Effekte von Apfelpolyphenolen auf die Modulation von entgiftenden Enzymsystemen als Biomarkers der Chemoprevention in humanen Kolonzellen

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena

von
Selvaraju Veeriah
Master of Science in biochemistry (M.Sc.)

geboren am 10.05.1974
in Pudukkottai, Indian

Effects of apple polyphenols on modulation of detoxifying enzyme systems as biomarkers of chemoprevention in human colon cells

Dissertation

for obtaining the degree of doctor rerum naturalium (Dr. rer. nat.) at the Faculty of Biology and Pharmacy, Friedrich-Schiller-University Jena

submitted by
Selvaraju Veeriah
Master of Science in biochemistry (M.Sc.)

Born on 10.05.1974
at Pudukkottai, India

Dedication

…to all of those who stood behind me, believed in my abilities, supported me in my intention, taught me, learned from me and contributed in any way to enrich my life experience at any point in time…

Disputation date: 18.06.2007

Reviewers:

1. Professor Dr. Beatrice L. Pool-Zobel
   Institute for Nutritional Sciences
   Department of Nutritional Toxicology
   Biology-Pharmaceutical Faculty
   Friedrich-Schiller-University Jena
   Dornburger Str. 24, D-07743 Jena, Germany

2. Professor Dr. Frank-D. Böhmer
   Institute of Molecular Cell Biology
   Medical Faculty
   Friedrich-Schiller-University Jena
   Drackendorfer Str. 1, D-07747 Jena, Germany

3. Prof. Dr. Dr. Dieter Schrenk
   Food Chemistry and Environmental Toxicology
   University of Kaiserslautern
   Erwin-Schrödinger Str. 52
   D-67663 Kaiserslautern, Germany

Day of the viva-voce: 18.07.2007

Day of the public defence: 08.08.2007

Contents

Abbreviations

AE	Apple extract
ACF	Aberrant crypt foci
AICR	American Institute for Cancer Research
AKT	V-akt murine thymoma viral oncogene homolog
AOM	Azoxymethan
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ARE	Antioxidant response element
B[a]P	Benzo[a]pyrene
BAX	BCL2-associated X protein
bp	Base pair
BPDE	Benzo(a)pyrene [B(a)P] diolepoxide
BRAF1	V-raf murine sarcoma viral oncogene homolog B1
CAT	Catalase
CIN	Chromosomal instability
COX	Cyclooxygenase
CRC	Colorectal cancer
Cum-OOH	Cumene hydroperoxide
CYP450	Cytochrome p450 enzyme
DAPI	4'-6-diamidino-2-phenylindole
DCC	Deleted in colon cancer
DMH	1,2-dimethylhydrazine
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERK1,2	Extracellular signal-regulated kinase 1 and 2
F-AE	Fermented apple extract
FAP	Familial adenomatous polyposis
GCL	Gamma-glutamylcysteine ligase
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione *S*-Transferase
H_2O	Water
H_2O_2	Hydrogen peroxide
HA	Heterocyclic amines
HNE	4-Hydroxy-2-nonenal
HNPCC	Hereditary nonpolyposis colorectal cancer
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1

K-ras	Kirsten rat sarcoma
LPH	Lactase-phloridzin hydrolase
MAPK	Mitogen-activated protein kinase
MIN	Microsatellite instability
MMR	Mismatch repair
MLH1	Mismatch repair protein 1; mutS homolog 1
MSH2	Mismatch repair protein 2; mutS homolog 2
MSH3	Mismatch repair protein 3; mutS homolog 3
MSH6	Mismatch repair protein 6; mutS homolog 6
MUTYH	MutY homolog (E. coli)
NADH	Nicotinamide adenosine dinucleotide
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear factor E2-related factor 2
PAH	Polycyclic aromatic hydrocarbons
PKC	Protein kinase c
PI3K	Phosphoinositide kinase-3
PRL3	Protein tyrosine phosphatase type IVA, member 3
RNA	Ribonucleic acid
SGLT1	Sodium/D-glucose cotransporter 1
SMAD4	SMAD family member 4
SOD	Superoxide dismutase
TGFBR2	Transforming growth factor, beta receptor II
TP53	Tumour protein p53
UGT	UDP-glucuronosyltransferase
WCRF	World Cancer Research Fund
WHO	World Health Organisation

1. Preface

Worldwide, approximately 10 million people annually are diagnosed with cancer and more than 6 million people die of the disease every year (Steward BW and Kleihues P, 2003). In the year 2000, malignant tumours were responsible for 12 % of the nearly 56 million deaths worldwide from all causes (Parkin, 2001). According to the World Cancer Report, the global cancer rates could increase by 50 % to 15 million by 2020 (World Health Organization, 2003). In many countries, more than a quarter of deaths are attributable to cancer. In the year 1981, Doll and Peto published their encyclopaedic analysis of the causes of cancer. The results of the analysis suggested that in 1970, 75 to 80 % of all cancers in the United States of America (USA) could have theoretically been avoided if the population of the USA could be like those of the countries in which the incidence of cancer was the lowest. What made the US population different from low-risk populations? The environmental (non-genetic) factors that differ between the United States and low risk populations are many and diverse, and include factors such as lifelong patterns of diet, weight gain, alcohol consumption, use of tobacco and use of pharmacological agents (Figure 1) (Doll and Peto, 1981). One out of every three Americans will be diagnosed with cancer at some time in their lifetime. Industrial nations like USA, UK, Italy, Australia, Germany, The Netherlands, Canada and France show the highest overall cancer rates. Developing countries like Northern Africa, Southern and Eastern Asia have the lowest cancer incidence. Current research indicates that the foods we eat can influence our susceptibility to certain types of cancer. It is estimated that up to 30 to 40 % of all cancers are preventable by changes in diet (Colditz *et al.*, 2006). Generally, high energy and high fat diets, which can lead to obesity, are thought to increase the risk of some cancers. Plant-based diets high in fresh fruits, vegetables, legumes and whole grains may help to prevent cancer (Gonzalez and Riboli, 2006). Diet is just one of the

lifestyle factors that influence the risk of developing cancer. Smoking, obesity, alcohol and physical activity levels are also important (Soerjomataram *et al.*, 2007). New research is strengthening the link between "healthy eating" and the prevention of certain types of cancer.

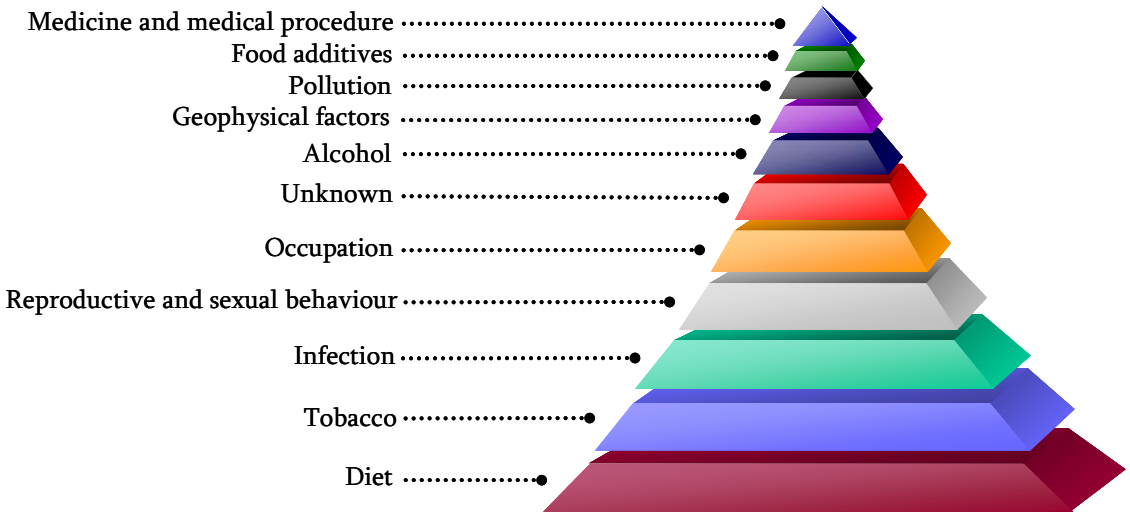


Figure 1. Proportion of cancer deaths attributed to non-genetic factors, as estimated by Doll and Peto, 1981.

1.1 Diet and colon cancer

Colorectal cancer (CRC) is the fourth most frequent cancer in the world. More than 940,000 cases occur annually worldwide, and nearly 500,000 die from it each year. In the year 2006, 148,610 cancer cases were diagnosed in USA and about 55,170 deaths were caused due to colorectal cancer (American Cancer Society, 2006). In Europe colorectal cancer is the second most common cancer, also it ranked second in frequency of deaths in both men and women (Figure 2). It is the second most frequent malignancy in affluent societies but is rare in developing countries (Bray *et al.*, 2002). Worldwide, the incidence of cancer of the colon varies 20-fold (highest in the USA, lowest in India) (Pisani *et al.*, 1999). There has also been a marked increase in the incidence of colon cancer in Japan over the past 40 years. Changes are unlikely in the

Japanese gene pool within 1-2 generations that could account for this increase, but it is possible that the Japanese susceptibility to colon cancer is nowadays unmasked by their changed diet (Tanaka and Imamura, 2006). This adds support to the conclusions and shows that the major causes for colon cancer are dietary habits. Genetic susceptibility appears to be involved in less than five per cent of cases. Up to 70 % of cases can be prevented by following a "healthy lifestyle" (Satia *et al.*, 2004). Physical activity and a diet high in vegetables and fibre have been shown to be protective, while a high red meat intake (especially processed meat) and alcohol may increase the risk (Bingham and Riboli, 2004). However, the link between dietary factors and cancer protection is still difficult to establish, and the protective role of fruits and vegetables is somewhat controversial (Hung *et al.*, 2004b; Schatzkin and Kipnis, 2004). It is therefore important to continue exploring possible interactions between dietary and potential cancer risk factors, and to appropriately stratify epidemiological studies (Schatzkin and Kipnis, 2004).

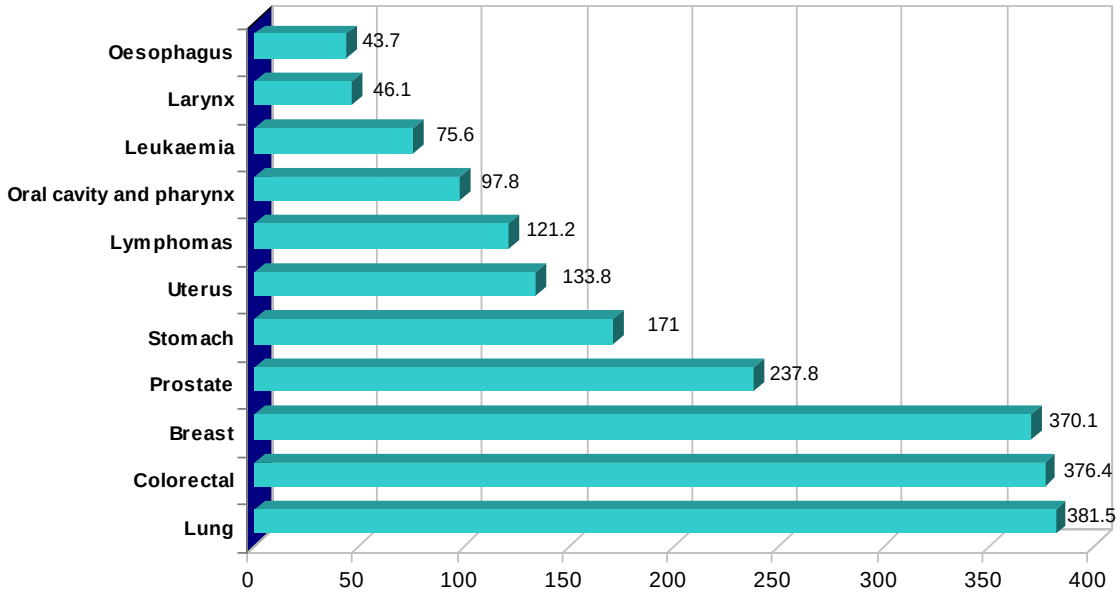


Figure 2. Estimates of number of incident cases of cancer in Europe (2004), both sexes combined (in thousands) (Boyle and Ferlay, 2005).

1.1.1 Genetics of colorectal cancer

Colorectal cancer (CRC) is usually observed in one of two specific patterns: sporadic and inherited. Sporadic disease, with no inherited predisposition, accounts for approximately 70 % of colorectal cancer in the population (Hisamuddin and Yang, 2004). These cancers are common in persons older than 50 years of age, probably as a result of dietary and environmental factors as well as normal aging (Heavey *et al.*, 2004). The two most common inherited syndromes associated with an increased risk of CRC are familial adenomatous polyposis coli (FAP) and hereditary non polyposis colorectal cancer (HNPCC) also called Lynch Syndrome. FAP is a rare autosomal dominant syndrome and least understood pattern of colon cancer development (de and Fernando, 1998). Up to less than 1 % of all cases of colon cancer may fall into this category. A germline mutation in the tumour suppressor gene for adenomatous polyposis coli (APC) results in FAP (Kinzler and Vogelstein, 1996). HNPCC is an inherited autosomal dominant syndrome (Jass *et al.*, 1994). Specific genetic mutations have been identified as the cause of HNPCC, these mutations are estimated to account for only 5-10 % of colorectal cancer cases overall (Figure 3). Although uncommon, these syndromes provide insight into the biology of all types of colorectal cancer. HNPCC is caused by a fault in DNA mismatch repair (MMR) genes, which include *MSH1*, *MLH2*, *MSH6*, *PMS2*, and *PMS1* (Grady, 2003; Lynch and Lynch, 2000).

Moreover, in the intestinal tract, several discrete familial syndromes characterised by multiple hamartomatous polyps have been described - these include the Peutz-Jeghers syndrome, Juvenile polyposis syndrome. Peutz-Jeghers syndrome is an autosomal dominant disorder and characteristics of this disease include the presence of pigmentation on the lips, buccal mucosa, hands, and feet; hamartomatous polyps throughout the gastrointestinal tract (Gruber *et al.*, 1998; Westerman *et al.*, 1999). Peutz-Jeghers syndrome is caused by germline mutations in *STK11/LKB1*, a serine-

threonine kinase gene. The cumulative risk of colon cancer is 39 %, with similar rates for gastric and pancreatic cancer (Brosens *et al.*, 2007). Juvenile polyps are distinctive hamartomas that have a smooth surface and are covered by normal colonic epithelium. Juvenile polyposis syndrome is defined by 10 or more colonic juvenile polyps or any number of juvenile polyps, with a family history of juvenile polyposis (Back *et al.*, 1999). The risk of colon cancer is increased in familial juvenile polyposis, with cancer occurring at an average age of 34 years. Most families with this syndrome have germline mutations of the *DPC4/SMAD4* gene, some families carry mutations in the *PTEN* gene (Brosens *et al.*, 2007; Jeter *et al.*, 2006).

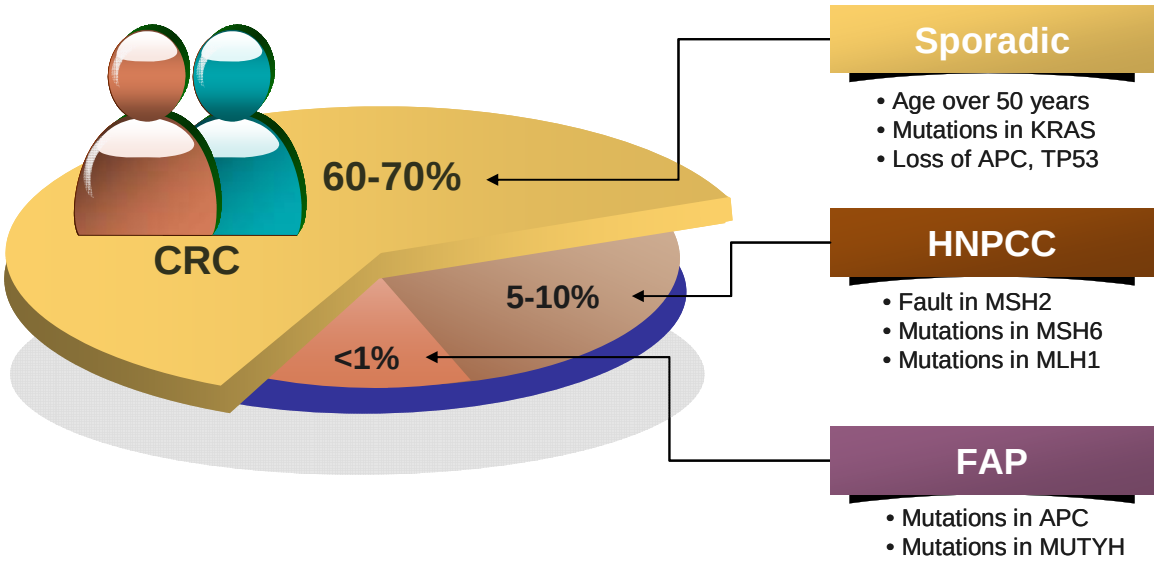


Figure 3. Factors associated with an increased risk of colorectal cancer (CRC) (Stark et al., 2006). *APC, Adenomatous polyposis coli; KRAS, Kirsten rat sarcoma; MLH1, Mismatch repair protein 1, mutS homolog 1; MSH6, Mismatch repair protein 6, mutS homolog 6; MUTYH, MutY homolog (E. coli); TP53,Tumour protein p53*

1.1.2 Overview of molecular alterations in human colorectal cancer

Tumorigenesis is a phenomenon in which transformation from normal to malignant mucosa is a multistep process and is called the adenoma-carcinoma sequence. This stepwise evolutionary process is mainly driven by selection of an increased mutation rate arising in a normal cell. It is estimated that at least four distinct genetic changes need to occur to ensure colorectal cancer evolution (Figure 4). The order is not always followed precisely, but the favoured sequences of events include inactivation of tumour suppressor genes by deletion or mutation and activation of proto-oncogenes by mutation. Adenomatous polyposis coli (APC) gene mutations and hypermethylation occur early, followed by *K-ras*, *BRAF1*, *SMAD4* mutations (Alazzouzi *et al.*, 2005; Rajagopalan *et al.*, 2002). Deleted in colon cancer (DCC) and *TP53* gene mutations occur later in the sequence (Bodmer, 2006). Inactivation of *APC* function seems to underlie both tumour initiation and progression in the colon. This leads to the earliest identifiable lesion in colon cancer formation, the aberrant crypt focus (ACF). Mutations in the *KRAS* oncogene and *APC, SMAD4* and *TP53* tumour suppressor genes are the main targets of colon carcinogenesis (Fearon and Vogelstein, 1990; Powell *et al.*, 1992). APC mutations disrupt the association of APC with β-catenin, resulting in excessive amounts of β-catenin and overactivation of the Wnt signaling pathway. Consequently, genes that promote tumour formation are transcribed (Behrens, 2005; Chung, 2000). Mutations in members of the transforming growth factor-β (TGF-β) signalling pathway are thought to have a rate limiting role in colorectal cancer. The TGF-β can stimulate or inhibit cell proliferation, differentiation, motility, adhesion or apoptosis (Blobe *et al.*, 2000). The most frequently targeted gene for mutation in this pathway is the TGF-β receptor type II tumour suppressor gene *(TGFBR2)*. Other less frequently targeted genes include the

BCL2-associated X protein *(BAX)* and DNA mismatch repair proteins *(MSH3, MSH6)* (Grady, 2003). Progression into metastatic CRC requires additional molecular changes in order for the tumour to invade surrounding tissues. The exact molecular events controlling CRC metastasis are not fully known. The involvement of, for example, *PRL3* and multiple factors in the WNT/β-catenin pathway has been suggested (Pai *et al.* 2004, Dhawan *et al.* 2005).

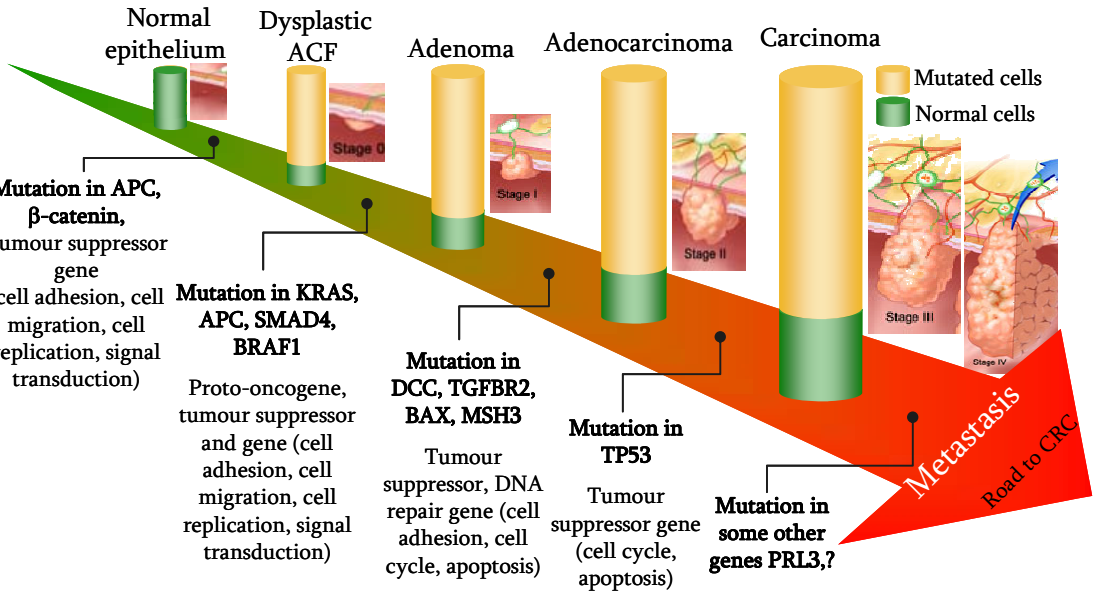


Figure 4. Proposed adenoma to carcinoma sequence in colorectal cancer (CRC) (Fodde et al., 2001b). *APC, adenomatous polyposis coli; BAX, bcl2-associated x protein; BRAF1, v-raf murine sarcoma viral oncogene homolog B1; DCC, deleted in colorectalcancer; K-ras, kirsten-ras; MSH3, muts, E. coli, homolog of 3; PRL3, protein-tyrosine phosphatase, type 3. SMAD4, mothers against decapentaplegic, drosophila, homolog of 4; TGFBR2, transforming growth factor-β receptor, type 2; TP53, tumour protein p53*

Moreover, the causes of molecular alterations in colorectal cancer can be grouped into two broad categories: chromosomal instability (CIN) and microsatellite instability (MIN) (Soreide *et al.*, 2006). CIN (85 %) is the hallmark of most colorectal cancers. CIN is characterized by the loss of heterozygosity (LOH) in tumour suppressor genes

(APC, TP53), defect in chromosome segregation and loss of the mitotic checkpoint gene BUB1 (Fodde *et al.*, 2001b). Mutated forms of APC, as present in colorectal cancers, have the ability to cause CIN (Fodde *et al.*, 2001a). It was therefore postulated that mutations in APC lead to spindle stress that can result in CIN through defective mitosis, and at the same time induce aberrant Wnt/β-catenin signalling activation, thus leading to both cell proliferation and genomic aberrations (Fodde *et al.*, 2001b) The MIN pathway involves the extensive accumulation of mutations of DNA mismatchrepair (MMR) genes *MLH1*, *MSH2*, *MSH6* and, rarely, *PMS2* (Hendriks *et al.*, 2006). This results in a mutator phenotype at the nucleotide level, and in a consequent instability of repetitive sequences such as microsatellites. Sporadic MIN tumours account for approximately 15 % of all colorectal cancers and it also occurs in patients with ulcerative colitis (Fodde, 2002; Lengauer *et al.*, 1998). Furthermore, microsatellite mutations have been observed in a number of putative MIN target genes and the tumorigenic implications of these mutations have been presented in some cases, such as *TGFβRII* and *BAX* (Ionov *et al.*, 2000).

1.1.3 Chemoprevention of cancer and mechanisms involved

In recent years, there has been an increased emphasis on chemoprevention. Chemoprevention of cancer is aimed to block, inhibit, or reverse either the initiation phase of carcinogenesis or the promotion of neoplastic cells. The initiation phase is characterised by the conversion of a normal cell to an initiated cell in response to DNA damaging agents and factors. The promotion phase is characterized by the transformation of an initiated cell into a preneoplastic cell, as a result of alterations in gene expression and cell proliferation. The progression phase involves the

transformation of the preneoplastic cell to a neoplastic cell population as a result of additional genetic alterations (Greenwald, 2002).

Dietary components may be effective chemopreventive agents and they might reduce the cancer risk through various mechanisms, affecting different stages of carcinogenesis (Kelloff *et al.*, 1999). According to Wattenberg (1985), chemopreventive agents can be classified into two main categories based on their mechanism of action, namely, "blocking agents" and "suppressing agents" (Wattenberg, 1985). Blocking agents can block or reverse the premalignant stage (initiation and promotion) of multistep carcinogenesis by increasing detoxification or by scavenging reactive carcinogenic compounds. Suppressing agents can inhibit the malignant transformation of initiated cells or at least retard the development and progression of precancerous cells into malignant ones (Figure 5) (Croce, 2001; Doucas *et al.*, 2006).

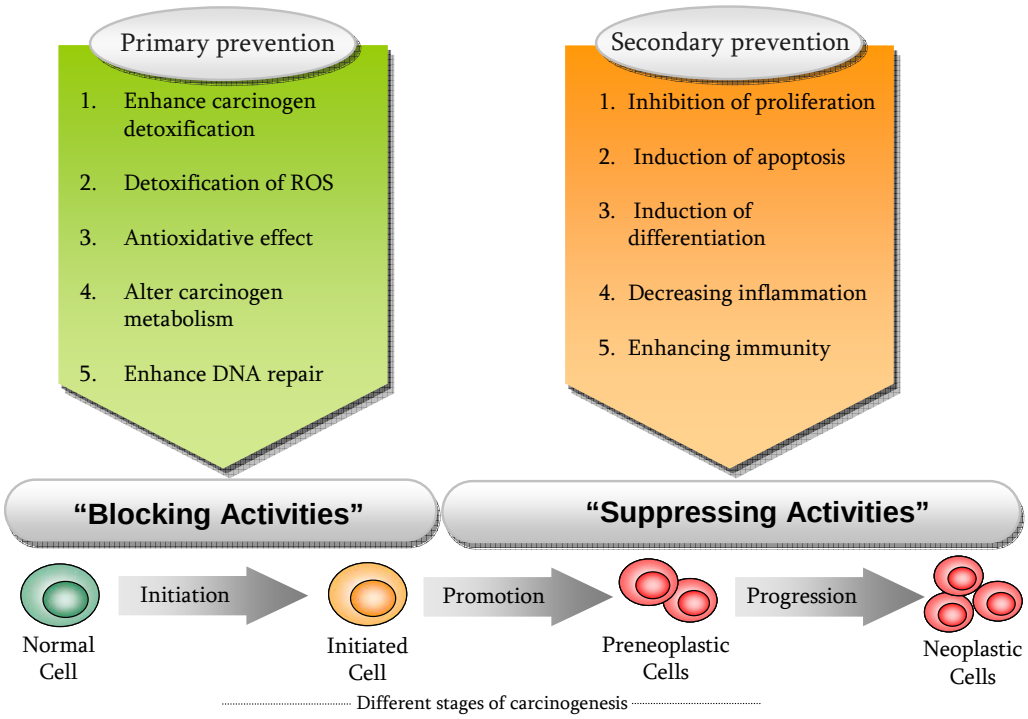


Figure 5. Carcinogenesis processes and chemoprevention strategies (Hursting et al., 1999).

1.2 Fruits, vegetables and colon cancer prevention

Epidemiological studies in humans that populations consuming diets high in fruits and vegetables are associated with reduced risks for many cancers including colon cancer (Block *et al.*, 1992; Fernandez *et al.*, 2006; Potter, 1999). According to the World Health Organisation's (WHO) report 2002, there are at least 2.7 million deaths globally per year of cancer, which are primarily attributable to low fruit and vegetable intake. However, the link between dietary factors and cancer protection is still difficult to establish, and the protective role of fruits and vegetables is somewhat controversial (Hung *et al.*, 2004a; Schatzkin and Kipnis, 2004). It is therefore, important to continue exploring possible interactions between dietary and potential cancer risk factors, and to appropriately stratify epidemiological studies (Schatzkin and Kipnis, 2004). Numerous components found in fruits and vegetables might contribute to their ability to reduce the risk of colon cancer, including dietary fibre, micronutrients, and various non-nutritive phytochemicals (Terry *et al.*, 2001). Many cell culture and animal model studies have been investigating the relationship between colon cancer risk and the consumption of specific type food items such as apples or onions that are rich in non-nutritive phytochemicals (Barth *et al.*, 2005; Gosse *et al.*, 2005). Results of these studies supported an inverse association between these non-nutritive phytochemicals, such as polyphenols, and colon cancer risk. Fruits are usually richer in polyphenols than vegetables, with a total phenolic content of 1–2 g/100 g fresh weight in certain fruits (Paganga *et al.*, 1999).

1.2.1 Polyphenols and their biological impact

Polyphenols are large, non-nutritive secondary metabolites of plants. Flavonoids are the largest class of phenolic compounds; over 5000 compounds have been described. They are mainly classified into flavones, flavanols (catechins), isoflavones, flavonols,

flavanones, and anthocyanins (Beecher, 2003). The structural basis for all flavonoids (Figure 6) is the flavone nucleus (2-phenyl-benzo-γ-pyrane) but, depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar (Kuhnau, 1976).

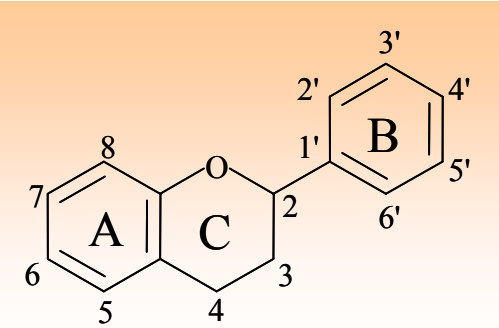


Figure 6. The typical structures of plant phenolics and numbering of the flavone nucleus (Beecher, 2003).

Polyphenols possess substantial anticarcinogenic and antimutagenic properties. They scavenge free radicals such as, reactive oxygen and nitrogen species generated in biological systems, thus breaking the free radical chain reaction of lipid peroxidation. Another antioxidative mechanism is the chelation of metals such as iron and copper ions, which prevent their participation in Fenton-type reactions and the generation of highly reactive hydroxyl radicals (Frei and Higdon, 2003). Polyphenols are also well recognized for their antiproliferative activities (Scalbert *et al.*, 2005).

Many polyphenols are considered to be cancer chemopreventive agents because they inhibit carcinogen activation, commonly catalysed by cytochrome p450 enzymes (CYP450) and they can induce phase II enzymes, *in vivo* and *in vitro* (Xu *et al.*, 2005). Induction of phase II enzymes may facilitate the elimination of certain carcinogens or of their reactive intermediates (Rushmore and Kong, 2002). Moreover, polyphenols can also induce apoptosis in cancer cells and inhibit the metabolism of arachidonic

acid. Metabolism of arachidonic acid (and linoleic acid) leads to the production of many proinflammatory or mitogenic metabolites such as certain prostaglandins and leukotrienes (Lambert *et al.*, 2005). The inhibition of phospholipase A2, COX, and lipooxygenase are potentially beneficial, and have been proposed as a mechanism in the chemopreventive action of polyphenols (Yang *et al.*, 2001). Opposite to this, there is also some evidence that polyphenols/antioxidant might cause some harmful health effects by their prooxidative effects. Oxidative stress can cause oxidative damage to large biomolecules such as proteins, DNA, and lipids, resulting in an increased risk for cancer (Galati and O'Brien, 2004; Halliwell, 2007).

1.2.2 Apple polyphenols and their biological activities

Apples are a good source of phenolic compounds (Eberhardt *et al.*, 2000). The total extractable phenolic content has been investigated and ranges from 110 to 357 mg/100 g of fresh apple (Podsedek *et al.*, 2000). The amounts of polyphenols are known to vary depending on the variety (Liu RH *et al.*, 2001). The most important flavonoids present in apples are flavanols (quercetin glycosides as the main representative) or catechins, flavonols, anthocyanidins, dihydrochalcones (e.g., phloridzin) and phenolic acids (e.g., chlorogenic acid, hydroxycinnamic acids) (Lister *et al.*, 1994). In the Western diet, apples are one of the main sources of flavonoids together with tea, wine, onions, and chocolate (Arts *et al.*, 2001). Apple polyphenolic compounds have strong antioxidant activity. The Vitamin C present in the apples is responsible for less than 0.4 % of the antioxidant activity; thus, the polyphenols may be the main cause of this effect. Apple juice consumption (700 ml) in human volunteers significantly ($p \leq 0.05$) increased the plasma antioxidant level and antioxidant capacity (Lotito and Frei, 2004; Netzel *et al.*, 1999).

The apple polyphenols may play a protective role against several cancer diseases including colon cancer as shown during *in vitro* and *in vivo* studies. It has been reported that apple extracts can inhibit the epidermal growth factor receptor (EGFR) in human colon carcinoma cell line (HT29) (Kern *et al.*, 2005). Polyphenol extracts from apples can inhibit the growth of human liver cancer and colon cancer cells *in vitro* (Eberhardt *et al.*, 2000). Apple juice consumption can prevent damage to human gastric epithelial cells in *vitro* and to rat gastric mucosa in *vivo* (Graziani *et al.*, 2005). Apple extracts effectively inhibited mammary cancer growth in the rat (Liu *et al.*, 2005). In addition, apple juice consumption decreases DNA-damage, hyperproliferation and aberrant crypt foci (ACF) development in the distal colon of 1,2-dimethylhydrazine dihydrochloride (DMH) initiated rats (Barth *et al.*, 2005). Moreover, another *in vivo* rat study showed that intervention with apple procyanidins reduced the number of aberrant crypt foci (ACF) and preneoplastic lesions initiated by azoxymethane (AOM) (Gosse *et al.*, 2005). The same study also indicated that polyphenols from apples can increase the expression of extracellular signal-regulated kinase 1 and 2 (ERK1, 2) and c-Jun N-terminal kinases (JNK) and activity of caspase-3, inhibit G2/M phase cell cycle arrest and suppress PKC in SW620 cells *in vitro*.

1.2.3 Metabolism and bioavailability of polyphenols

The bioavailability of polyphenols is an important determinant in understanding their biological activities. The dietary intake of polyphenols in northern Europe amounts to ~50-150 mg/day (Hollman and Arts, 2000). The bioavailability varies greatly between different polyphenols and depending on chemical properties, deconjugation/reconjugation in the intestine, intestinal absorption, and enzymes available for metabolism. For example, 52 % of the quercetin glycosides present in onions and 33 % of chlorogenic acid present in a supplement are absorbed (Hollman

et al., 1995). A commonly accepted concept is that the polyphenols are absorbed by passive diffusion. For this to occur, the glycosylated polyphenols need to be converted to the aglycone by glycosidases in the food or gastrointestinal mucosa, or from the colon microflora (Hollman *et al.*, 1999). Moreover, some intact glycosides are absorbed by the action of sodium-dependent glucose transporters (SGLT) in small intestine (Williamson *et al.*, 2000). A survey of the published bioavailability studies shows that human plasma concentrations of intact flavonoids do not exceed 1 μM when the polyphenols are given in doses similar to those consumed in our diets (Scalbert and Williamson, 2000).

Until now, few references are known about the bioavailability of polyphenols from whole foods, including apples. DuPont *et al.* demonstrated that the bioavailability of polyphenolic compounds from cider apples in humans (DuPont *et al.*, 2002). After drinking 1.1 l of cider apple juice, no quercetin was detected in the volunteer's plasma. Instead, low levels of 3'-methyl quercetin and 4'-methyl quercetin were measurable within 60 minutes. Moreover, the low amounts of catechin, epicatechin, and phloridzin contained in cider apples were not seen in the plasma at all. Hippuric acid and phloretin were both increased in the subject's urines but there was no evidence of quercetin, catechin, or epicatechin excreted in the urine samples (DuPont *et al.*, 2002). In another study involving human subjects, quercetin bioavailability from apples was only 30 % of the bioavailability of quercetin from onions (Hollman *et al.*, 1997). In this study, quercetin levels reached a peak after 2.5 hours in the plasma; however the compounds were hydrolysed prior to analysis, so the extent of quercetin conjugation in the plasma is unknown. The bioavailability differences between apples and onions most likely are from the differences in quercetin conjugates in the different foods.

A more recent study by Kahle *et al.* involving 11 human volunteers who ingested 1 l of apple juice showed that ~33 % of the ingested material was retrieved in the large intestine and the rest was probably absorbed in the small intestine. The majority of polyphenols reached the large intestine within 2 hours (Kahle *et al.*, 2005). Apples contain some quercetin glucoside which following hydrolysis by lactase-phloridzin hydrolase (LPH), would be available for uptake by intestinal cells. However, apples also contain other conjugates such as quercetin rhamnosides, quercetin xylosides, and quercetin galactosides that are not easily hydrolysed by LPH and most likely are not readily absorbed by small intestinal cells. Phloridzin, the glucoside conjugate of phloretin, is the major dihydrochalcone found in apples. Phloridzin is known to be a potent sodium/D-glucose cotransporter *(SGLT1)* inhibitor, but recently it has been discovered that phloridzin is also transported by *SGLT1* (Walle and Walle, 2003). Dietary phloridzin is known for their antioxidant properties and radical scavenging capacity. Still more research is needed to understand the bioavailability of polyphenolic compounds from whole foods. The exact mechanisms concerning the bioavailability of specific apple polyphenols are still unknown and becoming clearer as bioavailability research increases.

1.3 Biotransformation systems in humans

Biotransformation is the process by which both endogenous and exogenous substances are modified to facilitate their elimination. Biotransformation can convert lipophlic compounds to more water soluble metabolites that can be easily excreted. Basically there are two major biotransformation reaction systems (see Table 1 for the typical enzymes involved in biotransformation), which are called phase I (functional group modification) and phase II (conjugation) (Grubben *et al.*, 2001). Most pharmaceutical drugs are metabolised through phase I biotransformation reactions including oxidation, reduction, hydrolysis, dealkylation, deamination,

dehalogenation, ring formation, and ring breakage (Figure 7). Phase I reactions are catalysed by a multitude of enzyme activities (Table 1). The most important enzymes involve in phase I reactions are the CYP450 isoenzymes. So far, over 10 families of this phase I enzyme have been described in humans, which include at least 35 different genes (Liska, 1998). The CYP450 enzymes use oxygen and the reduced form of nicotinamide adenosine dinucleotide (NADH) as cofactor, to add a reactive group (i.e., hydroxyl radical) to the substrates. The result of this reaction is the generation of reactive molecules, which may be more reactive than the parent molecule, may cause damage to proteins, RNA, and DNA within the cell. Furthermore, phase I activities are also involved in detoxifying endogenous molecules, such as steroids (Grant, 1991).

Phase I enzymes	Phase II enzymes
Cytochrome P450 monooxygenases	Glutathione *S*-transferases
Flavin-containing monooxygenases	UDP-glucuronosyl transferases
Xanthine oxidases	Acetyltransacetylases
Alcohol dehydrogenases	Methyltransferases
Aldehyde dehydrogenases	Sulfotransferases
Aldehyde oxidases	Thioltransferases
Monoamine oxidases	
Esterases	

Table 1: Sample enzymes involved in biotransformation reaction systems in human (Liska, 1998).

The phase II detoxification reaction systems are highly complex, and involve multiple gene families. Generally xenobiotics (PAHs, epoxides, etc.), activated by phase I reactions are further metabolized by phase II conjugation reactions. Produced conjugates are more water-soluble and can be excreted. Several types of conjugation reactions occur in the body, including glucuronidation, sulfation, acetylation, methylation, and glutathione and amino acid conjugation (Figure 7). These reactions require cofactors which can be replenished through dietary sources. Moreover, phase

II reactions show a great amount of individual variability, due to the factors influencing detoxification activity such as, genetic polymorphisms, age and gender, diet and lifestyle, environment and disease (Pool-Zobel *et al.*, 2005a).

