research PRIN 2005, prot. 2005058197; Cariplo Foundation (2007.5810).

Acknowledgements

We are grateful to Di Stasi Company for the gift of broccoli used for the study. Dr Andrea Maestrelli and Dr Luigi Francesco Di Cesare of the Research Unit for Processing and Agrofood Industry (IAA) of the Agricultural Research Council (CRA) are acknowledged for their contributions on the initial processing of broccoli.

Conflict of interest statement: None declared.

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