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RESEARCH PAPER

Gene expression profiles in human peripheral blood mononuclear cells as biomarkers for nutritional in vitro and in vivo investigations

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Abstract Identification of chemopreventive substances may be achieved by measuring biological endpoints in human cells in vitro. Since generally only tumour cells are available for such investigations, our aim was to test the applicability of peripheral blood mononuclear cells (PBMC) as an in vitro primary cell model since they mimic the human in vivo situation and are relatively easily available. Cell culture conditions were refined, and the basal variation of gene expression related to drug metabolism and stress response was determined. Results were compared with profiles of an established human colon cell line (HT29) as standard. For biomarker development of nutritional effects, PBMC and HT29 cells were treated with potentially chemopreventive substances (chrysin and butyrate), and gene expression was determined. Key results were that relevant stress response genes, such as *glutathione S-transferase T2* (*GSTT2*) and *GSTM2*, were modulated by butyrate in PBMC as in HT29 cells, but the blood cells were less sensitive and responded with high individual differences. We conclude that these cells may serve as a surrogate tissue in dietary investigations and the identified differentially expressed genes have the potential to become marker genes for population studies on biological effects.

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Abbreviations

CAT	Catalase
COX-2	Cyclooxygenase-2
CV	Coefficient of variation
GSTM2	Glutathione S-transferase M2
GSTP1	Glutathione S-transferase P1
GSTT2	Glutathione S-transferase T2
H_2O_2	Hydrogen peroxide
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinine
ppm	Parts per million
SD	Standard deviation
SOD2	Superoxide dismutase 2, mitochondrial
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1

Introduction

A number of recent reports are showing that the latest technologies of genomics and proteomics have initiated a new era of biomarker development and many researchers are further developing the methods for cancer detection, treatment, and prevention [19, 46]. Different biomarker applications are based on blood cells such as PBMC, since they are obtained less invasively than biopsies and are suitable to measure systemic effects. Moreover, PBMC are used as surrogate tissues that may mimic effects occurring in remote target tissues of exposure [32, 37]. In vitro, these cells can be used as human primary cell models [15, 16].

For this, PBMC are normally isolated from leucocyte-enriched human blood preparations (buffy coats) and then treated with test substances over different periods before measuring distinct endpoints such as DNA damage. PBMC are suitable cells to analyse chemoprotective potentials of dietary food ingredients as has been shown in vivo after dietary interventions with fruit juices or water cress [14, 50]. In order to develop techniques for the evaluation of health effects in vitro further, it is necessary to assess the question how experimental conditions influence the selected parameters and how PBMC react to different stimuli in comparison to other target cells, such as a colon cancer cell line. For this approach, relevant genes related to stress response and drug metabolism were selected, namely *GSTP1*, *GSTT2*, *GSTM2*, *UDP glucuronosyltransferase 1 family, polypeptide A1* (*UGT1A1*), *catalase* (*CAT*), *superoxide dismutase 2* (*SOD2*), and *cyclooxygenase-2* (*COX-2*). The protein products of these genes are linked to both oxidative stress and carcinogenesis. Expression levels of these genes have been shown to be altered in human cells by dietary compounds and metabolites such as butyrate and the flavonoid chrysin. Butyrate, formed by gut fermentation of dietary fibre [12, 33, 38], is believed to act as a potential chemopreventive agent [40]. Luminal concentration of butyrate in humans is found to range from 11 to 25 mM, and concentrations in human portal veins are 1,000-fold lower and further decrease in the venous serum [17]. Butyrate (10 mM) treatment of primary colonocytes has been shown to modulate stress-related genes [38]. Gene expression of *CAT* was induced, while *COX-2* was reduced in colon cancer cells through histone deacetylases inhibition [43]. In addition, incubation of human colon cells of different transformation state with butyrate induced *GSTT2*, *GSTP1,* and *UGT1A1* [34]. Also, *GSTM2* was strongly induced by 4 mM butyrate in HT29 carcinoma cells [12]. PBMC were incubated with butyrate concentrations that reflect physiological concentrations for colonocytes but not for PBMC in order to test their suitability as surrogate tissue. Chrysin (5,7-dihydroxyflavone) is an inhibitor of the enzyme aromatase, which converts androgens to oestrogens, and a potent inducer of UGT1A1 catalytic activity in primary human hepatocytes, the human hepatoma cell line HepG2, and the human intestinal cell line CaCo-2 [13, 41].

In this study, we focused amongst others on phase II enzymes such as GSTs. These enzymes may be of importance in cancer development and therapy, and they can be modulated by dietary factors [20]. They catalyse nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom [33]. The different isoenzymes show diverse substrate specificities and are influenced by both intrinsic (GST polymorphisms) and extrinsic factors (consumption of fruits and vegetables) [49].