Recently, the antiporter activity (p-glycoproteins or multi-drug resistance) has been defined as the phase III detoxification system. The antiporter decreases the intracellular concentration of non-metabolized xenobiotics by pumping (energy-dependent efflux) xenobiotics out of a cell and back into the intestinal lumen and may allow more opportunity for phase I activity to metabolise the xenobiotic before it is taken into circulation (Chin *et al.*, 1993). Antiporter activity in the intestine appears to be co-regulated with intestinal phase I Cyp3A4 enzyme, suggesting that the antiporter may support and promote detoxification (Chin *et al.*, 1993; Liska, 1998).

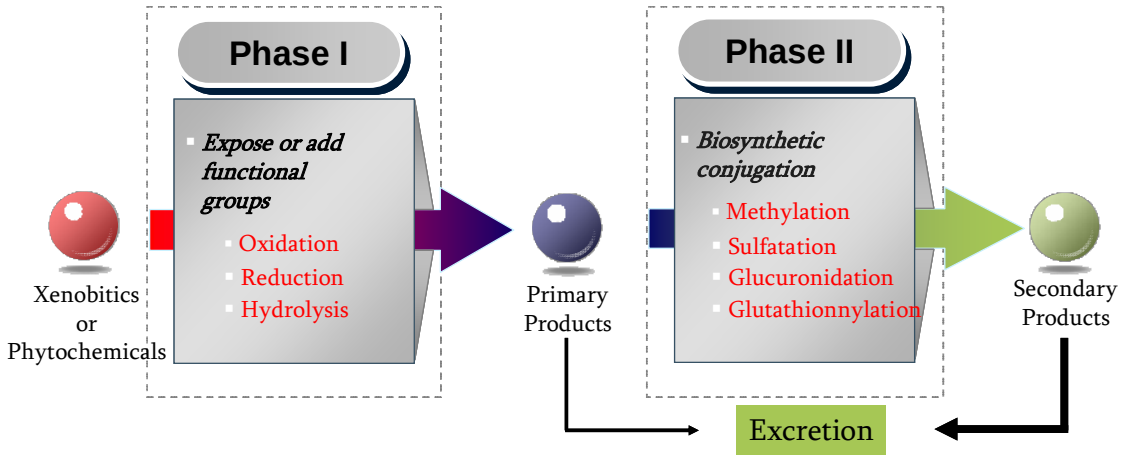


Figure 7: Biotransformation reactions (Liska, 1998). *Xenobiotics or phytochemicals are activated by phase I reactions (e.g. oxidation, reduction) and they are further metabolised by phase II conjugation reactions (e.g. methylation, glucuronidation) and the conjugates are excreted.*

1.3.1 Glutathione *S*-transferases (GSTs)

Glutathione *S*-transferases (GSTs) are a family of phase II metabolising enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Hayes and Pulford, 1995; Townsend and Tew, 2003). To date, human cytosolic GST superfamily contains at least 16 genes subdivided into seven distinct classes designated as: GST-Alpha (GSTA), GST-Mu (GSTM), GST-Pi (GSTP), GST-Theta (GSTT), GST-Zeta (GSTZ), GST-Sigma (GSTS) and GST-Omega (GSTO), whereas GST-kappa (GSTK) is located in the mitochondria as well as in peroxisomes. Each GST family is subdivided into several isoenzymes. The alpha, mu, pi and theta families are the most extensively studied one (Hayes and Strange, 2000). GSTs are constitutively expressed in a wide variety of tissues (Rowe *et al.*, 1997) and the expression levels of GSTs can vary markedly between individuals. Each GST family consists of isoenzymes which homo or hetero-dimerise to catalyse enzymatic reactions using different substrates (Hayes *et al.*, 2005). Sometimes overlapping substrate specificities exist. A number of studies demonstrated that high level expression of different GSTs detoxify many carcinogenic electrophiles, such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HAs), and can thus protect from DNA damage. PAHs such as, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) is a potent mutagenic and carcinogenic metabolite of benzo[*a*]pyrene (B[a]P). BPDE is metabolised by GSTA and GSTP class and then excreted (Fields *et al.*, 1998; Steiner *et al.*, 2007). The overexpression of GSTA4 isoenzyme may be relevant to protect against the genotoxicity of 4-hydroxynonenal (Knoll *et al.*, 2005).

Polymorphisms exist in many of the glutathione *S*-transferase genes, e.g., *GSTM1*, *GSTT1* and *GSTP1*. Deletion of the *GSTM1* and *GSTT1* genes results in a 'null' genotype characterized by a general deficit in enzymatic activity (Parl, 2005). About 50 % and 20 % of Caucasians have the null genotype of *GSTM1* and *GSTT1*,

respectively (Ates *et al.*, 2005). *GSTP1* null mice show an increased susceptibility to PAH-induced tumours (Dang *et al.*, 2005). In particular, allelic variants of the *GSTP1* gene has been associated with higher tumour susceptibility in organs exposed to PAH (Hemmingsen *et al.*, 2001). Modulation of these phase II detoxification enzymes play a critical role in protecting tissues from xenobiotics and carcinogens through a variety of reactions and are being investigated currently as biomarkers for decreasing colon cancer risk.

1.3.2 UDP-glucuronyltransferases (UGTs)

UGTs are endoplasmic reticulum membrane-bound enzymes that play an important role in the metabolism and detoxification of a large number of endogenous and exogenous nucleophilic substrates (Bock, 2003; Wells *et al.*, 2004). UGTs catalyse the transfer of a glucuronic acid moiety to a variety of acceptor groups such as phenols, alcohols, carboxylic acids, amines, carbamic acids, hydroylamines, hydroxylamides, carboxamides, sulfonamides, thiols, dithiocarboxylic acids, and nucleophilic carbon of 1,3-dicarbonyl compounds (Tukey and Strassburg, 2000). In humans, UGTs have been classified into two subfamilies UGT1 and UGT2; the latter was further subdivided into UGT2A and 2B (Mackenzie *et al.*, 2005). To date, 15 different UGTs have been identified in human. The UGT1 locus consists of nine functional UGT1A isoenzymes (*UGT1A1*, *UGT1A3*-*UGT1A10*) all derived from a single gene locus on chromosome 2. The UGT2 subfamily consists of 7 isoenzymes (2A1, 2B4, 2B7, 2B10, 2B11, 2B15 and 2B17). UGT1A enzymes are involved in the metabolism of exogenous compounds and UGT2 isoenzymes are involved mainly in the glucuronidation of endogenous compounds.

In humans, many UGTs are expressed in the liver and colon. *UGT1A8* and *UGT1A10* are predominantly expressed in the colon, whereas *UGT1A3* and *UGT1A9* are

expressed in both liver and colon. Most UGTs can glucuronidate more than one substrate, a promiscuity that may be typical for detoxifying enzymes (Burchell *et al.*, 1995). Several studies have demonstrated that UGTs exhibit a protective effect against exogenous and endogenous carcinogens. For example, food-derived mutagenic heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and *N*-OH-PhIP are glucuronidated by at least 7 *UGT1A* isoforms; *UGT1A3*, *UGT1A4*, *UGT1A6*, *UGT1A8*, *UGT1A9*, *UGT1A10* and *UGT2A1* (Strassburg *et al.*, 1999; Tukey and Strassburg, 2000). Benzo(a)pyrene (B(a)P) has been identified as substrate for several UGT isoenzymes such as, *UGT1A6*, *UGT1A7*, *UGT1A8*, *UGT1A9*, *UGT1A10*, and *UGT2B7* (Fang *et al.*, 2002; Zheng *et al.*, 2002). The UGT2B family preferentially glucuronidates endogenous substrates such as bilirubin, bile acids and steroid hormones in addition to xenobiotics (Hu and Wells, 1994). Hyodeoxycholic acid (HDCA), one of the bile acids serves as a substrate for *UGT2B4* and found to be more efficiently conjugated by *UGT2B7* (Strassburg *et al.*, 2000). Turgeon *et al.* recently reported that *UGT2B10* and B11 catalyse the glucuronidation of arachidonic and linoleic acid metabolites such as, 5-hydroxyeicosatetraenoic acid (HETE) and 13-hydroxyoctadecadienoic acid (HODE) (Turgeon *et al.*, 2003). Several functional polymorphisms in UGTs have been identified. Polymorphism in the UGT1A1 promoter results in reduced expression of gene and accounts for the most cases of "Gilbert's syndrome" results an elevated level of unconjugated bilirubin in the bloodstream. For example, Gilbert's syndrome is associated with abdominal pain, jaundice, severe diarrhoea and also reduces the liver's ability to detoxify certain drugs (Burchell and Hume, 1999). Moreover, UGT polymorphisms are associated with altered risks to certain cancers such as pancreatic cancer and breast cancer (Moghrabi *et al.*, 1993). Mutations in *UGT1A7* were suggested to increase the risk of colorectal cancer development (Strassburg *et al.*, 2002). Induction of the gene expression of

chemoprotective protective enzymes, such as UDP-glucuronyltransferases may be feasible as an approach to cancer prevention.

1.3.3 The effects of polyphenols on modulation of detoxification enzymes and mechanism involved

The phase I and phase II enzymes metabolise a large number of xenobiotics (Meyer, 1996). Phase I enzymes (Cyp450) generally activate the xenobiotics and thereby increase oxidative stress to cells. Whereas, phase II enzymes (GSTs, UGTs, GPXs, CAT, SODs, NQO1, GCL) are considered as detoxification or antioxidant enzymes and thus, protect against oxidative and electrophilic insults. Therefore, the balance between the phase I activating and phase II detoxifying enzymes plays an important role in determining initiation of carcinogenesis. The shift towards carcinogen inactivation or elimination by induction of these detoxifying enzymes protects cellular components from carcinogenic insults. Biochemical investigations of the flavonoid mechanisms of action have shown that these compounds can induce or inhibit a wide variety of enzymatic systems (Kuo, 2002), including expression of gene related to detoxification (phase II enzymes) enzymes (see Table 2) (Petri *et al.*, 2003; Sugatani *et al.*, 2004). Talalay *et al.* reviewed the protective effects of increased levels of phase II enzymes against oxidants and electrophiles (Kwak *et al.*, 2001; Talalay *et al.*, 2003). Steele *et al.* also showed an induction of phase II enzymes, in particular glutathione *S*-transferase (GST) by green tea polyphenols (Steele *et al.*, 2000). Polyphenolic compounds from grapes was modulated GST gene expression in human hepatocarcinoma cell line (Puiggros *et al.*, 2005). Recently, Hofmann *et al.* described the intervention with polyphenol-rich fruit juices may also increase GSTP1-1 protein expression in human leucocytes of healthy volunteers (Hofmann *et al.*, 2006). Moreover, treatment of human intestinal cell line (Caco-2) cell line with sulforaphane and the flavonoid, apigenin modulated gene expression including phase II detoxifying

enzymes, such as glutathione *S*-transferases (GST) and UDP-glucuronosyltransferases (UGT) *in vitro* (Svehlikova *et al.*, 2004). Another *in vitro* study by Galijatovic *et al.*, showed that the flavonoid chrysin and quercetin induced UGT expression in the Caco-2 cells (Galijatovic *et al.*, 2000).

Table 1. Overview on the effects of polyphenols on modulation of detoxification enzyme systems.

Agents	Model systems	Modulation of induction	References
Sulforaphane	Human intestinal cell line (Caco-2)	*GSTA1, UGT1A1* mRNA	Petri *et al*., 2003
Chrysin	Human hepatocarcinoma cell line (HepG2)	*UGT1A1* mRNA	Sugatani *et al*., 2004
Tea polyphenol	Human liver cells (Chang)	GST and NADPH:QR activity	Steele *et al*., 2000
Grape polyphenols	Human hepatocarcinoma cell line (HepG2)	*GST, GPx, GR* mRNA	Puiggros *et al*., 2005
Polyphenol-rich fruit juices	Human leucocytes	GSTP1-1 protein	Hofmann *et al*., 2006
Sulforaphane and apigenin	Human intestinal cell line (Caco-2)	*GSTA1 and UGT1A1* mRNA	Svehlikova *et al*., 2004
Chrysin and quercetin	Human intestinal cell line (Caco-2)	UGT1A6 protein	Galijatovic *et al*., 2000

The regulation of phase II gene expression addresses a wide variety of transcriptional regulators. One important mechanism which is critical for regulation of some, but not all phase II genes (including some GSTs or NADPH dependent quinone reductase) involves the antioxidant/electrophile-responsive response element (ARE/ERE) located within the 5' upstream regulatory region of the corresponding mouse, rat and human genes (Nguyen *et al.*, 2003; Rushmore *et al.*, 1991; Waleh *et al.*, 1998). A major transcription factor which can act on ARE is Nrf2 (nuclear factor E2-related factor 2). The critical role of Nrf2 for phase II gene regulation is strongly supported by the observation that Nrf2-deficient mice display not only a reduced expression of several phase II enzymes, but also a severely impaired tolerance against the toxic effects of carcinogens and inflammatory drugs (Nguyen *et al.*, 2003). Nrf2 interacts with the ARE in the promoter region of phase II detoxifying enzymes, can act as a master regulator of ARE-driven transactivation. It was demonstrated that Kelch-like ECH-associated protein1 (Keap1) - bound to actin protein and localised in the perinuclear space-sequesters Nrf2 in the cytoplasm by forming heterodimers and, inhibiting its

translocation to the nucleus, makes it unable to activate the ARE sequences. Inducers like polyphenols dissociate this complex, allowing Nrf2 to translocate to the nucleus and form a heterodimer with Maf protein resulting in an active Nrf2 binding to ARE. In addition, one or more mechanisms have been implicated for the Nrf2 activation by signalling via the upstream kinases pathways, including MAPKs, PI3K, PKC, and Akt (Pool-Zobel *et al.*, 2005a). Pinkus *et al.* demonstrated that polyphenols can also activate the activator protein-1 (AP-1) transcription factors that interact with AP-1 binding sites of target genes (*GSTP1* and *GSTA1)* to regulate transcription (Pinkus *et al.*, 1996).

The current state of our knowledge indicates that the selective induction of carcinogen-detoxifying enzymes (Phase I and/or Phase II enzymes) may be a useful approach for inhibiting carcinogenesis in chemoprevention. In this study, we have therefore examined if flavonoids from an apple extract contribute to reduce risks during colon carcinogenesis by inhibiting tumour cell growth or by favourably modulating expression of drug metabolism genes.

1.4 Objectives of the study

Several studies have shown evidence of associations between induced phase I and/or decreased phase II enzyme activities and an increased risk of disease, such as cancer. The contribution of phase II detoxification systems has received higher attention both in academical and clinical research. Currently little is known about the exact mechanism and role of the detoxification systems in metabolism of endogenous and exogenous compounds. Therefore, the objective of this study was to evaluate the effect of apple polyphenols on modulation of detoxifying enzyme systems as biomarkers of chemoprevention in human colon cells. To address this point the following questions were worked on:

- First, the antiproliferative effect of a natural polyphenolic apple extract (AE) was investigated on a colon carcinoma cell line (HT29) using cell proliferation assay (DNA staining with 4'6'-diimidazolin-2-phenylindole, DAPI). Furthermore, the effects were compared with major individual compounds in AE and a mixture of major AE compounds. Second, the effect of AE on modulation of detoxification enzyme systems was studied using cDNA gene array analysis (**Publication I).**

- The antiproliferative effect of different AEs with different polyphenolic compositions were investigated together with their corresponding fermentation products produced by incubation of the AEs with human gut flora under anaerobic conditions *in vitro*. Polyphenolic compositions of AEs and fermented AE (F-AEs) were compared. The effects on proliferation were determined in the colon carcinoma (HT29) and colon adenoma cell line (LT97) using cell proliferation (DAPI) assay **(Publication II).**

- The effect of short chain fatty acids (e.g. butyrate) produced during *in vitro* fermentation of AE on xenobiotics and stress related gene expression was studied in primary, colon carcinoma (HT29) and colon adenoma cell line (LT97) by means of gene array. Moreover, the modulation of gene expression by butyrate was compared to basal gene expression of primary cells **(Publication III).**

- The putative mechanism of expression of several genes (e.g. phase II genes) by polyphenols was reviewed based on currently available literature and our research evaluations **(Publication IV)**.

- The effects of AE on the modulation of detoxification enzyme systems and other gene functions related to tumour suppression, cell cycle, apoptosis and signal transduction pathways were investigated in colon adenoma (LT97) cells by cDNA-array analysis. In addition, the enzyme activities of glutathione *S*-transferases and UDP-glucuronosyltransferases were investigated **(Publication V).**

- In a pilot study to determine whether apple juice intervention in humans could affect genotoxin levels in the gut lumen and the effects of apple juice consumption in humans the protection against DNA-damage induced by carcinogens in *ex vivo* was measured by Comet assay. Furthermore, the capacity of those apple juice components which passed the small intestine for modulation of *GSTT2* mRNA expression, *GSTT2* promotor activity and for prevention of oxidative genotoxic stress was studied in HT29 cells using real-time PCR and reporter gene assay, respectively. Moreover, the samples

collected at different time points after intervention were characterised analytically using HPLC (**Publication VI).**

2 Publications

2.1 Publication I: *Veeriah S,* *Kautenburger T, Sauer J, Habermann N, Dietrich H, Will F, Pool-Zobel BL*. "Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics". *Mol Carcinog. 2006 Mar;45(3):164-74.*

Flavonoids from fruits and vegetables probably reduce risks of diseases associated with oxidative stress, including cancer. Apples contain significant amounts of flavonoids with antioxidative potential. The objectives of this study were to investigate such compounds for properties associated with reduction of cancer risks. HT29 cells were treated with apple extract (AE), with a synthetic flavonoid mixture mimicking the composition of the AE or with individual flavonoids. HT29 cell growth was inhibited by the complex extract and by the mixture. HT29 cells were treated with the AE and total RNA was isolated to elucidate patterns of gene expression using cDNA-microarray. Treatment with AE resulted in an upregulation of several chemopreventive genes. Some differentially modulated genes were confirmed with real-time PCR. On the basis of the pattern of differential gene expression found here, we conclude that apple flavonoids modulate toxicological defence against colon cancer risk factors. In addition to the inhibition of tumour cell proliferation, this could be a mechanism of cancer risk reduction.

Own contribution to the manuscript:

- Establishment of cDNA-microarray (Superarray) system in the lab
- Cell culture and measurement of HT29 cell proliferation
- Gene expression analysis with cDNA-microarrays and real-time PCR
- Data evaluation, interpretation and representation of the results

MOLECULAR CARCINOGENESIS 45:164–174 (2006)

Apple Flavonoids Inhibit Growth of HT29 Human Colon Cancer Cells and Modulate Expression of Genes Involved in the Biotransformation of Xenobiotics

Selvaraju Veeriah,[1] Tanja Kautenburger,[1] Nina Habermann,[1] Julia Sauer,[1] Helmut Dietrich,[2] Frank Will,[2] and Beatrice Louise Pool-Zobel[1]*

[1]*Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University, Jena, Germany*
[2]*Department for Wine Analysis and Beverage Research, Institute for Oenology and Beverage Research, Geisenheim, Germany*

Flavonoids from fruits and vegetables probably reduce risks of diseases associated with oxidative stress, including cancer. Apples contain significant amounts of flavonoids with antioxidative potential. The objectives of this study were to investigate such compounds for properties associated with reduction of cancer risks. We report herein that apple flavonoids from an apple extract (AE) inhibit colon cancer cell growth and significantly modulate expression of genes related to xenobiotic metabolism. HT29 cells were treated with AE at concentrations delivering 5–50 μM of one of the major ingredients, phloridzin (''phloridzin-equivalents,'' Ph.E), to the cell culture medium, with a synthetic flavonoid mixture mimicking the composition of the AE or with 5–100 μM individual flavonoids. HT29 cell growth was inhibited by the complex extract and by the mixture. HT29 cells were treated with nontoxic doses of the AE (30 μM, Ph.E) and after 24 h total RNA was isolated to elucidate patterns of gene expression using a human cDNA-microarray (SuperArray®) spotted with 96 genes of drug metabolism. Treatment with AE resulted in an upregulation of several genes (*GSTP1*, *GSTT2*, *MGST2*, *CYP4F3*, *CHST5*, *CHST6* and *CHST7*) and downregulation of *EPHX1*, in comparison to the medium controls. The enhanced transcriptional activity of *GSTP1* and *GSTT2* genes was confirmed with real-time qRT-PCR. On the basis of the pattern of differential gene expression found here, we conclude that apple flavonoids modulate toxicological defense against colon cancer risk factors. In addition to the inhibition of tumor cell proliferation, this could be a mechanism of cancer risk reduction.

Key words: apple flavonoids; colon cancer chemoprevention; cDNA-microarray; biotransformation enzymes; HT29 colon cells

INTRODUCTION

Colorectal cancer is the second leading cause of cancer deaths in men and women in Western countries. It is estimated that approximately 783 000 new cases will be diagnosed annually worldwide [1]. Epidemiological studies have shown that colorectal cancer incidence could be significantly modulated by dietary intake of flavonoids with fruits and vegetables [2,3]. Flavonoids probably contribute to prevention of various diseases associated with oxidative stress, including cancer, by their antioxidative properties [4–6]. Also, nonmicronutrient flavonoids are suspected of having tumor preventive efficacy by modulating signal transduction pathways which generate a stress response [7,8] and by inhibiting proliferation of cancer cells [9,10].

Apples are a significant part of the Western diet, and they are a major source of flavonoids. A Dutch Food Consumption Survey which included an analysis of flavonoid contents of fruits, vegetables, and beverages, reported that apples are the third largest contributors of flavonoids in the Dutch diet after tea and onions [11,12]. Similar orders of magnitude in terms of apple consumption are probably also valid for other countries of the European community. Flavonoids are the bioactive compounds in apples [13]. The total extractable phenolic content has been investigated and was found to range between 110 and 357 mg/100 g fresh apple [14]. Several studies have been performed to

Abbreviations: AE, apple extract; Ph.E, phloridzin-equivalents; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP4F3, cytochrome P450, family 4, subfamily F, polypeptide 3; CHST5, carbohydrate sulfotransferase 5; CHST6, carbohydrate sulfotransferase 6; CHST7, carbohydrate sulfotransferase 7.

*Correspondence to: Friedrich-Schiller-University Jena, Biology-Pharmaceutical Faculty, Institute for Nutritional Sciences, Department of Nutritional Toxicology, Dornburger Str. 25, 07743 Jena, Germany.

Received 3 May 2005; Revised 13 September 2005; Accepted 26 September 2005

DOI 10.1002/mc.20158

Published online 20 December 2005 in Wiley InterScience (www.interscience.wiley.com)

show possible health benefits of apple polyphenols (particularly flavonoids) [15]. Moreover, flavonoids in general, are known to have powerful antioxidant properties including the scavenging of free radicals and prevention of lipid peroxidation [16,17]. This is only partially in line with findings on selected dietary flavonoids in human colon cells in vitro. Some studies have shown that antioxidative polyphenols can protect colon epithelial cells from oxidative DNA damage in vitro, albeit other antioxidative analogs were not effective [18,19]. Therefore, it is necessary to more closely monitor potential protective mechanisms by compounds that have not yet been investigated in depth, such as apple flavonoids.

Biochemical investigations of the flavonoid mechanisms of action have shown that these compounds can induce or inhibit a wide variety of enzymatic systems [20], including expression of gene related to detoxification (Phase I and/or Phase II enzymes) enzymes [21,22]. Modulation of these enzymes has a very important role in decreasing colon cancer risk, because many chemical carcinogens which can initiate colon cancer enhance progression are metabolized by Phase I enzymes (e.g., members of the cytochrome p450 family) as well as by Phase II enzymes (e.g., GSTs, epoxide hydrolase, NAD(P)H: quinone reductase, and UDP-glucuronosyl transferase). The latter conjugate chemically reactive metabolites [23]. Talalay et al. reviewed the protective effects of increased levels of Phase II enzymes against oxidants and electrophiles [24,25]. The importance of Phase II enzymes for inactivating chemical carcinogens was highlighted in a study with nrf2 transcription factor deficient mice [26]. The current state of our knowledge indicates that the selective induction of carcinogen-detoxifying enzymes (Phase I and/or Phase II enzymes) may be a useful approach for inhibiting carcinogenesis in chemoprevention [27,28].

In this study, we have, therefore, examined if flavonoids from an apple extract (AE) contribute to reducing risks during colon carcinogenesis by inhibiting tumor cell growth or by favorably modulating expression of drug metabolism genes.

MATERIALS AND METHODS

Preparation of the Apple Polyphenol Extract

Clear apple juice was produced on an experimental scale. In compliance with the usual practice of the apple juice production, we used a defined mixture of table apples consisting mainly of the species Jonagold (20%), and the varieties *Topaz* (25%), cv. *Bohnapfel* (17.5%), cv. *Winterrambur* (22.5%), and cv. *Bittenfelder* (15%). This type of well-balanced mixture of cider and table apples is required to achieve adequate sensorial properties. Polyphenols of 100 L of that juice were retained on 5 L adsorber resin (XAD 16 HP, Rohm & Haas, Frankfurt, Germany) packed onto a Pharmacia glass column (BPG 100, 100×10 cm). Water soluble juice ingredients like sugars, organic acids, and minerals were washed out with six bed volumes of distilled water. Polyphenols were eluted with three bed volumes of ethanol (96%). The ethanolic fraction was gently concentrated by evaporation, transferred into the water Phase, and finally freeze dried [29].

HPLC Analyses of AE Polyphenols

AE polyphenols were separated after dissolution (1 g/L, 10% methanol) and 0.45 μm membrane filtration on an 1090 HPLC/PDA system (Hewlett-Packard; Böblingen, Germany) equipped with a 250×4.6 mm Aqua 5 μm C18 column and protected with a 4×3 mm C18 ODS security guard (Phenomenex; Aschaffenburg, Germany). Gradient elution was performed with an acetonitrile/acetic acid gradient according to Schieber et al. [30]. Detection wavelengths were 280 nm for flavonoids, 320 nm for phenolcarbonic acids, and 360 nm for the quercetin derivatives. Quantification was carried out using peak areas from external calibration curves. Phloretin-2′-xyloglucosid and 4-cumaroyl quinic acid were isolated with preparative HPLC from the AE. The two unknown dihydrochalcones (phloretin glycosides 1 and 2) were quantified using the calibration curve of commercially available phloridzin. Due to the lack of appropriate standards, the 3- and 5-isomers of cumaroyl quinic acid were quantitated with the calibration curve of the 4-isomer isolated on preparative scale [30].

Synthetic Apple Flavonoids and Preparation of the Mixture

The polyphenols and flavonoids identified in the AE (epicatechin, phloridzin, chlorogenic acid, caffeic acid, quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside) were HPLC purified (~90%–99%) and purchased from Sigma-Aldrich Chemical GmbH Co. (Munich, Germany). To assess effects on cell growth and cell survival, the individual flavonoids were dissolved in ethanol and stored at -20°C as a stock solution. A synthetic AE mixture was prepared, mimicking the composition of the complex AE. For this, a stock solution (1.4 mM in ethanol) was prepared, which contained equimolar concentrations of the phloridzin, which was identified as major polyphenol of the natural AE. The stock solution was divided into aliquots and stored -20°C. To assess effects on HT29 cell growth, all stock solutions were appropriately diluted and added to the cell culture medium yielding concentrations of 20–200 μM of individual flavonoids or of ''phloridzin-equivalents (Ph.E)'' (synthetic AE mixture). The nature of the 10% residual material in the HPLC samples could not be determined on account of the minute quantities

of material provided, but presumably they contained organic solvents. Therefore, each biological assay included a solvent control with ethanol to control for the residual material.

Cell Culture HT29 Cells

Human colon cancer cells HT29, that had been established by Fogh and Trempe [31] from a colon adenocarcinoma of a female Caucasian, were originally purchased from the American Tissue Culture Collection (Rockville, MD). HT29 cells were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen GmbH, 76131 Karlsruhe, Germany) supplemented with 10% FCS and antibiotics (1% penicillin/streptomycin (v/v); Roche Molecular Biochemicals, Meylan, France) according to our laboratory standard culture conditions. Adherent cultures were passaged two times in a week at subconfluent stage after trypsinization. Cultures were maintained under sterile conditions in incubators at 37°C in an atmosphere of 5% CO_2 and 95% air and were routinely checked to exclude mycoplasma or bacterial contaminations. Cell passages 27–46 were used for the experiments.

Determination of Cell Growth and Cell Survival

These studies were performed to compare basic biological activities of the test compounds and to find the doses to be used in the microarray analysis. HT29 colon tumor cells were seeded in 96-well plates (Nunc GmbH & Co., KG, Germany) at approximately 20% confluence (8000 cell/well) and allowed to attach for 48 h. The used medium was aspirated after 48 h and new medium (control) with test substance (treatment) was applied. Cells were treated with AE (delivered 5–100 μM phloridzin to the cell culture medium, that is "5–100 μM Ph.E"), with 10–200 μM individual compounds found to be apple ingredients (epicatechin, phloridzin, chlorogenic acid, caffeic acid, quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside) or with a synthetic mixture (concentration range from 5 to 100 μM, Ph.E) of these eight components composed according to the AE. The final concentration of ethanol in medium did not exceed 2% during treatments, which had no effect on cell growth. The growth of the cancer cells was determined 24, 48, and 72 h after addition of ethanol or of the apple compounds dissolved in ethanol. Remaining cells were quantified after cell lysis by staining the DNA with 4′,6-diamino-2-phenylindole (DAPI) and measuring the fluorescence intensity at 360 and 465 nm was using a computer-interfaced, 96-well microtiter plate reader (TECAN Spectrafluor GmbH, Crailsheim, Germany). All experiments were conducted using triplicate determinations per plate and each assay was repeated at least three times. The concentrations of extract, mixture and individual compounds at which 50% of the cells survived (EC_{50}) were determined. These concentrations were taken as the basis for choosing the subtoxic doses for the gene expression analysis.

Cell Treatment and RNA Preparation

HT29 colon cells (3×10^6) were precultured or allowed to adhere on the bottom of the T25 cell cultured flask (Falcon, Fisher Scientific, Loughborough, UK) for 48 h before treatment. Fresh medium was added and the cells were treated with AE at concentrations of 30 μM (Ph.E) for 24 h. After this time 6×10^6 cells had reached 80%–90% confluence. They were trypsinized and then harvested by resuspending in 5 mL of DMEM medium. Cell suspensions were centrifuged and the resulting cell pellets were resuspended again and washed twice with equal volumes of PBS containing BSA and EDTA (pH 7.0). Total cellular RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany). Prior to in vitro reverse transcription steps the integrity of the isolated total RNA was checked by agarose-formaldehyde denaturing gel electrophoresis. Contaminating DNA was eliminated by DNase-I treatment using the RNase-free DNase kit (Qiagen) according to manufacturer's instructions.

Analysis of Gene Expression Using cDNA-microarrays

cDNA-microarray analysis was conducted with Biotin-16-dUTP-labeled (Roche, Basel, Switzerland) cDNA probes which were specifically generated by single step linear polymerase reaction (LPR) using designed gene-specific primers (GEA primer mix, Primer A). Three micrograms of total RNA were in vitro transcribed with 200 U moloney murine leukemia (M-MLV) reverse transcriptase (Promega, Madison, WI). Expression of genes encoding enzymes of the biotransformation system was analyzed using the drug metabolism GEArray™ Q series cDNA expression array kits which were obtained from SuperArray®, Inc. (Cat no.: HS-011-N, SuperArray, Inc., Frederick, MD). This kit determines differential expression levels of multiple genes involved in a xenobiotic metabolism pathway containing 96 gene-specific cDNA fragments spotted on nylon membranes which were subdivided into three functionally characterized gene groups, namely (1) Phase I, (2) Phase II, and (3) Phase III metabolism. Detailed information including the mechanism and description of the gene probes are listed on the kit provider web page (http://www.superarray.com/gene_array_product/HTML/HS-011.html). The array membranes were subsequently hybridized with the denatured biotin-labeled cDNA probes at 60°C over night according to the manufacturer's protocol. The membranes were washed and chemilumilescence detection steps were performed by hybridizing the membranes with streptavidin-conjugated alkaline phosphatase and CDP-Star substrate. The signals were captured by CCD camera (Fujifilm LAS-1000,

Diana) and quantified using ''AIDA array'' Image analysis software version 3.50 (Raytest GmbH, D 75339 Streubenhardt, Germany). All signal intensities were PUC18 plasmid (negative control) DNA background subtracted, and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (positive control). Scatter plots were made from normalized signals. Relative gene expression levels were calculated as the ratio of the mean of all *GAPDH* signals of all experiments and the mean of *GAPDH* of each membrane. The relative expression level of each gene was based on the ratio of *GAPDH*.

Changes in gene expression can be illustrated as a fold increase/decrease. The cut-off fold induction determining expression was $\geq$1.5 or $\leq$0.6 fold-changes. Genes which suited both above criteria were considered to be induced or suppressed.

Changes in selected genes of interest were confirmed by quantitative real-time RT-PCR analysis. The cut off value of 1.5 was chosen, because our previous data on induction of GSTP1-1 and other GST-isoenzymes had shown that this increase by a factor of 1.5 significantly resulted in functional consequences [32,33]. This induction was measured using a number of different methods (determination of enzyme activities, protein expression with Western blot or ELISA, gene expression with Northern blot or semiquantitative PCR). The increase to 150% of an original value is especially meaningful for enzymes, which are highly available to begin with, such as GSTP1-1 and GSTT1-1 in HT29 cells [34].

PCR Primers and Cloning

The external controls were constructed with existing cDNA plasmid standards. The target and reference gene was PCR amplified with cDNA template from human colon tissues (BioChain, Hayward, CA) and the amplicons were analyzed by DNA-gels for specific products, followed by cloning into TOPO II vector according to manufacturer's protocol (Invitrogen GmbH, 76131 Karlsruhe, Germany). The primer sequences used for PCR amplification are as follows; for the target *GSTT2* sense, 5′-ggtggaacgcaacaggactgcc-3′ and antisense, 5′-gcctgataggcctctggtgagg-3′; *GSTP1* sense, 5′-ctgcgcatgctgctggcagatc-3′ and antisense, 5′-ggccaggaaggccttgagcttg-3′; and for the reference *GAPDH* sense, 5′-ccacccatggcaaattccatggc-3′ and antisense, 5′-tagacggcaggtcaggtccacc-3′.

Real-Time Quantitative RT-PCR Expression Analysis

The modulation of *GSTT2* and *GSTP1* mRNA was confirmed by an independent measure of mRNA levels. For quantitative RT-PCR-analysis we used the system of iCycler iQ® (Bio-Rad GmbH, München, Germany), enabling quantitative analysis of the mRNA expression levels. Therefore, aliquots of 3 μg total RNA from each sample were reverse transcribed using reagents from the SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. For each reaction 2 μL of cDNA equivalent to 100 ng total RNA was mixed with PCR master mix iQ™ SYBR® Green Supermix (2× SYBR Green I, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM $MgCl_2$, 20 nM fluorescein, and stabilizers, Bio-Rad), and the gene-specific primers for target *GSTT2* sense, 5′-tgacactggctgatctcatggcc-3′ and antisense, 5′-gcctcctggcatagctcagcac-3′; *GSTP1* sense 5′-ctgcgcatgctgctggcagatc-3′ and antisense, 5′-ttggactggtacagggtgaggtc-3′; and reference *GAPDH*, sense, 5′-ccacccatggcaaattccatggc-3′ and antisense, 5′-agtggactccacgacgtactcag-3′; (each 10 pmol) in a final volume of 25 μL. The PCR amplification efficiency of the target and reference gene was analyzed by standard curve using the plasmid template (2 μL) containing the cloned target and the reference-specific gene. All experiments were performed in triplicate and the PCR reaction mixture was set in an iCycler iQ 96-well PCR plate (Bio-Rad). The relative fluorescence signal was captured at primer nucleotide extension step of each cycle. At end of the reaction the melting curve analysis was conducted with temperature gradient from 60 to 95°C at 0.10°C/s to differentiate nonspecific primer dimer and specific amplicon.

The relative quantitation of *GSTT2* gene expression was performed using the plasmid standard (serial tenfold dilutions from 5 to 0.00005 ng curve method), using *GAPDH* as an endogenous reference. The iCycler iQ® optical v3.0a software was utilized for obtaining the relative threshold cycle number (C_T) and the data normalization and analysis was carried out by REST® tool programme [35]. Results were expressed as fold-change induction.

Biostatistical Analysis

All of the in vitro experiments were repeated at least three times and the results are presented as the means of the three separate experiments. The statistical analysis was carried out using GraphPad® Prism software Version 4.0 (GraphPad® Software, Inc., San Diego, CA). One way ANOVA and unpaired *t*-tests were used to assess differences between treated and control groups, as is stated in the legends of the Tables and Figures. Differences with p-values ≤ 0.05 were considered to be statistically significant (Table 3).

