Intervention with polyphenol-rich fruit juices results in an elevation of glutathione *S*-transferase P1 (hGSTP1) protein expression in human leucocytes of healthy volunteers

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Polyphenols are probably antigenotoxic on account of their antioxidant activities and might alter phase I and II enzymes in a way that results in chemoprotection. We investigated the hypothesis that polyphenols enhance expression of glutathione *S*-transferases (GSTs), which increases carcinogen detoxification and thereby provides protection against oxidative stress. HGSTP1 protein expression and GST polymorphisms were determined in leucocytes obtained during an intervention study with healthy subjects consuming two fruit juices in an 8 wk trial (polyphenol-free run in phase, juice intervention phase, washout phase, second juice intervention phase, each treatment regime lasted for 2 wk). The study had originally shown that juice intervention significantly reduced oxidative DNA damage in leucocytes at week 8 (Bub, A., Watzl, B., Blockhaus, M., Briviba, K. *et al*., *J. Nutr. Biochem.* 2003, *14*, 90 – 98). We reanalysed the levels of DNA damage based on GST genotypes. We also treated leucocytes *in vitro* with mixtures of polyphenols and determined cytotoxicity and expression of 96 genes related to drug metabolism. Key results with leucocytes of the intervention study were that the initial content of hGSTP1 protein was first suppressed at weeks 4 and 6. At week 8, however, hGSTP1 protein expression was significantly increased. HGSTP1 protein levels and DNA damage were inversely correlated ($p = 0.005$), but there was no difference for cells obtained from subjects with *hGSTM1*1* and *hGSTM1*0* genotypes, nor was there any difference between cells from subjects consuming the two different juices. The treatment of leucocytes with polyphenol mixtures *in vitro* did not result in modulated GST gene expression or total GST activity, but in an up-regulation of other biotransformation enzymes (*e.g.*, members of the cytochrom P450 and the sulphotransferase family). In conclusion, *in vitro* treatment of leucocytes led to a modulated mRNA expression of selected genes, not directly related to oxidative defence systems. *In vivo*, however, we observed a delayed enhancement of hGSTP1, which could be associated with an initial repression of oxidative DNA damage in leucocytes from human subjects, consuming juices with high levels of polyphenols.

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1 Introduction

Polyphenols are the major phytochemicals in fruits and vegetables [1] and may exert their effects *via* antioxidant properties [2]. A variety of studies has shown that polyphenols, such as flavonoids, have antioxidative activities *in vitro* [3, 4]. In cellular systems, they have been reported to have abundant biological activities related to chemoprotec-

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Abbreviations: Cy-3-*O*-g, cyanidin-3-*O*-glucoside; **EGCG,** (−)-epigallocatechin gallate; **GST,** glutathione *S*-transferase; **UGT,** UDP-glucuronosyltransferase

tive potentials. In particular, cell-based studies have shown that selected polyphenolic compounds affect signal transduction pathways, leading to inhibition of cell growth and transformation, enhanced apoptosis, reduced invasive behaviour and slowed angiogenesis. It is speculated that mechanisms of this type may be responsible for the anticarcinogenic activities that have, for instance, been shown for polyphenols such as curcumin, genistein and quercetin, as reviewed by Lambert *et al*. [5]. Whereas these aforementioned cellular effects probably contribute to secondary cancer prevention by retarding progression of tumorigenesis, additional mechanisms are important for primary cancer prevention [6]. These include blocking activities which directly or indirectly prevent chemical carcinogens from inducing mutations and thus cancer initiation or further progression [7]. Polyphenols, such as quercetin and myricetin, have also been shown to be active in this context, resulting in antigenotoxic effects in cellular systems [8, 9]. Similar effects have since been detected in humans *in vivo* after dietary intervention with polyphenol-rich fruit juices [10].

A straightforward explanation for the observed antigenotoxic properties could be that the polyphenols scavenge the reactive oxygen species which cause DNA damage and are thought to have a pivotal role for cancer causation [11]. Polyphenols, however, might also indirectly prevent DNA damage by altering phase I and II enzymes in a way that results in chemoprevention [12]. For example, several polyphenols, known to have anticarcinogenic activities in experimental systems, were able to modulate the activity of gastrointestinal UDP-glucuronosyltransferase (UGTs, EC 2.4.1.17) enzymes. The authors concluded that an induction of gastrointestinal UGT enzyme activity may contribute to a better detoxification of potentially carcinogenic compounds and subsequently to the prevention of gastrointestinal cancer [13].

Next to UGTs also glutathione *S*-transferases (GSTs, EC 2.5.1.18) are important phase II enzymes with detoxifying potential [14]. In rats dietary polyphenols (ellagic acid, flavone, coumarin and alpha-angelicalactone) have been shown to selectively enhance members of the GST detoxification system in the oesophagus, stomach and in the pancreas [15]. In humans, GST isoforms are also localized in different tissues with organ-specific expression patterns [16]. They constitute a complex supergene family that collectively metabolizes chemotherapeutic drugs, carcinogens and environmental pollutants, and play a protective pivotal role against xenobiotics, as has been reviewed in the past [17, 18]. Their levels of expression can have profound effects on susceptibility to chemical insult, with overexpression resulting in resistance and underexpression enhancing susceptibility [19, 20]. Human GSTs are characterized as cytosolic (Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega), mitochondrial (Kappa), and microsomal enzymes, which are membrane-associated proteins in eicosanoid glutathione metabolism (MAPEG) [17, 21]. Null genotypes for hGSTM1 and hGSTT1 occur in frequencies of approximately 50 and 20–50% of the population, respectively. They are important genotypes with potentially high impact on the metabolic capacity of the respective subjects, since they result in absence of the respective enzymes. The primary hypothesis has been that individuals with the GST-null genotypes are at higher risk for cancer because of a reduced capacity to eliminate activated carcinogens [22, 23]. Two linked polymorphisms were described in the *hGSTP1* gene, one in codon 105 and one in codon 114, of which the polymorph variant of codon 105 modifies the enzyme's specific activity [24]. HGSTP1 is also one of the main extrahepatic GSTs and is markedly expressed in peripheral leucocytes, where it may serve as a biomarker for induction of phase II enzymes by dietary intervention [25].