The first aim of this study was to investigate the applicability of gene expression in PBMC as biomarker for in vivo and in vitro investigations. In particular, it was of practical importance to determine how cryopreservation and thawing of isolated PBMC affects survival, viability, and responsiveness to different treatments when measuring gene expression as biological endpoint. Normally, isolated human PBMC do not proliferate in cell culture. For this, phytohemagglutinine (PHA), a lectin extract from the kidney bean (*Phaseolus vulgaris*), is often used in vitro to stimulate the growth of PBMC [18, 36]. Here, we investigated how PHA, which may improve the suitability of PBMC as surrogate tissue by inducing proliferation, affects parameters of gene expression. The second objective consisted of investigating patterns of gene expression of important drug metabolism and stress response enzymes and their modulation by nutritional factors. To this end, we used cDNA array and real-time PCR technology in the cellular model that mimics the human in vivo situation (thus quiescent PBMC) and compared the results with an established cancer cell line (HT29). Knowledge about the effects and comparison of both cell types might aid in validating the potential of using gene expression profiles of PBMC as biomarkers for human in vivo as well as in vitro studies, which could also be used on a large scale, i.e. during clinical trials or intervention studies.

Materials and methods

Test substances

PHA (Roche Diagnostics, Mannheim, Germany) was diluted in Milli-Q water to obtain a stock solution of 5 mg/mL and was further diluted in cell culture medium to yield a working solution of 10 µg/mL. Butyrate (Merck KGaA, Darmstadt, Germany) was directly diluted in cell culture medium to reach the concentrations 4 and 10 mM. For chrysin (Sigma, Deisenhofen, Germany), a stock solution (10 mM) in DMSO was prepared and further diluted in cell culture medium to reach a concentration of 10 µM. The final DMSO-concentration was 0.1%.

Cell culture of PBMC and HT29

A fraction of mononuclear cells was isolated from several anonymous buffy coat preparations by gradient centrifugation using HISTOPAQUE®-1077 (Sigma, Deisenhofen, Germany) as described earlier [6, 16]. All blood donors gave their informed consent. Cells were used either directly after isolation, kept in cell culture medium (PBMC medium: RPMI 1640 medium (Invitrogen GmbH, Karlsruhe, Germany) with 10% heat-inactivated foetal calf serum, 1%

penicillin/streptomycin (Invitrogen GmbH, Karlsruhe, Germany), and 1% L-glutamine (Invitrogen GmbH, Karlsruhe, Germany)) overnight prior to incubation, or were cryopreserved at −140°C (deep freezer QC 10140, Nunc, Wiesbaden, Germany). These cells were thawed rapidly in a water bath at 37°C prior to each experiment.

For gene expression investigations over different time periods, a sample subset from a study by Gill et al. [14] was used. We analysed three time points, namely the start of the intervention, after an eight-week control phase, where the subjects were asked to maintain their habitual diet, and again after a seven-week washout phase, without any restrictions for the subjects. Collection of blood samples and isolation of PBMC were performed as previously described in the original study [14].

The human colon adenocarcinoma cell line HT29 is derived from a moderately differentiated, grade II adenocarcinoma of the rectosigmoid colon. These cells were obtained from the American Tissue Culture Collection (ATCC HTB-38) and maintained as a subconfluent monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM; containing 4,500 mg/L glucose and 58 mg/L L-glutamine; Invitrogen GmbH, Karlsruhe, Germany), supplemented with 10% foetal calf serum (Invitrogen GmbH, Karlsruhe, Germany) at 37°C in a humidified incubator (5% CO_2, 95% humidity). We used passages 23–37 (after receipt from the ATCC) for the experiments reported in this paper.

Treatment of PBMC and HT29 cells

The number of PBMC was adjusted to 4×10^6 cells/mL, and cells were incubated with the test substances for 24 h in 25 cm^2 cell culture flasks. Cell number and viability of PBMC were determined before and after treatment with the test substances using a trypan blue solution (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) in a hemacytometer. HT29 cells were seeded in 25 cm^2 cell culture flasks (4×10^6 cells) and incubated 24 h later with the test substances for additional 24 h. Cell number and viability were also determined using a trypan blue solution.

Cytofluorometry

The expression of CD3, CD4, CD8, CD14, CD19, CD20, CD28, and CD56 was measured in PBMC (isolated from different buffy coats) by FACSCalibur® using CellQuest Pro Software (BDBiosciences, Heidelberg, Germany) and standard procedures. Antibodies were labelled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) and were obtained from different sources (CD3 and CD14 (Immunotech, Marseille, France), CD4 and CD8 (Beckman Coulter, Marseille, France), CD19 (BDBiosciences, Heidelberg, Germany), CD20 and CD28 (Serotec Düsseldorf, Germany), and CD56 (Biozol, Eching, Germany)).

RNA isolation

Total RNA from PBMC and HT29 cells was isolated from unfrozen cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 50 µL RNase free water and stored at −20°C. RNA quantification was done spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) or using a NanoDrop ND-1000 photometer (Thermo Fisher Scientific, Wilmington, United States) and either checked for integrity of the ribosomal RNA using formaldehyde denaturing RNA gel electrophoresis or Agilent Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, United States). The RNA Integrity Numbers (RINs) for the samples were obtained. Only RNA samples with RIN values of 7, or higher, were used for the analysis.