RESULTS

Apple Flavonoids (Extract and Mixtures) Inhibit Survival of Human Colon Tumor Cells

The AE inhibited growth of HT29 cells with EC_{50} values of 76.3, 31 and 20.5 μM (Ph.E, Table 2), after 24, 48, and 72 h treatment, respectively (Figure 1a–c). The survival of HT29 cells was not found to be

Table 1. Composition of Apple Flavonoids Analytically Characterized by HPLC Method

Apple extract (AE) polyphenols/flavonoids	Originally weighted (1 g/L)		Mean (mg/g)	Concentration (mM)/L
	Quantity-1	Quantity-2		
Procyanidin B1	5.5	8.5	7.0	0.012
Procyanidin B2	14.7	15.4	15.1	0.026
Epicatechin	24.8	13.5	19.2	0.066
Phloretinglycosid 1	24.2	25.2	24.7	[a]
Phloretinglycosid 2	8.3	9.7	9.0	[a]
Phloretinxyloglucosid	135.5	142.3	138.9	[b]
Phloridzin	26.8	29.0	27.9	0.064
Chlorogenic acid	180.0	183.0	181.5	0.512
Caffeic acid	4.8	4.8	4.8	0.027
3-Coumaroyl-quinic acid	9.5	9.5	9.5	[b]
4-Coumaroyl-quinic acid	76.9	77.6	77.3	[c]
5-Coumaroyl-quinic acid	10.3	10.4	10.4	[c]
Quercetin-3-rutinoside	2.5	2.7	2.6	0.004
Quercetin-3-galactoside	0.7	0.8	0.8	0.002
Quercetin-3-glucoside	1.2	1.5	1.4	0.003
Quercetin-3-rhamnoside	3.5	4.7	4.1	0.009
Summary of HPLC-value	529.2	538.6	533.9	

The abundance of all identified polyphenols in the total extract amounted to 53.4%.
[a]No commercial standard available, glycoside part unknown.
[b]No commercial standard available, isolated by preparative HPLC.
[c]No commercial standard available, quantitated with 3-isomer.

significantly different after treatment with AE alone, or in the presence of catalase (Figure 1a–c), which did not indicate that H_2O_2 formation was responsible for the biological efficacy [36]. The synthetic mixture effectively reduced cell growth as well, with EC_{50} values of ~111.0, 59.9, and 49.5 μM, Ph.E for 24, 48, and 72 h treatment, respectively (Table 2). This, however, indicated that the complete extract contains additional active components because it was more effective than the mixture of eight flavonoids. In contrast, the individual flavonoids (data are not shown, except caffeic acid, phloretin, and quercetin) showed hardly any inhibitory effects at all, with EC_{50} values ranging from 235.1 μM for caffeic acid to over 289.2 μM for chlorogenic acid after 48 and 72 h treatment. Even the most important apple ingredients, phloridzin and quercetin aglycons, inhibited HT29 cell growth only at relatively high concentrations, with EC_{50} value of 168.9, 147.3, and 148.4, 101.9 μM after 48

Table 2. EC_{50} Values ("–" not Present, "~" Approximately Calculated by the Software) for HT29 Cells Treated With Apple Flavonoids

Substances	EC_{50} (μM)		
	24 h	48 h	72 h
AE (phloridzin-equivalents, Ph.E)	~76.3	31.0	20.5
AE + catalase 1 U/mL (Ph.E)	~62.8	36.6	27.9
Synthetic AE (Ph.E)	~111.0	59.9	49.5
Caffeic acid	–	~235.0	189.3
Chlorogenic acid	–	–	~289.2
Epicatechin	–	–	–
Phloridzin	–	–	–
Phloretin	–	169.0	149.3
Quercetin	–	148.4	101.9
Quercetin-3-galactoside	–	–	–
Quercetin-3-glucoside	–	–	–
Quercetin-3-rhamnoside	–	–	–
Quercetin-3-rutinoside	–	–	–

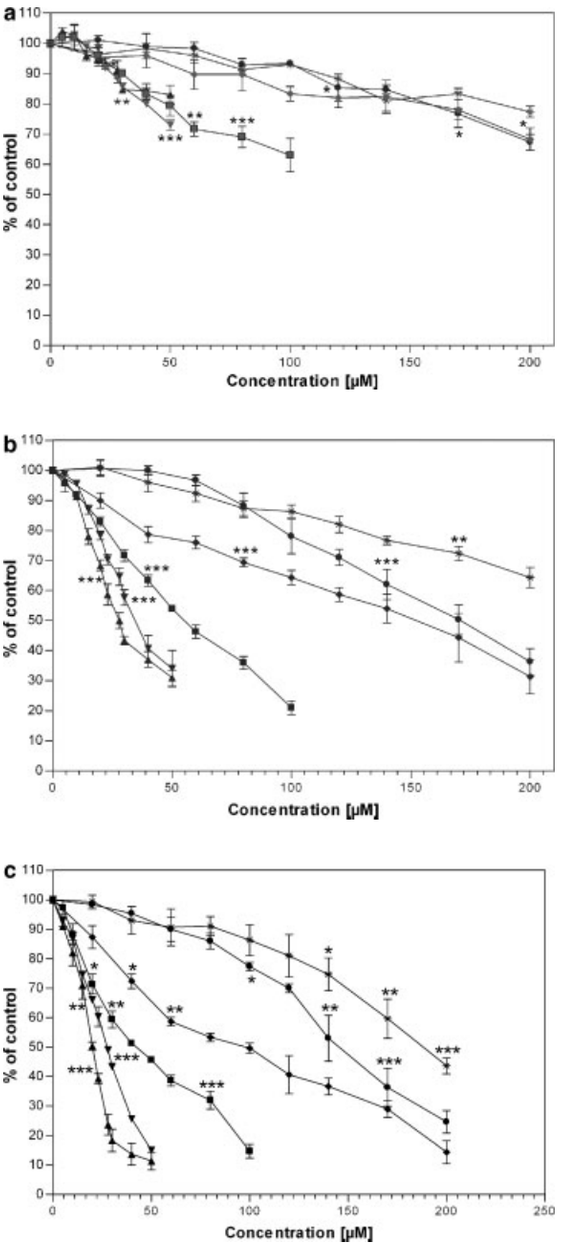


Figure 1. Proliferation of HT29 cells treated with apple flavonoids; (a) 24 h, (b) 48 h, and (c) 72 h incubation time periods: (—+—) caffeic acid, (—●—) phloretin, (—◆—) quercetin, (—■—) synthetic apple extract (AE) (phloridzin-equivalents, Ph.E), (—▼—) catalase + AE (Ph.E), (—▲—) AE (Ph.E), one-way ANOVA Bonferroni's posttest, $n = 3$.

and 72 h treatment, respectively (Table 2), which indicated more than additive effects by extract and mixture.

Apple Flavonoids Modulate Expression of the Genes Related to Drug Metabolism

For the gene expression analysis, mRNA was isolated from HT29 cells treated with a dose of 30 μM (Ph.E) for 24 h. The basis for the choice of this concentration was cell growth studies. It was equal to an EC_{25} value, which is considered to be in a subtoxic range.

The analysis of the human cDNA-macroarray (Figure 2) revealed that the AE significantly altered the expression of genes encoding enzymes related to xenobiotic metabolism. Figure 3 shows scatter plots that compare the expression of normalized signals from nontreated cells and cells treated with AE. The normalized data were clustered on the basis of three (Phase I, Phase II, and Phase II) major gene functions. Table 3 shows data for only genes that reached cut off signal $\geq$9.5 and for which the treatment with AE resulted in a fold change of $\geq$1.5 or $\leq$0.6. It is apparent that the AE-induced expression of at least seven genes (''fold change'' $\geq$1.5, $\leq$0.6) and inhibited one gene (*EPHX1*). Of these only three genes were shown to be significantly altered according to an unpaired *t*-test, namely *GSTP1*, *MGST2*, and *EPHX1*. When regarding only the less stringent criteria of a ''fold change,'' one of the upregulated genes belonged to the Phase I metabolism group, namely cytochrome P450, family 4, subfamily F, polypeptide 3 (*CYP4F3*) (P450 gene family). The rest belonged to the group of Phase II metabolism enzymes, namely *GSTP1*, *GSTT2*, *MGST2* (glutathione S-transferases), carbohydrate sulfotransferase 5 (*CHST5*), carbohydrate sulfotransferase 6 (*CHST6*), carbohydrate sulfotransferase 7 (*CHST7*) (sulfotransferases), which were increased in the AE-treated samples.

Modulation of *GSTT2* and *GSTP1* mRNA Transcript by Quantitative Real-Time RT-PCR

We performed additional mRNA expression analysis for selected genes of interest, namely *GSTP1* and *GSTT2*, by real-time qRT-PCR. We have developed the techniques to analyze these two genes in particular, because they appear to be the most abundantly expressed GSTs in the HT29 colon cells. Figure 4 shows that the AE (30 μM, Ph.E) significantly

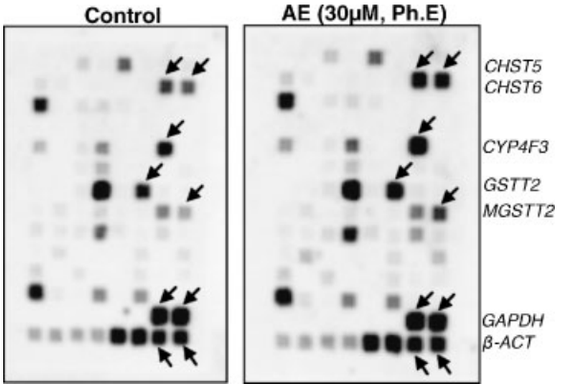


Figure 2. Pictures of cDNA membrane analysing gene expression; HT29 cells treated with AE or medium as control.

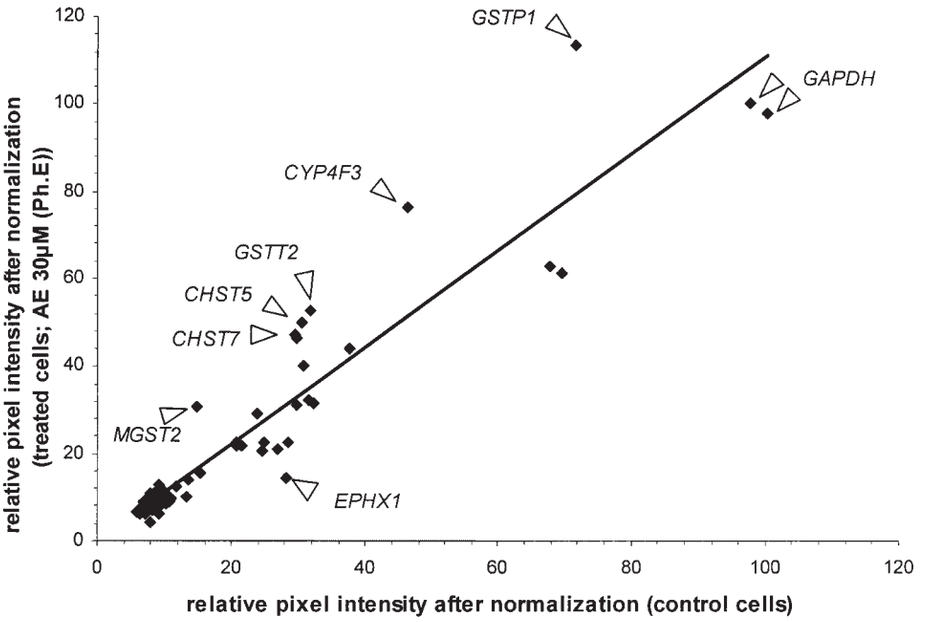


Figure 3. Scatter plot for HT29 cells treated with AE or medium as control.

induced mRNA expression of *GSTT2* 1.8 fold ($p \leq 0.0013$) and *GSTP1* 1.3 fold ($p \leq 0.0049$). This confirmed the significant increases of *GSTP1* (1.6 fold) from the cDNA-microarray analysis. The analysis also pointed an induction of *GSTT2*, which had shown only a nonsignificant 1.7 fold change in the microarray determinations.

DISCUSSION

Apples are one of the most important sources of polyphenol flavonoids in the Western diet, providing approximately 22% and 30% of the total phenols consumed per capita in the United States and in Europe, respectively. There are a number of polyphenolic substances in AEs [37], some of them have been analytically characterized as flavonoids. They have been shown to exert a wide range of effects in biological systems, including potent radical scavenging activities [38,39]. Eberhardt et al. [40], demonstrated that vitamin C in apples contributed to less than 0.4% of total antioxidant activity, suggesting that the complex mixture of phytochemicals in this type of fruit are of higher potency to protect from products of oxidative stress. Next to antioxidative activities, apple flavonoids have been shown to act antiproliferative in HepG(2) and Caco-2 cells [41]. Previous studies have shown that apple flavonoids can inhibit cancer cell growth in the liver [40].

Another hypothesis is that the apple antioxidants have concomitant prooxidative activities at higher concentrations and under certain in vitro conditions and thus lead to the formation of H_2O_2 [42]. Not the apple flavonoids, but the formed H_2O_2 was suggested to be the toxic component affecting cell proliferation, e.g., by causing a condensation of polyphenolic compounds such as flavonoids with commonly used cell culture media. We investigated this hypothesis by adding catalase (1 U/mL, Sigma) to our cell cultures, in order to degrade probably produced H_2O_2 [43]. However, we were not able to detect a reduced or an enhanced antiproliferative activity of our AE in the presence of catalase, suggesting that this mechanism is not relevant for our experimental conditions.

Hosokawa et al. [44] and Yoshida et al. [45] reported that quercetin aglycones arrested growth in cell lines derived from gastric, colonic, and leukemic cancers. Some of these compounds are also ingredients of apple flavonoid mixtures, such as quercetin aglycones and phloridzin aglycones that we investigated in our cellular system. We observed that the aglycones quercetin and phloretin significantly inhibited HT29 tumor cell growth (Figure 1a–c), suggesting that these components also contributed to the growth inhibitory properties of the complete AE. This is in line with other studies showing that the individual apple flavonoid aglycones possess strong cell growth inhibitory activities and are biologically more active than the glycoside derivatives [46,47]. An important, and so far unique, finding of our study was the observation that the individually tested apple flavonoids and their glycosides (Table 2) were hardly inhibitory on their own, but that equimolar concentrations applied as mixtures (mimicking the complete AE) were biologically active in that they resulted in an impairment of cell growth and survival.

Table 3. Gene Expression Profiling of HT29 Cells Treated With Medium Control and AE (30 μM, Ph.E; 24 h)

Functional gene groups	Gene	Signal ≥9.3 medium Mean	Signal ≥9.3 medium SD	AE 30 μM (Ph.E) Mean	AE 30 μM (Ph.E) SD	Fold change	*t*-test *p* values
Phase I, p450 family 5 of 25	*CYP2F1*	13.7	10.1	14.0	1.8	1.0	0.96
	CYP3A5	10.0	4.8	9.7	4.5	1.0	0.94
	CYP3A7	20.6	13.3	22.0	7.0	1.1	0.88
	CYP4F3*	**46.2**	**16.3**	**76.4**	**55.8**	**1.7**	**0.42**
	POR	8.8	4.0	10.8	3.5	1.2	0.55
Phase II, acetyltransferases 6 of 10	*ACAT1*	10.4	7.9	8.6	5.4	0.8	0.77
	ACAT2	10.7	9.3	9.1	3.7	0.9	0.80
	CRAT	11.1	4.7	9.5	2.6	0.9	0.64
	HAT1	8.3	4.1	10.4	3.4	1.3	0.53
	LOC51126	11.8	6.0	12.6	7.3	1.1	0.89
	MORF	7.8	3.7	10.8	5.5	1.4	0.48
Phase II, glutathione S-transferases 6 of 12	***GSTP1****	**71.6**	**14.6**	**113.2**	**21.4**	**1.6**	**0.05**
	GSTT1	8.5	3.9	9.8	3.2	1.2	0.67
	GSTT2	**31.7**	**12.1**	**52.6**	**26.4**	**1.7**	**0.28**
	MGST1	29.6	17.4	31.3	15.9	1.1	0.91
	MGST2*	**14.8**	**5.2**	**30.9**	**3.6**	**2.1**	**0.01**
	MGST3	9.1	7.3	12.9	11.3	1.4	0.65
Phase II, sulfotransferases 6 of 21	***CHST5***	**30.5**	**19.3**	**49.9**	**12.5**	**1.6**	**0.22**
	CHST6	**29.8**	**18.7**	**46.3**	**5.0**	**1.6**	**0.21**
	CHST7	**29.3**	**14.1**	**47.1**	**9.0**	**1.6**	**0.14**
	SULT1A1	10.3	8.0	11.0	2.7	1.1	0.89
	SULT2B1	8.4	3.9	10.8	4.0	1.3	0.51
	TPST1	10.5	7.7	10.6	4.1	1.0	0.98
Phase II, miscellaneous 5 of 13	*COMT*	10.7	5.8	9.3	3.0	0.9	0.72
	EPHX1[a,]***	**28.1**	**1.4**	**14.4**	**3.2**	**0.5**	**0.002**
	TPMT	37.6	14.5	44.2	21.0	1.2	0.68
	UGT1A1	20.6	4.8	22.6	2.2	1.1	0.56
	UGT2B	8.3	3.8	10.1	2.6	1.0	0.88
Phase III, drug transporters, metallothioneins 5 of 8	*MT1A*	23.8	11.8	29.4	14.8	1.2	0.54
	MT1E	7.9	3.3	9.3	2.6	1.2	0.63
	MT1G	15.3	9.3	15.6	1.9	1.2	0.61
	MT1L	9.6	4.3	12.0	1.5	1.0	0.96
	MTIX	13.4	11.8	10.0	4.2	1.3	0.40
Phase III, drug transporters, p-glycoproteins 2 of 7	*ABCC1*	30.8	10.2	40.2	24.9	0.7	0.66
	ABCC3	21.4	6.2	22.0	0.5	1.3	0.58
Total gene of 96		**35**				**8**	**4**

Only genes are shown which had mean signals of ≥9.3. Those which were modulated by factors of ≥1.5 or ≤0.6 fold are highlighted in bold letters.
Gene was downregulated by more than 0.5 fold; *,**genes were significantly modulated by AE (unpaired *t*-test).
*$p \leq 0.05$.
**$p \leq 0.01$.

Studies with a synthetic apple flavonoids mixture were performed to relate the effects found with individual components to the effects found with the complex mixtures to bridge the gap and to find an explanation for these apparent controversial findings. The biological activity in terms of growth inhibition was intermediary between individual compounds and complete extract. This suggests on one hand that there were additive or synergistic effects of the individual compounds, when applied as a mixture. On the other hand, it suggests that the complete AE contained additional components contributing to the growth arrest, which were not present in the synthetic apple mixture. This synergistic effect could have been a result of cleavage of glycosides yielding aglycones by apple phenolic acids (chlorogenic acid and caffeic acid) which were included in the synthetic mixture [48]. A number of studies relate the significant antiproliferative effects of apple flavonoids to their lipophilic properties which facilitate cell permeability [49]. Another reason for the enhanced efficacy of the complex extract could be that each ingredient has different molecular targets in the cells. The impairment of the individual targets on their own may have only minor functional consequences. However, when a number

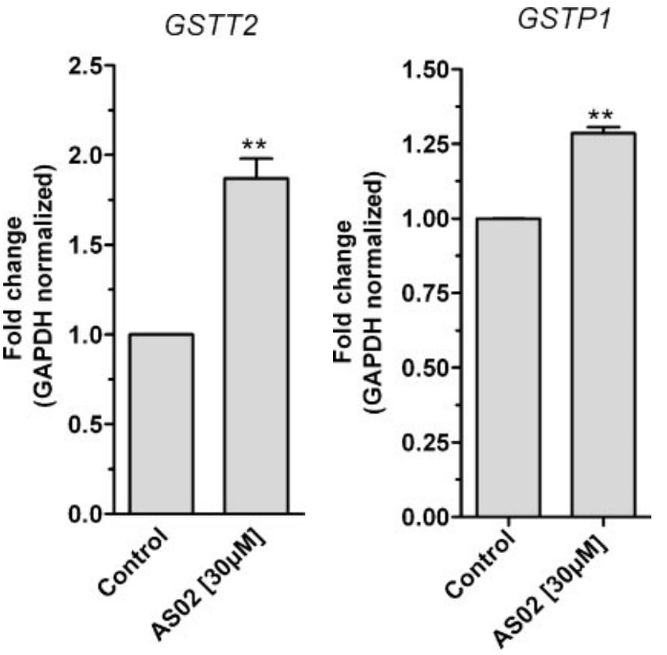


Figure 4. Real-time quantitative RT-PCR analysis of *GSTT2* (left, $**p \leq 0.0013$) and *GSTP1* (right, $**p \leq 0.0049$) mRNA transcripts for HT29 cells treated with AE 30 µM (Ph.E) or medium as control, unpaired *t*-test, $n = 3$.

of different ingredients impair a number of different signaling pathways, this could lead to more distinct functional consequences, reflected in as growth inhibition. We have not yet studied this in detail, but we are presently investigating the impact of our test samples on expression of genes related cell-cycle regulation and apoptosis, which may reveal more on the involved mechanisms. Altogether, this present part of our study shows that aglycones of the major apple flavonoids, especially quercetin and phloretin, inhibited growth of human colon cancer cells.

The present study investigated the potentials of apple flavonoids to modulate genes related to drug metabolism. Thirty five of 96 genes were differently expressed in HT29 cells treated with apple flavonoids, in comparison to the nontreated control cells. Of these more belonged to the group of Phase II biotransformation genes than to genes encoding Phase I and Phase III enzymes. HT29 cells treated with 30 µM AE (Ph.E) possibly expressed higher levels of *CYP4F3* (Phase I), *CHST5*, *CHST6*, *CHST7* (Phase II, sulfotransferases), and *GSTP1*, *GSTT2*, *MGST2* (Phase II, glutathione S-transferases) genes. This could be possibly related to chemoprevention [50], because the induction of many Phase II glutathione S-transferases has been suggested to serve as biomarker of reduced cancer risk and of chemopreventive response [51,52]. In contrast, for the example of one of the sulfotransferases [53], the induction of this enzyme group has been associated with an enhanced activation of promutagens. Whether or not this is also relevant for the specific sulfotransferases has not been investigated. The particular role of CHSTs seems to be related more to carbohydrate metabolism, than to xenobiotic metabolism. Our findings on induced cytosolic human *CHST5*, *CHST6*, and *CHST7* gene expression could also be considered to be beneficial to cells. An interesting finding of this study was that the Phase II epoxide hydrolase gene (*EPHX1*) was 1.95 fold downregulated. Because the enzyme product of this gene plays a role in metabolic activation of benzo(a)pyrene, its absence might convey protection in situations of exposure to polycyclic aromatic hydrocarbons [54]. The CYP450 monooxygenase plays a very important role in metabolic activation of numerous xenobiotics. Also, some species catalyze hydroxylation of endogenous substrates. The P450 isoform *CYP4F3* is known to contribute to the control of inflammation by inactivating the proinflammatory leukotriene LTB4 [55]. Thus, its induction *CYP4F3* by AE in HT29 cell lines may provide protection by inactivation of these proinflammatory mediators from inflammatory diseases. In the future, it will be interesting to assess the functional consequences of AE treatment in these cells. This will include the analysis of protein expression and of enzyme activity that would need to follow a similar pattern of response to AE as the transcriptional activity does, to be meaningful. In particular we will be investigating whether GST substrates, such as benzo(a)pyrene, may, e.g., loose their genotoxic potential as a result

of induced GSTP1. Similar observations have been made for HT29 cells treated with butyrate, which induces, e.g., GSTA4, GSTP1, GSTM2, and other GST isoenzymes. The genotoxicity of 4-hydroxynonenal, which is a highly-specific substrate of GSTA4-4 and of other GSTs, is inhibited in these cells [32].

In conclusion, here we have observed that treatment of HT29 cells with a well-characterized AE strongly inhibited growth of HT29 cells and markedly influenced expression of genes encoding xenobiotic enzymes in subtoxic concentrations. While the antiproliferative effect in tumor cells can be directly related to properties of chemoprotection, it is more difficult to speculate which functional consequences may arise from the altered pattern of gene expression. For the time being, when taking available knowledge into consideration, the upregulation of the genes *GSTP1*, *GSTT2*, *GSTM2*, *CHST5*, *CHST6*, *CHST7* and the downregulation of *EPHX1* can all be related to chemoprotection, due to an enhanced detoxification of some relevant risk factors or to their decreased metabolic activation. On the one hand, in tumor cells, this is probably not of advantage because it could give them an improved survival probability. On the other hand, the induced cellular protection could ward off risk factors, prevent the occurrence of additional molecular alterations, and thus retard further progression. In any case the target genes found to be affected here need to be investigated more in depth, and it remains to be resolved whether apple compounds may also favorably modulate expression of xenobiotic genes in nontransformed or in preneoplastic cells of the human colon. Altogether, the genes could be new targets for chemoprevention and this information adds more knowledge on particular beneficial effects of apples possibly also for colon cancer chemoprotection.

ACKNOWLEDGMENTS

This project is funded from Bundesministerium für Bildung und Forschung (BMBF), Germany (BMBF FKZ.01EA0103).

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin 1999;49:33–64.
2. Witte JS, Longnecker MP, Bird CL, et al. Relation of vegetable, fruit, and grain consumption to colorectal adenomatous polyps. Am J Epidemiol 1996;144:1015–1025.
3. Knekt P, Jarvinen R, Seppanen R, et al. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 1997;146:223–230.
4. Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr Cancer 1992;18:1–29.
5. Bal DG, Woolam GL, Seffrin JR. Dietary change and cancer prevention: What don't we know and when didn't we know it? CA Cancer J Clin 1999;49:327–330.
6. Tanaka T, Kohno H, Mori H. Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. Asian Pac J Cancer Prev 2001;2:165–177.
7. Briviba K, Pan L, Rechkemmer G. Red wine polyphenols inhibit the growth of colon carcinoma cells and modulate the activation pattern of mitogen-activated protein kinases. J Nutr 2002;132:2814–2818.
8. Lin JK. Cancer chemoprevention by tea polyphenols through modulating signal transduction pathways. Arch Pharm Res 2002;25:561–571.
9. Kuntz S, Wenzel U, Daniel H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. Eur J Nutr 1999;38:133–142.
10. Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. Cancer Res 1993;53:5815–5821.
11. Löwik MRH, Hulshof KFAM, van der Heijden LJM, et al. Changes in the diet in the Netherlands: 1987–1988 to 1992. Int J Food Sci Nutr 1998;49:S1–S64.
12. Hermann-Kunz E, Thamm M. Dietary recommendations and prevailing food and nutrient intakes in Germany. Br J Nutr 1999;81:S61–S69.
13. van der Sluis AA, Dekker M, Jongen WM. Flavonoids as bioactive components in apple products. Cancer Lett 1997;114:107–108.
14. Chinnici F, Gaiani A, Natali N, Riponi C, Galassi S. Improved HPLC determination of phenolic compounds in cv. golden delicious apples using a monolithic column. J Agric Food Chem 2004;52:3–7.
15. Boyer J, Liu RH. Apple phytochemicals and their health benefits. Nutr J 2004;3:5.
16. Hussain SR, Cillard J, Cillard P. Hydroxyl radical scavenger activity of flavonoids. Phytochemistry 1997;26:2489–2491.
17. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 1995;22:375–383.
18. Pool-Zobel BL, Adlercreutz H, Glei M, et al. Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells. Carcinogenesis 2000;21:1247–1252.
19. Aherne SA, O'Brien NM. Lack of effect of the flavonoids, myricetin, quercetin, and rutin, on repair of H_2O_2-induced DNA single-strand breaks in Caco-2, Hep G2, and V79 cells. Nutr Cancer 2000;38:106–115.
20. Kuo SM. Flavonoids and gene expression in mammalian cells. Adv Exp Med Biol 2002;505:191–200.
21. Sugatani J, Yamakawa K, Tonda E, et al. The induction of human UDP-glucuronosyltransferase 1A1 mediated through a distal enhancer module by flavonoids and xenobiotics. Biochem Pharmacol 2004;67:989–1000.
22. Petri N, Tannergren C, Holst B, et al. Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. Drug Metab Dispos 2003;31:805–813.
23. Windmill KF, McKinnon RA, Zhu X, et al. The role of xenobiotic metabolizing enzymes in arylamine toxicity and carcinogenesis: Functional and localization studies. Mutat Res 1997;376:153–160.
24. Talalay P, Dinkova-Kostova AT, Holtzclaw WD. Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv Enzyme Regul 2003;43:121–134.
25. Kwak MK, Egner PA, Dolan PM, et al. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. Mutat Res 2001;480–481:305–315.
26. Noda S, Harada N, Hida A, et al. Gene expression of detoxifying enzymes in AhR and Nrf2 compound null mutant mouse. Biochem Biophys Res Commun 2003;303:105–111.
27. Nelson KC, Armstrong JS, Moriarty S, et al. Protection of retinal pigment epithelial cells from oxidative damage by

oltipraz, a cancer chemopreventive agent. Invest Ophthalmol Vis Sci 2002;43:3550–3554.
28. Pool-Zobel BL, Veeriah S, Böhmer FD. Modulation of xenobiotic metabolising enzymes by anticarcinogens—Focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. Rev Mutat Res 2005; (in press).
29. Will F, Bauckhage K, Dietrich H. Apple pomace liquefaction with pectinases and cellulases—Analytical data of the corresponding juices. Eur Food Res Technol 2004;211(4):291–297.
30. Schieber A, Keller P, Carle R. Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. J Chromatogr A 2001;910:265–273.
31. Fogh J, Trempe X. Human tumor cells in vitro. In: Fogh J, editor. New York: Plenum Press; 1975. pp 115–159.
32. Ebert MN, Beyer-Sehlmeyer G, Liegibel UM, et al. Butyrate-induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxynonenal. Nutr Canc 2001;41:156–164.
33. Ebert MN, Klinder A, Schäferhenrich A, et al. Expression of glutathione S-transferases (GST) in human colon cells and inducibility of GSTM2 by butyrate. Carcinogenesis 2003;24:1637–1644.
34. Pool-Zobel BL, Selvaraju V, Sauer J, et al. Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, an approach in nutrigenomics. Carcinogenesis 2005;26(5):1064–1076.
35. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:e36.
36. Long LH, Clement MV, Halliwell B. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun 2000;273:50–53.
37. Alonso-Salces RM, Barranco A, Abad B, et al. Polyphenolic profiles of Basque cider apple cultivars and their technological properties. J Agric Food Chem 2004;52:2938–2952.
38. Lee KW, Kim YJ, Kim DO, Lee HJ, Lee CY. Major phenolics in apple and their contribution to the total antioxidant capacity. J Agric Food Chem 2003;51:6516–6520.
39. Bandoniene D, Murkovic M. On-line HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from apples (*Malus domestica* L.). J Agric Food Chem 2002;50:2482–2487.
40. Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. Nature 2000;405:903–904.
41. Liu RH, Sun J. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. J Agric Food Chem 2003;51:1718–1723.
42. Boyer J, Liu RH. Apple phytochemicals and their health benefits. Nutr J 2004;3:5.
43. Lapidot T, Walker MD, Kanner J. Can apple antioxidants inhibit tumor cell proliferation? Generation of H(2)O(2) during interaction of phenolic compounds with cell culture media. J Agric Food Chem 2002;50:3156–3160.
44. Hosokawa N, Hosokawa Y, Sakai T, et al. Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells. Int J Cancer 1990;45:1119–1124.
45. Yoshida M, Yamamoto M, Nikaido T. Quercetin arrests human leukemic T-cells in late G_1 phase of the cell cycle. Cancer Res 1992;52:6676–6681.
46. Kuo SM. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. Cancer Lett 1996;110:41–48.
47. Shen SC, Chen YC, Hsu FL, Lee WR. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. J Cell Biochem 2003;89:1044–1055.
48. Shen F, Weber G. Synergistic action of quercetin and genistein in human ovarian carcinoma cells. Oncol Res 1997;9:597–602.
49. Walle T. Absorption and metabolism of flavonoids. Free Radic Biol Med 2004;36:829–837.
50. Massaad L, de W I, Ribrag V, et al. Comparison of mouse and human colon tumors with regard to phase I and phase II drug-metabolizing enzyme systems. Cancer Res 1992;52:6567–6575.
51. Talalay P. Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 2000;12:5–11.
52. Clapper ML, Szarka CE. Glutathione S-transferases—Biomarkers of cancer risk and chemopreventive response. Chem Biol Interact 1998;111–112:377–388.
53. Meinl W, Meerman JH, Glatt H. Differential activation of promutagens by alloenzymes of human sulfotransferase 1A2 expressed in *Salmonella typhimurium*. Pharmacogenetics 2002;12:677–689.
54. Zhu QS, Qian B, Levy D. CCAAT/enhancer-binding protein alpha (C/EBPalpha) activates transcription of the human microsomal epoxide hydrolase gene (EPHX1) through the interaction with DNA-bound NF-Y. J Biol Chem 2004;279:29902–29910.
55. Le QV, Plee-Gautier E, Potin P, Madec S, Salaun JP. Human CYP4F3 are the main catalysts in the oxidation of fatty acid epoxides. J Lipid Res 2004;45(8):1445–1458.

2.2 **Publication II:** ***Veeriah S,*** *Hofmann T, Glei M, Dietrich H, Will F, Richling E, Pool-Zobel BL*. "Apple polyphenols and products formed in the gut differentially inhibit survival of human colon cell lines derived from adenoma (LT97) and carcinoma (HT29)". *J Agric Food Chem. 2007 Apr 18; 55(8):2892-900*

Colorectal tumour risks could be reduced by polyphenol-rich diets that inhibit cell growth. Here apple polyphenols were studied for effects on survival of colon adenoma (LT97) and carcinoma-derived (HT29) cell lines. Three apple extracts (AEs) from harvest years 2002-2004 were isolated (AE02, AE03, AE04) and fermented *in vitro* with human faecal flora. Extracts and fermentation products were analysed for polyphenols with HPLC. The cells were treated with AEs or fermented AEs (F-AEs) and cell growth was measured by DNA staining. All AEs contained high amounts of polyphenols and reduced cell survival (in LT97 > HT29). AE03 was most potent, possibly because it contained more quercetin and corresponding metabolite compounds. Fermentation of AEs resulted in an increase of short chain fatty acids, and polyphenols were degraded. Thus, by the fermentation of apple polyphenols through the gut flora, SCFA can be produced in the human colon. The F-AEs were ~3 fold less bioactive than the corresponding AEs, pointing to lower chemoprotective properties through fermentation.

Own contribution to the manuscript:

- Fermentation of different apple extracts
- Cell culture and determination of inhibition of LT97 and HT29 cell proliferation
- Data evaluation, interpretation and representation of the results

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Apple Polyphenols and Products Formed in the Gut Differently Inhibit Survival of Human Cell Lines Derived from Colon Adenoma (LT97) and Carcinoma (HT29)

SELVARAJU VEERIAH,[†] THOMAS HOFMANN,[†] MICHAEL GLEI,[†] HELMUT DIETRICH,[‡] FRANK WILL,[‡] PETER SCHREIER,[§] BASTIAN KNAUP,[§] AND BEATRICE LOUISE POOL-ZOBEL*,[†]

Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University, Dornburger Strasse 25, D-07743 Jena, Germany, Geisenheim Research Center, Section Wine Chemistry and Beverage Technology, Ruedesheimer Strasse 28, D-65366 Geisenheim, Germany, and Department of Food Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

Colorectal tumor risks could be reduced by polyphenol-rich diets that inhibit cell growth. Here, apple polyphenols were studied for effects on the survival of colon adenoma (LT97) and carcinoma-derived (HT29) cell lines. Three apple extracts (AEs) from harvest years 2002−2004 were isolated (AE02, AE03, and AE04) and fermented in vitro with human fecal flora. Extracts and fermentation products were analyzed for polyphenols with HPLC. The cells were treated with AEs (0−850 μg/mL) or fermented AEs (F-AEs, 0−9%), and survival was measured by DNA staining. All AEs contained high amounts of polyphenols (311−534 mg/g) and reduced cell survival (in LT97 > HT29). AE03 was most potent, possibly because it contained more quercetin compounds. Fermentation of AEs resulted in an increase of short chain fatty acids, and polyphenols were degraded. The F-AEs were ∼3-fold less bioactive than the corresponding AEs, pointing to a loss of chemoprotective properties through fermentation.

KEYWORDS: Antiproliferative activity; apple polyphenols; colon cancer chemoprevention; fermentation; colon cell line

INTRODUCTION

Epidemiological findings suggest that plant foods decrease colorectal tumor risks (*1*). This could be due to a number of different phytoprotectants, including polyphenolic flavonoids, which act chemopreventive by inhibiting the growth of tumor cells (*2*−*4*). It has also been shown that compounds such as flavonoids can affect processes that induce differentiation, cause apoptosis, and enhance anti-inflammatory responses in colorectal cancer cell lines (*5*−*7*). Apple flavonoids, such as phloridzin, quercetin, and (+)-catechin, have been shown to exert potent antiproliferative activities in several studies (*8*−*10*), and it has been shown that they are able to scavenge endogenous reactive oxygen species (ROS) (*11*). Other than acting as antioxidants (*12*) or scavenging carcinogens, the apple polyphenols may inhibit carcinogenesis by affecting molecular events in the initiation, promotion, and progression stages (*13*). Apples contain very high amounts of polyphenols (*14*), which vary depending on the variety (*15*). The total amount of polyphenols that can be extracted from 100 g of fresh apples ranges from 110 to 357 mg (*16*), and quercetin and its glycosides are the most abundant polyphenols found in apples (*17*).

Eberhardt et al. reported that an apple extract (AE) inhibited the proliferation of HepG2 liver tumor cells in vitro and suggested that these antiproliferative effects could have been due to the presence of a unique combination of phytochemicals in the apples in addition to ascorbic acid (*18*). We have performed similar studies with human colon carcinoma cells (HT29) and compared the effects of an AE, a synthetic AE (mixture of the major polyphenols that mimicked AE), and individual polyphenol compounds. It was shown that both mixtures significantly inhibited the growth of HT29 cells in a dose- and time-dependent manner (*10*). However, the effect differed between the test compounds (individual polyphenols < synthetic AE < natural AE). The findings thus suggested that complete mixtures of phytochemicals in fresh fruits were more antiproliferative in HT29 cells than the sum of individual ingredients when tested alone. Apples are a significant source of flavonoids in people's diet in the U.S. and Europe (*19*, *20*). The ability of the mammalian colon to absorb apple polyphenols has been shown in vivo and in vitro (*21*). About 0−33% of polyphenols reached the colon and were fermented by the gut bacteria (*22*). This means that both original apple polyphenols

* Corresponding author. Tel.: +49 3641 949670. Fax: +49 3641 949672. E-mail: b8bobe@uni-jena.de.
[†] Friedrich-Schiller-University.
[‡] Geisenheim Research Center.
[§] University of Würzburg.

10.1021/jf063386r CCC: $37.00
Published on Web 03/23/2007 **PAGE EST: 8.2**

as well as metabolites formed physiologically in the gut are present in the lumen and theoretically may interact with the enterocytes.

Therefore, here we had the aim of investigating unaltered AEs, as well as their counterparts fermented by the human gut flora. Our expectation was that the fermented apple polyphenol extracts (F-AEs) should differ from the unfermented polyphenol extracts (AEs). This was hypothesized because certain key flavonoids in the fresh, unfermented state are bound to sugar moieties and thus occur as glycosides, galactosides, rhamnosides, etc. The fermentation process is expected to liberate the aglycones of the flavonoids by cleaving the flavonoid sugar complexes. Alternatively, it is known from the literature (*23*) that polyphenols can be metabolized to short chain fatty acids (SCFA) of which butyrate and propionate have been shown to inhibit the growth of HT29 cells (*24*). The key question to be solved here was therefore to determine if the fermentation process leads to an alteration of the biological activities.

Different AEs with different polyphenolic compositions were investigated together with their corresponding fermentation products produced by incubation of the AEs with human gut flora under anaerobic conditions. Polyphenolic compositions of AEs and F-AEs were compared. Also, the effects on proliferation were determined in the human colon adenocarcinoma cell line HT29, as this was previously shown to be a sensitive model of biological activity (*25*). We also determined effects in the LT97 colon adenoma cell line, which represents an early premalignant stage of tumor development (*26*).

MATERIALS AND METHODS

Preparation of the Extract Containing Apple Polyphenols. Clear apple juice was produced on an experimental scale. In compliance with the usual practice of apple juice production, we used a defined mixture of apple varieties consisting of Jonagold (20%), Topaz (25%), Bohnapfel (17.5%), Winterrambur (22.5%), and Bittenfelder (15%). This type of well-balanced mixture of cider and table apples is required to achieve adequate sensorial properties. Polyphenols from 100 L of the resulting apple juice were retained on a 5 L adsorber resin (XAD 16 HP, Rohm and Haas, Frankfurt, Germany) that was packed in a Pharmacia glass column (BPG 100, 100 cm × 10 cm). Water soluble juice ingredients like sugars, organic acids, and minerals were removed by washing using six bed volumes of distilled water. Polyphenols were eluted with three bed volumes of ethanol (96%). The ethanolic fraction was gently concentrated by evaporation and transferred to an aqueous solution that was then freeze-dried (*27*).

Quantification of Polyphenols in AEs. A solution of the apple polyphenol extracts was prepared to yield a concentration of 1 g/L in 20% methanol. The solution was filtered (0.2 μm), and 20 μL was injected into an HP1090HPLC system equipped with a photodiode array detector (Hewlett-Packard, Palo Alto, CA). A Phenomenex aqua column (250 mm × 4 mm, Phenomenex, Aschaffenburg, Germany) was used at ambient temperature. Gradient elution was performed using an acetonitrile (ACN)/phosphoric acid gradient. Detection wavelengths were 280 nm for flavonoids, 320 nm for phenolcarbonic acids, and 360 nm for quercetin derivatives. Quantitation was carried out using peak areas from external calibrations with standard solutions (*27*). After isolation, the extracts were stored in a dark place at ambient temperature for 6 months, and this time, there were no significant differences of the polyphenols. Moreover, the proliferation assay and fermentation experiments were performed within 2 months after production of AEs. Also, the proliferation assay for F-AEs was done after only 1 month of storage (−80 °C).

Preparation of the Fermented Apple Polyphenol Extracts. All fermentations were conducted in vitro under anaerobic conditions (80% nitrogen, 10% carbon dioxide, and 10% hydrogen at 37 °C), basically according to described procedures (*28*). A mixture of fresh human feces from three healthy volunteers who had given their informed consent was prepared as a bacterial source. These were used to ferment the reconstituted AEs (AE02, AE03, and AE04). The volunteers consumed their normal, non-vegetarian diet without any restrictions. The study was approved by the Ethical Committee of the Friedrich-Schiller-University Jena.