In the following study we were interested in understanding whether polyphenols act by the mechanisms of altering phase II metabolism and whether this could be a reason why DNA damage is reduced by polyphenols in human leucocytes as observed previously [10]. The intervention study had been performed in healthy male subjects consuming two fruit juices, one rich in anthocyanidins and the other in green tea catechins. The study had shown us that juice intervention had no effect on single DNA strand breaks, but significantly reduced oxidative DNA damage in leucocytes. This was associated with an enhanced antioxidant status and stimulated immune cell functions, although the intervention for 2 wk did not result in elevated plasma polyphenols in subjects after overnight fasting. Using the remaining biological samples (cryopreserved peripheral leucocytes) of this previous study, the present study determined hGSTP1 expression and GST polymorphism to assess the effects of these parameters on oxidative damage. We also intended to more closely investigate the expression of genes related to biotransformation in peripheral leucocytes treated with the polyphenols of the fruit juices. Two individual mixtures of the components composed according to the two original juices [10] were prepared and investigated for different biological activities in leucocytes *in vitro*. In particular, we assessed the effects of the polyphenol mixtures on expression of genes related to drug metabolism *in vitro*.

2 Materials and methods

2.1 *Ex vivo* determinations

2.1.1 Study design and diet

The randomized, crossover study has been described in detail before [10]. It was divided into five periods each lasting 2 wk: weeks 1–2, run-in period, weeks 3–4, consumption of juice A or B (330 mL/day), weeks 5–6 washout per-

iod, weeks 7–8 consumption of juice B or A (330 mL/day) and weeks 9–10 washout period. The juices contained a mixture of apple, mango and orange juice. In addition, juice A (76% w/w water) was rich in anthocyanin-providing aronia, blueberries and boysenberries, while juice B (78% w/w water) contained flavanol-rich green tea, apricot and lime. During the 10-wk-study period the subjects were instructed to exclude polyphenol-rich foods from their diet. Each subject was his/her own control, since well-designed placebo-fruit juices (not containing polyphenols [26]) were not available at the time the study was performed. The study enrolled a total of 27 non-smoking men (mean age 35 years) with normal body weight (mean BMI 24 kg/m^2). It was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg. All participants gave their written consent.

2.1.2 Isolation of peripheral blood leucocytes

Samples used for the determinations described in this work were derived from the subjects immediately after the first run in phase (week 2), after the first intervention phase (week 4), after the first washout phase (week 6) and after the second intervention phase (week 8). The indicated time points were selected to cover the whole range of the intervention study. HGSTP1 protein determinations could not be performed for other time points, due to limiting quantities of the biological material. Leucocytes were isolated and frozen as described before [27]. Only 24 of the 27 subjects delivered sufficient amounts of leucocytes to perform the determinations described here. DNA damage was determined in fresh leucocytes immediately after they were available. HGSTP1 proteins were determined in batches, namely by simultaneously analysing all time points of each individual.

2.1.3 GST genotyping

Cryopreserved leucocytes (6×10^6 cells) were used to isolate DNA with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) as described in the manufacturer's manual. A multiplex PCR method was used to detect the presence or absence of the *hGSTM1* and *hGSTT1* genes [22, 28] using primers from MWG Biotech AG (Ebersberg, Germany) with the sequences and procedures as described in detail previously [25]. A fragment of the β-globin gene was coamplified as internal positive control in the PCR reaction.

2.1.4 DNA damage

DNA damage (single-strand breaks and oxidized DNA bases) was determined in fresh leucocytes immediately after isolation and followed the procedure as described in the original study [10, 29].

2.1.5 Glutathione *S*-transferase P1 (hGSTP1) protein and total protein

An ELISA kit was used to determine hGSTP1 in microtiter plates (HEPKIT-Pi, Biotrin, Sinsheim-Reihen, Germany), using a 1:4 dilution of the cytosol (corresponding to $2–3 \times 10^5$ cells). The test procedure is based on the sequential addition of sample, antibody-enzyme conjugate and substrate to microassay wells coated with anti-hGSTP1 IgG. The resultant colour intensity is proportional to the amount of hGSTP1. The assay range is 3.12–100 µg/L. The photometric detection of the coloured product was performed at 450 nm using 630 nm as reference (Microplate Reader Tmax, MWG-Biotech; Software: Softmax Version 2.34). Total protein content was measured using the method by Bradford with BSA as standard protein [30].