Real-time PCR analysis

Expression of mRNA was assessed by two-step SYBR Green I relative real-time PCR (iCycler iQ system, Bio-Rad GmbH München, Germany). Briefly, total RNA (1 µg) was converted into first-strand cDNA using SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer's protocol. Using oligo(dT) leads to specific transcriptions of mRNA. The PCR amplification reactions contained 2 µL of first-strand cDNA mixed with 12.5 µL of iQ SYBR Green Supermix (Bio-Rad GmbH München, Germany, master mixture: 2 × mix containing SYBR Green I dye, hot-start iTaq DNA polymerase, optimised buffer, and dNTPs qualified for quantitative PCR), 2.5 pmol of each of the specific primer pairs in a final reaction volume of 25 µL. All reactions were performed in duplicate. No-template controls (2 µL H_20) were included on each plate for each primer pair. The PCR profile consisted with initial denaturation of 2 min at 95°C, 40 cycles of 30 s at 94°C denaturing, 30 s at 60°C annealing, 40 s at 72°C extension, and followed by final extension step of 10 min at 72°C. Cumulative fluorescence was measured at the end of the extension phase of each cycle. Product-specific amplification was confirmed by melting curve analysis. Gene-specific primer sequences used for the quantification can be seen in Table 1. Confirmation of the gene specificity of primer nucleotides was performed using the NCBI-BLASTN search program and additionally after a test-PCR also with an agarose gel. Gene expression of all genes calculated based on the housekeeping gene *GAPDH* ($2^{[-Ct_{target\ gene}]}/2^{[-Ct_{GAPDH}]}$) and expressed as parts per million (ppm). The main

Table 1 Primer sequences (F forward, R reverse) for real-time PCR analysis

Gene	Locus ID		Primer sequences 5′ to 3′	Product (bp)
CAT	MN_001752	F	5′-TGG ACA AGT ACA ATG CTG AG-3′	144
		R	5′-TTA CAC GGA TGA ACG CTA AG-3′	
COX-2	MN_000963	F	5′-TCC TCC TGT GCC TGA TGA TTG C-3′	170
		R	5′-ACT GAT GCG TGA AGT GCT GGG-3′	
GAPDH	MN_002046	F	5′-ACC CAC TCC TCC ACC TTT GAC-3′	110
		R	5′-TCC ACC ACC CTG TTG CTG TAG-3′	
GSTM2	MN_000858	F	5′-AGC CGT ATG CAG CTG GCC AAA C-3′	149
		R	5′-GGA CAA AGG TGA TCT TGT CCC CA-3′	
GSTP1	MN_000852	F	5′-CTG CGC ATG CTG CTG GCA GAT C-3′	149
		R	5′-TTG GAC TGG TAC AGG GTG AGG TC-3′	
GSTT2	MN_000854	F	5′-TGA CAC TGG CTG ATC TCA TGG CC-3′	142
		R	5′-GCC TCC TGG CAT AGC TCA GCA C-3′	
UGT1A1	MN_000463	F	5′-TCA TGC TGA CGG ACC CTT TC-3′	145
		R	5′-CTG GGC ACG TAG GAG AAT GG-3′	
SOD2	MN_000636	F	5′-GCC CTG GAA CCT CAC ATC AAC-3′	111
		R	5′-CAA CGC CTC CTG GTA CTT CTC-3′	

reason for the choice of *GAPDH* as the reference gene is the fact that it is also spotted on our cDNA-arrays used in this study. For these arrays, the spot intensities of the target genes were also based on the signal intensities for *GAPDH*. Gene expression results were evaluated and presented in the ppm-format, which gives more information, compared to the fold change-format, particularly, when comparing basal gene expression in different cells and cells from different donors. An external control was used to analyse the effects of PHA on the expression of *GAPDH*.

cDNA array

Hybridisation was performed on 112 sites (blanks, negative reference spots, housekeeping genes, and 96 human genes related to drug metabolism) on two different cDNA gene macroarrays (Human Drug Metabolism Gene Array HS11 and Human Stress & Toxicity Gene Array HS12, SuperArray Bioscience Corporation, Frederick, MD, United States), as we have described before [46] according to the manufacturer's protocol. Spot intensities of the target genes were based on the signal intensities for *GAPDH*. Only *GAPDH* has been expressed with sufficient intensities in both cell lines and was reasonably stable in our experiments, when comparing different blood donors (data not shown).

Polymorphisms

Cryopreserved lymphocytes were used to isolate DNA with the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) as described in the manufacturer's manual. A polymerase chain reaction (PCR) method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes for every blood donor using original procedures [3, 28]. A fragment of the ß-globin gene was co-amplified as internal positive control in the PCR.

Statistics

Data were expressed as mean with standard deviation (SD) or single data points. The Prism software version 4.01 (Graph Pad, San Diego, United States) was used for two-sided paired and unpaired *t*-tests, one-way ANOVA with Bonferroni's multiple comparison test with selected pairs, and also two-way ANOVA with Bonferroni post-hoc tests. A P value below 0.05 was assumed to be significant.

Results

Characterisation and suitable cell culture conditions for PBMC

The two main subpopulations in PBMC, isolated from different buffy coats with a mean viability of 90 ± 9% ($n = 6$), were lymphocytes (76%) and monocytes (15%) (Table 2). The CD expression of the isolated blood cells is summarised in Table 2.