The fecal samples were immediately weighed and filled into one homogenizing bag. Potassium phosphate buffer (0.1 M, pH 7.0) was added (5:1 v/w), and the mixture was homogenized thoroughly in a Stomacher 400 (Seward, Worthing, UK). From the fecal homogenate, 40 mL aliquots were filled into 500 mL glass bottles. Apple polyphenol samples were dissolved in anaerobic potassium phosphate buffer to provide 20 g/L fermentable substances. A total of 40 mL of each polyphenol solution was added to separate bottles to obtain a final AE content of 10 g/L and a fecal suspension of 10% as recommended by Barry et al. (*29*). As a negative control (blank), potassium phosphate buffer was added to one bottle instead of apple polyphenols. Anaerobic conditions in the glass bottles were achieved by removing the air with an injected cannula (0.5 bar for 1 min). Subsequently, the bottles were filled with the fermentation gas mixture via the cannula (0.8 bar for 1 min). After 30 min (15 cycles repeated), the cannulas were removed, and the fermentation suspensions were incubated for 24 h in a shaking water bath at 37 °C. Afterward, the fermentation process was stopped by placing the suspensions on ice. Each sample was transferred to 50 mL tubes and centrifuged (4200*g*, 4 °C) for 30 min. The fermentation supernatants (F-AEs) were divided into aliquots and stored at −80 °C. Samples were sterilized by filtration (pore size 0.22 μm) before use in the cell culture experiments.

Analyses of Polyphenols or Metabolites in Fermented AE Samples. Only two (F-AE03 and F-AE04) of three fermentations samples were characterized analytically due to the limited sample size of F-AE02. Aliquots (2 mL) of the fermentation samples including the blank (without AEs) were lyophilized and dissolved in methanol. Polyphenol amounts in the fermentation samples were determined using the Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 712b autosampler (Waters, Eschborn, Germany). Data acquisition and evaluation were performed with Hewlett-Packard ChemStation software. A Hypersil Gold C18 column, 100 mm × 4.6 mm, with a 3 μm particle size (Thermo, Runcorn, UK), was used. The mobile phase consisted of aqueous 0.1% v/v formic acid and ACN (Lichrosolv, Merck, Darmstadt, Germany). The gradient applied was 1−99% ACN in 40 min at a flow rate of 1 mL/min, and 25 μL injection volumes were used. The peaks were identified by comparison of retention time and UV spectra (200−600 nm) with authentic references (*22*). Chlorogenic acid, caffeic acid, 4-*p*-coumaroylquinic acid, phloretin-2′-*O*-xyloglucoside, phloridzin, phloretin, procyanidin B_1, procyanidin B_2, (+)-catechin, (−)-epicatechin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, quercetin, and quercetin-3-*O*-rutinoside (100 mg/L each) in methanol were diluted. Dihydrochalcones, catechins, and procyanidins (B_1 and B_2) were determined at 280 nm, hydroxycinnamic acid derivatives at 320 nm, and flavonols at 360 nm. 3,4,5-Trimethoxycinnamic acid (Sigma, Steinheim, Germany) was used as a standard (IS) for quantification (50 mg/L). Calibration curves (at the appropriate wavelengths according to the absorption maximum of the compounds) were used for quantification. Compounds were quantified by means of calibration curves (peak area divided by IS area vs quotient of substance and IS concentration). Linearity was given for 0.4−600 mg/L; limits of quantification ranged from 0.4 to 0.9 mg/L and limits of determination from 0.2 to 0.4 mg/L with a signal-to-noise ratio of 3:1, respectively.

Analysis of SCFA. To determine the SCFA, the samples were mixed with an isocapronate standard (1:11 v/v), shaken, and centrifuged at 6400*g* for 10 min at 4 °C. Then, the gas chromatographic measurements (GC 17A, Shimadzu, Duisburg, Germany) were performed using a 15 m FFAP column (Phenomenex, Aschaffenburg, Germany) and a specific temperature program (starting temperature 130 °C, increase 35 °C/min, and final temperature 170 °C) (*30*).

Human Colon Cell Lines LT97 and HT29. LT97 cells were isolated from a micro-adenoma of a patient with familiar adenoma polyposis coli (*26*). These are cells of an early colon adenoma in the

premalignant stage of tumor development. Concerning their genetic specifications, they are characterized by homozygous mutations of the *APC* tumor suppressor gene and a homozygous *k-ras* oncogene. However, there are no genetic changes in the *p53* gene. LT97 cells were cultivated in MCDB medium with 2% fetal calf serum (FCS), 10 μg/mL insulin, 2×10^{-10} M triiodotyronin, 2 mg/mL transferrin, 1 μg/mL hydrocortisone, 5×10^{-9} M sodium-selenite, 30 ng/mL epidermal growth factor (EGF), and 1% penicillin/streptomycin. Adherent cultures were passaged at subconfluent stages by using PBS/EDTA (5 mM).

The HT29 cell line that had been established by Fogh and Trempe from a colon adenocarcinoma of a Caucasian female (*31*) was originally purchased from the American Tissue Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% FCS and antibiotics (1% penicillin/streptomycin (v/v); Roche Molecular Biochemicals, Meylan, France) according to our laboratory standard culture conditions. Confluent cultures were passaged 3 or 4 days after trypsinization.

Both cells lines were maintained under sterile conditions at 37 °C in a 95% humidified incubator (5% CO_2). Cells were routinely checked for mycoplasma contaminations using highly sensitive PCR analysis (Minerva Biolabs GmbH, Germany). For the experiments performed here, cells of passages 32−36 (LT97), and passages 30−41 (HT29) were used.

Determination of Cell Growth. Growth and survival of colon cells were determined in 96 well microtiter plates (Nunc GmbH & Co. KG, Berlin, Germany). A total of 72 h (LT97, ∼20−30% confluence) or 48 h (HT29 cells 8000 cell/well, ∼20−30% confluence) after seeding the cells, the cultures were treated with AEs (AE02, AE03, and AE04) and fermented AEs (F-AE02, F-AE03, and F-AE04) diluted in cell culture medium containing 0−850 μg/mL (dry mass) and 0−900 μg/mL (dry mass), respectively. After 24, 48, and 72 h of incubation time, the cells were lysed and fixed by methanol. Total cell counts were determined indirectly by staining DNA with DAPI (4′,6-diamino-2-phenylindole, Sigma, Germany), which becomes fluorescent after DNA binding. After 30 min, the DNA content, which reflects the number of cells, was detected by fluorimetrical analysis with Ex/Em 360/450 (TECAN Spectrafluor GmbH, Crailsheim, Germany). All data points were performed in triplicate, and each experiment was repeated independently at least 3 times for statistical evaluation. There were no additional substances added to the extracts. To measure the effect of AEs on cell growth, two controls were included in the proliferation assay. One was the no treatment control, for which the cells were incubated with only medium (0 μg/mL, 100%), and the second control was blank control, performed using different concentrations of extracts without cells. This second control (blank) was included to accommodate for the fluorescence of the extracts. Moreover, since DAPI can also produce fluorescence in the presence of extracts alone, the artifacts were normalized to the original fluorescence from cell DNA. The effective median doses (EC_{50}) of AEs and F-AEs that inhibited growth by 50% were determined and expressed as micrograms (μg) of AE.

Statistical Analysis. Statistical analysis was performed using the GraphPad Prism Version 4.0 for Windows (GraphPad Software Inc., San Diego, CA). Data shown in the tables and figures represent mean values ± SEM. Unless otherwise stated, these means were calculated from the means of triplicate replicates obtained in at least three independent experiments. Significant differences between treatment and control values were determined by one-way ANOVA and Bonferroni's post test.

RESULTS

Contents of Polyphenols in AE02, AE03, and AE04. The content of AE02 was reported in our previous publication (*10*), and it was used here to compare the content of new AEs (AE03 and AE04) additionally investigated here. AE02 contained the highest concentration of polyphenols, with a total amount of 533.9 mg/g of AE. AE04 contained 478.3 mg/g, whereas AE03 contained only 310.9 mg/g. **Table 1** shows the individual constituents in each of the three AEs. Chlorogenic acid was the most abundant phenolic compound detected in AE02 and AE04, each with concentrations of about 180 mg/g, whereas AE03 contained only about one-tenth of this amount. The phloretin sugars formed another abundant group of phenolic compounds in the AEs. AE02 contained the highest amount of phloretin glycosides with a total content of 200.5 mg/g. Phloridzin (phloretin-2′-*O*-β-D-glucoside) was the most common glycoside with 78.9 mg/g detected in AE03. AE02 had the smallest amount of phloridzin with only 27.9 mg/g. Quercetin derivates were detected in all three AEs. The total amounts of quercetin derivatives amounted to 116.2, 12.1, and 8.8 mg for AE03, AE04, and AE02, respectively. There are some uncertainties encountered for estimating the presence of unknown dihydrochalcones (see **Table 1** footnote), but according to the relevant literature, **Table 1** is more or less complete and contains all the major polyphenols. In addition, the concentrations of total sugar were also analyzed and are included in **Table 1**. Moreover, the extracts were analyzed for lipids after methanol/chloroform extraction. But, after a derivatization to fatty acid methyl ester (FAMEs) followed by gas chromatography−mass spectrometry (GC−MS) on a DB-Wax column, no fatty acids could be detected. Also, no phytosterols (silylation, GC−FID on DB-5) were detectable. Extracts were also analyzed for proteins, but after a 6 M HCl hydrolysis, no amino acids were detected in the hydrolysates (HPAEC/PAD, Dionex BioLC).

Table 1. Polyphenols in AE from Cultivar Years 2002, 2003, and 2004 (AE02, AE03, and AE04) Analytically Characterized by HPLC[a]

Substances	mean values (mg/g of AE)		
	AE02	AE03	AE04
280 nm			
procyanidin B1	7.0	6.2	n.d.
procyanidin B2	15.1	18.4	12.1
(+)-catechin	n.d.	2.7	n.d.
(−)-epicatechin	19.2	17.7	12.5
phloretin glycoside 1[b]	24.7	n.d.	n.d.
phloretin glycoside 2[b]	9.0	n.d.	n.d.
phloretin-2-*O*-xyloglucoside[c]	138.9	31.7	68.9
phloretin-2-*O*-xylogalactoside[c]		n.d.	4.2
phloridzin	27.9	78.9	48.0
320 nm			
chlorogenic acid	181.5	19.2	183.2
caffeic acid	4.8	4.0	7.5
3-coumaroyl-quinic acid[d]	9.5	3.0	9.4
4-coumaroyl-quinic acid[d]	77.3	5.0	66.0
5-coumaroyl-quinic acid[d]	10.4	3.8	39.8
p-cumaric acid	n.d.	4.2	2.6
cumaroyl-glucose	n.d.	n.d.	11.9
360 nm			
quercetine-3-*O*-rutinoside	2.6	49.1	4.5
quercetine-3-*O*-galactoside	0.8	8.1	1.8
quercetine-3-*O*-glucoside	1.4	12.3	1.5
quercetine-3-*O*-rhamnoside	4.1	25.1	4.3
quercetine-3-*O*-xyloside	n.d.	18.1	n.d.
quercetine-3-*O*-arabinopyranoside	n.d.	3.5	n.d.
total polyphenols	533.9	311.0	478.2
total polysaccharides	164.0	305.0	99.0

[a] Content of AE02 was reported in our previous publication (*10*). We have included the data here to compare the content of AE02 to the additionally investigated AEs (AE03 and AE04). n.d.: Polyphenols that were not detectable. [b] No commercial standard available, glycoside part unknown. [c] No commercial standard available, isolated by preparative HPLC, unknown dihydrochalcone. [d] No commercial standard available, quantitated with 3-isomer.

Contents of Polyphenols or Metabolites and SCFA in the Fermentation Samples. The polyphenols were hardly detectable

Table 2. Polyphenols and Metabolites Present in Fermented AE (F-AE03 and F-AE04) Analytically Characterized by HPLC[a]

	before fermentation		after fermentation	
polyphenols and metabolites (mg/g)	AE03	AE04	F-AE03	F-AE04
procyanidin B2	18.4	12.1	0.024	n.d.
catechin	2.7	n.d.	0.042	n.d.
phloretin-2-xyloglucoside	31.7	68.9	0.035	n.d.
phloridzin	78.9	48.0	n.d.	0.001
caffeic acid	4.0	7.5	n.d.	0.001
quercetin-3-rhamnoside	25.1	4.3	0.003	n.d.
phloroglucin	n.d.	n.d.	42.2	n.d.
3,4-dihydroxyphenylpropionic acid	n.d.	n.d.	33.3	41.3
total	160.80	140.80	75.60	41.30

[a] There were no polyphenols detected in the fermented blank control sample (without addition of AEs). n.d.: Polyphenols or metabolites were not detected.

in the fermentation samples (**Table 2**). Most of the polyphenols were degraded (approx. 99.9%) when compared to the total amount of non-fermented AEs. The total amounts were 0.10 and 0.002 mg/L of fermented F-AE03 and F-AE04, respectively. Only some of the polyphenols that have complex structures, namely, (+)-catechin (0.145 μmol/L), phloretin-2′-*O*-xyloglucosid (0.062 μmol/L), procyanidin B_2 (0.041 μmol/L), quercetin-3-*O*-rhamnoside (0.007 μmol/L), phloridzin (0.002 μmol/L), and caffeic acid (0.006 μmol/L) were retrieved in very minor amounts in the sample F-AE03 and F-AE04, respectively. There were no polyphenols detected in the fermented blank control samples (without addition of AEs). Moreover, the analysis of polyphenol metabolites in the fermentation samples showed that the total concentration of phloroglucin and 3,4-dihydroxyphenylpropionic acid was higher in F-AS03 (75.5 mg/L) than in F-AS04 (41.3 mg/L).

The formation of SCFA in the control sample (prepared without the addition of AE) was lower (11.4 mmol/L) than in the fermented samples with AE (**Figure 1**). The mean content of total SCFA in F-AE02 (44.5 mmol/L) was approximately 1.5-fold higher than in the other samples, which amounted to 28.9 mmol/L in F-AE03 and 35.4 mmol/L in F-AE04. Among all the analyzed SCFA, acetate (29.16 mmol/L) was found in high concentrations in all three F-AEs in comparison to the fecal control (blank), which had been produced without addition of the AEs. Butyrate (5.7 mmol/L) and propionate (4.5 mmol/L) were the second most abundant SCFA in all the F-AEs. The relative molar ratios for acetate, butyrate, and propionate were 65:19:16 for the blank, 74:15:11 for AE02, 69:15:16 for AE03, and 76:14:10 for AE04.

Modification of LT97 and HT29 Cell Growth by AEs. The effect of AE02 on HT29 cells was reported in our previous publication (*10*), and here, its activities were compared to AE03 and AE04. Treatment of LT97 and HT29 cells with all three AEs affected the cell growth in a time- and dose-dependent manner (**Figure 2**). After 24 and 48 h of treatment, the growth of the LT97 adenoma cells was more strongly inhibited by AEs than in the HT29 carcinoma cells. After 48 h, the EC_{50} values ranged from 240.8 ± 28.0 −to 454.8 ± 59.9 μg and from 380.8 ± 18.5 −to 634.5 ± 42.8 μg for the different AEs in the LT97 and HT29 cells, respectively (**Table 3**). AE03 had the highest antiproliferative activity as compared to AE02 or to AE04 and resulted in EC_{50} values of 240.8 ± 28.0 and 380.8 ± 18.5 μg after 48 h, in LT97 and HT29 cells, respectively. AE02 had the lowest antiproliferative activities with EC_{50} values of 454.8 ± 59.9 and 634.5 ± 42.8 μg after 48 h in LT97 and HT29 cells, respectively. Intermediate bioactivities were observed for AE04 (**Table 3**) with EC_{50} values of 290.6 ± 18.7 or 411.9 ± 30.8 μg in LT97 or HT29 cells. The values, however, were not significantly different from the values obtained for AE02 and AE03.

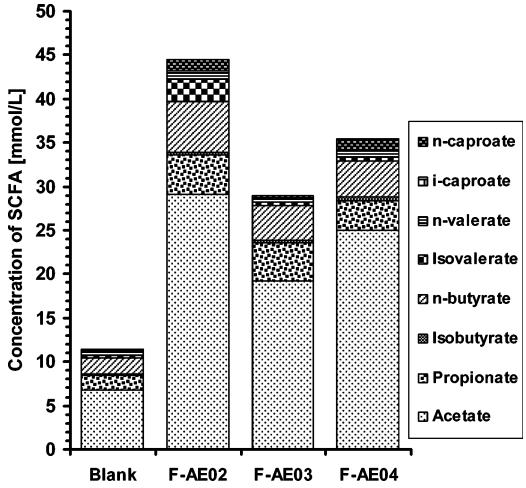


Figure 1. SCFA (mmol/L) in the fermentation samples of AEs (F-AE02, F-AE03, and F-AE04), $n = 2$.

Modulation of LT97 and HT29 Cell Growth by Fermented AEs. Figure 3 shows the effects of F-AEs on the growth of LT97 and HT29 cells after 24 and 48 h of treatment. The effect on cell growth of F-AEs and of the corresponding fermentation blank (without addition of AEs) was measured. Since there was a significant ($p \leq 0.01$) inhibition of cell growth by the blank (after 48 h), the data of F-AEs were normalized to the corresponding F-AE blank. All fermented samples also inhibited the growth of LT97 and HT29 cells in a time- and concentration-dependent manner. However, again, F-AE03 showed a more pronounced inhibitory effect than F-AE02 and F-AE04 and resulted in EC_{50} values of 404.1 ± 140.8 and 801.0 ± 44.0 μg after 48 h, in LT97 and HT29 cells, respectively (**Table 4**). The results show that the growth inhibition by fermented AEs did not directly reflect the amounts of SCFA found in the samples (**Figure 1**). Although the mean value of SCFA for F-AE02 was higher than that of F-AE03 and F-AE04, this did not result in a stronger growth inhibition. The LT97 cells were more sensitive than the HT29 cells toward the growth inhibitory activities of all F-AE. As compared to unfermented AEs, the F-AEs were approximately 3-fold less active.

DISCUSSION

AEs contain several compounds with antiproliferative potential (*10*). In this study, we used three different types of AEs, each containing different concentrations and types of polyphenols. The analyses of polyphenols again provided evidence for remarkable differences depending on cultivars, varieties, and harvest years.

The polyphenols, however, seem to be mostly degraded by fermentation mediated through the gut flora since some compounds were no longer detectable in fermented AEs. Exceptions were for larger, more complex polyphenols such as catechin and procyanidin derivatives, which were both still detectable, although only in very small amounts. Because of the complex structures of these particular compounds, they might be less susceptible to the action of the gut microflora enzymes (*32*). In addition, the fermentation process resulted in the formation of SCFA. Thus, by the fermentation of apple polyphenols through the gut flora, SCFA can be produced in the human colon, and

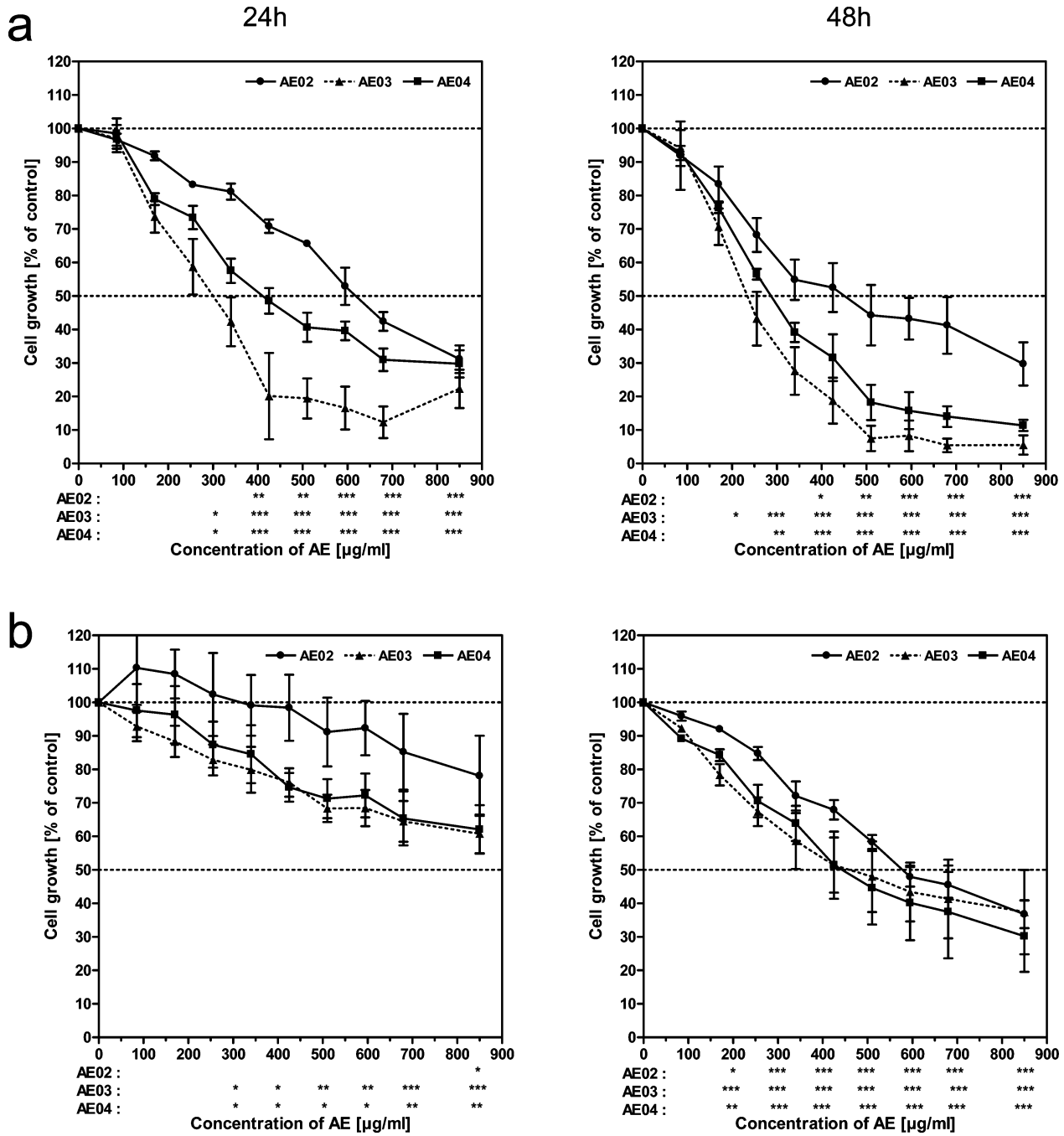


Figure 2. Growth inhibition of LT97 colon adenoma cells (**a**) and HT29 colon carcinoma cells (**b**) after 24 and 48 h incubation with AEs. The effect of AEs was normalized to the blank control (AEs without cells) and to the non-treated control (0 μg/mL, 100%) cells. One-way ANOVA and Bonferroni's post test gave *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$, $n = 3$.

Table 3. Antiproliferative Activities (EC_{50} Values, μg) of AEs Determined in LT97 and HT29 Cells[a]

	EC_{50} (μg/mL)					
AE	LT97			HT29		
treatment time (h)	24	48	72	24	48	72
AE02	650.9 ± 25.2	454.8 ± 59.9	361.0 ± 59.3	×	634.5 ± 42.8	462.4 ± 20.2
AE03	280.6 ± 45.3	240.8 ± 28.0	206.2 ± 19.5	×	380.8 ± 18.5	272.5 ± 8.1
AE04	430.8 ± 26.6	290.6 ± 18.7	224.2 ± 15.3	×	411.9 ± 30.8	454.7 ± 181.3

[a] ×: Denotes that EC_{50} values were not achieved, $n = 3$.

the formation depends on the amount of total polyphenols present in AEs. F-AE02, which had the highest amount of polyphenols before fermentation, also contained the highest amount of total SCFA. Interestingly, the fermentation of dietary fibers such as prebiotic long chain inulin-type fructans or arabinoxylans resulted in quite similar SCFA profiles (*33*, *34*).

We used DNA-DAPI staining as an indirect method to assess the total cell number in the proliferation assay. We have chosen

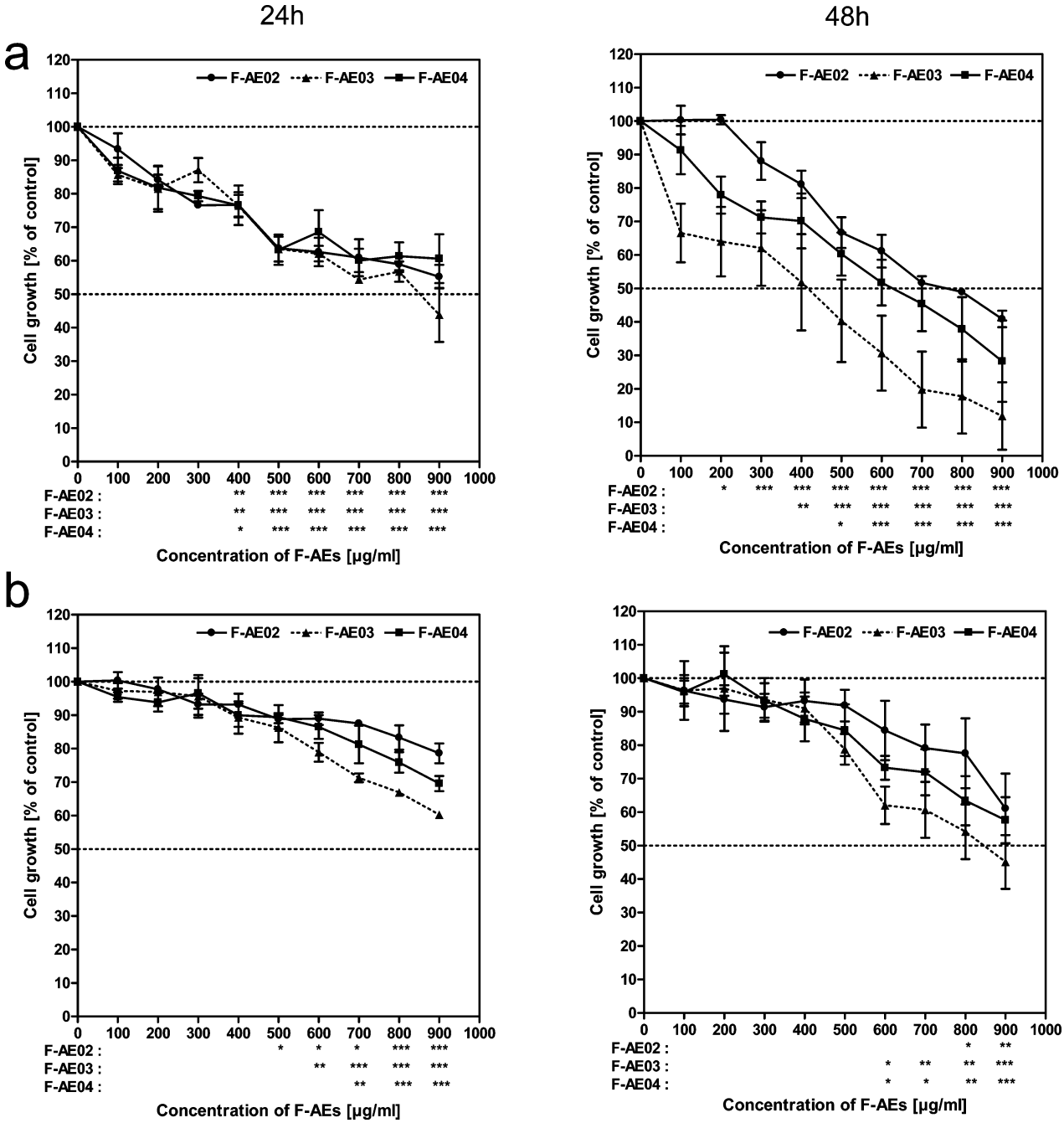


Figure 3. Growth inhibition of LT97 colon adenoma cells (**a**) and HT29 colon carcinoma cells (**b**) after 24 and 48 h incubation with F-AEs. The effect of AEs was normalized to the blank control (AEs without cells) and to the non-treated control (0 μg/mL, 100%) cells. One-way ANOVA and Bonferroni's post test gave *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$, $n = 3$.

Table 4. Antiproliferative Activities (EC_{50} Values, μg) of Fermented AEs Determined in LT97 and HT29 Cells[a]

	EC_{50} (μg/mL)					
F-AE	LT97			HT 29		
treatment time (h)	24	48	72	24	48	72
F-AE02	×	764.7 ± 9.3	628.8 ± 40.3	×	×	738.2 ± 13.2
F-AE03	821.4 ± 49.5	404.1 ± 140.8	565.8 ± 99.0	×	801.0 ± 44.0	687.7 ± 18.0
F-AE04	784.9 ± 44.1	572.4 ± 52.8	398.3 ± 106.4	×	×	724.9 ± 82.6

[a] ×: Denotes that EC_{50} values were not achieved, $n = 3$.

this method since it is faster, more reliable, and sensitive to determine the total cell number as compared to other methods used routinely in the laboratory. To exclude possible artifacts arising from fluorescence of the test substance, we included a blank control (test substances without cells). In our study, we used two in vitro cell model systems. One is a highly transformed human colon carcinoma cell line (HT29), and the second is a human colon adenoma cell line (LT97) that is

representative of preneoplastic cells. The rationale of the model choice was to compare the antiproliferative efficacy of apple polyphenols between transformed cells and partially transformed cells. If the effect was more pronounced in preneoplastic cells than highly transformed cells, one could expect that the effect would be higher in primary cells. However, since both cell types were grown and maintained in different cell culture media, some of the differences could also be attributed to culture conditions. The antiproliferative activity of AE03 was higher than that of AE04 and AE02 in both LT97 and HT29 cells after 24 and 48 h treatments. This was unexpected since AE03 (311.0 mg/g) contained only approximately 65 or 58% of the total polyphenols contained in AE04 or AE02, respectively. An explanation for this finding, however, could be that the pronounced antiproliferative activities of AE03 result from its higher quercetin concentrations, which were about 10- and 13-fold higher than the respective concentration of AE04 and AE02. This is supported by findings from previous studies with HT29 cells, showing that quercetin was the major compound contributing to the antiproliferative and antioxidative activities of AEs (*10*, *35*, *36*). This present part of the study confirms previous findings that both the mixture of major apple flavonoids as well as the amount of specific bioactive flavonoids are important factors for growth arrest in human colon cell lines (*37*).

Since the majority of the polyphenols was degraded (99.9%) during human gut flora-mediated fermentation, the growth inhibitory effects of F-AEs in LT97 and HT29 cells are probably not due to the same polyphenols as they are in the unfermented samples. The amounts of the detected polyphenols, such as (+)-catechin (0.145 μmol/L), phloretin-2′-*O*-xyloglucoside (0.062 μmol/L), procyanidin B_2 (0.041 μmol/L), and quercetin-3-*O*-rhamnoside (0.007 μmol/L), are possibly below the effective concentration ranges found in previous investigations, and some are lower than the reported concentration of apple polyphenols in human plasma (0.1−0.4 μmol/L) (*38*−*40*). It will, however, be of interest to assess in the future whether such low amounts of polyphenols, together with resulting metabolites, are able to inhibit growth or modulate the expression of relevant genes, as has, for example, been reported before (*41*, *42*). For example, it has also been shown that exposure of colon cells to catechins and procyanidins increases the expression of phase II enzymes, such as GSTs and UGTs, that are important for the biotransformation of carcinogens (*43*, *44*).

The SCFA, and especially butyrate, produced from AEs may play a role in the colon. Colon crypts may use SCFA as an energy source, whereas in tumor cells, SCFA stimulate pathways of growth arrest, differentiation, and apoptosis (*45*). Moreover, SCFA may also enhance toxicological defense in primary, adenoma, and tumor human colon cells by favorably modulating detoxifying enzymes (*46*). In our investigation, we did not find an association between the concentration of SCFA and antiproliferative properties of AEs. The F-AE03 contained only 80 and 63% of the SCFA contained in F-AE04 and F-AE02, respectively, but F-AE03 inhibited cell growth 1.6- and 1.4-fold more efficiently than F-AE04 and F-AE02 in LT97 cells. Similar directional but less pronounced effects were noticed with F-AE03 in comparison to F-AE04 and F-AE02 in HT29 cells with 1.2- and 1.1-fold differences, respectively. Thus, the amount of SCFA present in F-AEs did not directly reflect the differences in antiproliferative activities of F-AEs. Moreover, the total concentration of 3,4-dihydroxyphenylpropionic acid and phloroglucin, which are metabolites of proanthocyanidins and phloridzin (*47*, *48*) polyphenols, is higher in F-AS03 (75.5 mg/L) than in F-AS04 (41.3 mg/L), respectively. This might cause differences in antiproliferative effects by fermented AEs.

On the basis of equivalent apple concentrations, AEs were consistently about 10-fold more growth inhibitory than F-AEs in both LT97 and HT29 cells. Thus, the fermentation process reduced the effectiveness of AEs. The growth inhibition of adenoma-derived LT97 was more pronounced than carcinoma-derived HT29 cells after treatment with both AEs and F-AEs. LT97 cells may be representative of preneoplastic lesions in the human colon (*26*); thus, apple polyphenols might have a higher antiproliferative efficacy in the preneoplastic lesion than in carcinoma cells.

In conclusion, apple polyphenols were able to significantly suppress the growth of both adenoma (LT97) and carcinoma cells (HT29). The growth suppressing properties of AEs are due to their polyphenols but not due to the SCFA derived from the polyphenols during gut flora-mediated fermentation. The adenoma cells were more sensitive than the highly transformed carcinoma cells. This may mirror a higher chemoprotective potential of apple polyphenols in the preneoplastic lesion than in carcinoma. Our findings indicate that the adenoma and carcinoma cell proliferation is significantly inhibited by a specific combination of an apple polyphenol/flavonoid mixture from different cultivars. Collectively, these results imply that the antiproliferative effect by AEs might contribute to its overall chemoprotective function against colon carcinogenesis.

ABBREVIATIONS USED

AEs, apple polyphenol extracts; AE02, apple extract 2002; AE03, apple extract 2003; AE04, apple extract 2004; F-AEs, fermented apple polyphenol extracts; SCFA, short chain fatty acid.

ACKNOWLEDGMENT

We thank Prof. Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells. We are grateful to D. C. (F.H.) Peter Moeckel (Department of Nutritional Physiology of the University Jena) for SCFA analysis, Dr. Elke Richling (Molecular Nutrition Research Unit, University of Kaiserslautern) for analysis of polyphenols in the fermentation samples, and Dr. W. M. Pool for editorial assistance.

LITERATURE CITED

(1) Glade, M. J. Food nutrition and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997. *Nutrition* **1999**, *15* (6), 523−526.

(2) Boyer, J.; Liu, R. H. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, *3* (1), 5.

(3) Dragsted, L. O.; Strube, M.; Larsen, J. C. Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacol. Toxicol.* **1993**, *72* (Suppl. 1), 116−135.

(4) Terry, P.; Giovannucci, E.; Michels, K. B.; Bergkvist, L.; Hansen, H.; Holmberg, L.; Wolk, A. Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J. Natl. Cancer Inst.* **2001**, *93* (7), 525−533.

(5) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. Antiproliferative activity of flavonoids on several cancer cell lines. *Biosci. Biotechnol. Biochem.* **1999**, *63* (5), 896−899.

(6) Wenzel, U.; Kuntz, S.; Brendel, M. D.; Daniel, H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res.* **2000**, *60* (14), 3823−3831.

(7) Chan, M. M.; Ho, C. T.; Huang, H. I. Effects of three dietary phytochemicals from tea, rosemary, and turmeric on inflammation-induced nitrite production. *Cancer Lett.* **1995**, *96* (1), 23−29.
(8) Salucci, M.; Stivala, L. A.; Maiani, G.; Bugianesi, R.; Vannini, V. Flavonoid uptake and their effect on cell cycle of human colon adenocarcinoma cells (Caco2). *Br. J. Cancer* **2002**, *86* (10), 1645−1651.
(9) Ackland, M. L.; Van de Waarsenburg, S.; Jones, R. Synergistic antiproliferative action of the flavonols quercetin and kaempferol in cultured human cancer cell lines. *In Vivo* **2005**, *19* (1), 69−76.
(10) Veeriah, S.; Kautenburger, T.; Habermann, N.; Sauer, J.; Dietrich, H.; Will, F.; Pool-Zobel, B. L. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol. Carcinog.* **2006**, *45* (3), 164−174.
(11) Hanasaki, Y.; Ogawa, S.; Fukui, S. The correlation between active oxygen scavenging and antioxidative effects of flavonoids. *Free Radical Biol. Med.* **1994**, *16* (6), 845−850.
(12) Wolfe, K.; Wu, X.; Liu, R. H. Antioxidant activity of apple peels. *J. Agric. Food Chem.* **2003**, *51* (3), 609−614.
(13) Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu. Rev. Nutr.* **2001**, *21*, 381−406.
(14) Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **2002**, *50* (25), 7449−7454.
(15) Liu, R. H.; Eberhardt, M.; Lee, C. Antioxidant and antiproliferative activities of selected New York apple cultivars. *N.Y. Fruit Quarterly* **2001**, *9*, 15−17.
(16) Chinnici, F.; Gaiani, A.; Natali, N.; Riponi, C.; Galassi, S. Improved HPLC determination of phenolic compounds in cv. Golden Delicious apples using a monolithic column. *J. Agric. Food Chem.* **2004**, *52* (1), 3−7.
(17) Vrhovsek, U.; Rigo, A.; Tonon, D.; Mattivi, F. Quantitation of polyphenols in different apple varieties. *J. Agric. Food Chem.* **2004**, *52* (21), 6532−6538.
(18) Eberhardt, M. V.; Lee, C. Y.; Liu, R. H. Antioxidant activity of fresh apples. *Nature* **2000**, *405* (6789), 903−904.
(19) Vinson, J. A.; Su, X.; Zubik, L.; Bose, P. Phenol antioxidant quantity and quality in foods: fruits. *J. Agric. Food Chem.* **2001**, *49* (11), 5315−5321.
(20) Arts, I. C.; Hollman, P. C.; Feskens, E. J.; Bueno de Mesquita, H. B.; Kromhout, D. Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women. *Eur. J. Clin. Nutr.* **2001**, *55* (2), 76−81.
(21) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130* (Suppl. 8), 2073−2085.
(22) Kahle, K.; Kraus, M.; Scheppach, W.; Richling, E. Colonic availability of apple polyphenols—a study in ileostomy subjects. *Mol. Nutr. Food Res.* **2005**, *49* (12), 1143−1150.
(23) Blaut, M.; Schoefer, L.; Braune, A. Transformation of flavonoids by intestinal microorganisms. *Int. J. Vitam. Nutr. Res.* **2003**, *73* (2), 79−87.
(24) Beyer-Sehlmeyer, G.; Glei, M.; Hartmann, E.; Hughes, R.; Persin, C.; Bohm, V.; Rowland, I.; Schubert, R.; Jahreis, G.; Pool-Zobel, B. L. Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fiber sources. *Br. J. Nutr.* **2003**, *90* (6), 1057−1070.
(25) Schaeferhenrich, A.; Beyer-Sehlmeyer, G.; Festag, G.; Kuechler, A.; Haag, N.; Weise, A.; Liehr, T.; Claussen, U.; Marian, B.; Sendt, W.; Scheele, J.; Pool-Zobel, B. L. Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutat. Res.* **2003**, *526* (1−2), 19−32.
(26) Richter, M.; Jurek, D.; Wrba, F.; Kaserer, K.; Wurzer, G.; Karner-Hanusch, J.; Marian, B. Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro. *Eur. J. Cancer* **2002**, *38* (14), 1937−1945.
(27) Will, F.; Bauckhage, K.; Dietrich, H. Apple pomace liquefaction with pectinases and cellulases: analytical data of the corresponding juices. *Eur. Food Res. Technol.* **2000**, *211* (4), 291−297.
(28) Barry, J. L.; Hoebler, C.; Macfarlane, G. T.; Macfarlane, S.; Mathers, J. C.; Reed, K. A.; Mortensen, P. B.; Nordgaard, I.; Rowland, I. R.; Rumney, C. J. Estimation of the fermentability of dietary fiber in vitro: a European interlaboratory study. *Br. J. Nutr.* **1995**, *74* (3), 303−322.
(29) Barry, J. L.; Hoebler, C.; Macfarlane, G. T.; Macfarlane, S.; Mathers, J. C.; Reed, K. A.; Mortensen, P. B.; Nordgaard, I.; Rowland, I. R.; Rumney, C. J. Estimation of the fermentability of dietary fiber in vitro: a European interlaboratory study. *Br. J. Nutr.* **1995**, *74* (3), 303−322.
(30) Kiessling, G.; Schneider, J.; Jahreis, G. Long term consumption of fermented dairy products over 6 months increases HDL cholesterol. *Eur. J. Clin. Nutr.* **2002**, *56* (9), 843−849.
(31) Fogh, J.; Trempe, X. *Human Tumor Cells in Vitro*; Fogh, J., Ed.; Plenum Press: New York, 1975; pp 115−159.
(32) Spencer, J. P. Metabolism of tea flavonoids in the gastrointestinal tract. *J. Nutr.* **2003**, *133* (10), 3255−3261.
(33) Glei, M.; Hofmann, T.; Kuster, K.; Hollmann, J.; Lindhauer, M. G.; Pool-Zobel, B. L. Both wheat (*Triticum aestivum*) bran arabinoxylans and gut flora-mediated fermentation products protect human colon cells from genotoxic activities of 4-hydroxynonenal and hydrogen peroxide. *J. Agric. Food Chem.* **2006**, *54* (6), 2088−2095.
(34) Klinder, A.; Gietl, E.; Hughes, R.; Jonkers, N.; Karlsson, P.; McGlyn, H.; Pistoli, S.; Tuohy, K.; Rafter, J.; Rowland, I.; Van Loo, J.; Pool-Zobel, B. L. Gut fermentation products of chicory inulin-derived prebiotics inhibit markers of tumor progression in human colon tumor cells. *Cancer Epidemiol., Biomarkers Prev.* **2003**, *12* (11), 1339−1340.
(35) Schaefer, S.; Baum, M.; Eisenbrand, G.; Dietrich, H.; Will, F.; Janzowski, C. Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines. *Mol. Nutr. Food Res.* **2006**, *50* (1), 24−33.
(36) Kern, M.; Tjaden, Z.; Ngiewih, Y.; Puppel, N.; Will, F.; Dietrich, H.; Pahlke, G.; Marko, D. Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol. Nutr. Food Res.* **2005**, *49* (4), 317−328.
(37) Kuo, S. M. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. *Cancer Lett.* **1996**, *110* (1−2), 41−48.
(38) Graziani, G.; D'Argenio, G.; Tuccillo, C.; Loguercio, C.; Ritieni, A.; Morisco, F.; Del Vecchio, B. C.; Fogliano, V.; Romano, M. Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. *Gut* **2005**, *54* (2), 193−200.
(39) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1), 230−242.
(40) Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81* (1), 243−255.
(41) Gee, J. M.; Hara, H.; Johnson, I. T. Suppression of intestinal crypt cell proliferation and aberrant crypt foci by dietary quercetin in rats. *Nutr. Cancer* **2002**, *43* (2), 193−201.
(42) De Pascual-Teresa, S.; Johnston, K. L.; DuPont, M. S.; O'Leary, K. A.; Needs, P. W.; Morgan, L. M.; Clifford, M. N.; Bao, Y.; Williamson, G. Quercetin metabolites down-regulate cyclooxygenase-2 transcription in human lymphocytes ex vivo but not in vivo. *J. Nutr.* **2004**, *134* (3), 552−557.
(43) Chou, F. P.; Chu, Y. C.; Hsu, J. D.; Chiang, H. C.; Wang, C. J. Specific induction of glutathione *S*-transferase GSTM2 subunit expression by epigallocatechin gallate in rat liver. *Biochem. Pharmacol.* **2000**, *60* (5), 643−650.

