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Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet assay: a biomarker model

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Abstract
Methods are needed to assess exposure to genotoxins in humans and to improve understanding of dietary cancer prevention. The Comet assay was used to detect smoking-related exposures and dietary modulations in target tissues. Buccal scrapings, blood and faeces were collected from 38 healthy male volunteers (smokers and non-smokers) during a dietary intervention study with bread supplemented with prebiotics ± antioxidants. GSTM1-genotype was determined with PCR. Buccal and peripheral lymphocytes were analysed for DNA damage using the Comet assay. Genotoxicity of faecal water (FW) was assayed in human colon HT29 clone 19A cells. 'Tail intensity' (TI) was used as a quantitative indicator of DNA damage in the Comet assay. Intervention with bread reduced DNA damage in lymphocytes of smokers (8.3 ± 1.7% TI versus 10.2 ± 4.1% TI, $n = 19$), but not of non-smokers (8.6 ± 2.8% TI versus 8.3 ± 2.7% TI, $n = 15$). Faecal water genotoxicity was reduced only in non-smokers (9.4 ± 2.9% TI versus 18.9 ± 13.1% TI, $n = 15$) but not in smokers (15.5 ± 10.7% TI versus 20.4 ± 14.1% TI, $n = 13$). The Comet assay was efficient in the detection of both smoking-related exposure (buccal cells) and efficacy of dietary intervention (faecal samples). Smokers and non-smokers profited differently from the intervention with prebiotic bread ± antioxidants. Stratification of data by genotype enhanced specificity/sensitivity of the intervention effects and contributed important information on the role of susceptibility.

Keywords: *Biomarker, Comet assay, intervention study, faecal water, lymphocytes*

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Introduction

The mechanisms of cancer development are thought to include genetic alterations (mutations, amplifications and recombinations) in proto-oncogenes, tumour suppressor genes or DNA repair genes. For sporadic types of cancers (Fearon 1997), the accumulation of these alterations may occur in somatic tissues during a lifetime. The determination of genetic damage is therefore used as a biomarker to study cancer risks due to environmental and occupational exposures (Perera 1996, Levine et al. 1997, Wild and Pisani 1997, Perera et al. 2002) to genotoxins. Determination of genetic

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damage is probably also useful to study associations of diet and cancer induction since 20–60% of human tumours are dietary related (Doll and Peto 1981, Lippman et al. 1990, Doll 1991, 1996) and should be preventable by changing the food pattern resulting in a lower burden of toxic/genotoxic substances and a higher intake of protective compounds. Examples of such effects are reports on a reduced urinary excretion of modified DNA bases as a result of Brussels Sprouts ingestion (Verhagen et al. 1995), the oral exposure to reactive lipid peroxidation products using buccal cells (Dittberner et al. 1997), the exposure to genotoxins in the gut lumen by measuring faecal water genotoxicity (Glinghammer et al. 1997, Venturi et al. 1997) and modulation thereof by diet (Rafter et al. 1987, Rieger et al. 1999), or a reduced level of lymphocyte DNA damage after ingestion of vitamin supplements (Duthie et al. 1996). In the latter two examples, faecal water genotoxicity and genetic damage in peripheral lymphocytes were determined with the assay of single cell microgel electrophoresis, also referred to as the 'Comet assay'.

The Comet assay has a unique potential for human monitoring of genetic damage (Tice et al. 2000). It is the only technique to monitor DNA damage and repair at the level of single cells. Therefore, it can be used to analyse genotoxic effects in a few cells, as typically available from biopsies of human tumour target tissues (Pool-Zobel et al. 1994, 1999a). A demonstration of the usefulness of this technique during dietary intervention trials was shown recently (Pool-Zobel et al. 1997). The results showed that the consumption of whole foods (in this case vegetable juices) reduced lymphocyte DNA damage. Some subsequent trials e.g. with tomato puree (Riso et al. 1999), soy milk (Mitchell and Collins 1999) or with fruit juices (Bub et al. 2003) have also demonstrated a dietary-related reduction of genetic damage, whereas others using bread (Pool-Zobel et al. 2000) or vegetables and fruits (Moller et al. 2003) have not. This could be a hint that there are differences in the bioavailability of the active ingredients after ingestion of juices versus the ingestion of whole foods. This could have limited the sensitivity of the 'Comet assay biomarker' when using only lymphocytes as the surrogate tissues.