For first experiments, PBMC were divided into two groups. One was treated directly after isolation, and the other was first frozen for storage, thawed and then

Table 2 FACS analysis and Cluster of Differentiation (CD) expression in PBMC isolated from different buffy coats ($n = 3$)

Cells	Mean (%)	SD
Lymphocytes	75.6	4.0
CD3	71.0	1.8
CD4	41.7	17.2
CD8	33.1	14.9
CD19	10.8	2.6
CD20	8.7	0.4
CD28	48.4	21.0
CD56	21.8	17.3
Monocytes	15.4	4.5
Granulocytes	9.0	8.1

processed in identical fashion. Viability detected with trypan blue exclusion was not different between the buffy coats. The recovery of the cells after thawing was only 66 ± 15% ($P = 0.002$), but viability of the remaining cells was not reduced. Freshly isolated and thawed PBMC (4×10^6 cells/mL) were treated for 24 h with medium and PHA (10 µg/mL), and Table 3 shows the effects on different biological parameters. There were significantly reduced numbers of thawed PBMC in comparison to fresh PBMC after the 24-h incubation period (43 ± 15% calculated to the respective fresh PBMC). Viability of cryopreserved PBMC was reduced significantly in comparison to fresh PBMC. Treatment with PHA significantly reduced *GSTM2* and *CAT* gene expression in fresh and cryopreserved PBMC (Table 3). In continuative experiments, we tested the reliability of *GAPDH* as housekeeping gene in the target cells. Down-regulation of *CAT* and *GSTM2* was based on a significant up-regulation (4.5-fold in fresh PBMC, 2.4-fold in cryopreserved cells) of *GAPDH* after PHA treatment ($n = 3$). So, this effect was also more pronounced in fresh PBMC than in cryopreserved PBMC.

Basal gene expression in PBMC and HT29 based on two different platforms

Basal gene expression of selected target genes of drug metabolism and stress response in PBMC were compared with HT29 cells using two platforms, namely cDNA array and real-time PCR (Fig. 1). HT29 cells were used as a standard to determine whether the variations are based on the individual blood donors or technical variations. Lower variation of gene expression data in PBMC using cDNA array (Fig. 1a) may reflect the lower sensitivity of this particular cDNA technique in comparison to real-time PCR for which high inter-individual differences were detectable (Fig. 1b). For *UGT1A1*, high inter-individual variations (coefficient of variation (CV) 243%) were measured, for some donors, *UGT1A1* was hardly detectable. Beside this gene, all other genes were expressed in every blood donor. *GSTP1* and *CAT* were expressed at comparable levels in both PBMC and HT29 cells. *COX-2* was the only gene with distinctly higher expression levels in primary PBMC than in HT29 colon carcinoma cells. According to cDNA array, *GSTM2* was not expressed in HT29 cells. Real-time PCR investigations also showed quite low mRNA amounts for *GSTM2*.

Changes in individual basal gene expression over time

For several genes, high variations in gene expression in PBMC could be detected (Fig. 1b). In order to test whether these variations are just snap-shots or if they are specific for individuals, we analysed basal gene expression profiles for ten subjects after three different time points, namely at a starting point (week zero), after eight weeks, and again after 15 weeks. The results for three genes (*UGT1A1*, *SOD2*, and *GSTP1*) can be seen in Fig. 2. For all genes, subjects with a low expression of a certain gene retained this low expression, and people with a high expression showed a comparable high expression during the different time points.

Table 3 Effects of treatment for 24 h with PHA (10 µg/mL) on cell number, viability, and relative gene expression of *GSTM2* and *CAT* (based on *GAPDH*) in freshly isolated PBMC and cryopreserved PBMC

		Relative gene expression							
		Cell number (10^6/ml)		Viability (%)		GSTM2		CAT	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Fresh	Medium control	3.97a	1.05	96c	2	1.00e	0.00	1.00g	0.00
	PHA	3.56b	0.51	94d	4	0.44e	0.09	0.18g	0.01
Cryopreserved	Medium control	1.83a	0.67	82c	9	1.00f	0.00	1.00h	0.00
	PHA	1.26b	0.20	71d	3	0.41f	0.19	0.24h	0.06

Statistical analysis was performed with one-way ANOVA and Bonferroni's multiple comparison test with selected pairs, and differences between fresh and cryopreserved cells were tested with two-way ANOVA, *same letters* indicate significant differences, $n = 3$