(44) Sugatani, J.; Yamakawa, K.; Tonda, E.; Nishitani, S.; Yoshinari, K.; Degawa, M.; Abe, I.; Noguchi, H.; Miwa, M. The induction of human UDP-glucuronosyltransferase 1A1 mediated through a distal enhancer module by flavonoids and xenobiotics. *Biochem. Pharmacol.* **2004**, *67* (5), 989−1000.
(45) Heerdt, B. G.; Houston, M. A.; Augenlicht, L. H. Potentiation by specific short chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res.* **1994**, *54* (12), 3288−3293.
(46) Pool-Zobel, B. L.; Selvaraju, V.; Sauer, J.; Kautenburger, T.; Kiefer, J.; Richter, K. K.; Soom, M.; Wolfl, S. Butyrate may enhance toxicological defence in primary, adenoma, and tumor human colon cells by favorably modulating expression of glutathione *S*-transferases genes, an approach in nutrigenomics. *Carcinogenesis* **2005**, *26* (6), 1064−1076.
(47) Deprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A. Polymeric proanthocyanidins are catabolized by human colonic microflora into low molecular weight phenolic acids. *J. Nutr.* **2000**, *130* (11), 2733−2738.
(48) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003**, *35* (8), 837−844.

Received for review November 22, 2006. Revised manuscript received February 15, 2007. Accepted February 16, 2007. This project was funded by Bundesministerium für Bildung und Forschung (BMBF), Germany (BMBF FKZ.01EA0103).

JF063386R

2.3 **Publication III:** *Pool-Zobel BL,* ***Selvaraju V,*** *Sauer J, Kautenburger T, Kiefer J, Richter KK, Soom M, Wölfl S.* "Butyrate may enhance toxicological defence in primary, adenoma and tumour human colon cells by favourably modulating expression of glutathione *S*-transferases genes, an approach in nutrigenomics". *Carcinogenesis, 2005 Jun; 26(6):1064-76*

Butyrate, formed by bacterial fermentation of plant foods including polyphenols, has been suggested to reduce colon cancer risks by suppressing proliferation of tumour cells. Butyrate additionally has been shown to induce glutathione *S*-transferases (GSTs) in tumour cell lines, which may contribute to the detoxification of dietary carcinogens. In this study we have investigated the effects of butyrate on gene expression of 96 drug metabolism genes (cDNA-arrays) in primary human colon tissue, LT97 adenoma and HT29 tumour cells. In cells upon incubation with butyrate induced some GSTs that are known to be involved in defence against oxidative stress. We conclude that low GST expression levels were favourably altered by butyrate. An induction of the toxicological defence system possibly contributes to reported chemopreventive properties of butyrate, a product of dietary fibre fermentation in the gut.

Own contribution to the manuscript:

- Cell culture and RNA isolation, execution of the cDNA-arrays, gene expression analysis and verification of array genes by Northern blot and real-time PCR (was established in the lab)
- Data evaluation, interpretation and representation of the results

Carcinogenesis vol.26 no.6 pp.1064–1076, 2005
doi:10.1093/carcin/bgi059
Advance Access publication March 3, 2005

Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione *S*-transferases genes, an approach in nutrigenomics

Beatrice Louise Pool-Zobel*, Veeriah Selvaraju, Julia Sauer, Tanja Kautenburger, Jeannette Kiefer, Konrad Klaus Richter[1], Malle Soom[2] and Stefan Wölfl[2]

Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University, Dornburger Strasse 25, D-07743 Jena, Germany, [1]Department of General and Visceral Surgery, Clinic for Surgery and [2]Department of Molecular Medicine, Clinic for Internal Medicine II, Friedrich-Schiller-University, Erlanger Allee 101, D-07743 Jena, Germany

*To whom correspondence should be addressed. Tel: + 49 3641 949670; Fax: + 49 3641 949672;
Email: b8pobe@uni-jena.de

Butyrate, formed by bacterial fermentation of plant foods, has been suggested to reduce colon cancer risks by suppressing the proliferation of tumor cells. In addition, butyrate has been shown to induce glutathione *S*-transferases (GSTs) in tumor cell lines, which may contribute to the detoxification of dietary carcinogens. We hypothesize that butyrate also affects biotransformation in non-transformed colon cells. Thus, we have investigated the gene expression of drug metabolism genes in primary human colon tissue, premalignant LT97 adenoma and HT29 tumor cells cultured in an appropriate medium±butyrate. A total of 96 drug metabolism genes (including 12 GSTs) spotted on cDNA macroarrays (Superarray®; $n = 3$) were hybridized with biotin-labeled cDNA probes. To validate the expression detected with Superarray®, samples of LT97 cells were also analyzed with high density microarrays (Affymetrix® U133A), which include biotransformation genes that overlap with the set of genes represented on the Superarray®. Relative expression levels were compared across colon samples and for each colon sample±butyrate. Compared with fresh tissue, 13 genes were downregulated in primary cells cultivated *ex vivo*, whereas 8 genes were upregulated. Several genes were less expressed in LT97 (40 genes) or in HT29 (41 and 17 genes, grown for 72 and 48 h, respectively) compared with primary colon tissue. Butyrate induced *GSTP1*, *GSTM2*, and *GSTA4* in HT29 as previously confirmed by other methods (northern blot/qPCR). We detected an upregulation of GSTs (*GSTA2*, *GSTT2*) that are known to be involved in the defence against oxidative stress in primary cells upon incubation with butyrate. The changes in expression detected in LT97 by Superarray® and Affymetrix® were similar, confirming the validity of the results. We conclude that low GST expression levels were favourably altered by butyrate. An induction of the toxicological defence system possibly contributes to reported chemopreventive properties of butyrate, a product of dietary fibre fermentation in the gut.

Abbreviations: ARE, antioxidant responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSTs, glutathione *S*-transferases; HBSS, Hank's balanced salt solution; HDACs, histone deacetylases; Keapl, Kelch-like ECH-associated protein 1; PBS, phosphate buffered saline.

Introduction

The lifetime colorectal cancer risk in the general population is reported to be ~5%, with individual risk increasing significantly with age (1). Although a small proportion of colorectal tumors are caused by inherited genetic alterations (2), the greatest numbers of tumors are sporadic and probably the result of a life-long accumulation of genetic alterations in somatic tissues (3,4). These may be caused by carcinogenic compounds derived from foods that are putative risk factors for colorectal cancer (5,6). Carcinogenic compounds ingested with food may pass directly into the gut lumen or may reach the colon indirectly through the bile and/or the enterohepatic circulation after being metabolically activated and conjugated in the liver (7–9). The extent of dietary exposure, the ability to prevent DNA damage by inactivating dietary carcinogens and the capacity to repair the damage caused by dietary carcinogens all contribute to an individual's risk of developing cancer. A favourable balance of biotransformation enzymes, which include various phase I enzymes (10), phase III transport systems (11,12), as well as phase II enzymes, such as glutathione (GSH) *S*-transferases (13), UDP-glucuronosyl transferases (14,15), might protect tumor target cells from accumulating additional mutations. This mechanism of action by xenobiotics has been defined as 'blocking agent activity' as opposed to 'suppressing agent activity', which targets altered cells by e.g. inhibiting their growth or inducing apoptosis (16). Both mechanisms contribute to the chemopreventive action of compounds (17–19).

Levels of biotransformation enzymes have been associated with genetic polymorphisms (20), as well as with environmental factors (21). The induction of selected phase II enzymes that exhibit mainly detoxifying activities is an important target in dietary chemoprevention (22,23). A family of enzymes that plays an important role in detoxification is glutathione *S*-transferases (GSTs; EC 2.5.1.18), which catalyze the conjugation of many electrophilic compounds with reduced GSH. Based on their biochemical, immunological and structural properties, the GSTs are characterized as cytosolic, mitochondrial and microsomal enzymes. The cytosolic transferases are represented by classes Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega. The mitochondrial transferase is called class Kappa GST. The microsomal transferases form a unique MAPEG (membrane-associated proteins in eicosanoid and GSH metabolism) grouping of transferases (13,24).

In this context, we have been exploring the potential of physiologically available butyrate to modulate the expression levels of GSTs (25; T.Kautenburger, G.Beyer-Sehlmeyer, G.Festag, N.Haag, S.Kuechler, A.Kuechler, A.Weise, B.Marian, W.H.M.Peters, T.Liehr, U.Claussen, and B.L.Pool-Zobel, submitted for publication) and to confer resistance to human colon cells towards the exposure to colon cancer risk factors (26,27). Butyrate is a major product of dietary fibre fermentation by the gut microflora and evidence is

accumulating that it may also be formed from other ingredients of plant foods, such as polyphenols (28). In HT29 cells, butyrate was an efficient inducer of GSTs, particularly GSTP1-1, GSTM2-2 and GSTA4-4 (25–29), whereas in colon adenoma cells butyrate reduced the expression of GSTT1-1 protein, probably by destabilizing the GSTT1 mRNA (T.Kautenburger *et al*., submitted for publication). Additional GST genes may contribute to GSH conjugation within colon cells resulting in cellular protection (30).

In this study we (i) investigated whether GSTs and other biotransformation genes were expressed differently in human colon cells and (ii) determined differences in gene expression owing to butyrate. For this purpose we utilized two types of DNA arrays, both novel developments of functional genomics (31) and assessed the expression levels of 12 GSTs in colon epithelial tissue, primary human colon cells (32), premalignant human LT97 adenoma cells (33) and highly transformed HT29 tumor cells (34). All studied stages were considered to be relevant targets to study the dietary-related colon carcinogenesis, and particularly, HT29 cells have been used in many studies as a model for colon cancer cells. We aimed to enhance the knowledge of biotransformation capacities and the transcriptional regulation by butyrate. This type of nutrigenomics approach will help in expanding our understanding of the mechanisms that mediate the effects of chemopreventive diets in reducing the risk of colorectal cancer (35,36).

Materials and methods

Cell lines and culture condition

The human colon adenoma cell line LT97 was a kind gift from Professor Brigitte Marian (Institute for Cancer Research, University of Vienna, Austria) who established it from colon microadenomas of a patient with familial adenomatous polyposis (33). LT97 was maintained in a culture medium (MCDB 302) containing 20% of L15 Leibovitz medium, 2% FCS (fetal calf serum), 0.2 nM triiodo-L-thyronine, 1 μg/ml hydrocortisone (302 basic medium) supplemented with 10 μg/ml insulin, 2 μg/ml transferrin, 5 nM sodium selenite and 30 ng/ml EGF (epidermal growth factor). HT29 cells were isolated from a colon adenocarcinoma of a female Caucasian (34) and originated from an adenoma colon tissue. It was obtained from the American Tissue Culture Collection (ATCC), Rockville, MD, USA. The HT29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin (26). LT97 and HT29 cells were grown in T25 flasks and cultivated in a humidified incubator (5% CO_2; 95% humidity, at 37°C). Under these conditions, doubling time for LT97 was 72–96 h; and for HT29 cells, 24 h. Passages 29–32 and 29–46, respectively were used for the experiments.

Primary human colon tissue

Cells and RNA were isolated from patients who had given their informed consent after being admitted to hospital for surgery of colorectal tumors, diverticulitis and colon polyps (25). Mean age (±SD) of the six donors of tissue for direct RNA isolation was 58.5 ± 11.1 years; three of the donors were male and three were female. Mean age (±SD) of the three donors from which colon cells were first isolated before incubation and RNA isolation was 65.7 ± 20.2 years; one of the donors was male and two were female. The Ethical Committee of the Friedrich-Schiller-University of Jena approved the study. Non-tumor colon tissue was stored in HBSS (Hank's balanced salt solution; 8.0 g/l NaCl, 0.4 g/l KCl, 0.06 g/l $Na_2HPO_4 \times 2\ H_2O$, 0.06 g/l K_2HPO_4, 1 g/l glucose, 0.35 g/l $NaHCO_3$ and 4.8 g/l HEPES, pH 7.2), transported on ice to the laboratory within 1 h and worked up immediately. The human colon epithelium was separated from the tissue by a perfusion-supported mechanical disaggregation (32). Epithelial stripes were either conserved for RNA isolation or they were further incubated *in vitro* and treated with butyrate (see below).

Treatment with butyrate

Effects of butyrate on the growth properties of HT29 and LT97 cells and on the expression of *GSTP1*, *GSTM2*, *GSTA4* and *GSTT1* have been assessed in detail perviously (25). Based on these studies, each of the cell types was incubated and treated with the maximum butyrate concentration without affecting the viability and growth rates as had been described previously for LT97 and HT29 cells (T.Kautenburger *et al*., submitted for publication; 26), or as had been established during this study for primary colon tissues/cells. Therefore, the cell-specific, subtoxic and optimal conditions varied in terms of time between plating and treatment, duration of treatment and concentration of butyrate. HT29 cells were plated and after allowing attachment for 24 and 48 h, subjected to treatment with 4 mM butyrate or plain medium. LT97 cells were plated and after allowing attachment for 72 h, treated with 1 and 2 mM butyrate or plain medium. Both cell lines were harvested after a further 24 h treatment. Primary human colon tissue pieces were cultured in petri dishes (35 mm) and after allowing to settle for 15 min, subjected to treatment with 10 mM butyrate or plain medium. After 12 h treatment, the cells were isolated from the epithelial stripes by mincing and were incubated in 3 ml HBSS (60 min, 37°C) supplemented with 6 mg proteinase K (Sigma; Steinheim, Germany) and 3 mg collagenase P (Boehringer; Mannheim, Germany). The suspensions of primary human colon cells were diluted with HBSS, centrifuged and resuspended in PBS (phosphate-buffered saline; 8 g/l NaCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KCl and 0.2 g/l KH_2PO_4, pH 7.3). Viability and cell yields were determined with trypan blue.

RNA isolation

Total RNA was isolated from primary human colon cells, LT97 adenoma cells and HT29 tumors cells (up to 6×10^6 cells) using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 30–70 μl RNase free water and stored at −20°C. RNA was also isolated from surgical tissue samples, which had been placed into RNA Later solution (Qiagen, Hilden, Germany) immediately after excision. The integrity of the ribosomal RNA and DNA contamination was checked routinely using formaldehyde denaturing RNA gel electrophoresis (1.2%) before proceeding with the further macro and micro array analysis. Protein or phenol contamination and concentration of the total RNA was assessed by determining the ratio A 260:280 spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany).

Macro and micro array analysis

Superarray.® 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). Genes were classified into functional categories, representing phase I enzymes (cytochrome p450 family, epoxide hydroxylases), phase II enzymes (acetyltransferases, GST, sulfotransferases, and UDP-glucuronosyl transferases and miscellaneous others) and phase III enzymes (metallothioneins and *p*-glycoproteins). A detailed gene list is available on the company's website (http://www.superarray.com/) and in the accessory file to this manuscript. [The studies were done with c-DNA macroarrays, containing sequences of 96 genes related to drug metabolism, the data for the 12 spotted genes belonging to the family of the glutathione *S*-transferases are presented here in more detail, since confirmatory studies are available. Data for the other genes of drug metabolism are available from the accessory data file (http://www2.uni-jena.de/biologie/ieu/et/Dateien/Butyrate_gene.pdf).] Six arrays were used to determine the interindividual variation levels for RNA isolated from six different donors. Three arrays each were used for RNA isolated from three independently reproduced experiments consisting of medium controls and butyrate-treated samples of primary colon cells, LT97 cells and HT29 cells. HT29 cells were investigated both at 24 h after plating and at 48 h after plating to determine differences owing to culture conditions. Work-up of the array was performed according to the manufacturer's protocol. Single-stranded cDNA was synthesized from total RNA (1–3 μg) *in vitro* by using M-MLV reverse transcriptase (Promega, USA). By applying a single-step ampo linear polymerase reaction (LPR) labelling technique, the cDNA was labelled with dUTP-biotin. The cDNA macroarray was hybridized overnight at 60°C with the biotin-labelled cDNA. The hybridized membrane was subjected to chemiluminesence analysis for quantification of the conjugation signals with streptavidin-linked alkaline phosphatase and CDPstar. The resulting signals were captured by CCD camera equipment (Fujifilm LAS-1000, Diana, USA) and analyzed with AIDA array analysis (Raytest GmbH, Germany) program to comprehensively evaluate the differential gene expression of the various samples. 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 of the positive controls (household genes) were fixed to equal 100%. Thus, the data shown here represent the mean expression levels relative to negative and positive reference genes. Some genes may reach signals over those of the household genes and thus reach values >100%. Negative values are obtained for genes revealing signals below those of the six negative reference spots. Additionally, to enable other comparisons,

the data were also normalized according to two other criteria (data not shown). One was to set the lowest signal to equal 0% and the other was to set the means of signals of all genes to equal 100% (global normalization). The values of 'fold change', obtained for all three normalization procedures, were used to identify differentially expressed genes and butyrate-regulated genes, respectively. This comparison revealed that the first approach was the most sensitive and (based on all confirmatory data) also the most predictive one.

Affymetrix®. Hybridizations were done on Affymetrix U133A gene expression arrays containing probe sets recognizing >14 000 well-characterized human genes. A detailed list of genes is available on the Affymetrix website (http://www.affymetrix.com). Labelled probes for hybridization were prepared from total RNA obtained as described above from LT97 cells. To remove residual contamination with genomic DNA, total RNA samples were treated with DNAse I at 37°C for 30 min followed by repurification through RNeasy columns (Qiagen, Hilden, Germany). Labelling reactions were done following the suggested protocol for the preparation of fragmented biotinylated complementary RNA (cRNA). In short, with all variable points, 5 μg of total RNA, DNase I treated, was used for cDNA synthesis using the T7-promoter primer (Affymetrix). After a second strand synthesis, biotinylated cRNA was obtained by transcription from the double-stranded cDNA with T7-RNA-polymerase (Enzo). Biotinylated cRNA was fragmented by treatment with Mg^{2+} directly before hybridization. Hybridization and scanning were done on an Affymetrix array processing station and scanner. Primary data obtained scanning the signals of the micro arrays (Affymetrix U133A) were analyzed using the Affymetrix MicroArraySuite analysis package. The resulting signal intensities for each gene and the change of *P*-values were used for comparative evaluation.

Northern blot analysis of GSTP1 expression

Ten micrograms of LT97 and HT29 RNA were loaded on a 1.5% denaturing agarose gel, separated for 3–4 h at 80 V and blotted on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). Preparation of digoxygenin-labelled RNA probes for GSTP1 and GAPDH was performed as described previously (26). Hybridization occurred overnight at 72°C in standard high SDS hybridization buffer (containing 100 ng/ml of *GAPDH* and 67 ng/ml of *GSTP1* RNA probe). The signals were detected by incubating the membrane with anti-DIG alkaline phosphatase antibody (Roche Diagnostics, Mannheim, Germany), followed by CDP-Star substrate incubation. Afterwards, the blot was exposed for 10 min on X-ray film (Hyperfilm ECL, Amersham Biosciences, Freiburg, Germany) and photographed (Fluor-S® MultiImager, Bio-Rad, München, Germany). Evaluation of the band intensities proceeded with the Quantity One® 4.1 Software (Bio-Rad, München, Germany).

Real-time RT–PCR analysis of GSTT2 expression

Expression of *GSTT2* mRNA was assessed by the two-step SYBR Green I quantitative real-time RT–PCR by iCycler iQ system (Bio-Rad GmbH München, Germany). Briefly, 3 μg of total RNA from the butyrate-treated samples (LT97 and HT29 cells) were converted into first-strand cDNA using Superscript II (Invitrogen) according to the manufacturer's conditions. 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, iTaq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix, 10 mM $MgCl_2$, 20 nM fluorescein and stabilizers), 10 pmol stock of each of the specific primers (*GSTT2*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in a final reaction volume of 25 μl. All reactions were performed in triplicate. The PCR profile consisted of an initial denaturation of 5 min at 95°C, 40 cycles of 30 s at 95°C denaturing, 40 s at 63°C annealing, 45 s at 72°C extension and followed by a 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 and agarose gel electrophoresis analysis. Gene-specific primer sequences used for the quantification were as follows: *GAPDH*, forward, 5′-CCACCCATGG--CCACCCATGGCAAATTCCATGGC-3′ and reverse, 5′-AGTGGACTCC-ACGACGTACTCAG-3′; *GSTT2* forward, 5′-TGACACTGGCTGATCTC-ATGGCC-3′ and reverse, 5′-GCCTCCTGGCATAGCTCAGCAC-3′; PCR primer for target and reference gene cloning *GSTT2* forward, 5′-GGTGGA-ACGCAACAGGACTGCC-3′ and reverse, 5′-GCCTGATAGGCCTCTGGT-GAGG-3′; and *GAPDH* forward, 5′-CCACCCATGGCAAATTCCATGGC-3′ and reverse, 5′-TAGACGGCAGGTCAGGTCCACC-3′. Primer nucleotides confirmation of the total gene specificity was performed using the BLASTN search programme.

Relative quantification of unknown *GSTT2* mRNA gene expression was determined by using a series dilution of cDNA plasmid containing the inserted *GSTT2*, *GAPDH* and constructing a calibration curve. Wells with no template were used as negative control.

Statistical analysis

Superarray®. Comparisons were made for the directly excised tissue and for colon cells after cultivation in medium, which was a reflection of the baseline expression levels. Comparisons were also made for each of the three colon cells incubated with medium and with butyrate, which was a reflection of the modulated gene expression. Responses of drug metabolism genes spotted on the Superarray® membranes and Affymetrix® array were directly compared by using identical RNA aliquots of LT97 cells incubated in medium and with butyrate. Another comparison was made from a technical point of view, namely to compare the gene expression levels of HT29 cells, which were worked up 48 and 72 h after plating. Genes were clustered into functional entities and subjected to an analysis on a group basis, using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA). Values obtained after normalization were taken for an analyses of variance (ANOVA) test and Bonferroni's post-test was then used to identify genes that were statistically different between the groups. ANOVA calculations taken to compare biopsies and individual cell types were non-repeated measures, whereas ANOVA calculations, based on repeated measures, were used to determine the effects of butyrate. Additionally, unpaired *t*-tests (±Welch's correction for unequal variances) were used, as appropriate, to determine the differences of multiple genes on a group basis. All data were evaluated to establish the two-sided significance levels of independently reproduced determinations.

Affymetrix®. Before comparison of the signal intensities across all data sets, data were normalized using a global normalization approach supervised by the rank intensity distribution of the normalized signal intensities (37). Changes in gene expression were then calculated as fold changes with respect to the untreated reference (38). In cases, in which one gene is represented by different groups of probes, results were summarized when all probe sets gave the same results. In cases of discrepancy, probe set located at the 3′ end of the coding sequence were preferentially considered. In cases where no decision could be made, results for all probe sets were included in the presentation of results. The most likely explanation for these differences is that alternative processed and transcribed mRNA originated from the respective genes.

Real-time PCR. Final results were expressed as an *n*-fold difference in the *GSTT2* gene expression relative to the internal reference *GAPDH* and the calibrator. Statistical significance between control and treated cells was calculated by unpaired *t*-test and one-way ANOVA.

Results

Cellular parameters

Primary cells, isolated from colon tissue incubated *in vitro* (12 h), had a viability of 79 ± 13 and 76 ± 18% in the control medium and in the medium containing 10 mM butyrate, respectively. Confluence of LT97 cells before isolating RNA was ~70–80 and ~80–90% for medium control and for the butyrate-treated samples, respectively. Confluence of HT29 cells after 48 h attachment was 70–80 and 80–90%, and after 24 h attachment it was 60–70 and 70–80%, for medium controls and butyrate-treated samples, respectively. Viability of recovered HT29 and LT97 cells was always >95% for all experimental conditions.

Baseline expression levels

The baseline expression levels of the target genes were determined in freshly excised colon tissues from six individual donors. The data for the GST group of genes obtained for each donor are shown in Figure 1. (The accessory data file shows baseline values for all genes related to drug metabolism.) To enable a better discrimination of the expressed genes, the left panel shows GSTs with low signals and the GSTs with higher relative expression levels are grouped in the right panel of the figure. There was a considerable variation the of expression with total signal strength, which may vary depending on the probe characteristics. Altogether, the signal strength ranged from 482 (donor 3) to 972 (donor 5), which was a 2-fold difference for the sum of all GSTs.

The mean expression levels ($n = 6$ donors) of each individual gene were the basis for assaying differential expression

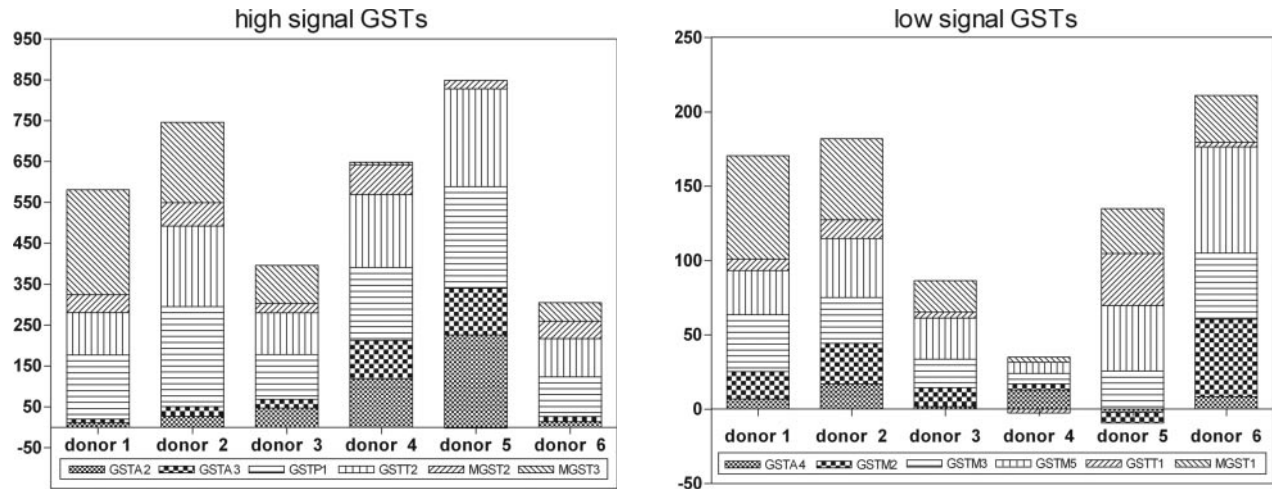


Fig. 1. Individual levels of GST mRNA-expression in freshly excised primary colon tissues derived from six different donors. The 12 GSTs were divided into two groups, namely one with low expression levels and in another one with high expression levels. The mean values and individual variations for the individual GSTs are shown in Table I.

across the human colon samples. For this comparative analysis, we took into account various parameters, namely, the absolute expression levels, the subtractive degree of change between groups, the fold change between groups and the reproducibility of the measurement. The cut-off values for the expression levels were chosen to be 10 [which was equal to the average variation (SEM) in the control genes from the colon tissue samples; $n = 6$]. The fold change to indicate upregulation was set at $\geq$2.0, which was previously found to be significant ($P < 0.05$, $n = 3$) for butyrate-mediated changes of GST activity, *GSTP1* mRNA and GSTP1-1 expression in HT29 cells (25). The fold change to indicate downregulation was set at $\leq$0.5, since this was previously found to be significant ($P < 0.001$, $n = 3$) for the butyrate-mediated inhibition of GSTT1-1 protein expression in LT97 cells (T.Kautenburger, *et al.*, submitted for publication). These numbers were therefore not arbitrary, since they had been shown to reflect significant changes for GST mRNA, GST protein and GST enzyme activity.

Table I shows the relative baseline expression levels for all differentially expressed genes (including GSTs), which were spotted on the membrane (accessory data file). Most genes yielded signals of >10. There were no differences of *GSTP1* and *GSTT1* expression in primary tissues, compared with the colon cells. The other GSTs were differently expressed, albeit with different patterns. With respect to the evaluation criteria described above, a subset of GSTs were less expressed in LT97 and in HT29 (72 h) than in colon tissues. However, in primary cells and in HT29 cells (48 h after plating), there were several examples of genes expressed more in the cells than in biopsies (Table I).

The evaluation of the data on group basis revealed that the expression levels of GSTs from LT97 were significantly ($P = 0.0317$, two-tailed paired *t*-test) different from HT29 (grown for 48 h after plating). Details comparing the expression levels of all genes are available from the accessory data sheets (accessory data file).

Effects of butyrate

According to the exclusion criteria, butyrate was an efficient inducer of GSTs, clearly resulting in an upregulation of *GSTA2* and *GSTT2* in primary cells, of *GSTM3*, *GSTT2* and *MGST3* in LT97 cells and of *GSTP1*, *GSTA4*, *GSTM2*, *GSTM5* and *MGST3* in HT29 cells [Table II (accessory data file)]. With only normalization procedure 1, upregulation of *GSTT2* in LT97 cells (2-fold at 2 mM butyrate) was significant ($P < 0.05$ two-way ANOVA, Bonferroni's post-test), as was the *GSTP1* upregulation (2.2-fold) in HT29 cells incubated for 72 h ($P < 0.001$). On a group basis, 1 and 2 mM butyrate treatment induced GSTs in LT97 ($P = 0.0104$ and $P = 0.0332$, respectively; two-way ANOVA). *MGST1* was efficiently (0.5-fold) downregulated in HT29 cells. It was one of the only few examples of a clear cut downregulation by butyrate, when taking into acount all the genes on the array. Details of all butyrate-mediated effects on GSTs are shown in Table II (accessory data file).

These experiments were independently reproduced three times, thus reducing the necessity of performing the obligatory confirmational experiments usually required for microarray analysis. However, we had some data (e.g. for *GSTP1, GSTM2* and *GSTA4* in HT29) available from previous work, as indicated in the legend of Table II and the expression levels of *GSTP1* in LT97 (no induction in the macro array) and in HT29 (induction in the macro array) were additionally confirmed by northern blot using aliquots of the same RNA. Figure 2 shows that the northern blot results are fully confirmatory of the microarray results. We also subjected an aliquot of the RNA isolated from LT97 (medium control, 1 or 2 mM butyrate) to expression analysis using Affymetrix®. Table III summarizes data for genes which were induced by butyrate using Superarray® ($n = 3$) and which were also spotted on Affymetrix® ($n = 1$). (Table III of the accessory data file shows additional comparisons for genes with signals >10 which were not induced according to Superarray analysis, but which were spotted on the Affymatrix array.) Of these 14 genes, all but one (*GSTT2*) gave results in the same direction. Therefore, *GSTT2* expression was additionally confirmed with real-time RT–PCR.

Analysis of the effect of butyrate on the colon adenoma cell line LT97 with Affymetrix® arrays also shows a significant regulation of a larger number of other genes (~500) connected with various other cellular processes. These data however, do not interfere with the discussed effect on the detoxifying genes and will be presented in another manuscript under preparation.

Table I. Baseline expression levels of drug metabolizing enzymes in human colon cells compared with tissues

Functional gene family	Number of expressed genes	Differentially expressed genes	Primary tissue* 0 h[a]		Primary cells 12 h[a]		LT97 adenoma cells 72 h[a]		HT29 tumour cells 72 h[a]		HT29 tumour cells 48 h[a]	
			Means	SEM	Down	Up	Down	Up	Down	Up	Down	Up
Phase I												
p450 Family	14/25	CYP2B	60.3	21.8	CYP2B		CYP2B		CYP2B		CYP3A4	
		CYP2F1	33.7	7.8			CYP2F1		CYP2F1			
		CYP3A4	36.3	17.5			CYP3A4		CYP3A4			
		CYP4F3	154.5	32.7			CYP4F3					
Phase II												
Acetyltransferases	10/10	ACAT1	89.8	16.1	CRAT	ACAT2	ACAT1		ACAT1		CHAT	DLAT
		ACAT2	331.5	63.3		LOC51126	ACAT2		ACAT2		NAT1	HAT1
		CHAT	134.8	44.2			CHAT		CHAT			HBOA
		CRAT	41.9	12.1			CRAT		CRAT			LOC51126
		DLAT	27.8	7.4			DLAT		DLAT			
		HAT1	17.6	6.6			NAT1		NAT1			
		HBOA	3.2	4.7			LOC51126		LOC51126			
		MORF	17.2	5.1								
		NAT1	82.4	16.6								
		LOC51126	48.2	12.9								
Glutathione *S*-transferases	12/12	GSTA2	73.7	34.4	GSTA2	GSTM2	GSTA2		GSTA2		GSTA2	GSTA4
		GSTA3	45.8	18.8	GSTA3	GSTM3	GSTA3		GSTA3		GSTA3	MGST1
		GSTA4	7.4	2.9	MGST1	GSTM5	GSTM3		GSTM3		GSTM3	
		GSTM2	17.9	8.4			GSTM5		GSTM5		GSTM5	
		GSTM3	27.5	5.5			GSTT2		GSTT2			
		GSTM5	36.7	8.6			MGST2		MGST2			
		GSTT2	152.2	25.1			MGST3		MGST3			
		MGST1	35.1	9.7								
		MGST2	43.7	8.2								
		MGST3	99.3	43.3								
Sulfotransferases	15/21	CHST5	37.8	18.1	CHST5	TPST1	CHST6		CHST6		SULT1B1	CHST5
		CHST7	72.1	45.8	CHST7		HNK-1ST		HNK-1ST			CHST7
		HNK-1ST	56.4	7.9	SULT1B1		SULT1A1		SULT1A1			
		SULT1A1	31.8	11.9			SULT1A2		SULT1A2			
		SULT1A2	27.5	9.5			SULT1B1		SULT1B1			
		SULT1B1	52.4	20.4			TPST1		TPST1			
		TPST1	40.2	4.8								
Miscellaneous	12/13	UGT1A1	151.5	54.0	UGT2A1	NNMT	UGT1A1		UGT1A1	TPMT	UGT2A1	UGT2B
		UGT2A1	28.0	10.0	UGT2B4		UGT2A1		UGT2A1		UGT2B10	TPMT
		UGT2B	59.1	9.7	TPMT		UGT2B		UGT2B		UGT2B4	EPHX1
		UGT2B10	105.5	15.8			UGT2B10		UGT2B10		NNMT	
		UGT2B4	38.8	13.7			UGT2B4		UGT2B4			
		COMT	32.4	8.6			HNMT		COMT			
		HNMT	169.9	20.9			NNMT		HNMT			
		NNMT	87.8	18.3					NNMT			
		TPMT	33.6	25.7								
Phase III												
Metallothioneins	8/8	MT1A	125.5	33.1		MT1G	MT1A		MT1A		MT1A	
		MT1G	257.1	23.2			MT1G		MT1G		MT1G	
		MT1H	196.7	32.6			MT1H		MT1H		MT1H	
		MT1L	267.5	34.1			MT1L		MT1L		MT1L	
		MT2A	87.9	23.6			MT2A		MT2A		MTIX	
		MT3	21.2	4.3			MTIX		MT3			
		MTIX	386.0	42.5					MTIX			
p-Glycoproteins	3/7	ABCC2	127.1	70.4	ABCC2		ABCC2		ABCC2			ABCC3
		ABCC3	45.7	13.0	ABCG2		ABCG2		ABCG2			
		ABCG2	131.0	39.6								
Housekeeping genes	10/10	GAPD	311.2	30.3			PPIA	RPL13A	PPIA	RPL13A		RPL13A
		GAPD	275.3	17.7			PPIA	RPL13A	PPIA	RPL13A		RPL13A
		PPIA	89.5	7.6			PPIA	ACTB	PPIA	ACTB		ACTB
		PPIA	88.4	11.0			PPIA	ACTB		ACTB		ACTB
		PPIA	97.7	6.8								
		PPIA	107.0	4.9								
		RPL13A	−0.3	5.1								
		RPL13A	0.9	4.4								
		ACTB	15.3	7.1								
		ACTB	14.9	7.6								

Primary colon cells isolated from surgical material, LT97 human colon adenoma cells and HT29 cells were plated and grown in medium for 12 h, 72 h, and/or 48 h prior to work-up. Only those genes for which the baseline expression levels reached a signal of $\geq$10 at least in one cell type are show in the table. Regulation is based on expression levels in primary tissue. Downregulation is defined as a ratio $\geq$2 (tissue/cells), and a subtractive difference $>$20. Upregulation is defined as a ratio $\leq$0.5 (tissue/cells). Data were calculated using the relative signals obtained after normalization from samples of six different donors (biopsies) from three independently reproduced experiments (colon cells).