Research is strongly needed to assess the impact of antioxidant plant ingredients in various tissues of healthy humans and especially to improve understanding of the cancer-preventive functions of whole fruits and vegetables (Machlin 1995). Therefore, new sensitive and predictive biomarkers, which are more specific for individual exposure routes or tumour types, could be of advantage for this research (Branca et al. 2001). We have addressed this question by comparing 'Comet assay' responses using different tissues from human volunteers consuming bread during a dietary intervention study. We measured DNA damage in peripheral lymphocytes, as a measure of systemic exposure, DNA damage in buccal lymphocytes (Osswald et al. 2003), as a reflection of direct exposure via the *per oral* route of dietary ingestion, and the genotoxicity of faecal water (Osswald et al. 2000), to estimate some of the effects caused by gut fermentation of the fibre ingredients in bread. The subjects were characterized for *GSTM1*1* and *GSTM1*0* genotypes since this is a common type of genetic variability. Human glutathione S-transferases (GSTs) are considered to be particularly important for detoxifying many carcinogenic compounds and reactive intermediates that may be breast carcinogens (Hayes and Pulford 1995). Subjects with different GST genotypes may therefore have different susceptibilities to environmental exposures. About 50% of the Caucasian population carry a homozygous deletion of the GSTM1 locus, resulting in an inactive gene product (null genotype), and the lack

of functional GSTM1 enzyme activity (Caporaso et al. 1999, Perera et al. 2002). It was of interest to determine in the *GSTM1*0* genotype whether the lack of this phase II enzyme was associated with an enhanced level of genetic damage, since individuals who inherit the *GSTM1*0* genotype are not capable of conjugating and detoxifying specific substrate epoxide intermediates (Wiencke et al. 1995).

Materials and methods

Bread

The bread used in this study was a standard sourdough bread supplemented with ingredients of enhanced functional activities. As shown in Table I, the control bread consisted of the basic mixture with wheat flour, coarsely ground rye grain, malt flour, sour dough, apple fibre, salt and wheat glue. One of the intervention breads was supplemented with inulin, linseed and soy flours whilst the other was additionally supplemented with antioxidative ingredients, namely selenium-rich wheat, tomato extract, as well as green tea and spice extracts, all of which had been characterized for antioxidative and functional cellular activities previously (Beyer-Sehlmeyer et al. 2003, Glei et al. 2003).

Intervention study

Forty-one subjects were randomly allocated to two intervention arms. After three volunteers dropped out due to failure in compliance, the study group consisted of 38 healthy male volunteers (18 non-smokers and 20 smokers) aged 20–55 years. A selection of their vital data, comparing non-smokers to smokers, is shown in Table II.

Table I. Selected properties of the breads used for the intervention study.

	Control bread	Prebiotic-only bread	Prebiotic and antioxidative bread
Composition (%)			
Apple fibre	2.0	2.0	2.0
Sourdough	3.0	3.0	3.0
Malt flour	0.8	0.8	0.8
Sunflower seeds	10.0	10.0	10.0
Wheat flour	66.9	50.9	48.1
Rye bran	15.0	15.0	15.0
Salt	2,3	2,3	2,3
Soya	–	6.0	6.0
Inulin	–	4.0	4.0
Linseed	–	4.0	4.0
Wheatglue	–	2.0	4.0
Green tea	–	–	0.5
Spices	–	–	0.7
Tomato	–	–	0.5
Wheat (Se rich)	–	–	1.0
Antioxidant activity ((mmol l^{-1})/100 g)			
TEAC (hydrophile)	0.441 ± 0.015	0.758 ± 0.002	1.274 ± 0.033
TEAC (lipophile)	0.036 ± 0.002	0.068 ± 0.026	2.185 ± 0.041

Table II. Selected clinical characteristics of the subjects.

Parameter	All volunteers ($n=38$)	Non-smokers ($n=18$)	Smokers ($n=20$)
Age (years)	27.4±7.5	26.3±4.8	28.4±9.2
Height (m)	1.8±0.1	1.8±0.1	1.8±0.1
Body mass (kg)	75.0±8.5	72.9±6.8	77.0±9.5
BMI (kg m^{-2})	23.2±2.8	22.7±2.2	23.7±3.4
Total cholesterol (mmol l^{-1})	3.5±1.1	3.4±0.9	3.7±1.3

The design was based on 5-week intervention periods, which were chosen to ensure sufficient time for the absorption and distribution of the micronutrients. After an initial 2-week run in period (defined below), the study started with a control period (defined below), which was then followed by the two parallel intervention periods, each lasting 5 weeks. A follow-up analysis of selected subjects was carried out several weeks after the intervention trial had ended (post-phase). The run in phase was for recruitment, during which the volunteers consumed a free diet. In the control period, subjects consumed the basic bread (control bread phase) and during the intervention periods, 17 participants ate the prebiotic bread while the other 21 participants consumed the prebiotic/antioxidant bread. The subjects were maintained on a standardized diet (all foodstuffs were provided) for the last weeks of each control and intervention phases to keep variation of the biomarker responses as low as possible (Oberreuther-Moschner et al. 2004). Fasting blood and faeces from 5 days was collected at the end of each period, and after the post-phase, during which the volunteers also had consumed their free diets again.