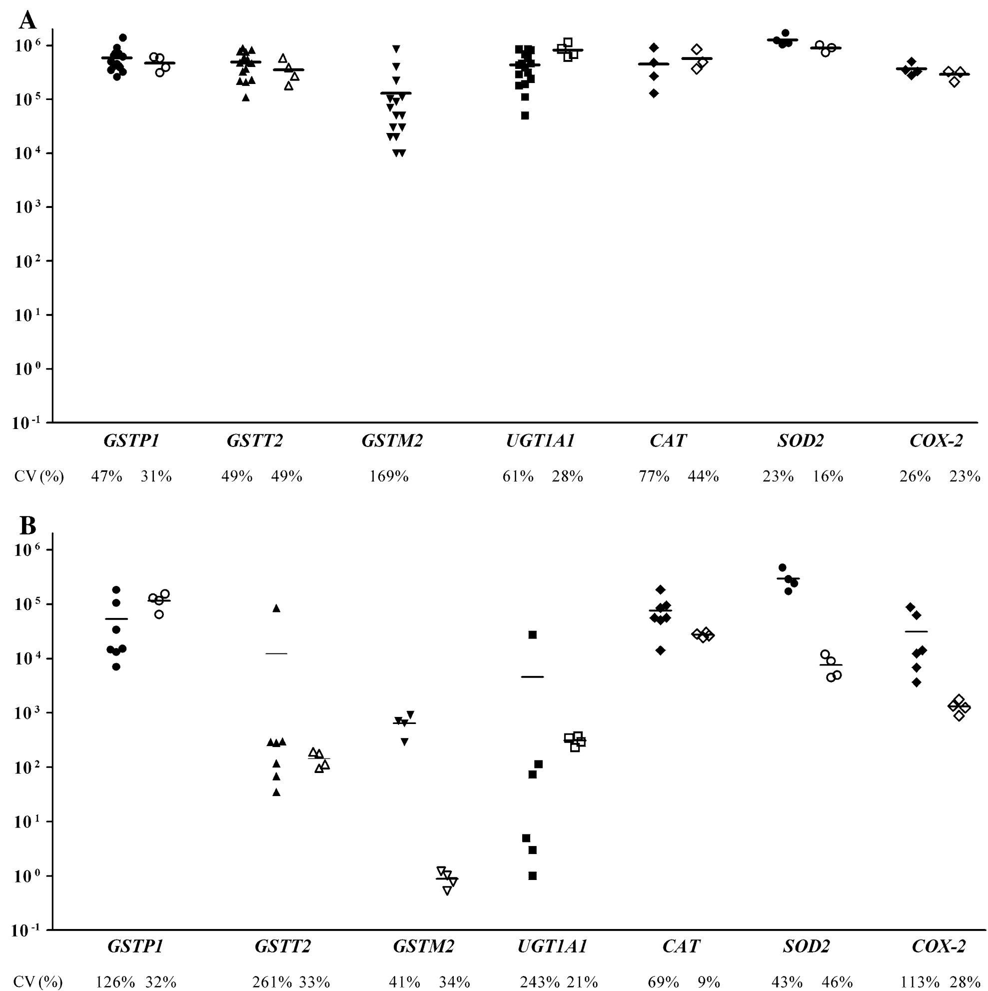

Fig. 1 Relative gene expression (based on expression of housekeeping gene *GAPDH* [ppm]) of target genes in untreated cells in two different platforms: **a** cDNA array, **b** Real-time PCR. Basal gene expression in PBMC (*filled characters*) was characterised by high individual variation, whereas HT29 cells (*open characters*) demonstrated low variability. Variation was detected more sensitive in real-time PCR experiments. *GSTM2* was not detectable in HT29 cells using cDNA array

Modulation of gene expression in HT29 and PBMC after incubation with butyrate and chrysin

In analogy to previous studies [38], we incubated both cell types with either 4 mM or 10 mM butyrate. These concentrations are physiological for the colon cells [17] and are non-toxic for both cells types. For both concentrations, there was no significant influence on cell viability or on the cell amount detectable (data not shown). After 24 h, relevant stress response genes such as *GSTT2* and *GSTM2* were significantly modulated in the same direction in HT29 cells and in PBMC (Fig. 3a, b). Butyrate (10 mM) significantly increased *GSTT2* and *GSTM2* gene expression in both cell types with much stronger effects on HT29. PBMC seemed to be less sensitive compared to colon cells and showed high variations from donor to donor. Chrysin, which is considered to be a potent *UGT1A1* inducer, showed a significant effect on *GSTM2* expression, but no significant induction of *UGT1A1*, only a similar trend (fold change in PBMC 3.5, in HT29 1.5) could be detected in both cell types.

GSTM1 and T1 genotypes

Null polymorphisms (*GSTM1*0* and *GSTT1*0*) of all blood donors ($n = 8$) used for the in vitro experiments were analysed to possibly explain individual responses. The frequencies of *GSTM1* and *GSTT1* null genotypes were 0.5 and 0.25, respectively. One donor was negative for both enzymes (*GSTM1*0* and *GSTT1*0*). The stratification of subjects with different genotypes revealed no differences for in vitro experiments.

Discussion

The so-called surrogate tissue analysis is not a new concept, but using new DNA and RNA techniques could bring a multiplicity of important information and a better understanding of cellular pathways. Only a few studies in the recent years looked at gene expression profiles in human blood cells, with a focus on different diseases such