*Means $\pm$ SEM, $n = 6$. The baseline data for this table are in Table I of the accessory data file.

[a]Total culture period.

Table II. Overview on the modulation of expression of drug metabolizing enzymes by butyrate in primary colon cells isolated from surgical material in LT97 human colon adenoma cells and in HT29 cells

Functional gene family	Total number of genes	Primary 12 h[a]	LT97 72 h[a]		HT29 72 h[a]	48 h[a]
		10 mM	1 mM	2 mM	4 mM	
Phase I						
p450 Family	25	**CYP2F1** **CYP3A4** **CYP4F3**** **CYP7A1**	CYP4F3***	**CYP4F3*****	**POR**	POR
Phase II						
Acetyltransferases	10	**ACAT1** CHAT	**ACAT1** CRAT DLAT **NAT1** **LOC51126**	ACAT1 **CRAT** **DLAT** **NAT1** **LOC51126**		**CRAT**
Glutathione *S*-transferases	12	**GSTA2** **GSTT2**	GSTM3 GSTT2 MGST3	**GSTM3** **GSTT2*** **MGST3**	**GSTP1***** *MGST1*	GSTA4 **GSTM2** GSTM5 *MGST1* MGST3
Sulfotransferases	21	HNK-1ST		**CHST5** CHST7 **TPST1**		**TPST1*****
Miscellaneous	13		**UGT1A1*** **TPMT****	UGT1A1 UGT2B **TPMT*****	TPMT**	**COMT**
Phase III						
Metallothioneins	8	MT1E MT1L MT3	**MT1A** MT1E **MT1G** **MT1H** **MT1L** **MT2A** **MTIX**	**MT1A** MT1E **MT1G** **MT1H** **MT1L** **MT2A** **MTIX**	**MT1A** **MT1E** **MT1G** MT1H **MT1L** **MT2A** **MTIX**	MT1A MT1E MT1G MT1L MT3 MTIX
p-Glycoproteins	7	**ABCC2** **ABCC3** **ABCG2**	ABCB1 **ABCG2**	**ABCB1**		

Treatment was for 12 h immediately after explantation (primary cells) or for the last 24 h of the whole cultivation period (LT97 and HT29 cells). Only those genes are shown for which the baseline expression levels reached a signal of $\geq$10. Bold letters: butyrate-mediated change was $\geq$2-fold with subtractive differences $>$20s. Normal letters: butyrate-mediated change was at least 1.5-fold and/or with subtractive differences at least 15. *MGST1* was the only gene which was downregulated with a butyrate-mediated change of 0.5-fold. Data were calculated using the mean relative signals obtained after normalization from three independently reproduced experiments. Individual genes were significantly different from medium controls ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; repeated measures ANOVA with Bonferroni's post-test to compare replicate means by row). The baseline data for this table are in Table II of the accessory data file.
[a]Total culture period.

Confirmatory studies of array GSTT2 gene expression by real-time RT–PCR

The modulation of the *GSTT2* gene was confirmed by an independent measure of mRNA levels. Relative mRNA levels using cDNA macroarray were reasonably consistent with relative mRNA levels determined using real-time RT–PCR, which is more sensitive than northern blot analysis. We found that the relative *GSTT2* expression level was 2.73, 2.52-fold and 2.08-fold in the cells treated with 1 or 2 mM (LT97) and 4 mM (HT29) butyrate, respectively (Figure 3a and b). The increased expression of the *GSTT2* gene was statistically significant (one-way ANOVA and unpaired *t*-test).

Discussion

Colon cell systems

In vitro studies provide important tools to enhance our understanding of hazardous effects by chemicals and to predict the potential consequences of exposure to humans (39). There is also an increasing need to investigate chemicals for mechanisms of beneficial effects on health using *in vitro* methods (40). Colorectal cells and cell lines are highly useful in studying the genotoxic potentials of cancer risk factors (32,41–43), properties of chemoprotective components (27,44–46), as well as their interactions (26,38,47). The majority of such *in vitro* studies have utilized tumor cell lines. Whilst this may be feasible for studies on chemotherapeutic potentials, primary or premalignant cells are needed for studies on chemoprevention. However, it has been hardly possible to study early changes affecting the normal colonic epithelial cells owing to the lack of manageable culture methods for those cells (33). We have recently demonstrated the validity of using intact primary colonic epithelial cells (for 30 min–1 h) as models to assess the genotoxicity of risk factors (32,41,48). We have now extended our methodology to first cultivate the intact tissue *in vitro* and then to isolate cells, which was profoundly successful for retaining cell viability. Thus, we were able to treat primary tissue with butyrate for up to 12 h and then isolate viable cells in sufficient quantity and quality for expression analysis.

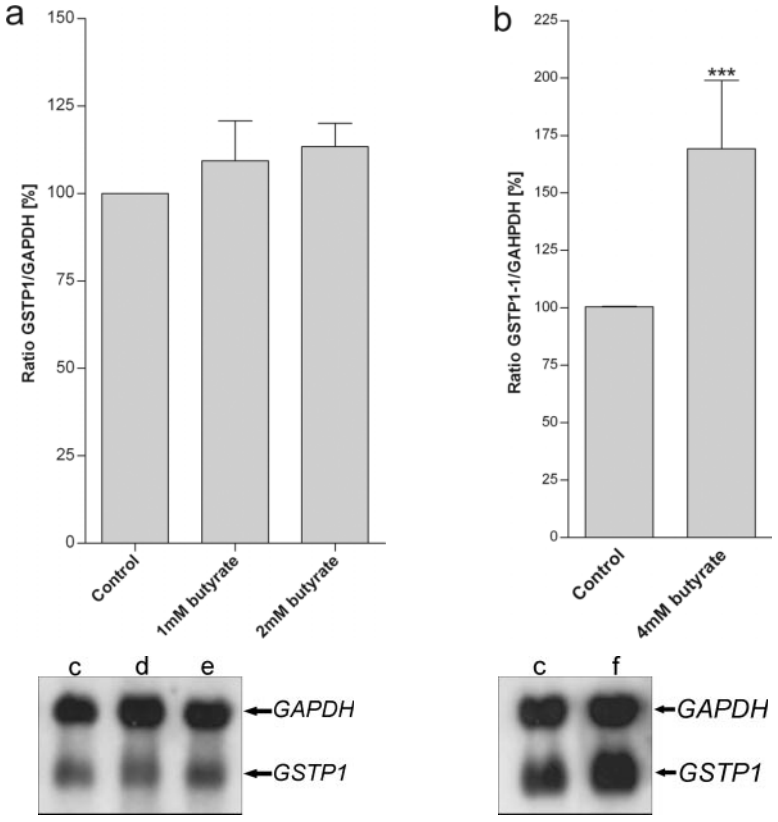


Fig. 2. Northern blots showing the expression of *GSTP1* mRNA in human adenoma LT97 (**a**) and HT29 colon cancer cells (**b**). LT97 and HT29 cells were treated with 1, 2 or 4 mM butyrate. Lane c = control cells, d = 1 mM, e = 2 mM butyrate and lane f = 4 mM butyrate. Values are expressed as mean ±SEM, $n = 3$, $^{***}P < 0.001$ (a: one-way ANOVA, b: unpaired *t*-test).

We had previously also reported novel findings on how a newly established cell line (LT97), consisting of epithelial cells representing an early premalignant phenotype and genotype, could be used as an experimental model to investigate the impact of risk factors (42). The LT97 cells have typical genetic traits of adenoma, such as loss of both *APC* tumor suppressor gene alleles and a mutated *Ki-Ras*-allele, but normal *TP53* (33). This cell line was investigated along with primary and HT29 tumor cells, since there was a need to understand how cell models from these three different stages (non-malignant primary cells, premalignant adenoma cells and malignant tumor cells) would respond to the gut-lumen specific environmental factors.

GSTs in colon tissue

Tissue specimens were included for reference purposes, since this type of material is commonly used to understand gene expression in the human colon and reflects more the expression levels of the *in vivo* situation than cells in culture (49). Here, we have focused our attention on the expression of GSTs (accessory data file), since phase II metabolism is decisive for cell and tissue-specific susceptibility. The activities of both toxic and antitoxic agents are probably highly dependent on the expression of such biotransformation enzymes by which they can be detoxified and which, in turn, they may modulate (50,51). Information on gene expression levels is only available sporadically and data usually pertain to only selected individual genes. We have therefore used expression genomics to enhance our understanding on GSTs [and other drug metabolism systems (accessory data file)] in colon tissue and cells. These advanced methods are very powerful in that they can generate expression data for a large number of genes simultaneously across multiple samples. Here, we have been able to show the GST gene expression levels in tissue directly excised from the colon of six different individuals. The variation on transcriptional level was in a similar order of magnitude as we had previously observed for GST protein expression in colon samples obtained from 15 donors (25). In these previous studies, some samples contained 2- to 4-fold higher GST protein levels than others and in some of the samples, not even the most common colonic GST form, namely GSTP1-1 was available in abundant amounts. The results of this study (mRNA from 12 GST isoenzymes, 6 donors) also show a 2-fold difference between the person with the lowest and highest values of GST expression. On the basis of our present knowledge, we may conclude that a considerable number of subjects could be at higher risk on account of low GST expression levels.

GSTs in cell models compared with tissues

We have now, for the first time, been able to study the expression levels in cells isolated from the tissues (primary cells) incubated *in vitro* for a period up to 12 h. This was the longest duration of *in vitro* culture yielding sufficient viable cells that had succeeded in our hands so far. Several genes were differently expressed in the primary cells, compared with tissues. Three of the 12 investigated GSTs (*GSTA2, GSTA3,* and *MGST1*) were expressed less, whereas three GSTs (*GSTM2, GSTM3* and *GSTM5*) were expressed more in the cells than in the tissue, reflecting changes probably owing to the cultivation

Table III. Modulation of gene expression in LT97 adenoma cells by butyrate

Gene		Superarray Fold change			Affymetrix Fold change		
		1 mM	2 mM		1 mM	2 mM	
Phase I							
p-450 family	CYP3A7	1.7	**2.2**	CYP3A7	0.7	0.7	211843_x_at
	CYP4F3	1.7	**2.4**	CYP4F3	**3.7**	**6.5**	206514_s_at
Phase II							
Acetyltransferases	ACAT1	**4.0**	**3.5**	ACAT1	1.7	1.9	205412_at
	CRAT	**2.4**	**2.6**	CRAT	**2.2**	1.9	209522_s_at, 205843_x_at
	DLAT	**3.9**	**4.9**	DLAT	1.1	1.1	213149_at, 212568_s_at
	HAT1	**2.4**	**2.7**	HAT1	0.6	1.0	203138_at
	LOC51126	**3.4**	**4.4**	LOC51126	1.4	1.5	203025_at
	NAT1	**3.6**	**4.5**	NAT1	**2.1**	**3.6**	214440_at
Glutathione *S*-transferases	GSTA4	**6.1**	**2.7**	GSTA4	**2.2**	**1.7**	202967_at
	GSTM2	**3.2**	**3.5**	GSTM2	**0.6**	**0.7**	204418_x_at
	GSTM3	**5.8**	**7.3**	GSTM3	**3.9**	**4.3**	202554_s_at
	MGST3	**2.0**	**2.7**	MGST3	1.1	1.1	201403_s_at
	GSTT2	1.5	**2.0**	GSTT2	0.9	1.8	205439_at
Sulfotransferases	CHST5	1.4	**2.0**	*CHST5*	1.2	0.8	64900_at
	TPST1	**2.7**	**3.9**	*TPST1*	1.6	1.9	204140_at
	CHST7	1.2	1.6	CHST7	**6.7**	**10.4**	206756_at
Miscellaneous	EPHX1	**2.9**	**2.9**	EPHX1	**2.6**	1.5	202017_at
	LTA4H	**3.2**	**3.7**	LTA4H	1.9	**2.3**	208771_s_at
	UGT1A1	**2.5**	1.8				
	COMT	**2.3**	**2.5**	COMT	**0.3**	**0.4**	208818_s_at, 208817_at
	HNMT	**2.1**	**2.0**	HNMT	**0.4**	**0.2**	204112_s_at, 211732_x_at
	TPMT	1.6	**2.2**	TPMT	1.7	1.4	203671_at, 203672_x_at
Metallothioneins	MT1A	**3.8**	**2.9**				
	MT2A	**6.8**	**4.9**	MT2A	**4.8**	**5.7**	212185_x_at, 212859_x_at
	MT1L	**5.0**	**4.4**				
	MT1G	**2.7**	**2.3**	MT1G	1.9	**4.5**	204745_x_at, 210472_at
	MT1H	**2.6**	**2.2**	MT1H	**4.9**	**6.1**	206461_x_at
	MT3	**2.6**	**4.2**	*MT3*	**2.9**	**4.5**	205970_at
	MTIX	**4.1**	**3.8**	MT1X	**6.3**	**8.8**	204326_x_at, 208581_x_at
Phase III							
p-Glycoproteins	ABCB1	**169.7**	**205.4**	ABCB1	**2.4**	1.5	209994_s_at, 209993_s_at
	ABCC3	0.7	**0.4**	ABCC3	**0.1**	**0.0**	208161_s_at, 209641_s_at
	ABCG2	−19.7	−14.6	ABCG2	**2.2**	**2.0**	209735_at

Comparison of two array methods for those genes which were changed (≥2 fold in comparison to the respective medium control). For the Superarray® membranes, all genes (including those with expression levels <10) were regarded in this evaluation. Bold lettering means that the values are ≥2- or ≤0.5-fold change (butyrate-treated sample/medium control), which is defined as induction or inhibition, respectively. Fold change—mean value of probe sets named in Affymetrix® array (Superarray® $n = 3$; Affymetrix® $n = 1$).

in vitro. In comparison, LT97 and HT29 cells cultivated for 72 h before work-up revealed more striking differences to the tissue samples. Of the 12 GSTs, 7 and 7 respectively, were expressed less in the cells than in the tissue. The differences, however, were not cell line specific, since in HT29 cells cultivated for only 48 h, four genes were expressed less and two additional genes were expressed more. These results again clearly show that the *in vitro* cultivation conditions had marked influence on gene expression, and thus these need to be carefully controlled during experiments using cells *in vitro*.

GST upregulation as a mechanism of chemoprevention

The described comparative analyses (tissue versus colon cells) were needed as a basis to set up the experimental conditions of further studies. These studies had the aim to define specific GST expression patterns in human colon cells of various origins, to determine whether these could be modulated by butyrate, and to assess whether the modulation would be likely to confer protection against diet-associated risk factors. Thus, we have now found that butyrate is an efficient inducer of several GSTs in cells from all three stages of malignancy. Treatments with the maximal tolerated doses of butyrate resulted in an upregulation of *GSTP1, GSTM2*, *GSTA4, MGST3* and others in HT29 cells, of *GSTM2, GSTM3*, *GSTT2* and *GSTA4* in LT97 cells and of *GSTA2* and *GSTT2* in primary cells, with a marked downregulation of *MGST1* in HT29 cells. According to the available databases, the products of these genes inactivate endogenous α, β-unsaturated aldehydes, quinones, epoxides and hydroperoxides formed as secondary metabolites during oxidative stress and protect from food contaminants, such as polycyclic aromatic hydrocarbons (24). For instance the gene product GSTA4-4 was previously reported to have a high affinity for the substrate 4-hydroxynonenal (HNE) (52,53), which is a cytotoxic and mutagenic lipid peroxidation product associated with oxidative stress (54). GSTA2-2 may be of similar importance as GSTA4-4, but with different substrate specificity, resulting in the detoxification of other products, such as cumene hydroperoxide, dibenzo[*a*,*l*]pyrene diol epoxide, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (24). GSTP1-1 may inactivate benzo[*a*]pyrene-9,12-diolepoxide, the reactive intermediate of benzo[*a*]pyrene (55), which is of dietary relevance, since it may be found in cooked foods (56). Other preferential substrates for GSTP1-1 are acrolein, base propenals,

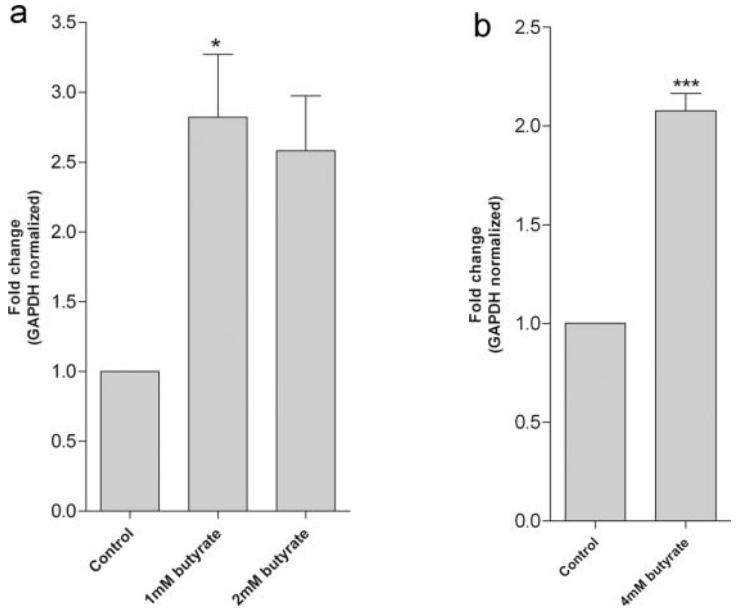


Fig. 3. Quantitative analysis of *GSTT2* mRNA transcript by real-time RT–PCR in LT97 cells (**a**) and HT29 cells (**b**) treated with 1, 2 and 4 mM butyrate, respectively. The relative gene expression analysis in HT29 cells showed 2.08-fold ($^{***}P < 0.001$) and in LT97 cells 2.73 ($^{*}P < 0.05$), 2.52-fold ($^{*}P < 0.05$) (a: one-way ANOVA, b: unpaired *t*-test) increase compared with control cells.

chlorambucil, crotonyloxymethyl-2-cyclohexenone (COMC-6), ethacrinic acid (EA) and thiotepa (24). GSTM2-2 is known to efficiently detoxicate *O*-quinones (e.g. aminochrome), the oxidation products of catecholamines, which may be involved in the development of the Parkinson's disease (57). Other GSTM2-2 substrates are COMC-6, DCNB, dopa *O*-quinone and prostaglandins (24). The human class GSTTs display activity against a broad range of compounds, including methyl halides and sulfate esters. The activity of recombinant GSTT2-2 with a range of secondary lipid peroxidation products, as well as its reported glutathione peroxidase activity with organic hydroperoxides, suggests that it may play a significant role in protection against the products of lipid peroxidation (58), and its substrates cumene hydroperoxide and menaphthyl sulfate (24). Finally, the *MGST3* gene encodes an enzyme, which catalyzes the conjugation of leukotriene A4 and reduced GSH to produce leukotriene C4. This enzyme also demonstrates GSH-dependent peroxidase activity towards lipid hydroperoxides (59) and conjugates CDNB and (*S*)-5-hydroperoxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid (24). *MGST1* encodes a protein that catalyzes the conjugation of GSH to electrophiles and the reduction of lipid hydroperoxides (60). Particular substrates are CDNB, cumene hydroperoxide, hexachlorobuta-1,3-diene (24). *MGST1* was inhibited in HT29 cells, and thus the only example of a clear cut downregulation by butyrate when regarding all genes on the array. The consequences of the downregulation of this GST are not predictable and need to be studied in more detail.

Altogether, a number of the butyrate target genes can be associated with potential chemoprotection, since they should have the ability to ward off risk factors associated with oxidative stress and genotoxic risks (61). Their life-long upregulation in primary cells by dietary butyrate may therefore contribute to the prevention of carcinogenesis, which may be mediated by genotoxic products of oxidative stress (62,63). The ingestion of dietary fibres providing sufficient luminal butyrate concentrations may accordingly be considered to substantially contribute to an effective strategy of dietary cancer chemoprevention.

Confirmatory data

Altogether the evaluation of our expression analysis data was based on $n = 3$ independent experiments (cell culture experiments) or on the data obtained by $n = 6$ individual donors, strengthening the validity of the measurement. We compared different normalization procedures and stick to the one basing the 100% value on the means of all housekeeping genes. The reported responses found with GSTs using this normalization have largely been confirmed independently with other methods. For example, we have previously found that GSTP1-1, GSTM2-2, GSTA1/2 proteins and *GSTM2* mRNA were induced in HT29 cells treated with 2–4 mM butyrate (25), as were *GSTA4* mRNA and GSTA4-4 protein (29). In LT97 cells, GSTP1-1 protein was not induced by butyrate pretreatment (T.Kautenburger *et al*., submitted for publication), the genes found to be modulated in this study (*GSTM3, GSTT2, MGST3*) had not been investigated since antibodies were not available. Other confirmatory data were generated here, such as *GSTP1* induction in HT29 and non-induction in LT97 using northern blot analysis.

Interestingly, the independent evaluation of RNA aliquots using two different array methods gave often similar results, which is to our knowledge, the first direct comparison of this type. An exception was *GSTT2* which was induced according to Superarray®, but not to Affymetrix®. We therefore additionally investigated *GSTT2* expression using another aliquot of the RNA by real-time qRT–PCR. The results confirmed the responses observed for *GSTT2* with Superarray® but not with Affymetrix®. The inability to detect the induction of *GSTT2* on Affymetrix® array is most possibly owing to the characteristics of the *GSTT2* probe set. The signal given by *GSTT2* is so weak that it is assumed by the analysis software as absent both in control and treated cells. However, the signal intensity increased 1.8 times in cells treated with 2 mM butyrate versus control cells.

Potential mechanisms of GST induction in human cells

One important mechanism which is critical for regulation of some, but not all phase II genes (including some GSTs or NADPH-dependent quinone reductase) involves the antioxidant/electrophile-responsive element (ARE/ERE) located within the 5′ upstream (consensus sequences 5′-GTGACNNNGC-3′) regulatory region of the corresponding mouse, rat and human genes (64,65). A major transcription factor which can act on ARE is Nrf2 (nuclear factor E2-related factor 2) (66,67). As a key regulator of Nrf2 activity, which links Nrf2-mediated ARE activation to cellular exposure to oxidants and chemoprotective agents, the BTB- and Kelch-domain containing protein Keap1 (Kelch-like ECH-associated protein 1) has been identified. Keap1 anchors the transcription factor Nrf2 in the cytoplasm and targets it for ubiquitination and proteasome degradation, thereby repressing its ability to induce phase II genes (68). Inducers of ARE-mediated gene expression disrupt the Keap1–Nrf2 complex, leading to an increase in Nrf2 levels, and allowing Nrf2 to translocate into the nucleus (69). Some of the GST-encoding genes contain ARE motif and can be induced in an ARE-mediated manner (24). ARE sequences in the promoter of *GSTA2* are required for basal expression and for its induction by phenolic antioxidants (70). These compounds activate GST-encoding genes, however, also through the AP-1 family of transcription factors, which include Jun, Fos, Maf, ATF and Fra proteins (71). AP-1-binding sites have been identified in the promotor regions of the *GSTA1*, *GSTA4* and *GSTP1* genes (72,73). In addition, C/EBPβ (CCAAT/enhancer binding protein β), which is a member of the C/EBP bZip class of transcription factors, may serve as a more common transcriptional factor for the induction of phase II enzymes and cancer chemoprevention.

The mechanisms by which butyrate probably mediates gene expression in human colon tumor cells are by activation of the mitogen-activated protein kinase (MAPK) signalling transduction pathway (26), and by modifying the acetylation of histones at the N-terminal lysine rich tails (74,75). Two classes of enzymes can affect the acetylation of histones, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). The classes of compounds that are identified as HDAC inhibitors now include: short–chain fatty acids, such as butyrate and several analogues (76). HDAC inhibitors from several different structural classes exhibit clinical activity against a variety of human malignancies, and have also attracted interest as potential chemopreventive agents. Butyrate inhibits HDAC activity and cell growth at millimolar concentrations. Our own studies have shown that treatment of human colon cells HT29 leads to the marked accumulation of acetylated histone 4 (H4), which could be related to enhanced levels of GST-encoding gene expression in these cells (77). More elaborate studies by Mariadason *et al*. (78) conclude a tight correspondence between the kinetics of altered histone acetylation and kinetics of altered expression for genes in specific clusters and that changes in HDAC activity underlie the changes in expression for these genes. Whether or not promoter areas of GSTs are actually targeted by this mechanism is not known and will be an important subject of research in the near future.

Double-edged sword/GST induction in tumor cells

It remains to be elucidated, whether the typical expression patterns can afford chemoresistance of the cells to appropriate substrates, some of which may be colon cancer risk factors. It must also be clarified for each of the target genes in more detail by which mechanisms their butyrate-mediated induction proceeds on molecular level, e.g. by inhibiting the deacetylation of histones (79) and/or through MAPK pathways leading to the transcriptional activation of antioxidative response element (19).

In this context, we must also again consider the concept of a double-edged sword. 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. Connected to this is a reduced probability of cancer initiation in the underlying stem cells. In tumor and in adenoma cells, on the other hand, GST induction could counteract cancer chemotherapy by causing resistance to therapeutic agents, thereby enhancing the survival of transformed cells (26). However, this adverse situation, may not be probable *in vivo*, since the luminal millimolar concentrations of butyrate could be much too high (exceeding 2–4 mM) to result in GST induction. Instead physiological gut luminal butyrate concentrations would impair tumor cell or adenoma cell growth and thus decrease availability of such cells for GST induction. The physiologically available butyrate amounts may also be efficient in inducing apoptosis in tumor cells and thus additionally remove them from the tissue. Another reflection is that, not only butyrate, but also propionate is produced during gut fermentation, and this short chain fatty acid adds on to the growth inhibitory properties of gut luminal products (80,81). Finally, according to all available information, the concentrations found in the gut lumen are much higher (10–20 mM) than the concentrations used here (82,83), albeit *in vivo* colon tissue is probably more protected from the gut luminal components by barrier functions of the mucosa (84) than they are *in vitro* in cell culture. However, it may still be speculated that emerging premalignant and malignant cells will be removed owing to the toxic and growth inhibitory properties of SCFA before GST induction can occur.

Physiological butyrate concentrations may indeed retard tumor progression and lead to a reduced tumor incidence, as has been suggested by the results of a number of animal studies. Dietary fibres, which are fermented to yield high amounts of butyrate, have been associated with a higher efficacy of protecting from AOM-induced colon tumors in animals (85–88). In particular, an *in vivo* study by Perrin demonstrated that those fibres, which promoted a stable butyrate-producing colonic ecosystem decreased the rate of aberrant crypt foci in rats, thus adding on to the line of evidence that a stable butyrate producing colonic ecosystem related to dietary plant foods reduces risks of developing colon cancer (87).

Conclusions

A considerable number of subjects could be at higher risk on account of low GST expression levels in their colonocytes. The hypothesis is that butyrate may mediate in colonocytes an enhanced expression of GSTs and other systems, which protect from products of oxidative stress. We have now been able to add evidence to support this possible mechanism using new systems of *in vitro* toxicology, namely, primary human colon cells. The favourable modulation of toxicological defence systems in these cells is expected to contribute to protection during early stages of carcinogenesis by resulting

in an enhanced cellular protection from cancer risk factors. Butyrate also has the potential to inhibit growth of emerging premalignant and malignant cells, which could conceivably retard tumor progression. When translated to the *in vivo* situation, it must be first of all be taken into account that results on gene expression regulation in some conditions *in vitro* may misrepresent the status of regulation of the same genes *in vivo*. However, the results are also promising in that they could also mean that a life-long supply with butyrogenic dietary plant foods may contribute substantially to dietary colon cancer chemoprevention. This is a feasible hypothesis, which will be needed to be proved in human clinical trials.

Acknowledgements

We thank Prof. Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells, to all donors of biopsies and tissue samples for giving their informed consent and supporting the work. We are grateful to Dr Volker Mai for valuable discussion and editorial comments. We were supported by the German Research Council, Deutsche Forschungsgemeinschaft, Germany (DFG PO 284/8-1) for work on primary cells, Bundesministerium für Forschung und Technologie, Germany (BMBF FKZ.01EA0103) for work on LT97 adenoma cells, ORAFTI, Tienen, Belgium (PRECANTOO) for work on butyrate and HT29 cells.

Conflict of Interest Statement: None declared.

References

1. Pisani,P., Parkin,D.M., Bray,F. and Ferlay,J. (1999) Estimates of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer*, **83**, 18–29.
2. Lynch,H.T. and de la Chapelle,A. (2003) Hereditary colorectal cancer. *New Engl. J. Med.*, **348**, 919.
3. Fearon,E.R. and Vogelstein,B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767.
4. Fodde,R., Smits,R. and Clevers,H. (2001) APC signal transduction and genetic instability in colorectal cancer. *Nat. Rev. Cancer*, **1**, 55–67.
5. Wakabayashi,K., Nagao,M., Esumi,H. and Sugimura,T. (1992) Food-derived mutagens and carcinogens. *Cancer Res.*, **52** (Suppl.), 2092s–2098s.
6. World Cancer Research Fund and American Institute for Cancer Research. (1997) *Food, Nutrition and the Prevention of Cancer: A Global Perspective*. American Institute for Cancer Research, Washington, DC.
7. Eisenbrand,G. and Tang,W. (1993) Food-borne heterocyclic amines. Chemistry, formation, occurrence and biological activities. A literature review. *Toxicology*, **84**, 1–82.
8. Turesky,R.J., Lang,N.P., Butler,M.A., Teitel,C.H. and Kadlubar,F.F. (1991) Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon. *Carcinogenesis*, **12**, 1839–1845.
9. Malfatti,M.A. and Felton,J.S. (2001) *N*-Glucuronidation of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and *N*-hydroxy-PhIP by specific human UDP-glucuronosyltransferases. *Carcinogenesis*, **22**, 1087.
10. Langouet,S., Coles,B., Morel,F., Becquemont,L., Beaune,P., Guengerich,F.P., Ketterer,B. and Guillouzo,A. (1995) Inhibition of CYP1A2 and CYP3A4 by oltipraz results in reduction of aflatoxin B1 metabolism in human hepatocytes in primary culture. *Cancer Res.*, **55**, 5574–5579.
11. Ishikawa,T. (1992) The ATP-dependent glutathione *S*-conjugate export pump. *Trends Biochem. Sci.*, **17**, 463–468.
12. Davies,R.J., Sandle,G.I. and Thompson,S.M. (1991) Inhibition of the Na^+-K^+-ATPase pump during induction of experimental colon cancer. *Cancer Biochem. Biophys.*, **12**, 81–94.
13. Hayes,J.D. and Pulford,D.J. (1995) The glutathione *S*-transferase supergene family. regulation of GST* and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol.*, **30**, 445–460.
14. Brockton,N.T. (2002) UGT1A1 polymorphisms and colorectal cancer susceptibility. *GUT*, **50**, 749.
15. Strassburg,C.P., Vogel,A., Kneip,S., Tukey,R.H. and Manns,M.P. (2002) Polymorphisms of the human UDP-glucuronosyltransferase (UGT) 1A7 gene in colorectal cancer. *GUT*, **50**, 851–856.
16. Wattenberg,L.W. (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.*, **52** (Suppl.), 2085s–2091s.
17. Sporn,M.B. and Suh,N. (2000) Chemoprevention of cancer. *Carcinogenesis*, **21**, 525.
18. Johnson,I.T., Williamson,G. and Musk,S.R.R. (1994) Anticarcinogenic factors in plant foods: a new class of nutrients? *Nutr. Res. Rev.*, **7**, 175–204.
19. Suhr,Y.J. (2003) Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer*, **3**, 768–780.
20. Houlston,R.S. and Tomlinson,I.P.M. (2001) Polymorphisms and colorectal tumor risk. *Gastroenterology*, **121**, 282–301.
21. Hoensch,H.P. and Hartmann,F. (1981) The intestinal enzymatic biotransformation system: potential role in protection from colon cancer. *Hepatogastroenterology*, **28**, 221–228.
22. Kensler,T.W. and Helzlsouer,K.J. (1995) Oltipraz: clinical opportunities for cancer chemoprevention. *J. Cell Biochem.*, **22** (Suppl.), 101–107.
23. Roebuck,B.D., Curphey,T.J., Li,Y., Baumgartner,K.J., Bodreddigari,S., Yan,J., Gange,S.J., Kensler,T.W. and Sutter,T.R. (2003) Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. *Carcinogenesis*, **24**, 1919–1928.
24. Hayes,J.D., Flanagan,J.U. and Jowsey,I.R. (2005) Glutathione Transferases. *Annu. Rev. Pharmocol. Toxicol.*, **45**, 51–88.
25. Ebert,M.N., Klinder,A., Schäferhenrich,A., Peters,W.H.M., Sendt,W., Scheele,J. and Pool-Zobel,B.L. (2003) Expression of glutathione *S*-transferases (GST) in human colon cells and inducibility of GSTM2 by butyrate. *Carcinogenesis*, **24**, 1637–1644.
26. Ebert,M.N., Beyer-Sehlmeyer,G., Liegibel,U.M., Kautenburger,T., Becker,T.W. and Pool-Zobel,B.L. (2001) Butyrate-induces glutathione *S*-transferase in human colon cells and protects from genetic damage by 4-hydroxynonenal. *Nutr. Cancer*, **41**, 156–164.
27. Abrahamse,S.L., Pool-Zobel,B.L. and Rechkemmer,G. (1999) Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration in isolated rat colon cells. *Carcinogenesis*, **20**, 629–634.
28. Blaut,M., Schoefer,L. and Braune,A. (2003) Transformation of flavonoids by intestinal microorganisms. *Int. J. Vitam. Nutr. Res.*, **73**, 79–87.
29. Knoll,N., Ruhe,C., Glei,M., Gallagher,E.P., Sauer,J., Veeriah,R. and Pool-Zobel,B.L. (2005) Associations of butyrate-mediated glutathione *S*-transferase (GSTA4-4) induction, cellular glutathione depletion and genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells. *Toxicol. Sci.*, in press.
30. Peters,W.H.M., Roelofs,H.M.J., Nagengast,F.M. and van Tongeren,J.H.M. (1989) Human intestinal glutathione *S*-transferases. *Biochem. J.*, **257**, 471–476.
31. Butte,A. (2004) The use and analysis of microarray data. *Nat. Rev. Drug Discov.*, **1**, 951–960.
32. Schäferhenrich,A., Sendt,W., Scheele,J., Kuechler,A., Liehr,T., Claussen,U., Rapp,A., Greulich,K.O. and Pool-Zobel,B.L. (2003) Endogenously formed cancer risk factors induce damage of *p53* in human colon cells obtained from surgical samples. *Food Chem. Toxicol.*, **41**, 655–664.
33. Richter,M., Jurek,D., Wrba,F., Kaserer,K., Wurzer,G., Karner-Hanusch,J. and Marian,B. (2002) Cells obtained from colorectal microadenamas mirror early premalignant growth patterns *in vitro*. *Eur. J. Cancer*, **38**, 1937–1945.
34. Fogh,J. and Trempe,X. (1975) New human cell line. In Fogh,J. (ed.) *Human Tumor Cells In Vitro*. Plenum Press, New York, pp. 115–159.
35. Lupton,J.R. (2004) Microbial degradation products influence colon cancer risk, the butyrate controversy. *J. Nutr.*, **134**, 479–482.
36. Daniel,H. (2002) Genomics and proteomics: importance for the future of nutrition research. *Br. J. Nutr.*, **87**, s305–s311.
37. Kroll,T.C. and Wolfl,S. (2002) Ranking: a closer look on globalisation methods for normalization of gene expression arrays. *Nucleic Acids Res.*, **30**, e50.
38. Hardwick,J.C., Van Santen,M., Van Den Brink,G.R., Van Deventer,S.J. and Peppelenbosch,M.P. (2004) DNA array analysis of the effects of aspirin on colon cancer cells: involvement of Rac1. *Carcinogenesis*, **25**, 1293–1298.
39. Eisenbrand,G., Pool-Zobel,B.L., Baker,V. *et al.* (2002) Methods of *in vitro* toxicology. *Food Chem. Toxicol.*, **40**, 193–236.
40. Verhagen,H., Aruoma,O.I., van Delft,J.H.M., Dragsted,L.O., Ferguson,L.R., Knasmüller,S., Pool-Zobel,B.L., Poulson,H.E., Williamson,G. and Yannai,S. (2003) The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in *in vitro* experiments and animal studies *in vivo*. *Food Chem. Toxicol.*, **41**, 603–610.
41. Pool-Zobel,B.L. and Leucht,U. (1997) Induction of DNA damage in human colon cells derived from biopsies by suggested risk factors of colon cancer. *Mutat. Res.*, **375**, 105–116.