Isolation and cryopreservation of human lymphocytes

Lymphocytes were isolated from blood of the volunteers by gradient centrifugation with HISTOPAQUE®-1077 (Sigma, Deisenhofen, Germany) (Boyum 1968). A total of 5 ml phosphate-buffered saline (PBS) was added to 5 ml blood, gently mixed and layered onto 5 ml HISTOPAQUE®-1077. After centrifugation at 400*g* for 30 min at room temperature, the second opaque layer was transferred into a new clean centrifuge tube with a Pasteur pipette and diluted to 20 ml with PBS. The mixture was centrifuged at 300*g* for 10 min at room temperature and the lymphocyte pellet was resuspended in 5 ml PBS. Cell number and viability were determined in a Neubauer haemocytometer using trypan blue. A total of 6×10^6 lymphocytes ml^{-1} in PBS were centrifuged at 380*g* for 8 min at room temperature, the supernatant was removed and the pellet resuspended in freezing medium, consisting of 90% foetal calf serum (FCS) (Gibco, Karlsruhe, Germany) and 10% dimethyl sulfoxide (DMSO) (Fluka, Steinheim, Germany). The cells were frozen to -80°C using a Nalgene Cryo 1°C freezing container with isopropylalcohol (Sigma) to achieve a -1°C min^{-1} rate of cooling. They were stored at -140°C. Cells were thawed rapidly before each experiment in a water bath at 37°C.

Collection of mouth washings and isolation of buccal lymphocytes

Volunteers were asked to rinse their mouths with Hanks buffered saline solution (HBSS) for 1 min. Mouth wash samples were collected in centrifuge tubes. After this initial sampling, volunteers were then required to stimulate the oral mucosa with a cell

scraper and rinse their mouth a second time for 1 min. The second mouthwash sample was collected in the same centrifuge tube. Cells were concentrated by centrifugation for 8 min at 400*g*. Pellets were resuspended in 5 ml cell culture medium (RPMI 1640; Invitrogen GmbH, Karlsruhe, Germany). The cells were stored on ice. Cell numbers and viabilities were determined as described above. The discrimination of epithelial cells and lymphocytes was achieved by microscopic inspection of their characteristic shapes and sizes. Buccal lymphocytes were isolated and characterized as described (Osswald et al. 2003) using density gradient centrifugation. Therefore buccal cell samples were layered onto the HISTOPAQUE®-1077 and handled as described above. Aliquots of isolated lymphocytes were stained with trypan blue, for determination of cell numbers and viabilities.

Collection of faeces and isolation of faecal water

Total faeces of one passage were collected from the subjects, when possible, in the early morning and stored in a cooled container for transport to the laboratory. Work up proceeded, as described (Osswald et al. 2000), by homogenizing the samples in a plastic bag under a well-vented fume hood. The samples were transferred to aluminium cap-locked polycarbonate tubes (tubes: 25 × 89 mm, maximum volume 26.3 ml, maximum 371 000*g*, Beckmann No. 355681; caps: Beckmann No. 355654) and centrifuged (Optima LE-80K Ultracentrifuge, Beckmann) at 25 000*g* for 2 h at 4°C. The supernatants, representing the actual faecal water fractions, were aliquoted in portions of <100 µl and stored at -80°C until further work up.

Treatment of HT29 clone 19A cells with faecal samples

HT29 clone 19A cell line is a permanently differentiated sub-clone derived from the carcinoma cell line HT29 treated with sodium butyrate (Augeron and Laboisse 1984). HT29 clone 19A was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin (Ebert et al. 2001). Under given laboratory conditions, HT29 clone 19A cells doubled their number within 24 h. Passages 27–48 were used for the experiments. Faecal water was added in amounts of 11.1–100 µl cell suspension containing 4×10^5 HT29 clone 19A cells, thus resulting in a final concentration of 10% faecal water (Rousset 1986). The suspensions were incubated for 30 min in a shaking water bath at 37°C. Aliquots were taken for determination of viability using trypan blue and the remaining cell suspensions were then centrifuged. The pellets were taken up in agarose, distributed onto slides and then processed according to our protocol for the microgel electrophoresis (Pool-Zobel et al. 1999b) as described below.