2.2 *In vitro* determinations

2.2.1 Test chemicals

(+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate (EGCG), quercetin, rutin, quercitrin, myricetrin, kampferol, hesperidin, naringenin, gallic acid, protocatechinic acid, gentisic acid, chlorogenic acid, caffeic acid and phloridzin had been purified by HPLC (~90–99%) by the supplier and all were obtained from Sigma-Aldrich Chemie (Munich, Germany). Isoquercitrin was from Fluka (Taufkirchen, Germany), eriodictyol was from Roth (Karlsruhe, Germany) and ferulic acid was from ICN (Eschwege, Germany).

Cyanidin-3-*O*-glucoside (Cy-3-*O*-g) was isolated from the pale of black currants. In a first step anthocyans were extracted with methanol/acetic acid (19:1) and concentrated with an Amberlite XAD-7 column. Afterwards Cy-3-*O*-g was isolated from the extract with high-speed countercurrent chromatography (HSCCC) [31]. The procyanidin mixture consisted of 69.1% procyanidin B2, 20.0% procyanidin B3 and 10.9% oligomere procyanidins) and was isolated by the gentle separation technique of countercurrent chromatography (CCC).

2.2.2 Preparation of polyphenol mixtures that simulated the fruit juices

The polyphenol mixtures were prepared using concentrations, that simulated contents of the complex fruit juices of the *in vivo* intervention study (Table 1) [10]. For this, the 22 ingredients (solid powder) were mixed in the predetermined ratio and stored at −20°C. The dry mixtures were dissolved in DMSO stock solutions (40 mM in DMSO), which contained the molar concentrations of the identified major polyphenols as previously identified in the natural fruit juices [10]. To assess biological activities in leucocytes, stock solutions were appropriately diluted and added to the cell culture medium yielding concentrations based on the

Table 1. Chemical composition of polyphenol mixtures (in μM) mimicking fruit juices (in mg/L) of the *in vivo* intervention study [10]

Chemical	Mixture A concentration			Mixture B concentration		
	Juice A mg/L	μM	μM at 40 μM Cy-3-*O*-g	Juice B mg/L	μM	μM at 40 μM EGCG
(+)-Catechin	10	34.5	3.2	15	51.7	6.1
(−)-Epicatechin	22	75.8	7	33	113.7	13.4
(−)-Epigallo-catechin	0	0	0	5	16.3	1.9
EGCG	0	0	0	155	338.2	40
Procyanidins	35	60.5	5.6	27	46.7	5.5
Cy-3-*O*-g	210	433.2	40	0	0	0
Quercetin	5	16.5	1.5	1	3.3	0.4
Rutin	9	14.7	1.4	4	6.6	0.8
Isoquercitrin	37	79.7	7.4	0	0	0
Quercitrin	15	33.5	3.1	3	6.7	0.8
Myricetrin	7	22	2	0	0	0
Kampferol	4	14	1.3	0	0	0
Hesperidin	13	21.3	2	47	77	9.1
Eriodictyol	28	97.1	9	40	138.8	16.4
Naringenin	26	95.5	8.8	16	58.8	7
Gallic acid	14	82.3	7.6	10	58.8	7
Protocatechuic acid	44	285.5	26.4	0	0	0
Gentisic acid	41	266	24.6	270	1751.8	207.2
Chlorogenic acid	157	443.1	40.9	30	84.7	10
Caffeic acid	23	127.7	11.8	6	44.3	5.2
Ferulic acid	10	51.5	4.8	1	5.2	0.6
Phloridzin	4	9.2	0.8	2	4.6	0.5
Total amounts	714	2263.6	209	665	2807.2	332

Mixture A was prepared according to an anthocyanidins-rich juice, mixture B contained ingredients simulating a green tea/isoflavonoid-rich juice.

molar concentration of Cy-3-*O*-g (mixture A) and EGCG (mixture B). The highest DMSO concentration in the cell cultures was ≤0.1%.

2.2.3 *In vitro* culture of peripheral blood leucocytes

Peripheral blood leucocytes were isolated from an enriched cell preparation (Buffy coats obtained from whole blood of one blood donor), cryopreserved and genotyped for *hGSTT1* and *hGSTM1* polymorphisms as described above. In order to perform the *in vitro* experiments, the cells were thawed rapidly to avoid toxic impacts of the freezing medium and were washed once with PBS before using them in cell culture. We used and refined our previously described methods for long-term *in vitro* culture of peripheral human leucocytes [27]. The *in vitro* investigations were performed to assess putative mechanisms, using established and characterized *in vitro* cell culture techniques. Since the maximal exposure duration is self-evidently shorter than *in vivo* we included higher concentrations to have comparable doses (which are a function of both exposure times and of concentrations). We also included concentrations as those found in the plasma of subjects consuming polyphenols.

2.3 Parameters of toxicity

Cell integrity of the treated leucocytes was assessed with the CellTiter-Blue™ assay (Promega, Mannheim, Germany), a method which can be used for nonadherent cell suspensions, such as leucocytes, to estimate the number of viable cells. The CellTiter-Blue™ assay was used to study the effects of polyphenol mixtures on cell viability. This assay uses the dye resazurin that only viable cells reduce into the highly fluorescent resorufin. The assay was carried out in 96-well microtiter plates with fluorescence measurements (Tecan, Spectra Fluor Plus, Austria, Em/Ex 520/595 nm) at different time points, namely after 0–72 h.