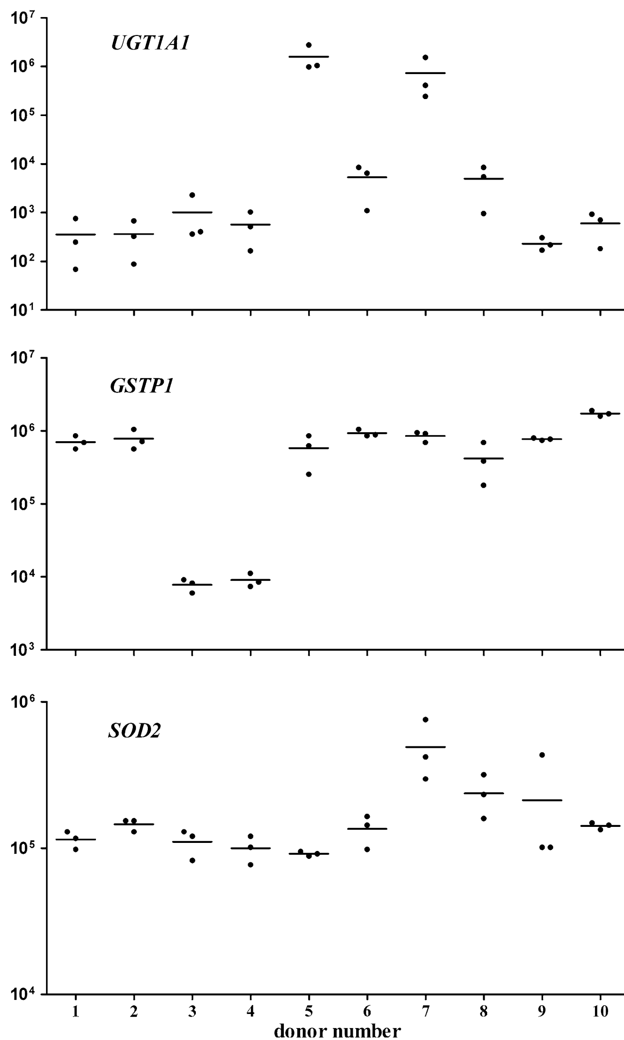

Fig. 2 Real-time PCR analysis of selected genes that were investigated in PBMC. Shown are the basal gene expression profiles for 10 subjects after three different time points, namely at a starting point (week zero), after 8 weeks, and again after 15 weeks. *GAPDH* (housekeeping gene) was used as internal reference control (gene expression related to *GAPDH* [ppm])

as multiple sclerosis, cancer, or coronary heart disease [1, 7]. Nutritional-related ex vivo transcriptomic modulations in human PBMC are not often investigated, with a few exceptions such as studies on *COX-2* modulation by quercetin [11] or the activation of the nuclear receptor peroxisome proliferator–activated receptor α (PPARα) [4]. Our data help to define the extent and nature of the normal variability in gene expression in human blood cells and the feasibility of modulated gene expression in vitro. In this study, PMBC are used as a primary cell model as they are relatively easy to obtain, reflect individual differences and, importantly, give information for their potential use as target cells for further in vivo studies.

For practical reasons, PBMC obtained ex vivo are often cryopreserved until use. These cryopreserved PBMC are suitable and accepted biomarkers, e.g. to analyse effects of intrinsic and extrinsic factors on oxidative DNA damage [30, 39]. Since modulation of gene expression was the endpoint in this study, we investigated how storage and the mitotic stimulation affect the responsiveness of these cells. The lectin PHA was included in this study since it is often used for detecting DNA damage or DNA repair in PBMC, because the ability of mammalian cells to remove DNA damage is correlated with cell activity [26, 48]. On the other hand, PHA possibly superposes effects of or interferes with the test substance. In our study, PHA significantly up-regulated the housekeeping gene *GAPDH* by a factor of five after a 24-h incubation, a modulation which was also found by Vereninov et al. [47] in proliferating PBMC. Especially the regulation of housekeeping genes seems to be inappropriate for in vitro gene expression studies with PBMC. Significant induction of *GAPDH* by PHA was lower in cryopreserved PBMC, which indicates less responsiveness of cryopreserved PBMC to substances that modulate gene expression. Taken the results together, it seems to be advisable to use unfrozen and unstimulated PBMC for in vitro cell culture experiments.

We measured the expression of seven important drug metabolism and stress response genes simultaneously with two different platforms in PBMC and in the colon cancer cell line HT29. The measurement of basal expression in PBMC has been performed without any previous inductions. All investigated genes were expressed in each individual, with the exception of *UGT1A1* in one donor. Thus, these blood cells fulfil one important qualification to be considered as a suitable surrogate tissue, which may indicate the status of cellular detoxification systems in humans and the possible modulation by food ingredients. The differences between individual donors were higher compared to HT29 (Fig. 1). Since the colon cancer cells are derived from one clone and should have a stable gene expression, we used them as reference to make statements possible about the individual differences in PBMC. In the blood cells, particularly high variations could be detected for *UGT1A1* (CV 243%) and for *GSTT2* (CV 261%). For *UGT1A1*, these high variations may be caused by the numerous polymorphisms in the *UGT1A1* gene [10]. The most common polymorphism in Caucasian populations is a TA insertion in the $TA(TA)_6TAA$ sequence in the promoter region, which is responsible for a decreased transcription rate [35]. The baseline gene expression level in PBMC could be also of particular importance for nutritional interventions. One hypothesis, which is supported by studies by Chow et al. [9] is that differential effects on activity of detoxifying enzymes are based on baseline enzyme level. In addition, it is interesting that basal gene expression seems to be quite conserved in individuals, since the expression levels of three investigated genes