DNA damage and oxidized DNA bases

DNA damage was measured with the single-cell microgel electrophoresis assay, also known as the 'Comet assay' (Singh et al. 1988). We used our protocol for the Comet assay and additionally the modification with endonuclease III or formamidopyrimidine glycosylase, to detect levels of oxidized pyrimidine or purine bases (Duthie et al. 1996). A total of 10^5 lymphocytes were distributed with 30 µl low melting point agarose on microscope slides and after 10 min covered with another layer of agarose. Nine slides from each donor were placed into a lysis bath (100 mM Na_2EDTA,

1%Triton X-100, 2.5 mM NaCl) for 1–2 h. After at least 1 h six slides were washed with endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 mg ml^{-1} bovine serum albumin fraction V, pH 8.0) and then incubated with formamidopyrimidine glycosylase (three slides) or endonuclease III (three slides) in buffer sealed with a cover slip for 30 or 45 min at 37°C, respectively. Subsequently, all slides of one donor were placed into an electrophoresis chamber containing alkaline solution (1 mM Na_2EDTA, 300 mM NaOH) for DNA unwinding. After 20 min, the current was switched on and electrophoresis carried out at 25 V, 300 mA for 20 min. The slides were removed from the alkaline buffer, placed on a tray and washed three times for 5 min with neutralization buffer (0.4 mM Tris, pH 7.5). Subsequently, the slides were stained with 30 µl SYBR Green®. Thus for each donor and intervention phase, triplicate values for DNA strand breaks and oxidative DNA damage (slides treated with endonuclease III or formamidopyrimidine glycosylase) were available for evaluation. All steps beginning with the isolated lymphocytes were conducted under red light. Evaluation of the images on the slides was performed by microscopic analysis. Using the imaging software of Kinetic Imaging (Nottingham, UK), 50 images were evaluated per slide and the percentage of fluorescence in the tail was scored. The value for slides without endonuclease III or formamidopyrimidine glycosylase reflected the amount of DNA breaks, alkali labile sites, apurinic and apyrimidinic sites, and single stranded DNA resulting from repair or replication (Collins et al. 1995, 1996). The percentage of fluorescence in the tail for slides with repair specific enzymes minus the values from corresponding slides was the measure for oxidized DNA bases.

Determination of GSTM1 genotype

Cryopreserved lymphocytes (6×10^6 cells ml^{-1}) were used to isolate DNA with the QIAamp® DNA Mini Kit (QIAGEN) as described in the manufacturer's manual. A polymerase chain reaction (PCR) method was used to detect the presence or absence of the *GSTM1* gene (Bell et al. 1993) using primers from MWG Biotech AG (Ebersberg, Germany) with the sequences and procedures as described in detail previously (Pool-Zobel et al. 1998). A fragment of the *ß-globin* gene was co-amplified as internal positive control in the PCR reaction.

Statistical evaluation

Each comet assay slide was scanned to evaluate 50 images per slide. For the presentation of this data, the measure 'per cent fluorescence in tail' from three slides was chosen as the base to calculate the means $\pm$SD for each data point. Data points were statistically evaluated using ANOVA with Bonferroni's post-tests and *t*-tests (GraphPad Prism® 4.0), as appropriate and as indicated in the respective tables and figures.

Results

Intervention effects

Human subjects increased their bread intake from approximately 166 ± 61 g day^{-1} (run in phase) to 202 ± 57 g day^{-1}, when given the control breads. This quantity of

bread was consumed during the intervention phases (prebiotic bread or prebiotic bread + antioxidants) as well (209 ± 69 g day^{-1}).

Genetic damage in lymphocytes

Figure 1 compares the baseline levels of cellular damage in different lymphocytes from smokers and non-smokers after a controlled, given diet. It shows there were significant differences in the amounts of single strand breaks in buccal lymphocytes compared with the corresponding peripheral cells only in smokers (smokers: 14.2 ± 3.5% TI versus 8.3 ± 1.7% TI; non-smokers: 8.1 ± 1.6% TI versus 8.3 ± 2.7% TI). In contrast, oxidative DNA damage of buccal cells was higher than in peripheral lymphocytes in both test groups (smokers: 7.7 ± 3.1% TI versus 1.9 ± 2.1% TI; non-smokers: 6.6 ± 4.2% TI versus 2.9 ± 4.0% TI). Damage (strand breaks) in buccal cells from smokers (14.2 ± 3.5% TI) was also significantly higher than damage in buccal cells from non-smokers (8.1 ± 1.6% TI). The data was obtained from a subset of individuals, smokers and non-smokers for whom both peripheral and buccal lymphocytes were available. The stratification into subjects with *GSTM1*1* ($n = 20$) and *GSTM1*0* ($n = 18$) genotypes revealed no differences for strand breaks, whereas oxidized bases tended to be higher in *GSTM1*0* than in *GSTM1*1* (tail intensities of 7.6 ± 1.9% versus 5.9 ± 1.5% for smokers and 8.7 ± 2.0% versus 6.8 ± 1.0% for non-smokers). Intervention related effects in buccal lymphocytes were not observed (results not shown).