Other types of cytotoxic effects of the polyphenol mixtures were investigated using the trypan blue exclusion assay, which measures the effects on membrane integrity. For the *in vitro* analysis, leucocytes were incubated in 96-well-plates with the two polyphenol mixtures for 0–72 h. Cells were stained with trypan blue, counted and checked for trypan blue staining (dead cells) or exclusion (viable cells) in a haemocytometer.

The 'Comet Assay', which reveals DNA damage on a single cell level, was performed, according to our described proce-

dures [27]. Leucocytes (final concentration 2×10^6 cells/mL) were incubated with different concentration of polyphenols. One group of cells was treated with H_2O_2 (75 μM, 5 min), as the positive control.

2.3.1 Gene expression with c-DNA array

Leucocytes were precultured in T25 cell culture flasks for 24 h before treatment. Cells were harvested, centrifuged and fresh medium with the polyphenol mixtures was added to the cells (2×10^6 cells/mL). After 24 h cells were again harvested, centrifuged and the resulting cell pellets were washed twice with equal volumes of PBS. Total cellular RNA was isolated using RNeasy mini kit (Qiagen). Prior to *in vitro* reverse transcription steps the integrity of the isolated total RNA was checked by agarose formaldehyde denaturing gel electrophoresis. Two arrays each were used for RNA isolated from leucocytes of two independently reproduced experiments each consisting of a medium control, DMSO control and four treatment groups. Hybridization was performed on 112 sites (3 blanks, 3 negative reference spots, 10 household genes and 96 human genes related to drug metabolism) on cDNA gene macroarrays (GEArray Q Series Human Drug Metabolism Gene Array HS11, SuperArray® Bioscience Corporation, Frederick, MD, USA), as we have described before [32] according to the manufacturer's protocol. A detailed gene list is available on the company's website (http://www.superarray.com/). Raw data were normalized between 0 and 100% expression, where the signals of the means of the negative controls (areas without spotted gene sequences or with genes not expressed in human cells) equalled 0% and the means of the signals all spotted house hold genes were fixed to equal 100%.

2.3.2 Total GST activity measurements

Cells were treated with the polyphenol mixtures in different concentrations for 6 and 24 h and cytosol was obtained as described earlier [19]. Total GST activity in the cytosol was determined spectrophotometrically at 340 nm using 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM glutathione as substrates at a temperature of 30°C [33].

2.3.3 Statistical analysis

The evaluation of all data was based on individual or mean values with cells from 24 human individuals or for the *in vitro* experiments from at least three independently reproduced experiments (one exception were the c-DNA arrays with $n = 2$). The Prism software version 4.01 (Graph Pad, San Diego, USA) was used for establishing two-sided significance levels using paired or unpaired *t*-tests, with and without Welch's corrections for unequal and equal variances, respectively. Two- and one-way ANOVA with Bonferroni's post-test were used, as appropriate, and as indicated in the results sections. Treatment-related effects were considered statistically significant at $p < 0.05$.

3 Results

3.1 *Ex vivo* determinations

Leucocytes were available from 24 of the total 27 subjects that had originally participated in the intervention study [10]. Of these 12 consumed juice A and 12 consumed juice B in the first intervention phase and then switched to juice B/A in the second intervention phase. There were 17 subjects with *hGSTM1*1*, 7 subjects with *hGSTM1*0* genotypes and 17% were *hGSTT1*0* carriers. Altogether, the numbers of subjects in these subgroups of polymorphisms were too small to assess possible differences in susceptibility. That is why we only aimed to discriminate some selected biological effects in drinkers of juices A and B, or of subsets with the *hGSTM1*1* and *hGSTM1**0 genotypes.

Table 2 shows the degrees of DNA damage and the levels of hGSTP1 protein expression in all 24 subjects or in the subgroups, it shows these two responses depending on the type of the consumed fruit juice or on the genotype. Since polyphenols may induce additional proteins other than hGSTP1, we chose to present the data as 'μg hGSTP1 protein *per* 10^6 cells' since we have previously found this to be a more valid demonstration of inducibility than 'μg hGSTP1 *per* total protein' [34]. It was apparent that there is a significant reduction of DNA damage in the leucocytes of all 24 donors after the second phase of intervention with the fruit juice, as had also been reported previously [10]. There were no differences between subjects just consuming juice A or B. There were also no differences between subjects with *hGSTM1*1* and *hGSTM1*0* genotypes. The only reason for the lacking significance of the results at week 8 in the *hGSTM1*0* genotypes (after the second phase of the fruit juice intervention) was probably the limited number of subjects in this group (*hGSTM1*0*, $n = 7$; *hGSTM1*1*, $n = 17$).

HGSTP1 protein expression was relatively higher before the fruit juice intervention than after the first 2 wk of treatment and after the 2 wk wash out period (Table 2). It seemed as if both interventions resulted in an inhibition of hGSTP1 in the peripheral blood leucocytes for all subjects taken together. There were no differences of response between the two juice interventions. There were also no apparent differences when comparing the subgroups with the different genotypes, but again the limited number of samples from subjects with *hGSTM1*0* genotype may be the reason for the lacking significance of the hGSTP1 increase at week 8. Altogether, the results point to an induction of hGSTP1 in peripheral leucocytes of subjects treated with the two fruit juices. This induction seemed to reflect an enhanced recovery of hGSTP1 protein subsequent to its initial inhibition following week 4 (after the first 2 wk of intervention with fruit juice) and week 6 (after the first wash out period). To see if there is any connection between DNA damage and hGSTP1 protein, we correlated hGSTP1