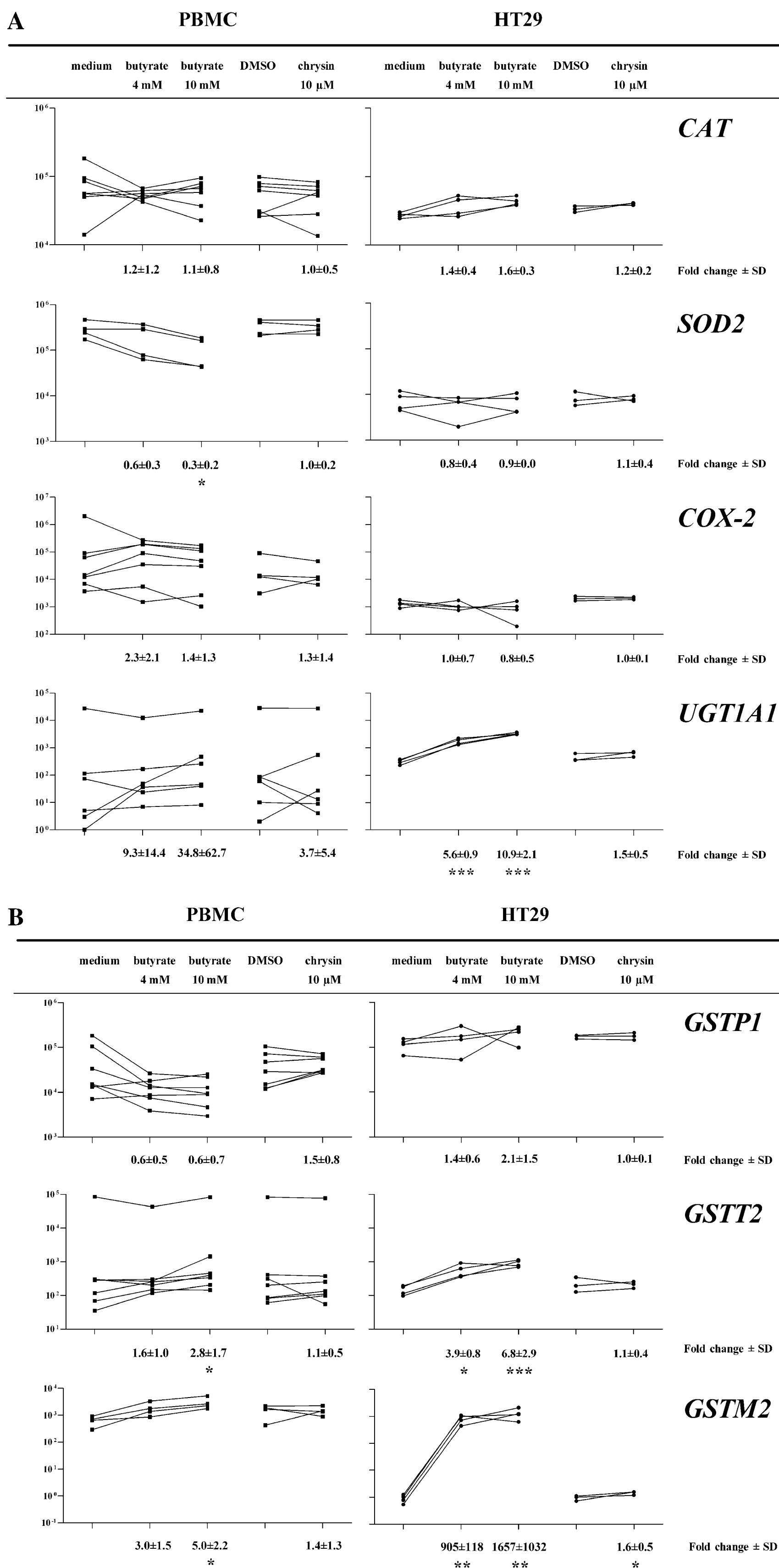

Fig. 3 **a** and **b** Real-time PCR analysis of selected genes that were investigated in PBMC (*left side*) and HT29 cells (*right side*). Shown are effects of butyrate and chrysin after 24-h incubation on the expression levels of *CAT, SOD2*, *COX-2, UGT1A1*, *GSTP1*, *GSTT2*, and *GSTM2*. The modulations of mRNA levels were obtained by comparing the treatment groups to the medium controls and calculating the fold changes. *GAPDH* (housekeeping gene) was used as internal reference control (gene expression related to *GAPDH* [ppm]). Significant differences to the controls were calculated by a one-way ANOVA with Bonferroni's post-test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, $n = 4$–7 for PBMC and $n = 3$–4 for HT29)

showed just minimal intra-individual but high inter-individual changes. Our data suggest the hypothesis that there are no individuals with a general low baseline detoxification capacity; rather every individual has a specific pattern for different detoxification enzymes.