Effects of smoking, intervention and GSTM1-genotype on genetic damage in peripheral blood lymphocytes

Figure 2 shows that intervention related differences were apparent for DNA damage (measured as strand breaks + endonuclease III-sensitive sites) in peripheral lymphocytes from smokers ($n = 20$) receiving prebiotic bread ± antioxidants (run in: 11.4 ± 5.1% TI versus intervention: 8.4 ± 4.0% TI).

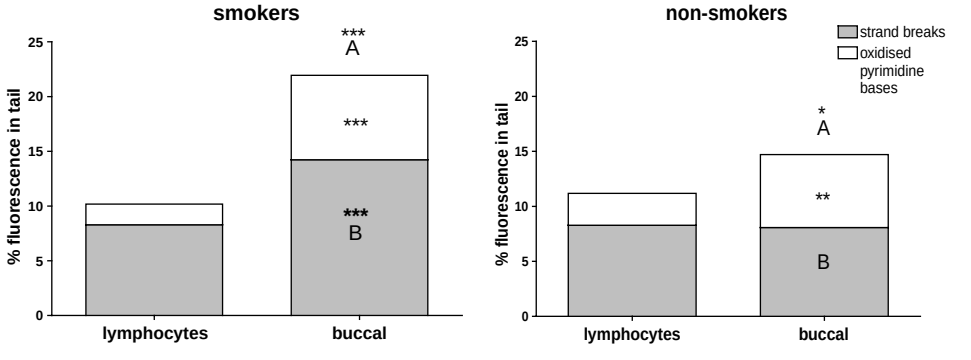

Figure 1. Comparison of DNA damage after the control bread phase. Damage (endonuclease III-sensitive sites) is shown as DNA strand breaks and oxidized pyrimidine bases in buccal and human peripheral lymphocytes from the same subjects (subset of all subjects, since buccal cells were not isolated from all of the volunteers). Significant differences between buccal and peripheral blood lymphocytes for the individual damage types and for the total damage, respectively, are depicted by asterisks within the bar of the buccal lymphocytes or above it (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two-way ANOVA with Bonferroni post-tests). Differences between buccal lymphocytes of smokers and non-smokers are labelled with A or B for total damage and for strand breaks, respectively.

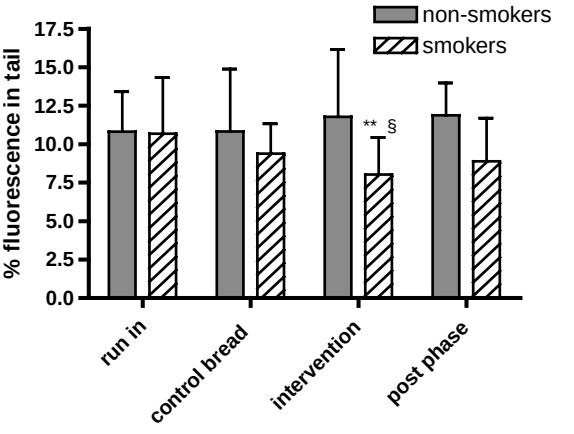

Figure 2. Effect of intervention with bread on the levels of DNA damage (endonuclease III-sensitive sites) in peripheral lymphocytes isolated from male smokers and non-smokers. All subjects were grouped together since there were no apparent differences between the responses in the prebiotic only or prebiotic with antioxidant-supplemented breads. $^{**}p < 0.01$, significantly different from non-smokers, Significantly different from smokers after the run-in phase.

Table III shows that *GSTM1*0* non-smokers had significantly higher levels of DNA damage in their lymphocytes than in *GSTM1*1* non-smokers after the test bread. After the test bread phase, their damage was also significantly higher than in *GSTM1*0* smokers. Finally, statistically significant intervention effects were apparent only in the *GSTM1*0* smokers receiving both bread types (control bread: $9.5 \pm 3.1\%$ TI versus test bread: $8.4 \pm 3.2\%$ TI), although a similar trend was present for *GSTM1*1* smokers as well.