Table 2. DNA damage, expressed as '% fluorescence in tail'

Time point (week)	Parameter	Juice A			Juice B			All			*hGSTM1*1*			*hGSTM1*0*		
		Mean	SEM		Mean	SEM		Mean	SEM		Mean	SEM		Mean	SEM	
2	DNA	48	6		43	7.3		45	4.7		48	5.7		39	7.9	
4	damage	41	6.7	n.s.	32	3.4	n.s.	37	3.8	n.s.	38	5.1	n.s.	32	4.6	n.s.
6		36	3.8	n.s.	34	4.4	n.s.	35	2.8	n.s.	35	3.8	n.s.	35	3.4	n.s.
8		26	2.8	$p = 0.0039$	27	3.4	$p = 0.0074$	27	2.1	$p = 0.0011$	26	2.7	$p = 0.0025$	29	3.4	n.s.
2	hGSTP1	23	2.5		24	2.1		23	1.6		21	1.6		28	3.3	
4	ng/10^6 cells	14	2	$p = 0.0110$	15	1.5	$p = 0.0002$	15	1.2	$p < 0.0001$	15	1.5	$p = 0.0063$	13	2.2	$p = 0.0030$
6		14	2.2	$p = 0.0105$	15	2.1	$p = 0.0006$	15	1.5	$p = 0.0002$	12	1.4	$p = 0.0002$	19	3.5	n.s.
8		34	2.9	$p = 0.0106$	34	3.9	$p = 0.0341$	34	2.4	$p = 0.0009$	35	2.5	$p < 0.0001$	30	5.6	n.s.
				$p < 0.0001$			$p = 0.0004$			$p < 0.0001$			$p < 0.0001$			n.s.

Shown are mean values of endonuclease sensitive sites (consisting of DNA strand breaks, oxidized pyrimidines and other lesions) as determined using the comet assay with endonuclease III [54]). Total protein levels were 8 ± 1.2 and 7 ± 0.8 μg/10^6 cells for the volunteers consuming juices A and B, respectively. There was a significant increase of total protein (10 ± 1.2) in the cells of *hGSTM1*1* genotoypes, which was not considered to be of biological significance. Significant differences were assessed with unpaired *t*-test with Welch's correction for unequal variances. Numbers of volunteers were $n = 12$ for juices A and B, $n = 17$ for *hGSTM1*1* and $n = 7$ for *hGSTM1*0*.

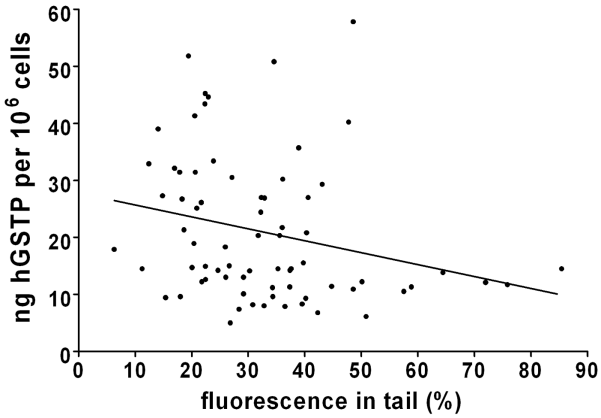

Figure 1. Individual strand breaks and oxidized pyrimidine bases *versus* hGSTP1 for all subjects ($n = 24$) and time points, which are significantly different from week 2, namely weeks 4, 6 and 8 ($n = 72$). The correlation (Spearman) is significant with $p = 0.005$.

levels against DNA damage using data from all time points that were significantly different from week 2, namely weeks 4, 6 and 8 ($n = 72$). Figure 1 shows that hGSTP1 levels and DNA damage were inversely correlated with a *p*-value of 0.005.

3.2 *In vitro* determinations

In vitro determinations with isolated human peripheral blood leucocytes and with polyphenols mixtures, mimicking the composition of the fruit juices, were carried out to enhance our understanding of effects of polyphenols in these types of target cells. For this, however, we first needed to determine noncytotoxic concentration ranges. For all investigations the *hGSTT1* and *hGSTM1* polymorphisms were determined (13% *hGSTT1*0* and 61% *hGSTM1*0),* but due to small sample sizes we could not see any differences between the subgroups for the *in vitro* determinations.

Cell integrity of the leucocytes, treated with different concentrations of polyphenols, was assessed with the CellTiter-Blue™ assay. Solutions were prepared and added in amounts delivering concentrations of 0.1–40 μM Cy-3-*O*-g or 0.1–40 μM EGCG to the final solution ('Cy-3-*O*-g equivalents' or ‚EGCG equivalents'), respectively, for mixtures A and B. The total polyphenol concentration available at, *e.g.*, 40 μM 'equivalents' is shown in Table 1. Table 3 shows that cell viabilities decreased significantly by at the highest employed concentration of polyphenol mixture B (40 μM EGCG-equivalents) and after the longest incubation periods (48 and 72 h). In comparison to mixture B, mixture A had a lower impact on cell viability.