42. Schäferhenrich,A., Beyer-Sehlmeyer,G., Festag,G. *et al*. (2003) Human adenoma cells are highly susceptible to the genotoxic action of 4-hydroxy-2-nonenal. *Mutat. Res*., **9496**, 1–14.
43. Glei,M., Klinder,A., Latunde-Dada,G.O., Becker,T.W., Hermann,U., Voigt,K. and Pool-Zobel,B.L. (2002) Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. *Mutat. Res*., **519**, 151–161.
44. Glei,M., Matuschek,M., Steiner,C., Böhm,V., Persin,C. and Pool-Zobel,B.L. (2003) Initial in vitro toxicity testing of functional foods rich in catechins and anthocyanins in human cells. *Toxicol. in vitro*, **17**, 723–729.
45. Agullo,G., Gamet-Payrastre,L., Fernandez,Y., Anciaux,N., Demigné,C. and Rémésy,C. (1996) Comparative effects of flavonoids on the growth, viability and metabolism of a colonic adenocarcinoma cell line (HT29 cells). *Cancer Lett*., **105**, 61–70.
46. Ciacco,P.J., Shen,H., Jaiswal,A.K., Lyttle,M.H. and Tew,K.D. (1995) Modulation of detoxification gene expression in human colon HT29 cells by glutathione *S*-transferase inhibitors. *Mol. Pharmacol*., **48**, 639–647.
47. Steele,V.E., Kelloff,G.F., Balentine,D., Boone,C.W., Mehta,R., Bagheri,D., Sigma,C.C., Zhu,S. and Sharma,S. (2000) Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by *in vitro* bioassays. *Carcinogenesis*, **21**, 63–67.
48. Pool-Zobel,B.L., Abrahamse,S.L., Collins,A.R., Kark,W., Gugler,R., Oberreuther,D., Siegel,E.G., Treptow-van Lishaut,S. and Rechkemmer,G. (1999) Analysis of DNA strand breaks, oxidized bases and glutathione *S*-transferase P1 in human colon cells. *Cancer Epidemiol. Biomarkers Prev*., **8**, 609–614.
49. Glebov,O.K., Rodriguez,L.M., Nakahara,K. *et al*. (2003) Distinguishing right from left colon by the pattern of gene expression. *Cancer Epidemiol. Biomarkers Prev.,* **12***, 755–762*.
50. Arturrson,P. and Borchardt,R. (1997) Intestinal drug absorption and metabolism in cell cultures: caco-2 and beyond. *Pharm. Res*., **14**, 1655–1660.
51. Ito,K., Iwatsubo,T., Kanamitsu,S., Nakajima,Y. and Sugiyama,Y. (1998) Quantitative prediction of in vivo drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annu. Rev. Pharmacol. Toxicol*., **38**, 461–499.
52. Hubatsch,I., Ridderström,M. and Mannervik,B. (1998) Human glutathione transferase A4-4: an Alpha class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. *Biochem. J*., **330**, 175–179.
53. Bruns,C.M., Hubatsch,I., Ridderström,M., Mannervik,B. and Tainer,J.A. (1999) Human glutathione transferase A4-4 crystal structures and mutagenesis reveal the basis of high catalytic efficiency the toxic lipid peroxidation products. *J. Mol. Biol*., **288**, 427–439.
54. Esterbauer,H., Eckl,P. and Ortner,A. (1990) Possible mutagens derived from lipids and lipid precursors. *Mutat. Res*., **238**, 223–233.
55. Hu,X., Herzog,C., Zimniak,P. and Singh,S.V. (1999) Differential protection against benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide-induced DNA damage in HepG2 Cells stably transfected with allelic variants of p class human glutathione *S*-transferases. *Cancer Res*., **59**, 2358–2362.
56. Goldman,R. and Shields,P.G. (2003) Food mutagens. *J. Nutr*., **133**, 965S–9973.
57. Baez,S., Segura-Aguilar,J., Widersten,M., Johansson,A.S. and Mannervik,B. (1997) Glutathione transferases catalyse the detoxication of oxidized metabolites (*o*-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes. *Biochem. J*., **324**, 25–28.
58. Tan,K.L. and Board,P.G. (1996) Purification and characterization of a recombinant human Theta-class glutathione transferase (GSTT2-2). *Biochem. J*., **315**, 727–732.
59. Schroder,O., Sjostrom,M., Qiu,H., Stein,J., Jakobsson,P.J. and Haeggstrom,J.Z. (2003) Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. *Biochem. Biophys. Res. Commun*., **312**, 271–276.
60. Jakobsson,P.J., Morgenstern,R., Mancini,J., Ford-Hutchinson,A. and Persson,B. (1999) Common structural features of MAPEG—a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism. *Protein Sci*., **8**, 689–692.
61. Hayes,J.D. and Strange,R.C. (1995) Potential contribution of the glutathione *S*-transferase supergene family to resistance to oxidative stress. *Free Radic. Res*., **22**, 193–207.
62. Cerutti,P.A. (1985) Prooxidant states and tumor promotion. *Science*, **227**, 375–381.
63. Toyokuni,S., Okamoto,K., Yudoi,J. and Hiai,H. (1995) Persistent oxidative stress in cancer. *FEBS Lett*., **358**, 1–3.
64. Waleh,N.S., Calaoagan,J., Murphy,B.J., Knapp,A.M., Sutherland,R.M. and Laderoute,K.R. (1998) The redox-sensitive human antioxidant responsive element induces gene expression under low oxygen conditions. *Carcinogenesis*, **19**, 1337.
65. Rushmore,T.H., Morton,M.R. and Pickett,C.B. (1991) The antioxidant responsive element. *J. Biol. Chem*., **18**, 11632–11639.
66. Chui,D.H., Tang,W. and Orkin,S.H. (1995) C-DNA cloning of murine Nrf2 gene, coding for a p45 NF-E2 related transcription factor. *Biochem. Biophys. Res. Commun*., **209**, 40–46.
67. Itoh,K., Chiba,T., Takahashi,S. *et al*. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antu oxidant response elements. *Biochem. Biophys. Res. Commun*., **236**, 313–322.
68. Zhang,D.D. and Hannink,M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell Biol*., **23**, 8137–8151.
69. Wakabayashi,N., Dinkova-Kostova,A.T., Holtzclaw,W.D., Kang,M.I., Kobayashi,A., Yamamoto,M., Kensler,T.W. and Talalay,P. (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl Acad. Sci. USA*, **101**, 2040–2045.
70. Chen,C., Yu,R., Owuor,E.D. and Kong,A.N. (2000) Activation of antioxidant-response element (ARE), mitogen activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch. Pharm. Res*., **23**, 605–612.
71. Pinkus,R., Weinert,L.M. and Daniel,V. (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-*k*B, and glutathione *S*-transferase gene expression. *J. Biol. Chem*., **271**, 13422–13429.
72. Desmots,F., Rauch,C., Henry,C., Guillouzo,A. and Morel,F. (1998) Genomic organization, 5′-flanking region and chromosomal localization of the human glutathione transferase A4 gene. *Biochem. J*., **336**, 437–442.
73. Whalen,R. and Boyer,T. (1998) Human glutathione *S*-transferases. *Semin. Liver Dis*., **18**, 345–358.
74. Boffa,L.C., Lupton,J.R., Mariani,M.R., Ceppi,M., Newmark,H.L., Scalmati,A. and Lipkin,M. (1992) Modulation of colonic epithelial cell proliferation, histone acetylation and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats. *Cancer Res*., **52**, 5906–5912.
75. Marks,P.A., Rifkind,R.A., Richon,V.M., Breslow,R., Miller,T. and Kelly,W. (2001) Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer*, **1**, 194–202.
76. Marks,P.A., Richon,V.M. and Rifkind,R.A. (2000) Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J. Natl. Cancer Inst*., **92**, 1210–1216.
77. Kiefer,J., Beyer-Sehlmeyer,G. and Pool-Zobel,B.L. (2002) Wirkung von Na-Butyrat und Trichostatin A auf den Histon-Acetylierungsstatus in HT29 Kolonkarzinom-Zellen. In *Proceedings of the 19th Annual Conference of the German Society for Nutrition*, Friedrich-Schiller Universität, Jena, March 9–11, p. 34.
78. Mariadason,J.M., Corner,G.A. and Augenlicht,L.H. (2000) Genetic reprogramming in pathways of colonic cell maturation induced by shortchain fatty acids; comparison with trichostatin A, sulindac and curcumin, and implications for chemoprevention of colon cancer. *Cancer Res*., **60**, 4561–4572.
79. Marks,P.A., Rifkind,R.A., Richon,V.M., Breslow,R., Miller,T. and Kelly,W. (2001) Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer*, **1**, 194–202.
80. Beyer-Sehlmeyer,G., Glei,M., Hartmann,F., Hughes,R., Persin,C., Böhm,V., Rowland,I.R., Schubert,R., Jahreis,G. and Pool-Zobel,B.L. (2003) Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Br. J. Nutr*., **90**, 1057–1070.
81. Klinder,A., Gietl,E., Hughes,R. *et al*. (2004) Gut fermentation products of inulin-derived prebiotics inhibit markers of tumour progression in human conon tumour cells . *Int. J. Cancer Prev*., **1**, 19–32.
82. Cummings,J. (1981) Short–chain fatty acids in the human colon. *GUT*, **22**, 763–779.
83. Cummings,J., Pomare,E.W., Branch,W.J., Naylor,C.P.E. and Macfarlane,G.T. (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *GUT*, **28**, 1221–1227.
84. Caderni,G., Femia,A.P., Giannini,A., Favuzza,A., Luceri,C., Salvadori,M. and Dolara,P. (2003) Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. *Cancer Res*., **63**, 2388–2392.

85. Compher,C.W., Frankel,W.L., Tazelaar,J., Lawson,J.A., McKinney,S., Segall,S., Kinosian,B.P., Williams,N.N. and Rombeau,J.L. (1999) Wheat bran decreases aberrant crypt foci, preserves normal proliferation, and increases intraluminal butyrate levels in experimental colon cancer. *J. Parenter. Enteral. Nutr.*, **23**, 269–277.
86. McIntyre,A., Gibson,P.R. and Young,G.P. (1993) Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. *GUT*, **34**, 386–391.
87. Perrin,P., Pierre,F., Patry,Y., Champ,M., Berreur,M., Pradal,G., Bornet,P., Meflah,K. and Menenteau,J. (2001) Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. *GUT*, **48**, 53–61.
88. McIntosh,G.H., Royle,P.J. and Pointing,G. (2001) Wheat aleurone flour increases cecal ß-glucuronidase activity and butyrate concentration and reduce colon adenoma burden in azoxymethane treated rats. *J. Nutr.*, **131**, 127–131.

Received August 13, 2004; revised February 10, 2005; accepted February 20, 2005

2.4 **Publication IV:** *Pool-Zobel BL,* ***Veeriah S,*** *Böhmer FD.* "Modulation of xenobiotic metabolising enzymes by anticarcinogens - focus on glutathione *S*-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis". *Mutat Res. 2005 Dec 11; 591(1-2):74-92*

A wide variety of antioxidant or phase II detoxifying enzymes such as GSTs contribute to a fundamental cellular defence system against oxidative and electrophilic insult. One important mechanism of GST induction involves transcriptional activation of Nrf2 transcription factors and an antioxidant-responsive element (ARE) and this may protect cells from oxidative damage. Many chemoprotective phytochemicals have been found to enhance cellular antioxidant capacity through activation of this particular transcription factor, thereby blocking initiation of carcinogenesis. The modulation of cellular signalling by anti-inflammatory phytochemicals hence provides a rational and pragmatic strategy for molecular target based chemoprevention. This review summarises the modulation of detoxification enzyme systems including GSTs by several dietary factors and describes the recently identified molecular targets of phytochemicals. It is hoped that continued research will lead to development of phytochemicals as an anticancer agent.

Own contribution to the manuscript:

- Information on molecular mechanisms of regulation of phase II detoxification genes was collected and represented

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Mutation Research 591 (2005) 74–92
Fundamental and Molecular Mechanisms of Mutagenesis
www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Modulation of xenobiotic metabolising enzymes by anticarcinogens—focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis

Beatrice Pool-Zobel [a,*], Selvaraju Veeriah [a], Frank-D. Böhmer [b]

[a] *Department of Nutritional Toxicology, Institute for Nutrition, Friedrich Schiller University Jena, 07743 Jena, Germany*
[b] *Institute of Molecular Cell Biology, University Hospital, Friedrich Schiller University Jena, 07743 Jena, Germany*

Received 4 February 2005; received in revised form 8 April 2005; accepted 10 April 2005
Available online 3 August 2005

Abstract

There is evidence that consumption of certain dietary ingredients may favourably modulate biotransformation of carcinogens. Associated with this is the hypothesis that the risk for developing colorectal cancer could be reduced, since its incidence is related to diet. Two main groups of biotransformation enzymes metabolize carcinogens, namely Phase I enzymes, which convert hydrophobic compounds to more water-soluble moieties, and Phase II enzymes (e.g. glutathione S-transferases [GST]), which primarily catalyze conjugation reactions. The conjugation of electrophilic Phase I intermediates with glutathione, for instance, frequently results in detoxification. Several possible colon carcinogens may serve as substrates for GST isoenzymes that can have marked substrate specificity. The conjugated products could be less toxic/genotoxic if GSTs are induced, thereby reducing exposure. Thus, numerous studies have shown that the induction of GSTs by antioxidants enables experimental animals to tolerate exposure to carcinogens. One important mechanism of GST induction involves an antioxidant-responsive response element (ARE) and the transcription factor nuclear factor E2-related factor 2 (Nrf2), which is bound to the Kelch-like ECH associated protein 1 (Keap1) in the cytoplasm. Antioxidants may disrupt the Keap–Nrf2 complex, allowing Nrf2 to translocate to the nucleus and mediate expression of Phase II genes via interaction with the ARE. GSTs are also induced by butyrate, a product of gut flora-derived fermentation of plant foods, which may act via different mechanisms, e.g. by increasing histone acetylation. GSTs are expressed with high inter-individual variability in human colonocytes, which points to large differences in cellular susceptibility to xenobiotics. Enhancing expression of GSTs in human colon tissue could therefore contribute to reducing cancer risks. However, it has not been demonstrated in humans that this mechanism is associated with cancer prevention. In the future, it will be useful to determine GSTs during dietary intervention studies to enhance our understanding of this protective mechanism.

Keywords: Chemoprevention; Colon cancer; Phase II enzymes; Biotransformation; Detoxification

* Corresponding author. Tel.: +49 3641 949670; fax: +49 3641 949672.
E-mail address: b8pobe@uni-jena.de (B. Pool-Zobel).
URL: www.uni-jena.de/biologie/ieu/et.

0027-5107/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2005.04.020

1. Colorectal carcinogenesis (CRC)

1.1. Genes and environment

There is increasing evidence that human cancers can be prevented not only by avoiding exposure to carcinogens but also by favouring the intake of protective factors that modulate the defense mechanisms of the host organism. This preventative strategy, referred to as chemoprevention [1], can be pursued either by means of pharmacological agents [2] and/or by dietary factors [3]. One of the cancers that could be successfully prevented, especially by dietary ingredients, is colorectal cancer (CRC), since there is strong evidence for both positive and inverse associations with diet [4]. Colorectal cancers are the second to third most frequent causes of death due to cancer in Europe, with 190,000 new cases occurring per year, affecting 6% of men and women by the age of 65 years [5]. The risk of developing CRC rises exponentially with age, commencing at 40 years, increasing to 50 years, and doubling with each decade, where it peaks at 70 years [6]. Several risk factors have been identified, including red meat intake [7,8], smoking [9,10], and alcohol intake [11]. Individuals with chronic inflammatory diseases of the digestive tract and family histories, e.g. with ulcerative colitis and Crohn's disease, are at increased risk. Molecular mechanisms linking chronic inflammation and colon cancer have recently been disclosed [12]. Another proportion of colorectal tumors are associated with family history and caused by inherited genetic alterations [13–15]. High-penetrance mutations confer predisposition to colorectal cancer in Lynch syndrome (previously designated as hereditary non-polyposis colon cancer [HNPCC], which involves mutations in mismatch-repair genes) and in familial adenomatous polyposis (which involves mutations in the APC tumor suppressor, a protein which sequesters cytoplasmic beta-catenin and targets it for degradation). Together, these conditions account for 5% or less of all cases of colorectal cancer [16,17]. Low-penetrance mutations probably account for a high proportion of all colorectal cancers, in both familial and sporadic cases. These mutations are more difficult to identify, but are increasingly being detected and characterized [17,18].

The sporadic tumors are likely the result of life-long accumulation of genetic alterations in somatic tissues. Multiple genetic lesions in the affected cells lead to the acquisition of host-threatening biological phenotypes such as growth factor-independence, apoptosis deficiency, metastatic potential, and pro-angiogenic activity [19]. These may be caused by carcinogenic risk factors found in foods or in tobacco smoke [4,20]. Many molecular lesions which contribute to the development of colon cancer have been characterized, including mutations in APC, Ras, and p53 [21]. Aberrations in further genes with potential relevance for colon carcinogenesis are being identified based on different types of high-throughput studies [22,23]. For example, allele loss and point mutations in genes encoding protein-tyrosine phosphatases suggest a role of members of this class of molecules as tumor suppressors [23–25]. The extent of exposure, the ability to prevent DNA damage by inactivating carcinogens, and the capacity to repair the damage caused by carcinogens all contribute to an individual's risk of developing cancer. Gene–gene and gene–environment interactions have a significant influence on susceptibility to cancer [26]. In terms of dietary risk-reduction of colon tumors in populations, consuming "Western style diets", it is desirable to find ways to better utilize dietary factors to enhance life-long chemoprevention.

1.2. Dietary prevention by modulation of biotransformation

A mechanism by which dietary ingredients could be of benefit is that they modulate biotransformation in such a manner that carcinogens are less active and thus exert less harm in the target cells of cancer. Numerous examples exist on how the modulation of Phase I and Phase II enzymes by dietary agents are related to chemopreventive potential [27]. In the context of dietary colon cancer prevention, the hypothesis is difficult to prove, since the issues are extremely complex. There are a number of open questions such as which carcinogens induce colon cancer and how are these carcinogens metabolically transformed in the target cells. Largely unknown is the question of how food components modulate the biotransformation in a favourable manner, so that unavoidable carcinogens are no longer able to cause the type of damage which enhance carcinogenesis. It is very difficult to resolve these questions in detail, due to the very complex exposure situations encountered with diet.

The proposed intake of a balanced diet with high variety of vegetable food, for instance, provides a large variety of compounds that may interact in an additive, synergistic or inhibitory manner. Also, the individual susceptibilities, e.g. based on genetic polymorphisms of populations consuming the diets are highly interactive with modulator activities of the food components. These associations can be quite different for different types of cancers. Since the evidence for the modulation of biotransformation systems on the whole has rarely been investigated, in this review, we will focus on one group of biotransformation enzymes, namely the Phase II enzyme family of glutathione S-transferases (GSTs; EC.2.5.1.18). This family of enzymes has recently been reviewed in detailed excellence by Hayes et al. [28]. Here, we will address the question on how GSTs could contribute to modulating risks in colorectal cancer.

1.3. Plant foods, CRC risks and glutathione S-transferases

Associations between colon and rectal cancer and intakes of vegetables, other plant foods, and fiber have stimulated much debate. Many studies of colon cancer have shown inverse associations with intakes of plant foods and fibre, although other studies have not [29–31]. A recent study investigated whether habitual consumption of fruits and vegetables, especially citrus fruits and *Brassica* and allium vegetables, is positively associated with parameters reflecting the activity of the GST enzyme system in human rectal mucosa [32]. GST enzyme activity, GST isoenzyme levels of GST-alpha (A1-1, A1-2, and A2-2), -mu (M1-1) and -pi (P1-1), and glutathione (GSH) levels were measured in rectal biopsies from 94 subjects. Diet, lifestyle, *GSTM1*, and *GSTT1* null polymorphisms were assessed. Consumption of citrus fruits was positively associated with GST enzyme activity, as was found with *Brassica* vegetables but only among carriers of the *GSTM1*1* genotype and not among *GSTM1*0* individuals. Consumption of allium vegetables was not associated with GST enzyme activity, but negatively with GSTP1-1 levels. Associations were similar among those with the *GSTT1*1* and *GSTT1*0* genotype. Based on their studies, the authors were able to conclude that variations in habitual consumption of fruits, particularly citrus fruits, and of vegetables, in particular *Brassica* vegetables, among those subjects with the *GSTM1*-plus genotype, may contribute to variations in human rectal GST enzyme activity [32].

2. Biotransformation

Biotransformation is the "process whereby a substance is changed from one chemical to another (*transformed*) by a chemical reaction within the body" [33]. The involved enzymes regulate toxic, mutagenic, and neoplastic effects of environmental and endogenous carcinogens, in two main phases of conversions. Phase I enzymes (cytochromes P450 and flavin-dependent monooxygenases) convert hydrophobic compounds to reactive electrophiles by oxidation, hydroxylation and reduction reactions to prepare them for reaction with water-soluble moieties. Phase II enzymes (e.g. GSTs, UDP-glucuronosyltransferases (UGTs), sulfotransferases, *N*-acetyltransferases) primarily catalyze conjugation reactions. Phase I enzymes, particularly the cytochrome P450s, frequently result in bioactivation, compared with the inactivation that most often results from Phase II reactions. This has led to the concept that selective induction of expression of genes encoding Phase II enzymes may have the potential to protect against chemical carcinogenesis [34].

3. Glutathione S-transferases

3.1. GSTs—state of the art

GSTs (EC 2.5.1.18) are ubiquitous enzymes found in bacteria, yeast, nematodes, insects, fish, birds, and mammals [35]. In humans, they are localized in different tissues with organ specific expression patterns [36]. They constitute a complex supergene family that collectively metabolizes chemotherapeutic drugs, carcinogens, and environmental pollutants, and plays a protective pivotal role against xenobiotics, as has been reviewed in the past [35,37]. Their levels of expression can have profound effects on susceptibility to chemical insult, with over expression resulting in resistance and under expression enhancing susceptibility [35,37]. The induction of hepatic GSTs by antioxidants or other anticarcinogenic xenobiotics enables experimental animals to tolerate exposure to carcinogens [38,39]. Therefore, the induction of GST expression by chemicals

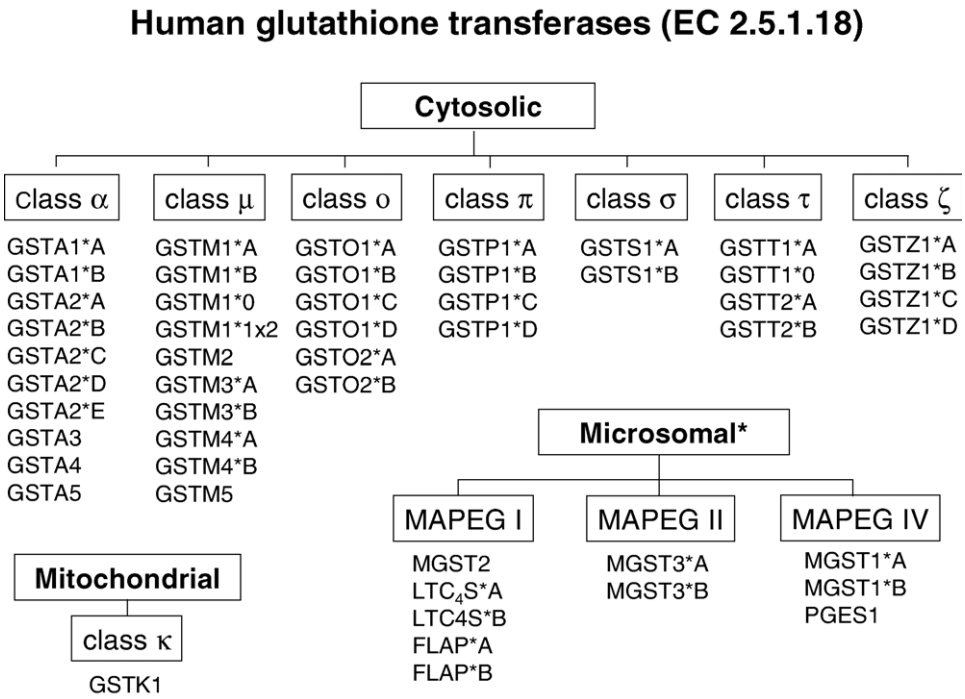


Fig. 1. Schematic overview of genes encoding human glutathione transferases (EC 2.5.1.18), adapted from Hayes et al. [28]. GSTs catalyze nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom. There are three major families of proteins with glutathione transferase activity. Two of these, the cytosolic and mitochondrial GST, comprise soluble enzymes that are only distantly related [150,151]. The third family comprises microsomal GSTs, now referred to as membrane-associated proteins in eicosanoid and glutathione (MAPEG) metabolism [152]. Cytosolic and mitochondrial GST share some similarities in their three-dimensional fold [150], but are structurally distinct from the MAPEG enzymes [153]. The similarities of the GSTs are that all three families contain members that conjugate glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) and exhibit glutathione peroxidase activity toward cumene hydroperoxide (CuOOH) [28]. A number of variants have been identified which comprise of sequence polymorphisms (GSTA1, GSTA2, GSTM1, GSTM3, GSTM4, GSTO1, GSTO2, GSTP1, GSTT2, GSTZ1), deletion polymorphisms (GSTM1, GSTT1), and duplication polymorphisms (GSTM1). (*) A higher number of single nucleotide polymorphisms have been reported for individual population groups as reviewed in [28]. Nomenclature for other MAPEG enzymes: LTC4S, leukotriene C4 synthase (conjugates leukotriene A4 with GSH); FLAP, 5-lipoxygenase-activating protein (arachidonic acid-binding protein required for 5-lipoxygenase to exhibit full activity); PGES1, prostaglandin E2 synthase 1 (catalyzes GSH-dependent isomerization of PGH2 to PGE2) [152].

and by diet holds promise to enhance the toxicological defence system of colon tissue, and thus retard development of cancer, by reducing exposure to carcinogens. One of these exposures is oxidative stress which leads to molecular damage in cells that is linked to many degenerative diseases including cancer [40]. GSTs contribute to resistance against oxidative stress [41]. The inactivation of toxic and mutagenic alkene products of free radical reactions and oxidative processes, catalyzed by GSTs, is a major detoxication pathway protecting cells and tissues [42]. GSTs catalyze the conjugation of the tripeptide glutathione via its sulphur atom to many toxins containing an electrophilic functional group, allowing these compounds to be excreted from the body [35]. GSTs also exhibit peroxidase activity toward organic hydroperoxides and serve to combat oxidative stress [41]. Based on their biochemical, immunological, and structural properties, the GSTs are characterised as cytosolic, mitochondrial, and microsomal enzymes (Fig. 1). The cytosolic transferases are represented by classes Alpha, Mu, Pi, Sigma, Theta, Zeta, and Omega. The mitochondrial transferase is called class Kappa GST. The microsomal transferases form a unique “membrane-associated proteins in eicosanoid and glutathione metabolism” (MAPEG) grouping of transferases [28,35]. Null geno-

types for *GSTM1* and *GSTT1* occur in frequencies of approximately 50% and 20–50% of the population, respectively, and 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. A pooled analysis for *GSTM1* revealed no association with colorectal adenoma or cancer, whereas the *GSTT1* null genotype was associated with a small increase in colorectal cancer risk [18]. Two linked polymorphisms were described in the *GSTP1* gene, one in codon 105 and one in codon 114, of which the polymorph variant of codon 105 modifies the enzyme's specific activity [43]. No association with colorectal cancer was observed for either polymorphisms [18]. These results are consistent with larger scale analyses [44].

3.2. GST expression levels in human colon cells

It has been argued that studies focusing on polymorphisms in the low-penetrance genes may have limitations in studying cancer susceptibility and that appropriately designed studies using validated functional parameters should be more predictive of the biotransformation capacity of a given tissue [45]. Recently, we have investigated gene expression, protein expression, and GST activities of drug metabolism genes in primary human colon tissue, premalignant LT97 adenoma and HT29 tumor cells cultured in appropriate medium ± butyrate, which could be a chemopreventive product formed during fermentation of dietary fibre by the gut flora [46]. Expression analysis was carried out with 96 drug metabolism genes (including 12 GSTs) spotted on cDNA microarrays (Superarray®; $n = 3$), and confirmed with Northern and Western blot or real-time PCR techniques [47–49]. Relative expression levels were compared across colon samples and for each colon sample. *GSTP1* was the most abundant GST subunit of all colon cells, including colon fibroblasts, whereas *GSTA1/2* was expressed least. Differences were also detected for cells in different degrees of malignant transformation. Compared to primary colon cells, several genes were not expressed in the preneoplastic adenoma cell line LT97 or in HT29 tumor cells. Our findings further showed that GSTs are expressed with high inter-individual variability in human colonocytes, which points to large differences in cellular susceptibility to xenobiotics. GST gene and protein expression levels were determined in tissue directly excised from the colon of different individuals. The analysis of GST proteins from 15 donors showed that some samples contained two- to four-fold higher GST protein levels than others and in some of the samples, not even the most common colonic GST form, namely GSTP1-1 was available in abundant amounts [48]. The results of a further study on variations of transcriptional levels (mRNA from 12 GST isoenzymes, six donors) also revealed a two-fold difference between the person with lowest and highest values of GST expression [49]. On the basis of our present knowledge, we may conclude that a considerable number of subjects could be at higher risk on account of low GST expression levels, in spite of the lacking associations between GST polymorphisms and cancer risks. The studies also support endeavours to find ways to increase GSTs in the colon as a potential strategy for chemoprevention.

3.3. Colon cancer risk factors as substrates for GSTs

Any strategy involving modulation of biotransformation necessitates knowledge on the exposing chemicals, since these will be the substrates for the target proteins. In particular, it is of interest to identify the carcinogens that are metabolically converted by enzymes of biotransformation. For the purpose of this review, here we present putative carcinogens that are possibly detoxified by GSTs, which have marked substrate specificity related to their protein structure [50]. In the context of risk factors for CRC, the study of nutritionally caused human diseases has traditionally focussed on food borne toxicants [51]. Cooking, pan frying, and grilling of meat cause the formation of mutagens or carcinogens (heterocyclic amines, polycyclic aromatic hydrocarbons) [52]. The impact of these contaminants, especially of the heterocyclic amines, was previously considered to be very low on account of the exposure situation and the respective carcinogenic potencies [53]. However, they are classified IARC-carcinogens of category II and therefore are expected contribute to the overall risk [54]. The genotoxic heterocyclic amines must undergo metabolic activation to exert genotoxic effects. Studies have shown that cytochrome P450s as well as UGTs are key enzymes involved in metabolic activation and

deactivation of one of the most abundant heterocyclic amines which is 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) [55,56], whereas one of its less abundant metabolites, *N*-acetoxy-PhIP, is detoxified by GSTs [57].

Data from both in vitro and in vivo studies suggest that exposure to polycyclic aromatic hydrocarbons (PAHs), such as benzo(*a*)pyrene, may be important determinants of colorectal cancer risk [54,58]. Dietary PAHs are derived from animal fats [59,60] and are also present at significant levels in tobacco smoke [61]. There is a close association of colorectal cancer risk and cigarette smoking [10,62,63], possibly due to transversion mutations in K-ras [64]. PAHs are known to be metabolized by a variety of Phase I and Phase II drug metabolizing enzymes, including cytochrome P450s, glutathione *S*-transferases, and *N*-acetyl transferases. The reactive metabolite of benzo(*a*)pyrene, namely benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) is, e.g. a substrate for GSTs of the alpha class [28]. It also a substrate for GSTP1 with different affinities, depending on whether the protein product is encoded by the wild-type or genetic variant form of the GSTP1 gene [65].

Next to the food contaminants, endogenously formed mutagens and carcinogens may increase risk as well. The compounds include nitroso compounds (apparent total nitroso compound (ATNC)), formed in the gut subsequent to nitrate intake [66]. ATNC formation also increases with increasing meat intake [67]. However, there are only few data on the identities or biological activities of ATNC [68] and it is possible, if the method for detection is subject to interference, that the species being measured are not in fact *N*-nitroso compounds [69]. However, nitroso compounds are amongst the most carcinogenic compounds known [70], and older studies report that some representatives are possibly detoxified by GSTs [71].

Iron, alcohol metabolism, and/or inflammatory bowel conditions may lead to oxidative stress in the gut lumen or in the colon epithelium, and oxidative stress leads to lipid peroxidation. For iron, the mechanisms could involve the production of reactive oxygen species (ROS) from peroxides via the Fenton Reaction [72], by which it could serve as a risk factor for colorectal carcinogenesis [73]. Iron together with hydrogen peroxide, or the resulting ROS, cause cell damage including mutations and are indirectly genotoxic in colon cells [74,75]. Recent studies have shown that increased iron intake, consumed in the form of ferrous sulphate capsules, causes a significant increase in free radical generation in ex vivo incubations [76]. Similarly, diets rich in fat and meat have been shown to increase faecal free radicals [77]. Free radical generation is also significantly correlated with the level of iron in the faeces. Using a refined HPLC method, it has now been reported that ROS are produced by (an as yet unidentified) soluble factor within the faecal stream rather than by the indigenous bacteria [78]. However, uncertainties are apparent for the role of iron in meat as an actual risk factor via ROS production as heme iron is one of the most effectively absorbed forms of dietary iron in the small intestine. It may be that only very high meat consumption carries any real risk. A recent study has indicated that faecal water from rats fed heme iron is more cytotoxic and hyperproliferative in colon cells than that from rats fed inorganic iron [79]. Obviously, more studies are needed to analyse the prooxidative effects of heme iron, when available from the gut lumen and to assess the susceptibilities of colon cells to this factor. The supergene family of GSTs are well recognized to contribute to resistance against these and other factors of oxidative stress, whereby they may also exert peroxidase activities [28,41].

In an extension of these mechanisms, it must be noted that ROS-mediated attacks of lipids (derived from the cellular membranes or from high fat diets) may result in oxidation products (aldehydes, 4-hydroxyalkenals) that can interact directly with DNA of mammalian cells [80]. These factors could serve as endogenous colon risk factors, when available from the gut lumen. It has been reported that 2-alkenals are genotoxic in colon cells of the rat [81]. α,β-Unsaturated aldehyde products from radical reactions and lipid peroxidation may be efficiently conjugated by several GST isoenzymes, resulting in their detoxification [42]. The genotoxicity of one prominent compound, 4-hydroxynonenal, is largely dependent on the activity and presence of the GST/GSH system [47] and has high affinity for the isoenzyme GSTA4-4 [82], by which it is efficiently conjugated.

These examples presented above are a selection of the putative cancer risk factors, which may mutually contribute to dietary associated colorectal carcinogenesis. Collectively, the examples include substrates for glutathione S-transferases, and thus are relevant risk

factors that could be less toxic/genotoxic if GSTs are induced.

4. Phase II gene expression

4.1. Molecular mechanisms of regulation

Many xenobiotics, or products of them generated by Phase I enzymes, are able to induce the expression of Phase II genes. The regulation of Phase II gene expression addresses a wide variety of transcriptional regulators. One important mechanism which is critical for regulation of some, but not all Phase II genes (including some GSTs or NADPH dependent quinone reductase) involves the antioxidant/electrophile-responsive response element (ARE/ERE) located within the 5′ upstream (consensus sequences 5′-GTGACNNNGC-3′) regulatory region of the corresponding mouse, rat, and human genes [83–85]. A major transcription factor which can act on ARE is nuclear factor E2-related factor 2 (Nrf2, Fig. 2) [86,87]. Nrf2 belongs to the NF-E2 family of nuclear basic leucine zipper (bZip) transcription factors. The key regulator of Nrf2 activity, which links Nrf2-mediated ARE activation to cellular exposure to oxidants and chemoprotective agents, has been identified as the BTB- and Kelch-domain containing protein Keap1 (*K*elch-like *E*CH-*a*ssociated *p*rotein 1). Under basal conditions, Keap1 binds to Nrf2 and mediates association with a Cul3-dependent ubiquitin ligase complex [88–90]. Keap1 thus anchors the transcription factor Nrf2 in the cytoplasm, and targets it for ubiquitination and proteasome degradation, thereby repressing its ability to induce Phase II genes [91]. Inducers of ARE-mediated gene expression disrupt the Keap1–Nrf2 complex, leading to increased Nrf2 levels and translocation into the nucleus. [92]. Keap1 functions as a redox sensor, in that modification of one (or more) Keap1 cysteine residue(s) by the inducing agents destabilizes Nrf2 binding and releases Nrf2 from the ubiquitin-conjugating complex [88].

Multiple further signalling pathways induced by xenobiotics have been reported to stimulate or modulate ARE-mediated gene expression [85,93]. They can feed into the described system at least at three levels: Modulation of Nrf2–Keap1 complex stability, Nrf2 activity and heterodimer formation, and activation of other transcription factors interacting with ARE motifs or motifs embedded into AREs in certain promoters (Fig. 2). An in vitro study in HepG2 cells showed that the dimerization of Nrf2 (589 amino acid) with c-Jun is more effective than Nrf1 (742 amino acid) with c-Jun in regulation of ARE-mediated gene expression. Also, the authors reported that the level of intracellular c-Jun is important and that higher levels of c-Jun repressed the activation of ARE due to formation of a c-Jun and c-Fos complex that interferes with the binding of the Nrf2/c-Jun complex to ARE [94]. Homodimers of Juns (c-Jun, Jun-D, and Jun-B) or heterodimers of Jun with c-Fos or Fras (Fra1 and Fra2) bind to AP1 promoter sites contained in genes like *GSTP1* and *GSTA1* [95]. Kim et al. demonstrated that sulforaphane induces ARE in HepG2 cells and that the intracellular level of GSH was inversely related to ARE activation [96].

4.2. Induction of hGST gene expression

Some of the GST-encoding genes, which contain ARE motifs (see Table 1) can be induced in an ARE-mediated manner. Thus, it has been shown that Oltipraz ((5-[2-pyrazinyl]-4-methyl-1, 2-3-thione), an antischistosomal (parasite killing) agent, which protects against chemically induced carcinogens induces gastric and hepatic GST activity and mRNA in Nrf2 wild-type but not in Nrf2-deficiant mice. This allowed the authors to conclude that Nrf2 is the main factor for regulation of GSTs [97]. Relatively little is known about the relevance of ARE-mediated transcriptional regulation for expression of human GSTs. ARE sequences in the promoter of h*GSTA2* are required for basal expression and for its induction by phenolic antioxidants, like green tea phenols [98]. These compounds activate GST-encoding genes, however, also via the AP-1 family of transcription factors, which include Jun, fos, Maf, Nrl and Fra proteins [99]. As is shown in Table 1, AP-1-binding sites have been identified in the promotor regions of the *GSTA1*, *GSTA4* and *GSTP1* genes [100,101]. C/EBPβ (CCAAT/enhancer binding protein β) is a member of the C/EBP bZip class of transcription factors with important roles for cell survival and cell proliferation. The C/EBP family comprises four isoforms (α, β, ε, and δ), which form homodimers and heterodimers and bind to a consensus C/EBP binding DNA motif [26]. Oltipraz induces *GSTA2* mRNA and protein expression in a rat hepatocyte-derived cell line (H4IIE), via PI3-kinase-mediated nuclear translo-

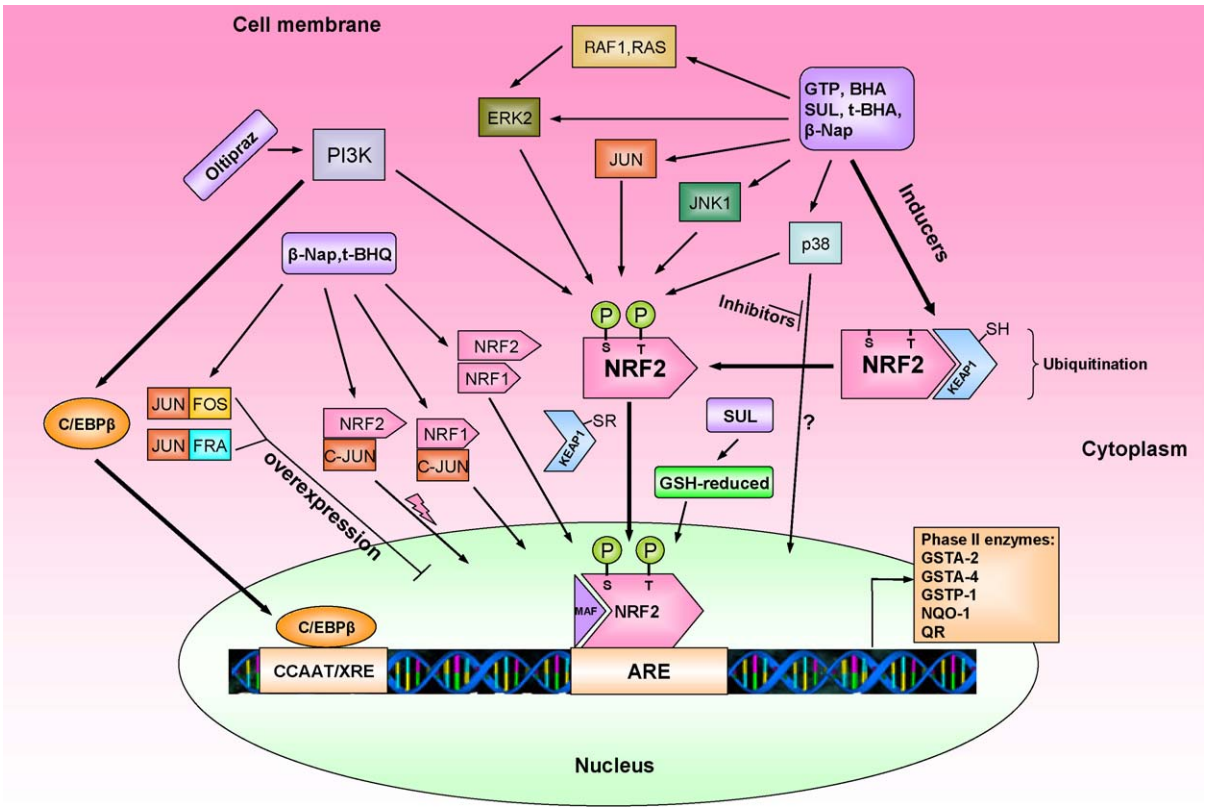


Fig. 2. Schematic diagram for Phase II enzyme regulation by different substances adapted according to Surh [3]. Abbreviations: C/EBPβ, CCAAT/enhancer-binding protein beta; JUN, mitogen-activated protein kinase; ERK2, extracellular signal-regulated kinase; J NK1, c-Jun N-terminal kinases 1; AP-1, activator protein-1; JUN, FOS, and FRA, transcription factor complex; KEAP1, Kelch-like ech-associated protein 1; NF-E2, nuclear factor erythroid 2; NRF2 and NRF1; NF-E2related factor-2 and -1; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; p38, cysteine-rich MAPK kinase kinase; NQO-1, NAD(P)H:quinone oxidoreductase 1; QR, quinone reductase; Raf-1, Ras, GTPase-activating protein; NF-kB, nuclear factor kappa-b; STAT, signal transducer and activator of transcription; GATA, gata-binding protein; Sp1, specific protein-1; PPAR, peroxisome proliferator-activated receptor; GSH, glutathione; GSTA-2 and GSTA-4, glutathione S-transferase alpha-2 and 4; GSTP1, glutathione S-transferase Pi-1; GSTM-1 and GSTM-3, glutathione S-transferase Mu-1 and 3; GTP, green tea polyphenols; BHA, butylated hydroxyanisole; SUL, sulforaphane; *t*-BHA, *tert*-butylhydroquinone; β-Nap, β-naphthoflavone and Oltipraz. A major mechanism for induction of Phase II gene expression is transcriptional activation through promoters containing ARE motifs. ARE motifs bind Nrf2, a transcription factor of the basic leucine zipper (bZip) transcription factor family. By interaction with the redox sensor Keap1 in its reduced state, Nrf2 is maintained in the cytoplasm and targeted for degradation in the proteasome. Modification of Keap1 cysteine(s) by xenobiotics leads to disruption of the complex, stabilization, and nuclear translocation of Nrf2. Nrf2 has activity only as heterodimer with other transcriptional regulators, as indicated. Its activity can further be modified by different signal transduction pathways, which, for example, mediate Nrf2 phosphorylation. The illustrated pathways are explained in more detail in the text.

cation and binding of to the C/EBP response element (TTGCGAA) in the *GSTA2* gene promoter. The authors searched the GenBank database for the C/EBP response elements in the regulatory regions of other Phase II enzymes. The result revealed that the genes containing C/EBP as a core sequence include human γ-glutamylcysteine synthetase, mouse quinone reductase, human GSTα, and human heme oxygenase-1. Therefore, C/EBPβ may serve as a more common transcriptional factor for the induction of Phase II enzymes and cancer chemoprevention. Other transcription factor binding sites (NF-κB, SP-1, AP-2, GRE, and AP-2) have been described for *GSTP1* and/or *GSTA1*, but it is clear from the available data that much further experimentation is necessary to complete understanding on the induction of GSTs by different mechanisms.