Faecal water effects in HT29 clone 19A colon cells

Faecal water genotoxicity was assessed in available individuals, 13 smokers and 15 non-smokers. Table IV combines the subjects of both intervention (test bread) groups, and shows that the DNA damaging potential of faecal water was markedly reduced after the control bread phase only in non-smokers (run in: $19.0 \pm 13.0\%$ TI versus control bread: $8.8 \pm 2.9\%$ TI).

When regarding subjects with *GSTM1*1* (eight smokers, seven non-smokers) and *GSTM1*0* (five smokers, seven non-smokers) genotypes, interesting differences were apparent as is shown in Figure 3. In non-smokers of both genotypes, intervention with bread significantly decreased faecal water genotoxicity. For smokers, only *GSTM1*0* genotypes had a reduced faecal water genotoxicity after bread intervention. In contrast, the excretion of faecal genotoxins remained high in *GSTM1*1* smokers, regardless of the dietary intervention.

Comparison of individual genetic damage in peripheral lymphocytes and faecal water genotoxicity

There was no apparent association detectable between the measured DNA damage in peripheral lymphocytes and the genotoxic potential of the faecal waters of the subjects.

Table III. Genetic damage in peripheral lymphocytes isolated from the subjects of the intervention trial and analysed according to genetic enzyme polymorphisms. The subjects of both intervention arms (bread supplemented with prebiotics and with prebiotics+antioxidants) are grouped (test bread). Results are shown for endonuclease III-sensitive sites, which include strand breaks and oxidized DNA pyrimidine bases.

	*GSTM1*1*						*GSTM1*0*					
	Non smokers			Smokers			Non smokers			Smokers		
	Means	SD	*n*	Means	SD	*n*	Means	SD	*n*	Means	SD	*n*
Run in	9.8	2.3	7	###11.0	5.1	10	11.4	2.0	8	#10.8	4.3	10
Control bread	§§10.8	3.3	7	8.9	1.9	10	10.7	4.3	8	9.5	3.1	10
Test bread	9.4	2.9	7	8.6	3.1	10	§§§*12.3	4.6	8	#8.4	3.2	10
Post-phase	11.4	2.4	5	8.6	3.1	6	12.3	1.9	5	9.8	2.8	6

*Significant differences of *GSTM1*0* to the corresponding *GSTM1*1* genotypes, whereas '#'points to values different from those obtained in the control bread phase of the same column (two-way ANOVA and Bonferroni's post-test); §differences between non-smokers and smokers of the same genotype; *n*, number of subjects.

Summary

Table V summarizes the different results of the study to give an overview of the key data. In summary, the diverse biomarkers used here were capable of detecting (1) exposure effects, e.g. enhanced DNA damage in buccal cells in smokers compared to non-smokers; (2) effects of dietary intervention with bread, e.g. reduced faecal water genotoxicity in all non-smokers and in smokers with *GSTM1*0* genotype; reduced DNA damage in lymphocytes of smokers; reduced proportion of oxidized bases in lymphocytes of non-smokers after intervention with bread containing antioxidants; and (3) effects of genetic susceptibility, e.g. more lymphocyte DNA damage in *GSTM1*0* than *GSTM1*1* subjects, high genotoxicity of faecal water from *GSTM1*1* smokers.

Discussion

The present study used sourdough breads that were supplemented with prebiotics (inulin, linseed and soy) and with additional antioxidants from tomato, selenium-rich

Table IV. Faecal water genotoxicity: DNA strand breaks (per cent fluorescence in tail) obtained after incubating HT29 clone 19A cells with faecal water (isolated from a randomly selected subset of subjects during the study).

Faecal water	Strand breaks induced in HT29 clone 19A cells					
	Non smokers			Smokers		
	Means	SD	*n*	Means	SD	*n*
Run in	19.0	13.0	15	20.5	14.1	13
Control bread	#8.8	2.9	15	15.5	10.7	13
Test bread	#9.5	2.9	15	*21.0	15.4	13
Post-phase	13.6	9.6	9	19.2	19.0	5

*Significant difference between smokers and non-smokers (two-way ANOVA and Bonferroni's post-test), whereas '#'points to values different from those obtained in the run in phase of the same column; *n*, number of subjects.