Cytotoxic effects of the polyphenol mixtures were additionally directly investigated using the trypan blue exclusion assay, which measures the effects on membrane integrity. For the *in vitro* analysis, leucocytes were incubated with the two polyphenol mixtures and cell viability was measured as described in Section 2. After 2 h of incubation, the cells of the medium control had a viability of 95 ± 2% and the cells of the DMSO control had a viability of 92 ± 3%. These values were retained for up to 72 h of in *vitro* incubation, after which 92 ± 3% viability was scored in the medium control and 89 ± 3% in the DMSO control. Neither of the polyphenols had an effect on viability either, since even at the highest concentrations (40 μM equivalents) and longest durations of treatment (72 h) with mixtures A and B, cells were still viable at 91 ± 4 and 87 ± 2%, respectively.

DNA damage was determined as a sensitive marker of toxic effects reflecting the loss of DNA integrity. Leucocytes were incubated for 1 or 24 h at 37°C with different concentration of polyphenols, yielding 0.1–40 μM equivalents of Cy-3-*O*-g (mixture A) and EGCG (mixture B). There were

Table 3. Cell integrity, as measured using CellTiter-Blue™ assay, of human leucocytes after incubation with two polyphenol mixtures ($n = 3$)

	µM equivalents of reference compound	Duration of *in vitro* treatment 1 h Mean	SD	2 h Mean	SD	12 h Mean	SD	24 h Mean	SD	48 h Mean	SD	72 h Mean	SD
		% of the medium control											
Medium		100	0	100	0	100	0	100	0	100	0	100	0
DMSO		121	25	98	4	102	11	107	7	112	22	97	0
Mixture A[a)]	0.1	110	12	100	5	102	8	112	17	112	13	99	9
	1	103	8	100	6	104	9	113	6	106	4	109	6
	5	103	7	102	3	101	3	110	9	102	4	103	3
	10	108	12	107	13	101	10	110	11	101	1	105	3
	20	106	8	94	5	102	13	103	8	102	3	102	7
	40	104	12	98	3	98	8	97	5	90	4	79	25
Mixture B[b)]	0.1	85	5	97	6	101	5	106	8	98	2	99	2
	1	96	8	102	5	102	8	109	9	98	2	98	2
	5	108	10	105	16	101	13	106	10	100	3	99	3
	10	102	6	103	4	97	7	104	7	94	8	92	7
	20	106	6	102	7	103	7	100	12	81	14	80	29
	40	114	22	98	7	96	16	79	20	44	42	34	44

Concentrations were calculated for equivalents of Cy-3-*O*-g (mixture A) and EGCG (mixture B) in µM.
a) Reference compound was of Cy-3-*O*-g.
b) Reference compound was EGCG. The results were not significantly different from each other (one-way ANOVA with Dunnett's multiple comparison post-test) with the exception of mixture B 40 µM for 48 h and 72 h ($p < 0.01$).

no compound or time related increases of DNA damage (data not shown).

On the basis of the cytotoxicity measurements we were able to choose nontoxic but physiologically relevant concentrations for the gene expression analysis, which is an important requirement prior to assessing functional effects *in vitro* [35].

3.2.1 Gene expression with c-DNA array

To study functional effects, peripheral leucocytes were incubated for 24 h with 0.1 and 1 µM equivalents of Cy-3-*O*-g (mixture A) and EGCG (mixture B), equalling 0.52 and 0.83 µM or 5.2 and 8.3 µM total polyphenols for mixtures A and B, respectively (see Table 1). These incubation conditions and concentrations were noncytotoxic, as demonstrated above and are within the maximum plasma concentrations attained after a polyphenol-rich meal, (0.1–10 µM) [36]. Leucocytes expressed a number of genes related to drug metabolism. Of the 96 genes spotted on the membrane, 58 were expressed according to our guidelines (see Section 2). These included 11 of the 12 members of the GST family (*hGSTA2*, *hGSTA3*, *hGSTA4*, *hGSTM2*, *hGSTM3*, *hGSTM5*, *hGSTP1*, *hGSTT1*, *hGSTT2*, *hMGST2* and *hMGST3*) and three of the five spotted UGTs (*UGT1A1, UGT1A4* and *UGT2B10*), but only 9 of the 24 CYPs (*CYP2C9*, *CYP2C19*, *CYP2F1*, *CYP3A4*, *CYP3A5*, *CYP3A7*, *CYP4F3, CYP20A1* and *CYPOR*). On the basis of our gene-array results, *hGSTP1* is by far the major isoenzyme of all investigated *hGSTs*. Fold changes over the factor of 2, compared to the DMSO-control, were considered as regulated [32]. After incubation with mixture B (1 µM EGCG equivalents) seven genes (*ABCC2, CYP2F1, CYP3A4, CYP3A5, NNMT, SULT1C2 and SULT2A1)* meets this criteria (Table 4). The hGSTs and UGTs spotted on the membrane gave sufficient signals to indicate that they were well expressed (see Supplementary Material), but the polyphenols only tended to up-regulate these genes.

3.2.2 Enzyme activity measurements

Freshly isolated leucocytes were used for these determinations, since our previous studies had shown that there was a loss of hGSTP1 protein and of GST activity in cryopreserved cells [27]. Table 5 shows the enzyme activities in peripheral leucocytes incubated for 6 and 24 h with 0.1, 1, 10 and 40 µM equivalents of Cy-3-*O*-g (mixture A) and EGCG (mixture B). Neither of the polyphenol mixtures had an influence on GST activity. There was also no effect of the two mixtures on total protein content.