Beside the basal expression of the investigated genes, another requirement for biomarker development is the responsiveness of cells to in vivo/in vitro stimuli. After incubation with 10 mM butyrate, there was a significant up-regulation of *GSTT2* and *GSTM2* in both cell types. GSTM2 catalyses the detoxification of oxidised metabolites (*o*-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes [2]. The observation that *GSTM2* mRNA was not detectable in untreated HT29 cells is in line with the observation of Ebert et al. [12] who could neither detect *GSTM2* mRNA nor GSTM2 protein in unstimulated HT29 cells. GSTT2 has peroxidase activity towards t-butyl hydroperoxide and most pronounced against cumene hydroperoxide, whereas there is no detectable activity towards H_2O_2 [21, 24, 42]. *GSTT2* polymorphisms are associated with colorectal cancer risk [25], and GSTT2 protein levels can be enhanced in rat colon by administration of acetyl salicylic acid, which has been shown to reduce rat colon cancer risk [45]. Therefore, the enhanced levels of *GSTT2* and *GSTM2* products possibly result in an elevated detoxification of peroxides or other oxidised metabolites. This suggests a better protection of butyrate-treated cells during situations of metabolic and oxidative stress. It remains to be elucidated whether the typical expression patterns may afford chemoresistance of the cells to appropriate substrates, some of which may be cancer risk factors. On one hand, an induction of GSTs in primary cells seems straightforward and favourable since this should result in an enhanced detoxification of risk factors. In tumour cells such as HT29 on the other hand, GST induction could counteract cancer chemotherapy by causing resistance to therapeutic agents, thereby enhancing the survival of transformed cells. *GSTP1* is one of the main extrahepatic GSTs and is markedly expressed in PBMC, where it may serve as a biomarker for induction of phase II enzymes by dietary intervention [31]. The decrease in *GSTP1* gene expression in PBMC is possibly specific for these cells, since other studies also reported a reduction in *GSTP1* on mRNA and protein level after fruit or vegetable consumption [22, 29].

To our knowledge, this is the first study on PBMC, which shows that phase II enzymes can be modulated in unstimulated cells by food metabolites such as butyrate in vitro. This indicates that PBMC react in vitro to physiological stimuli, which is a necessary prerequisite for the development of gene expression as a suitable biomarker. One important question for the future is the involved mechanism that is critical for regulation of theses enzymes. Some GSTs show an antioxidant/electrophile-responsive element (ARE/ERE) with the nuclear factor E2-related factor 2 (Nrf2) that can act on the ARE element and Nrf2 is important for the expression of many GSTs [8]. Thimmulappa et al. could show an Nrf2-dependent induction of antioxidative genes in PBMC by triterpenoids. Whether Nrf2 and/or ARE are important for the induction of GSTs by butyrate in PBMC is not known and is an important question for further studies.

In humans, the glucuronidation of small lipophilic agents is catalysed by the UGTs in the endoplasmic reticulum. This metabolic pathway leads to the formation of water-soluble metabolites originating from normal dietary processes, cellular catabolism, or exposure to drugs and xenobiotics. A polymorphism in the promoter region of the *UGT1A1* gene with seven instead of six thymine adenine (TA) repeats is responsible for the activity of this enzyme [35]. In a study by Hu et al., a greater than 200-fold inter-individual variability was observed in both the glucuronidation and covalent binding of toxic metabolites in human PBMC [23]. Similar to these huge differences, we could also detect enormous variations in the modulation of gene expression after incubation with butyrate. Four of six blood donors showed a strong induction of *UGT1A1* in PBMC. In addition, the flavonoid chrysin, which is a potent UGT1A1 inducer in many colon cell lines and liver cells [13], had modulatory effects on gene expression in PBMC of just a few blood donors.

Gene expression analyses in PBMC reflected huge differences in inducability of selected enzymes by the different food factors, indicating a possible subject-specific nutrient response, a hypothesis which is also concluded by other authors [5]. The differences were quite obviously much higher compared to cell culture cells such as HT29. This could not be explained until now by analysis of different polymorphisms (e.g. *GSTT1*, *GSTM1*). The number of blood donors, which have to be investigated for in vivo experiments, should be higher than the number of cell culture experiments. In addition, it will be important to find out the reasons for the different modulation patterns in the individual blood donor.

In this study, PBMC were used as surrogate target cells for effects that may also occur in well-established cell culture models as well as in the target organs in vivo. Since nearly all investigated drug metabolism and biotransformation enzymes are expressed on mRNA-level in PBMC and some genes could be modulated by food ingredients, PBMC seem to be a suitable tool for in vitro and ex vivo gene expression investigations. This hypothesis is also supported by a study by van Baarsen et al. [44] that could show that responses of in vitro stimulated PBMC isolated prior to treatment are consistent with those of the ex vivo

results. A critical question is whether these effects in PBMC at gene expression level are comparable to that in the target organs. Liew et al. [27] could show that approximately 80% of genes expressed in many tissues such as brain, liver, lung, or stomach were also found expressed in blood cells, which is a first criteria of being a successful surrogate tissue. Also, preliminary investigations from our group (data not shown) with cDNA-arrays showed that there is a significant correlation between gene expression of PBMC as surrogate and primary colonocytes as target tissue of the same donor (mean $r_{pearson} = 0.78$).

In summary, our study demonstrates that nutritional food ingredients modulate gene expression in human PBMC in vitro and that this ex/in vitro method is a suitable nutrigenomics biomarker approach. Furthermore, the identified differentially expressed genes could become marker genes for population studies on biological effects of an intervention in humans. One of the questions that need to be answered in the future is whether the genes involved in stress response and drug metabolism can be modulated by nutrition in vivo, if the modulation is associated with antigenotoxic effects and if the changes in the blood cells reflect changes that occur in the specific target tissue.

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