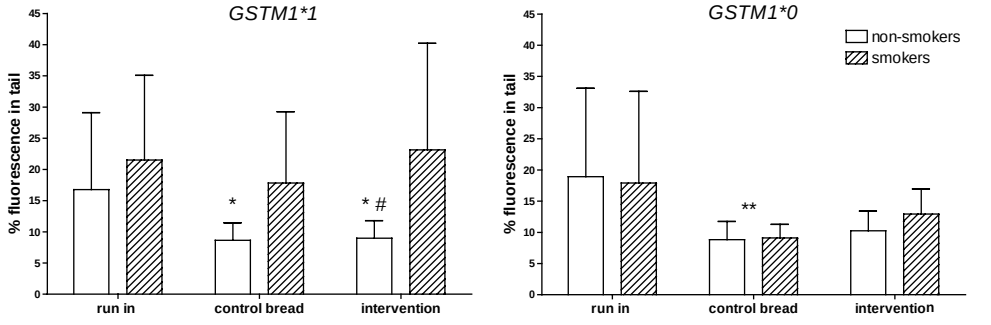

Figure 3. Faecal water genotoxicity in GSTM1*1 and GSTM1*0 smokers and non-smokers during the intervention trial. Data for the post-phase were excluded due to limited sample sizes. *$p < 0.05$ significantly different from smokers after the run-in phase (paired t-test), #$p < 0.05$ significantly different from non-smokers (two-way ANOVA with Bonferroni post-tests).

wheat, spices and green tea (Glei et al. 2003) to investigate the influence of a dietary intervention on different biomarkers. Baking did not destroy all of the antioxidative capacity of this functional bread (Table I). Our study had a parallel design, since every subject passed through a control phase before they were randomized and entered one intervention arm. Thus, an extra blind control was not included, which might have been a limitation. On the other hand, an advantage of our approach was to maintain our subjects on a standardized diet (all foodstuffs were provided) for the last week of each control and intervention phase. This was done to keep the variation of the biomarker responses as low as possible and accordingly all the measurable effects should mainly result from the bread intervention.

By using the multiple tissues monitoring approach, local and systemic effects influenced by bread were detected. Several different interesting results became

Table V. Summary of the main results showing the effects of smoking, bread intervention and genetic susceptibility on biomarker responses.

	Exposure genotype intervention	Type of DNA damage		
		Strand breaks	Oxidized bases	All damage
Buccal cells	smoking	↑	↑	↑
	*GSTM1*0*	–	(↑)	(↑)
	bread	–	–	–
Lymphocytes	smoking	–	–	–
	*GSTM1*0*	–	(↑)	↑
	bread	–	↑↓[a]	↓[b]
Faecal water	smoking	↑	n.d.	n.d.
	*GSTM1*1*	↑	n.d.	n.d.
	bread	↓[c]	n.d.	n.d.

↑,↓Significant findings that indicate a modulation of DNA damage during the specified intervention phase; symbols in parentheses are findings of marginal significance ($p < 0.1$ but > 0.05), whereas '–' points to no change of DNA damage, in comparison with the respective standards, no intervention, non-smoker, *GSTM1*1*, n.d., not determined.

[a]Intervention with prebiotics-only bread increased and intervention with prebiotics+antioxidant bread decreased the proportion of oxidized DNA bases in non-smokers.

[b]Reduction of damage was only apparent in smokers.

[c]Reduction was only apparent in non-smokers (both genotypes) and in smokers with *GSTM1*0* genotype.

apparent by performing of many comparisons (based on smoking habits, genotype and bread intervention), even though some failed to reach statistical significance and revealed only trends. The most distinct impact was the modulation of faecal water genotoxicity. The DNA damaging potential of the aqueous faecal extracts was reduced to almost half by the control bread. This marked effect was found only in the non-smokers. In smokers, control bread, prebiotics-only bread or prebiotics bread with antioxidants had no impact at all. However when regarding subjects with *GSTM1*0* and *GSTM1*1* genotypes, obvious differences were observed. In smokers with the null gene polymorphism (*GSTM1*0*), fewer genotoxins were excreted in the faeces after intervention with bread. In contrast, smokers with the gene (*GSTM1*1*) were apparently not protected by fibre intervention. These subjects excreted faeces with similar levels of genotoxins as before intervention. These results were unexpected at first glance, since individuals who are carriers of the homozygous deletion in the GSTM1 gene (*GSTM1*0*) were expected to have an increased cancer risk due to lower detoxification capacity. For instance, evidence is available that GSTM1*0 increases risks for the development of some cancers, e.g. in lung and bladder (Rebbeck 2003). For the example of colorectal cancer, however, the associations are not as straightforward, and two studies even suggest increased disease risks in subjects with high meat intake and *GSTM1*1* genotype (Cotton et al. 2000). When considering pharmacological aspects of the exposure situations, these findings are not controversial at all. Thus, it is known that smokers are exposed to polycyclic aromatic hydrocarbons (PAH), which can be conjugated by GSTM1-1. Smokers with an intact GSTM1-1 metabolism therefore are expected to produce high amounts of GST-conjugates, some of which will be excreted via the faeces. Since the GST conjugates are not completely stable, this would explain why we observed a higher genotoxic activity of faeces from *GSTM1*1* smokers than from *GSTM1*0*/*GSTM1*1* non-smokers. Additionally, the increased level of genotoxins in faecal water could result from an enhanced rate of PAH bioactivation (Hecht 2003) and consequently in a higher amount of conjugated metabolites in the faeces of *GSTM1*1* smokers. At present, it is not possible to conclude that this would present a higher or lower risk for the exposed individuals. However, in a recent study on faecal water genotoxicity of rats, we showed that (1) faecal water genotoxicity reflected genotoxic exposure in the caecum, that (2) tumour incidence and faecal genotoxicity were directly related, and that (3) an intervention with a prebiotics type of dietary fibre reduced tumour risks by reducing exposure to genotoxins in the gut (Klinder et al. 2004). For humans there are no reports indicating that faecal water genotoxicity is associated with tumour risk. However, a few reports have shown that the *GSTM1*0* genotype enhances colorectal cancer susceptibility in smokers (Lin et al. 1995), whereas others have shown no associations (Katoh et al. 1996). No study was available taking the fourth factor, dietary fibre, into account, for the associations between colorectal cancer, smoking, and *GSTM1* genotype. Thus it will be interesting in the future to assess smoking and *GSTM*-genotype related colon cancer risks in humans based on dietary fibre intake.