4 Discussion

Polyphenols inhibit DNA damage *in vivo* [10], although there are some studies with vegetable and fruits that do not show this association [37]. As has been discussed in detail, discrepancies could be due to type of polyphenols used during the intervention, the food matrix and bioavailability, dose and time point of analysis [38]. The present study how-

Table 4. Modulation of gene expression for genes related to drug metabolism in human leucocytes after incubation (24 h) with different polyphenol mixtures

Symbol	GeneBank	Description	Medium		DMSO		Mixture A 0.1 μM[a]			Mixture A 1 μM[a]			Mixture B 0.1 μM[b]			Mixture B 1 μM[b]		
			Mean	SD	Mean	SD	Mean	SD	Fold change	Mean	SD	Fold change	Mean	SD	Fold change	Mean	SD	Fold change
ABCC2	NM_000392	ATP-binding cassette, subfamily C, member 2	48.7	50.2	51.6	57.3	38.7	64.0	0.8	72.3	88.2	1.4	66.4	68.5	1.3	119.2	127.3	**2.3**
CYP2F1	NM_000774	Cytochrome P450, family 2, subfamily F, polypeptide 1	32.4	16.7	24.6	17.6	13.4	12.2	0.5	22.6	1.7	0.9	36.2	30.5	1.5	51.8	17.7	**2.1**
CYP3A4	NM_017460	Cytochrome P450, family 3, subfamily A, polypeptide 4	7.9	1.8	7.8	3.0	4.4	2.8	0.6	9.9	6.4	1.3	13.6	3.3	1.7	17.8	9.4	**2.3**
CYP3A5	NM_000777	Cytochrome P450, family 3, subfamily A, polypeptide 5	5.8	2.5	5.8	6.5	5.3	4.0	0.9	7.7	5.4	1.3	8.4	4.2	1.5	14.8	11.7	**2.5**
MGST3	NM_004528	Microsomal GST 3	14.5	0.6	22.6	15.4	19.7	18.2	0.9	8.3	4.5	**0.4**	11.6	19.4	0.5	16.1	26.1	0.7
NNMT	NM_006169	Nicotinamide *N*-methyltransferase	45.3	22.2	36.5	5.6	27.7	3.5	0.8	47.0	3.5	1.3	45.8	6.6	1.3	73.8	3.0	**2.0**
SULT1C2	NM_006588	Sulphotransferase family, cytosolic, 1C, member 2	5.7	3.6	4.1	0.3	4.2	1.0	1.0	5.5	0.1	1.3	5.1	0.7	1.2	10.0	0.5	**2.4**
SULT2A1	NM_003167	Sulphotransferase family, cytosolic, 2A, member 1	5.6	2.6	4.1	0.9	5.7	2.2	1.4	6.4	2.4	1.5	5.5	0.1	1.3	9.9	3.7	**2.4**

a) Reference compound was Cy-3-*O*-g.
b) Reference compound was EGCG. The data are mean values ± SD; fold changes were calculated to the DMSO control; fold changes below 0.5 and over 2 are marked bold; $n = 2$.

Table 5. Total GST enzyme activity (nM/min/10×10^6 cells) detected in peripheral blood leucocytes incubated with polyphenols mixtures for 6 and 24 h

	μM equivalents of reference compound	Duration of *in vitro* treatment 6 h Mean	6 h SD	24 h Mean	24 h SD
		nmol/min/10×10^6 cells			
Medium		1.6	0.6	2.3	0.9
DMSO		3.1	0.6	2.5	1.2
Mixture A[a)]	0.1	2.4	0.9	2.5	0.6
	1	2.3	1	2.4	1
	10	3	0	2.6	0.6
	40			3.1	1.8
Mixture B[b)]	0.1	3	0.7	2.4	0.5
	1	2.6	0.4	3	1.1
	10	3.1	0.2	3	1.2
	40			2.3	0.6

a) Reference compound was Cy-3-*O*-g.
b) Reference compound was EGCG. The data are mean values ± SD; $n = 3$ for 6 h and $n = 3$–6 for 24 h duration of treatment. The results were not significantly different from each other (one-way ANOVA with Dunnett's multiple comparison post-test).

ever was based on a specific intervention trial. The outcome of the study was that the consumption of two fruit juices providing 236 and 226 mg/day polyphenols resulted in improved antioxidant status, reduced level of oxidative DNA damage and enhanced immune functions with no differences between the two juices [10]. Postintervention measurement of oxidized DNA bases revealed a similar level of oxidized DNA bases as compared to baseline, when subjects were neither receiving fruit juice supplementation nor consuming a low-polyphenol diet. This may indicate that juice consumption was related to the reduction in DNA damage. The mechanisms however are not clear since both juices with totally different compositions (anthocyanin *vs.* green tea (catechins)) show identical results. This could mean that it was not the action of polyphenols thus was causing the effects but the fruit juice consumption itself. Even though the effects cannot be attributed to specific compounds of the juices, the findings are in line with our previous studies on the protective activities of vegetable juices using a similar set of biomarkers [29]. In another human intervention study with diabetic subjects the intake of polyphenol-rich food (110 mg/day) for 14 days also significantly reduced DNA damage in leucocytes [39], and other studies, *e.g.*, an human intervention study performed with red mixed berry juice showing these types of associations as well [26]. Here we were interested in determining whether a modulated state of chemoprotection reflected by an enhancement of toxicological defence systems, such as GSTs, contributes to these effects of reduced DNA damage.

We have previously determined that reduced genetic DNA damage in leucocytes may be due to the enhancement of cytosolic hGSTP1 by tomato and carrot juices [25]. In the present study, we now found that hGSTP1 is first suppressed in leucocytes obtained after the initial 2-wk intervention with the fruit juices. The suppression is still apparent after the first washout period. After another 2-wk period of intervention with the other fruit juice, however, hGSTP1 is then significantly induced. This confirms the previous observations on the time delay between the intake of polyphenols and other measurable and beneficial changes of

physiological functions [10]. The time point, at which hGSTP1 induction is significant, is the same as the time point of significant inhibition of oxidative DNA damage. This could suggest a causal relationship, at least for week 8 when the measurements were made. Before that there are no significant effects on DNA damage, but markedly reduced hGSTP1 levels. There was also a significant correlation ($p = 0.005$) between hGSTP1 protein levels and oxidative DNA damage, which agrees well with other studies showing that increased hGSTP1 values were accompanied by a reduction of DNA damage in human leucocytes [40]. The immediate decrease of hGSTP1 protein expression is probably not due to the direct toxicity of the fruit juice polyphenols, since our *in vitro* studies do not indicate a particular vulnerability of the peripheral leucocytes after polyphenol incubation. However, also a treatment of K562 leukemia cells with the natural compound curcumin leads also to an inhibition of the hGSTP1 protein *via* AP-1 and NFκB transcription factors [41]. It is possible that this reduction is specific for human blood cells like leucocytes since also Persson *et al*. [42] reported a reduction of hGSTP1 on mRNA and protein level after 250 g of additional mixed vegetables *per* day.

As recommended previously for performing *in vitro* studies with polyphenols, our functional studies were carried out at concentrations within the range of the maximum concentrations that have been detected in plasma of human volunteers consuming a polyphenol-rich meal. These levels are reported to be in the range of 0.1–10 μM [35]. For example, plasma EGCG levels were 0.17 μM [43] or 0.72 μM in humans consuming green tea solids (dissolved in water) [44]. We have previously shown that GST protein expression, namely hGSTT1, is inhibited by butyrate [45]. This observation in human colon adenoma cells was attributed to an enhanced degradation of *hGSTT1* mRNA thus reducing protein expression. Whether or not this could be a mechanism, by which the fruit juices first reduced hGSTP1 expression *in vivo* is not known, and the results of our *in vitro* investigations performed here do not support the mechanism.

Hepatic GSTs have been shown to be induced by carcinogenic xenobiotics, which however is not necessarily involved with a chemopreventive benefit [46]. Alternatively antioxidants or other anticarcinogenic xenobiotics may induce GSTs, which enables experimental animals to better tolerate exposure to carcinogens [47, 48]. Recent analysis for *GST* polymorphisms revealed heterogeneous associations indicating that the *GST* genotype could be associated with increases in cancer risks or that there were no associations, depending on tissue, on exposure situations and on coupling of polymorphisms for other genes [49–52].

The new findings presented here showed that oxidative DNA damage in response to polyphenol-rich fruit juices were not different in *hGSTM1*1* and *hGSTM1*0* subjects. This is different from another study by our group where we did observe more leucocyte DNA damage in *hGSTM1*0* than in *hGSTM1*1* subjects (12.3 ± 4.6 *vs.* 9.4 ± 2.9, $p < 0.05$) after intervention with bread rich in prebiotics ± antioxidants [53]. In the present study, the total levels of endonuclease III sites were higher, probably a reflection of higher activity of the batch of endonuclease III used in these studies, which was from a different source.

We have also recently shown that the continuous presence of EGCG can reduce radical induced DNA damage in primary leucocytes [27]. On the basis of the experimental protocol we were able to conclude that this is possibly due to a combination of different mechanisms. For one, it was hypothesized that EGCG may favourably modulate expression of antioxidative systems as could occur during the 18 h pretreatment and secondly it was concluded that EGCG could scavenge free radicals. Ellagic acid, ferulic acid, Brussels sprouts, quercetin, α-angelicalactone, tannic acid, coumarin, fumaric acid, curcumin and flavone, separately, and combinations of α-angelicalactone and flavone given to rats in the diet increased either hepatic or intestinal (proximal, mid and distal small intestine and colon) UGT enzyme activities, or both [13]. In the present study, we had addressed the question of whether UGTs could also be induced in peripheral leucocytes by polyphenols mixtures that were composed to mimic the fruit juices of the *in vivo* study. The original aim was to elucidate whether the specific polyphenols from fruit juice could contribute to mechanisms resembling chemopreventive activities. Since effects, however, were not found it will be necessary in the future to investigate also the whole juice (up to the limits of toxicity) and to identify additional components with similar biological activities in leucocytes, as well as in hepatocytes and in gastrointestinal cells.

In conclusion, here we have presented new *in vivo* evidence showing a modulated content of hGSTP1 protein in peripheral leucocytes from human subjects who had been consuming fruit juices with high levels of polyphenols. Interestingly, polyphenol mixtures that we composed to mimic the polyphenol concentrations in the juices did not modulate GSTs on mRNA and on enzyme activity level *in vitro*. They did not inhibit nor enhance expression of genes related to this group of enzymes. This could either be due to the *in vitro* situation, which is not able to mimic the delayed response observed *in vivo* (*e.g.*, 24 h exposure *vs.* 8 wk), or to the mixture of polyphenols, which may not contain the (as yet unidentified) key ingredients responsible for the effects on hGSTP1 by the complex whole fruit juice *in vivo.*

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