The positive association between an increased uptake of dietary fibre and cancer protection in the human colon has been a controversial subject in the past, but newer results tend to support the theory that sufficient amounts of dietary fibre, as consumed in this study, reduce risks for developing colorectal cancer (Bingham et al. 2003, Ferguson and Harris 2003). Our observed fibre-mediated reduction of genotoxicity in

faecal waters from non-smokers and *GSTM1*0* smokers support this suggestion and point to straightforward mechanisms involved, such as scavenging of reactive intermediates (Ferguson et al. 2001) or reduction of co-carcinogenic secondary bile acids in stool (De Kok and Van Maanen 2000), which are able to induce DNA damage in colon adenocarcinoma cells (Venturi et al. 1997).

The analysis of DNA damage in lymphocytes of all subjects showed that the high fibre intake (22 g day^{-1} during the run in phase versus 29 g day^{-1} during the bread intervention phases) reduced baseline levels of genotoxicity in smokers, with no obvious differences between genotypes. This could mean that via high intake of dietary fibre, an efficient metabolic conversion or scavenging of smoking-related genotoxins may reduce the systemic exposure load and thus result in less lymphocyte damage in all smoking subjects. Interestingly, when additionally taking the genotype into account it seems as if the lack of GSTM1 protein in non-smokers leads to a higher systemic genotoxic burden of the body than smoking, since we detected a significantly higher level of base damage in the peripheral lymphocytes of *GSTM1*0* non-smokers than in *GSTM1*1* non-smokers and smokers. However, further investigations will be necessary to explore the plausibility and background of this observed effect. Interestingly, endogenous oxidative DNA base damage was reduced in non-smoking subjects consuming prebiotic bread supplemented with antioxidants. This effect was moderate, but in line with our previous reports on a reduced degree of oxidative base damage in healthy, non-smoking subjects consuming carrot and tomato juices (Pool-Zobel et al. 1997) or fruit juices (Bub et al. 2003).

The last set of biomarker analyses used the major oral cells obtained in mouthwashes. We had previously shown that these consist of equal proportions of non-viable epithelial cells and viable lymphocytes, of which the lymphocytes can be isolated in sufficient quantities and quality for further investigations (Osswald et al. 2003). There were marked differences in DNA damage of cells from smokers and non-smokers. Smokers had more damage than non-smokers, reflected as an increased level of strand breaks. These probably have their origin from alkali labile sites and thus could be a reflection of damage caused by genotoxic electrophilic carcinogens derived from tobacco smoke. Buccal lymphocytes of all subjects had more oxidized bases than the peripheral lymphocytes. Therefore, the buccal cells were suitable for detecting both exogenous (smoking-related strand breaks $\pm$ oxidized DNA bases) and endogenous (oxidized bases) types of exposures in human subjects. Neither types of damage, nor the overall yields of DNA damage, however, were modulated by the dietary interventions studied here.

In conclusion, by using various target tissues of the body, we were able to determine exposure and diet related modulation of genetic damage in human subjects and to assess the influence of genetic susceptibility. The Comet assay was a rapid and sensitive means to detect genotoxic and chemoprotective agents, which allowed versatile applications. By employing it in multiple tissues, all derived relatively non-invasively, we were able to obtain new information on meaningful associations between diet, lifestyle, susceptibility and potential exposure risks or risk reductions.

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