Table 1
Transcription factor binding sites in the promoter regions of genes encoding selected biotransformation enzymes

Gene name	Transcription factor binding site	Species	Reference
Glutathione S-transferase Pi-1 (GSTP1)			
	NF-kB	Human	Xia et al. [139]
	AP-1		Morceau et al. [140]
	SP-1		
NAD(P)H:quinone oxidoreductase (NQO-1)			
	HSE	Human	Hayes et al. [141]
	NF-kB		Venugopal et al. [142]
	CCAAT		
	AP-1		
	AP-2		
Glutathione S-transferase Alpha-1 (GSTA1)			
	AP-2	Human	Lorper et al. [143]
	AP-1	Rat	Falkner et al. [144]
	GRE		
	TCF1		
	NF-E2		
	HNF		
Glutathione S-transferase Alpha-2 (GSTA2)			
	C/EBP	Human	Lorper et al. [143]
	PPAR		Hayes et al. [141]
	NF-E2		Park et al. [145]
Glutathione S-transferase Alpha-4 (GSTA4)			
	NF-kB	Human	Desmots et al. [100]
	STAT		
	GATA		
	AP-1		
	SP-1		
Glutathione S-transferase Mu-1 (GSTM1)			
	MYP	Mouse	Bartley et al. [146]
Glutathione S-transferase Mu-2 (GSTM2)			
	ETS	Mouse	Kumar et al. [147]
	MYP		
	AP-2		
	NF-E2		
	AP-1		
	SP-1		
Glutathione S-transferase Mu-3 (GSTM3)			
	SP-1	Human	Patskovsky et al. [148]
Glutathione S-transferase Theta-1 subunit Yrs (GSTT1)			
	SP1	Rat	Ogura et al. [149]
	PU1		
	PEA3		
	AP-2		
Glutathione S-transferase Alpha-2 (GSTA2)[a]			
	ARE	Human	Chen et al. [98]

Transcription factors were functionally characterized for the regulation of target genes [100,139–149].

[a] Further ARE elements identified in the promoters of rat GSTA2, rat GSTP1, and mouse GSTP1 are described in Nguyen et al. [85].

In summary, numerous pathways occur, by which GST-encoding genes can be transcriptionally activated and studies are needed to assess which compounds have the potential to mediate one or more of these mechanisms in human colon cells and how this relates to GST activity and detoxification of colon cancer risk factors.

4.3. Modulation of GSTs in human colon cells by butyrate

Low GST expression levels in the colon may be compensated by the presence of butyrate, an important product of gut flora mediated fermentation of dietary fibers. Our recent studies have shown that butyrate, one of the three most abundant SCFA produced in the gut [102], induced *GSTP1*, *GSTM2*, and *GSTA4* in HT29 cells [48,103]. We also detected an up regulation of GSTs (e.g. *GSTA2*, *GSTT2*) in primary human colon epithelial cells upon incubation with butyrate [49]. Thus, low GST expression levels were favourably altered by butyrate. The mechanisms by which butyrate has been found to mediate gene expression in human colon tumor cells are by activation of the MAPK signalling transduction pathway [103], but also by modifying the acetylation of histones at the N-terminal lysine rich tails [104,105]. Two classes of enzymes can affect the acetylation of histones, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). Altered HAT or HDAC activity has been identified in several cancers and the modification of the structure of N-terminal tails of histones by acetylation or deacetylation is crucial in modulating gene expression, as it affects the interaction of DNA with transcription-regulatory non-nucleosomal protein complexes. The classes of compounds that are identified as HDAC inhibitors now include short-chain fatty acids, such as butyrate and several analogues [106]. HDAC inhibitors from several different structural classes exhibit clinical activity against a variety of human malignancies, and have also attracted interest as potential chemopreventive agents [107,108]. Butyrate inhibits HDAC activity and cell growth at millimolar concentrations. Our own studies have shown that treatment of human colon cells HT29 leads to the marked accumulation of acetylated Histone 4 (H4), which could be related to enhanced levels of GST-encoding gene expression in these cells [109]. Whether or not promoter areas of GSTs are being targeted by this mechanism, will be an important sub-

ject of research in the near future. Additional work is also necessary to disclose whether the anticarcinogenic properties of dietary fibres, which upon ingestion give rise to enhanced butyrate formation in the gut lumen [110], may in part be protective on account of this mechanism. These dietary fibres also include the prebiotic food ingredients, inulin-type fructans (β(2-1)-fructans) extracted from chicory roots (*Cichorium intybus*), which are fermented to lactic acid and short-chain fatty acids (SCFA) and which have marked anticancer properties [46]. The products formed during in vitro fermentation of inulin-type fructans and human gut flora have different chemoprotective properties, including modulation of GSTs and other enzymes of biotransformation in human colon tumor cells and in a colon adenoma cell line [111,112].

5. GSTs as targets of chemoprevention

Cancer chemoprevention can be defined as the use of naturally occurring or synthetic agents to prevent, inhibit, or reverse the process of carcinogenesis [113]. A large body of experimental data has shown that this approach is feasible. As described above, chemopreventive agents transcriptionally induce a battery of genes whose protein products can protect cells from chemical-induced carcinogenesis. Enhanced detoxification of cancer causing agents may contribute to reduced cancer risk. Whether or not an enhanced expression of GSTs contributes to cancer prevention has not been demonstrated in humans conclusively. But there are several lines of evidence that support this assumption: (1) A large number of animal experiments have shown that cancer reducing activities of chemicals can be associated with increases in Phase II metabolism [114]. In particular, it has been shown that the antishistosomal agent Oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-3-thione) inhibits in animal experiments carcinogenesis induced by aflatoxin B1, polycyclic aromatic hydrocarbons and *N*-nitrosamines [38,115] and at the same time results in marked elevations of the activities of Phase II enzymes in hepatic and extrahepatic tissues [116]. Fig. 3 summarizes data on the chemoprotective efficacy of oltipraz; (2) in human cells an enhanced GST-level is associated with less damage by the putative cancer risk factor 4-hydroxynonenal (HNE) [103], whereas lower GST-levels are associated with more damage [117]; (3) individuals with null polymorphisms [118] for *GSTM1*, *GSTT1* or other types of genetic predispositions [18] are at higher risk for developing tumors in the case of specific exposure situations [119–121], albeit the associations with colorectal cancer are not as clear [44]. Alternatively, sequence polymorphisms for *GSTP1* protect from benzo(*a*)pyrene mediated genotoxic damage [65] and genetic polymorphisms, like *GSTM1*0* and *GSTT1*0* appear to be associated with an improved beneficial effect of cruciferous vegetables (see below) [122].

6. GSTs as targets of dietary prevention

Phytochemicals in plant foods modulate activities of biotransformation enzymes, one mechanism by which fruits and vegetables, and cruciferous vegetables, in particular, could act chemoprotective [116,123].

6.1. Brassica vegetables

It has been reported that the chemoprotective effect of cruciferous vegetables is due to their high glucosinolate content and the capacity of glucosinolate metabolites to modulate biotransformation enzyme systems (e.g. cytochromes P450 and conjugating enzymes) [122]. Glucosinolates (β-thioglycoside-*N*-hydroxysulfates) are hydrolysed by the plant enzyme myrosinase releasing the biologically active isothiocyanates (ITC). Thiol conjugates of ITC are formed by conjugation with glutathione, and elimination proceeds by cleavage of glutamine and glycine and acetylation to produce *N*-acetyl-cysteine ITC conjugates (mercapturic acids); which are excreted in urine, and the mechanisms of action by which they may profoundly affect drug metabolism have been reviewed [124]. Accordingly, ITCs inhibit specific cytochrome P450 (P450) enzymes involved in the activation and detoxification of carcinogens. Inhibition of the metabolic activation of carcinogens is probably an important component of ITC inhibition of carcinogenesis. Other investigations, however, show that ITCs also induce Phase II enzymes such as glutathione S-transferases and quinone reductase. This may increase the detoxification of electrophilic metabolites formed during Phase I metabolism which results less DNA damage [125]. According to Talalay and co-workers, ITCs thus

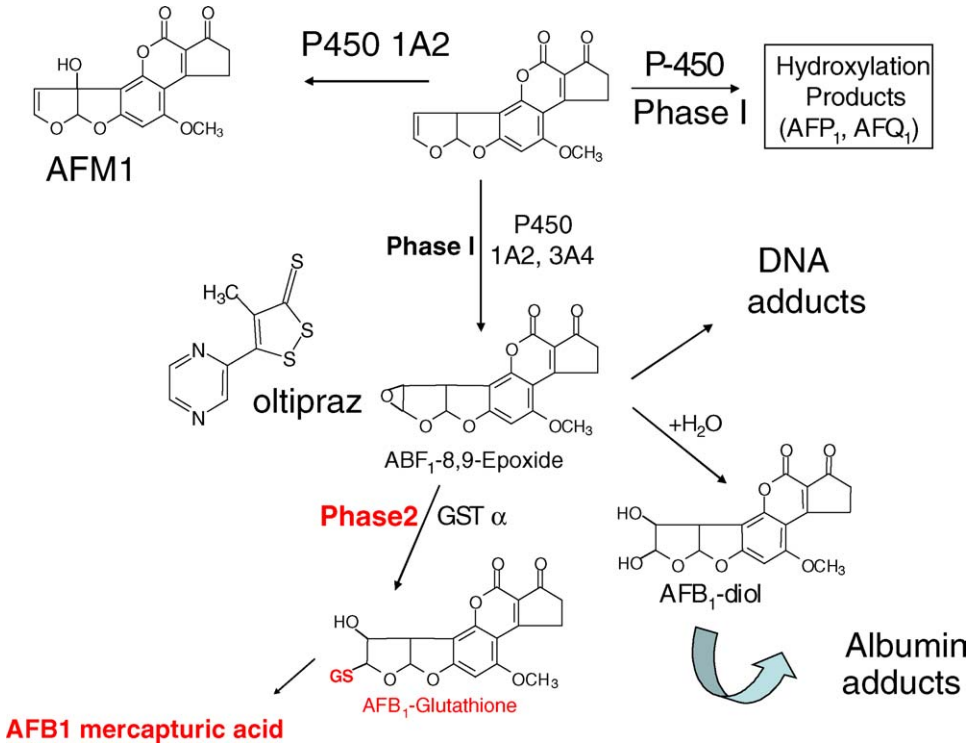


Fig. 3. Overview on key biotransformation pathways of aflatoxin B1 (AFB1) and steps that oltipraz might affect its enzymatic conversion (adapted according to Zhang et al. [154]). Oltipraz was extensively evaluated as a treatment for schistosomiasis in the early 1980s when it was found that single doses markedly elevated the activities of Phase II enzymes in hepatic and extrahepatic tissues [155]. The subsequent analyses of chemopreventive activities showed that oltipraz indeed inhibited cancers in breast, colon, pancreas, lung, forestomach, skin, bladder, and liver tumor models [115]. Roebuck et al. [38] reported that dietary administration of oltipraz completely protected against aflatoxin-induced hepatocarcinogenesis, probably due to induced activities of glutathione S-transferases which facilitates conjugation of glutathione to aflatoxin-8,9-oxide, thereby enhancing its elimination and coordinately diminishing DNA adduct formation [156]. Molecular studies indicated that initial increases in hepatic glutathione S-transferase mRNA and protein levels in response to oltipraz were mediated through transcriptional activation of transferase genes in rodent hepatocytes [157,158] and in human hepatocytes [159]. These findings were the basis for a randomized, placebo-controlled, double-blind intervention trial conducted in residents of Qidong, People's Republic of China, who are at high risk for exposure to aflatoxin and development of hepatocellular carcinoma. The major conclusions of the resulting study were that intermittent, high-dose oltipraz inhibited Phase I activation of aflatoxins, and sustained low-dose oltipraz increased Phase II conjugation of aflatoxin, yielding higher levels of aflatoxin–mercapturic acid. The study thus highlighted the feasibility of inducing Phase II enzymes as a chemopreventive strategy in humans [160]. AFM1, the metabolite aflatoxin M1; GSH, glutathione.

block carcinogenesis by dual mechanisms, of which the Phase II response, which includes GST-induction, contributes most importantly to reducing cellular susceptibility to carcinogens [126]. Since ITCs are present in substantial quantities in human diets, the agents are ideal candidates for the development of effective dietary chemoprotection of humans against cancer.

There are three ways by which ITC may modulate GSTs of which some may result in chemoprevention. Firstly, GSTA1-1, GSTP1-1, GSTM1-1, and GSTM2-2, may conjugate ITCs with glutathione in humans, albeit substrate specificity can vary greatly [127]. Also, the induction capacity is clearly related to cellular uptake [128]. Secondly, ITCs induce expression of Phase I and Phase II enzymes and also directly inhibit P450 [129]. Some ITCs induce Phase I enzymes, others induce only Phase II enzymes, and some induce both [130,131] and the properties could be associated with chemoprotection. It has been demonstrated that the addition of watercress to diets of smokers protected from genotoxic insults by tobacco smoke-related carcinogens [132]. It is, however, a complex endeavour

to evaluate or predict protective properties by other representatives of *Brassica*, since individual species contain a variety of glucosinolates, each with different properties [133]. Thirdly, GST polymorphisms seem to greatly affect the beneficial role of *Brassica* vegetables. Subjects with *GSTM1*0* genotypes seem to benefit most [133], as was shown in a randomized crossover study of four controlled diets (basal diet with no vegetables or fruit, and the basal diet supplemented with cruciferous, allium or apiaceous vegetables). Serum GSTA concentration, an enzyme induced by ITC, increased significantly in response to cruciferous vegetable feeding, but only in *GSTM1*-null individuals [122]. In the absence of serological evidence for hepatocellular damage, e.g. measured as serum alanine aminotransferase levels, increased serum GSTs can be correlated to tissue levels [123]. Therefore, the results indicated that the relationships between cruciferous vegetable intake and cancer risk are influenced by genetic polymorphisms of GSTs [122].

Human intervention studies have been performed to examine effects of cruciferous vegetable supplementation on metabolism of carcinogens. Broccoli and Brussels sprouts increased metabolism of cooked meat derived heterocyclic aromatic amines (i.e., reduced urinary excretion of 2-amino-3,8-dimethyl- imidazo[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazol[4,5-*b*]pyridine), implicating the induction of both CYP1A2 and Phase II enzymes involved in heterocyclic amine metabolism [131]. More studies are needed to understand whether the consequences of this type of bifunctional induction of enzymes of biotransformation result in overall metabolic inactivation or activation [134].

7. Effects of Brassica and ITC in the human colon

The results of 74 case-control studies (reviewed in [135]) on the association between *Brassica* consumption and cancer risk showed for 64% an inverse association between consumption of one or more *Brassica* vegetables and risk of cancer at various sites, and colon was one of the sites for which the association appeared to be most consistent. It was not possible to resolve whether associations could be attributed to *Brassica* vegetables per se or to vegetables in general. In a more recent study by Seow et al. [136], middle-aged men and women were enrolled for a nested case-control analysis. Two hundred and thirteen incident cases of colorectal cancer were compared to 1194 controls. Information on dietary ITC intake from cruciferous vegetables was combined with *GSTM1*, *T1*, and *P1* genotype data. The analysis revealed that there were no overall associations between *GSTM1*, *T1*, or *P1* genotypes and colorectal cancer risk. However, among individuals with both *GSTM1* and *T1* null genotypes, a 57% reduction in risk among high versus low consumers of ITC was observed for colon cancer. Studies by Johnson [137] have addressed which effects glucosinolates may have in the gut lumen. Their work has shown that the glucosinolate breakdown product sulforaphane can inhibit DNA adduct formation induced by a heterocyclic amine in a dose-dependent manner, possibly acting through the induction of Phase II detoxification enzymes such as GSTs and UGTs (see above). Another study has investigated the effects of indols and ITCs in human colon cancer cell lines, in which the natural isothiocyanates sulforaphane, benzyl isothiocyanate, and phenethyl isothiocyanate stimulated apoptosis in human colon adenocarcinoma cell lines [138]. Natural indols were inducers of cytochrome P450, but not of gamma-glutamylcysteine synthetase heavy subunit (γGCS(h)), In contrast, treatment of the cells with ITCs did not result in an induced expression of *CYP1A1*, but caused a marked increase of the protein levels of NAD(P)H:quinone oxidoreductase 1 (NQO1) and γGCS(h). Thus, evidence was presented that indoles and ITCs stimulate either xenobiotic response element- or antioxidant response element-driven gene expression. The induction was associated with chemoresistance against benzo(*a*)pyrene, supporting the protective consequences of altered metabolic activities [138].

8. Conclusions

There is increasing evidence that human cancers can be prevented by favouring the intake of protective factors that modulate the defense mechanisms of the host organism. The careful co-ordination and balance of Phase I and Phase II metabolizing enzymes is necessary for cellular protection against xenobiotics. Especially the complex supergene family of GSTs that collectively metabolizes chemotherapeutic drugs, car-

cinogens and environmental pollutants plays a protective pivotal role against xenobiotics and carcinogens. Their levels of expression can have profound effects on susceptibility to chemical insult, with over expression resulting in resistance and under expression enhancing susceptibility. GSTs are expressed with high inter-individual variability in human colonocytes, which points to large differences in cellular susceptibility to xenobiotics. Thus, dietary, putative cancer risk factors, such as nitrosamines, heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, and products of oxidative stress collectively include substrates for glutathione S-transferases. A considerable number of subjects could therefore be at higher risk on account of low GST expression levels. Together this supports endeavours to find ways to increase GSTs in the colon as a potential strategy for chemoprevention. Cancer prevention experts generally recommend that people maintain a healthy weight, exercise, and eat diets high in fruits, vegetables, and fibre. And there is a growing body of evidence showing that plant foods can enhance GST expression levels. In particular, e.g. it has been shown that isothiocyanates (ITCs) of *Brassica* vegetables block carcinogenesis by dual mechanisms, namely inhibition of Phase I and induction of Phase II, including GST. Since ITCs are present in substantial quantities in human diets, the agents are ideal candidates for the development of effective dietary chemoprotection of humans against cancer. Moreover, other studies have shown that variations in habitual consumption of fruits, particularly citrus fruits, and of vegetables, in particular *Brassica* vegetables, may contribute to variations in human rectal GST enzyme activity. Numerous pathways occur, by which GST-encoding genes can be transcriptionally activated. These include pathways mediated by antioxidants, via an antioxidant-responsive element, or by the gut fermentation product butyrate via modulation of genomic histone acetylation. However, studies are needed to assess which compounds have the potential to mediate one or more of these mechanisms in human colon cells and how this relates to GST activity and detoxification of colon cancer risk factors. Although there is strength of associations between dietary factors and cancer, the question remains how such factors affect individuals. One growing area of research is therefore to develop markers to measure effectiveness of dietary intervention. One of these markers could be the detection of an altered state of chemoprotection due to modulation of Phase I and Phase II enzymes, in general, and an induction of GSTs, in particular. Since efficient life-long mechanisms of chemoprevention could include a favourable modulation of biotransformation systems in human tissues by diet, this type of measurement could help researchers select the most promising dietary regimens for clinical trials and public health issues. The evaluation of this marker necessitates more knowledge on the types of involved carcinogens and their biotransformation and how the dietary mediated alteration of biotransformation is related to cancer initiation and progression. Altogether, the study of these associations warrants close attention in the future and may eventually lead to a more precise understanding on these types of protective mechanisms.

Acknowledgements

Authors' research on GSTs is supported by EU proposals (PHYTOPREVENT, QLK1-CT-2000-00266; SEAFOOD plus, FQS-506359; SYNCAN, QLRT-1999-00346), by a National Network of Molecular Nutrition Research (BMBF No. FKZ: 01EA0103), by the German Research Foundation, Deutsche Forschungsgemeinschaft (DFG PO 284/8-1; DFG FlavoNet PO 284/9), and by the German Cancer Help Foundation, Deutsche Krebshilfe (Deutsche Krebshilfe 70-2165-PO2).

References

[1] W.K. Hong, M.B. Sporn, Recent advances in chemoprevention of cancer, Science 278 (1997) 1073–1078.
[2] M.B. Sporn, N. Suh, Chemoprevention of cancer, Carcinogenesis 21 (2000) 525.
[3] Y.-J. Surh, Cancer chemoprevention with dietary phytochemicals, Nat. Rev. Cancer 3 (2003) 768–780.
[4] World Cancer Research Fund, American Institute for Cancer Research. Food, Nutrition and the Prevention of Cancer: A Global Perspective, American Institute for Cancer Research, Washington, DC, 1997.
[5] S.A. Bingham, Epidemiology and mechanisms relating diet to risk of colorectal cancer, Nutr. Res. Rev. 9 (1996) 197–239.
[6] P.M. Heavey, D. McKenna, I.R. Rowland, Colorectal cancer and the relationship between genes and the environment, Nutr. Cancer 48 (2004) 124–141.
[7] W.C. Willett, M.J. Stampfer, G.A. Colditz, B.A. Rosner, F.E. Speizer, Relation of meat, fat and fiber intake to the risk of

colon cancer in a prospective study among women, N. Engl. J. Med. 323 (1990) 1664–1672.
[8] E. Giovannucci, E.B. Rimm, M.J. Stampfer, G.A. Colditz, A. Ascherio, W.C. Willett, Intake of fat, meat and fiber in relation to risk of colon cancer in men, Cancer Res. 54 (1994) 2390–2397.
[9] E. Cho, S.A. Smith-Warner, D. Spiegelman, W.L. Beeson, P.A. van den Brandt, G.A. Colditz, A.R. Folsom, G.E. Fraser, J.L. Freudenheim, E. Giovannucci, R.A. Goldbohm, S. Graham, A.B. Miller, P. Pietinen, J.D. Potter, T.E. Rohan, P. Terry, P. Toniolo, M.J. Virtanen, W.C. Willett, A. Wolk, K. Wu, S.S. Yaun, A. Zeleniuch-Jacquotte, D.J. Hunter, Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies, JNCI Cancer Spectr. 96 (2004) 1015–1022.
[10] E. Giovannucci, An updated review of the epidemiological evidence that cigarette smoking increases risk of colorectal cancer, Cancer Epidemiol. Biomarkers Prev. 10 (2001) 725–731.
[11] H.K. Seitz, G. Pöschl, F. Stickel, Alcohol and colorectal cancer, in: W. Scheppach, M. Scheuerlein (Eds.), Exogenous Factors in Colonic Carcinogenesis, Kluwer Academic Publisher, 2003, pp. 128–141.
[12] H. Clevers, At the crossroads of inflammation and cancer, Cell 118 (2004) 671–674.
[13] B. Vogelstein, E.R. Fearon, S.R. Hamilton, S.E. Kern, A.C. Preisinger, M. Leppert, Y. Nakamura, R. White, A.M.M. Smits, J.L. Bos, Genetic alteration during colorectal-tumor development, N. Engl. J. Med. 319 (1988) 525–532.
[14] E.R. Fearon, Human cancer syndromes: clues to the origin and nature of cancer, Science 278 (1997) 1043–1050.
[15] H.T. Lynch, A. de la Chapelle, Hereditary colorectal cancer, N. Engl. J. Med. 348 (2003) 919.
[16] R. Fodde, R. Smits, H. Clevers, APC signal transduction and genetic instability in colorectal cancer, Nat. Rev. Cancer 1 (2001) 55–67.
[17] A. de la Chapelle, Genetic predisposition to colorectal cancer, Nat. Rev. Cancer 4 (2004) 769–780.
[18] M.M. de Jong, I.M. Nolte, G.J. te Meerman, W.T.A. van der Graaf, E.G.E. de Vries, R.H. Sijmons, R.M.W. Hofstra, J.H. Kleibeuker, Low-penetrance genes and their involvement in colorectal cancer susceptibility, Cancer Epidemiol. Biomarker Prev. 11 (2002) 1332–1352.
[19] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57–70.
[20] K. Wakabayashi, M. Nagao, H. Esumi, T. Sugimura, Food-derived mutagens and carcinogens, Cancer Res. 52 (Suppl.) (1992) 2092S–2098S.
[21] K.W. Kinzler, B. Vogelstein, Lessons from hereditary colorectal cancer, Cell 87 (1996) 159–170.
[22] A. Bardelli, D.W. Parsons, N. Silliman, J. Ptak, S. Szabo, S. Saha, S. Markowitz, J.K.V. Willson, G. Parmigiani, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, Mutational analysis of the tyrosine kinome in colorectal cancers, Science 300 (2003) 949.
[23] Z. Wang, D. Shen, D.W. Parsons, A. Bardelli, J. Sager, S. Szabo, J. Ptak, N. Silliman, B.A. Peters, M.S. van der Heijden, G. Parmigiani, H. Yan, T.L. Wang, G. Riggins, S.M. Powell, J.K.V. Willson, S. Markowitz, K.W. Kinzler, B. Vogelstein, V.E. Velculescu, Mutational analysis of the tyrosine phosphatome in colorectal cancers, Science 304 (2004) 1164–1166.
[24] C.A.L. Ruivenkamp, T. van Wezel, C. Zanon, A.P.M. Stassen, C. Vlcek, T. Csikós, A.M. Klous, N. Tripodis, A. Perrakis, L. Boerrigter, P.C. Groot, J. Lindeman, W.J. Mooi, G.A. Meijjer, G. Scholten, H. Dauwerse, V. Paces, N. Van Zandwijk, G.J.B. van Ommen, P. Demant, Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers, Nat. Genet. 31 (2002) 295–300.
[25] C.A.L. Ruivenkamp, M. Hermsen, C. Postma, A. Klous, J. Baak, G.A. Meijjer, P. Demant, LOH of PTPRJ occurs early in colorectal cancer and is associated with chromosomal loss of 18q12-21, Oncogene 22 (2003) 3472–3474.
[26] S.A. Bingham, E. Riboli, Diet and cancer—the European prospective investigation into cancer and nutrition, Nat. Rev. Cancer 4 (2004) 206–215.
[27] G.J. Hammons, B.D. Lyn-Cook, Modulation of biotransformation enzymes in cancer chemoprevention, Int. J. Cancer Prev. 1 (2004) 3–14.
[28] J.D. Hayes, J.U. Flanagan, I.R. Jowsey, Glutathione transferases, Annu. Rev. Pharmocol. Toxicol. 45 (2005) 51–88.
[29] S.A. Bingham, N.E. Day, R. Luen, P. Ferari, N. Slimani, T. Norat, F. Clavel-Chapelon, E. Kesse, A. Nieters, H. Boeing, A. Tjonneland, K. Overvad, C. Martinez, M. Dorronsoro, C.A. Gonzalez, T.J. Key, A. Trichopoulu, A. Naska, P. Vineis, R. Tumino, V. Krogh, H.B. Bueno-de-Mesquita, P.H.M. Peeters, G. Berglund, G. Hallmans, E. Lund, G. Skeie, R. Kaaks, E. Riboli, Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study, Lancet 361 (2003) 1496–1501.
[30] C.S. Fuchs, E. Giovannucci, G.A. Colditz, D.J. Hunter, M.J. Stampfer, B. Rosner, F.E. Speizer, W.C. Willett, Dietary fiber and the risk of colorectal cancer and adenoma in women, N. Engl. J. Med. 340 (1999) 169–176.
[31] H.C. Hung, K.J. Joshipura, R. Jiang, F.B. Hu, D. Hunter, S.A. Smith-Warner, G.A. Colditz, B. Rosner, D. Spiegelman, W.C. Willett, Fruit and vegetable intake and risk of major chronic disease, JNCI Cancer Spectr. 96 (2004) 1577–1584.
[32] P.A. Wark, M.J.A.L. Grubben, W.H.M. Peters, F.M. Nagengast, E. Kampman, F.J. Kok, P. van't Veer, Habitual consumption of fruits and vegetables: associations with human rectal glutathione S-transferase, Carcinogenesis 25 (2004) 2135–2142.
[33] National Library of Medicine, Toxicology Tutor II, Toxicokinetics. http://www.sis.nlm.nih.gov/ToxTutor/Tox2/a41.htm, 2003. U.S. Department of Health and Human Services, Ref Type: Electronic Citation.
[34] L.W. Wattenberg, Inhibition of carcinogenesis by minor dietary constituents, Cancer Res. 52 (Suppl.) (1992) 2085s–2091s.
[35] J.D. Hayes, D.J. Pulford, The glutathione S-transferase supergene family regulation of GST* and the contribution of the

isoenzymes to cancer chemoprotection and drug resistance, Crit. Rev. Biochem. Mol. Biol. 30 (1995) 445–460.
[36] Y.C. Awasthi, R. Sharma, S.S. Singhal, Human glutathione S-transferases, Int. J. Biochem. 26 (1994) 295–308.
[37] G.J. Beckett, J.D. Hayes, Glutathione S-transferases: Biomedical applications, Adv. Clin. Chem. 30 (1993) 282–380.
[38] B.D. Roebuck, Y.L. Liu, a.R. Rogers, J.D. Groopman, T.W. Kensler, Protection against aflatoxin B1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz): predictive role for short-term molecular dosimetry, Cancer Res. 51 (1991) 5501–5506.
[39] J.D. Hayes, R. McLeod, E.M. Ellis, D.J. Ulford, L.S. reland, L.I. cLellan, D.J. Udah, M.M. Anson, G.E. Eal, Regulation of glutathione S-transferases and aldehyde reductase by chemoprotectors: studies of mechanisms responsible for inducible resistance to aflatoxin B1, in: B.W. Steward, D.B. McGregor, P. Kleihues (Eds.), Principles of Chemoprevention, International Agency for Research on Cancer, Lyon, 1996, pp. 175–188.
[40] P.A. Cerutti, Prooxidant states and tumor promotion, Science 227 (1985) 375–381.
[41] J.D. Hayes, R.C. Strange, Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress, Free Rad. Res. 22 (1995) 193–207.
[42] K. Berhane, M. Widersten, A. Engstrom, J.W. Kozarich, B. Mannervik, Detoxication of base propenals and other a,b-unsaturated aldehyde products of radical reactions and lipid peroxidation by humane glutathione transferases, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 1480–1484.
[43] M.A. Watson, R.K. Stewart, G.B. Smith, T.E. Massey, D.A. Bell, Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution, Carcinogenesis 19 (1998) 275–280.
[44] S.C. Cotton, L. Sharp, J. Little, N. Brockton, Glutathione S-transferase polymorphisms and colorectal cancer: a HuGE review, Am. J. Epidemiol. 151 (2000) 7–32.
[45] H. Ahsam, A.G. Rundle, Measures of genotype versus gene products: promise and pitfalls in cancer prevention, Carcinogenesis 24 (2003) 1429–1434.
[46] B.L. Pool-Zobel, Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data, Br. J. Nutr. 92 (2004) 1–20.
[47] M.N. Ebert, G. Beyer-Sehlmeyer, U.M. Liegibel, T. Kautenburger, T.W. Becker, B.L. Pool-Zobel, Butyrate-induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxynonenal, Nutr. Cancer 41 (2001) 156–164.
[48] M.N. Ebert, A. Klinder, A. Schäferhenrich, W.H.M. Peters, W. Sendt, J. Scheele, B.L. Pool-Zobel, Expression of glutathione S-transferases (GST) in human colon cells and inducibility of GSTM2 by butyrate, Carcinogenesis 24 (2003) 1637–1644.
[49] B.L. Pool-Zobel, R. Veeriah, T. Kautenburger, J. Kiefer, K.K. Richter, J. Sauer, M. Soom, S. Wölfl, Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, submitted for publication.
[50] R.N. Armstrong, Structure, catalytic mechanism, and evolution of the glutathione transferases, Chem. Res. Toxicol. 10 (1997) 2–18.
[51] C.S. Reddy, A.W. Hayes, Food-borne toxicants, in: A.W. Hayes (Ed.), Principles and Methods of Toxicology, Raven Press Ltd., New York, 1994, pp. 317–360.
[52] G. Eisenbrand, W. Tang, Food-borne heterocyclic amines. Chemistry, formation, occurrence and biological activities. A literature review, Toxicology 84 (1993) 1–82.
[53] D.W. Layton, K.T. Bogen, M.G. Knize, F.T. Hatch, V.M. Johnson, J.S. Felton, Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research, Carcinogenesis 16 (1995) 39–52.
[54] T. Sugimura, Nutrition and dietary carcinogens, Carcinogenesis 21 (2000) 387–395.
[55] S. Langouet, A. Paehler, D.H. Welti, N. Kerriguy, A. Guillouzo, R.J. Turesky, Differential metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in rat and human hepatocytes, Carcinogenesis 23 (2002) 115–122.
[56] M.A. Malfatti, J.S. Felton, N-Glucuronidation of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and N-hydroxy-PhIP by specific human UDP-glucuronosyltransferases, Carcinogenesis 22 (2001) 1087.
[57] B. Coles, S.A. Nowell, S.L. MacLeod, C. Sweeny, N.P. Lang, F.F. Kadlubar, The role of human glutathione S-transferases (hGSTs) in the detoxification of the food-derived carcinogen metabolite *N*-acetoxy-PhIP, and the effect of a polymorphism in hGSTA1 on colorectal cancer risk, Mutat. Res. 482 (2001) 3–10.
[58] C. Sachse, G. Smith, M.J.V. Wilkie, J.H. Barrett, R. Waxman, F. Sullivan, D. Forman, D.T. Bishop, C.R. Wolfl, The Colon Cancer Study Group. A pharmacogenetic study to investigate the role of dietary carcinogens in the etiology of colorectal cancer, Carcinogenesis 23 (2003) 1839–1849.
[59] W. Lijinsky, P. Shubik, Benzo(*a*)pyrene and other polynuclear hydrocarbons in charcoal-broiled meat, Science 145 (1964) 53–55.
[60] W. Lijinsky, The formation and occurrence of polynuclear aromatic hydrocarbons associated with food, Mutat. Res. 259 (1991) 251–261.
[61] IARC IARC Monographs on the evaluation of carcinogenic risks to humans, vol. 38, Tobacco smoking, IARC, Lyon, 1986.
[62] E. Giovannucci, E.B. Rimm, M.J. Stampfer, G.A. Colditz, A. Ascherio, J. Kearney, W.C. Willett, A prospective study of cigarette smoking and risk of colorectal adenoma and colorectal cancer in U.S. men, J. Natl. Cancer Inst. 86 (1994) 183–191.
[63] A. Chao, M.J. Thun, E.J. Jacobs, S.J. Henley, C. Rodriguez, E.E. Calle, Cigarette smoking and colorectal cancer mortality in the cancer prevention study II, JNCI Cancer Spectr. 92 (2000) 1888–1896.
[64] B. Diergaarde, A. Vrieling, A.A. van Kraats, G.N.P. van Muijen, F.J. Kok, E. Kampman, Cigarette smoking and genetic alterations in sporadic colon carcinomas, Carcinogenesis 24 (2003) 565.
[65] X. Hu, C. Herzog, P. Zimniak, S.V. Singh, Differential protection against benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide-

induced DNA damage in HepG2 cells stably transfected with allelic variants of p class human glutathone S-transferases, Cancer Res. 59 (1999) 2358–2362.
[66] I.R. Rowland, T. Granli, O.C. Bockman, P.E. Key, R.C. Massey, Endogenous N-nitrosation in man assessed by measurement of apparent total N-nitroso compounds in feces, Carcinogenesis 12 (1991) 1395–1401.
[67] K.R. Silvester, S.A. Bingham, J.R.A. Pollock, J. Cummings, I. O'Neill, Effect of meat and resistant starch on fecal excretion of apparent N-nitroso compounds and ammonia from the human large bowel, Nutr. Cancer 29 (1998) 13–23.
[68] R. Hughes, A.J. Cross, J.R.A. Pollock, S. Bingham, Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation, Carcinogenesis 22 (2001) 199.
[69] S.A. Bingham, B. Pignatelli, J.R.A. Pollock, A. Ellul, C. Malaveille, G. Gross, S. Runswick, J.H. Cummings, I.K.O. O'Neill, Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? Carcinogenesis 17 (1996) 515–523.
[70] R. Preussmann, B.W. Steward, N-nitrosocarcinogens, in: C.E. Searle (Ed.), Chemical Carcinogens, ACS Monograph Series No 182, American Chemical Society, Washington, DC, 1984, pp. 643–823.
[71] T. Gichner, J. Veleminsky, Mechanisms of inhibition of N-nitroso compounds-induced mutagenicity, Mutat. Res. 202 (1988) 325–334.
[72] J. Imlay, S.M. Chin, S. Linn, Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro, Science (1988) 640–642.
[73] I.T. Johnson, The potential role of iron in colorectal cancer, ECP-News 31 (1997) 18–20.
[74] O.I. Aruoma, B. Halliwell, E. Gajewski, M. Dizdaroglu, Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates, J. Biol. Chem. 264 (1989) 20509–20512.
[75] M. Glei, A. Klinder, G.O. Latunde-Dada, T.W. Becker, U. Hermann, K. Voigt, B.L. Pool-Zobel, Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A, Mutat. Res. 519 (2002) 151–161.
[76] E.K. Lund, S.G. Wharf, S.J. Fairweather-Tait, I.T. Johnson, Oral ferrous sulfate supplements increase the freee radical-generating capacity of feces from healthy volunteers, Am. J. Clin. Nutr. 69 (1999) 250–255.
[77] J.G. Erhardt, S.S. Lim, J.C. Bode, C. Bode, A diet rich in fat and poor in dietary fiber increases the in vitro formation of reactive oxygen species in human feces, J. Nutr. 127 (1998) 706–709.
[78] R.W. Owen, B. Spiegelhalder, H. Bartsch, Generation of reactive oxygen species by the faecal matirx, GUT 46 (2000) 225–232.
[79] A.L.A. Sesink, D.S.M.L. Termont, J.H. Kleibeuker, R. Van der Meer, Red meat and colon cancer: the cytotoxic and hyperproliferative effects of dietary heme, Cancer Res. 59 (1999) 5704–5709.
[80] H. Esterbauer, P. Eckl, A. Ortner, Possible mutagens derived from lipids and lipid precursors, Mutat. Res. 238 (1990) 223–233.
[81] C. Janzowski, V. Glaab, E. Samimi, J. Schlatter, B.L. Pool-Zobel, G. Eisenbrand, Food relevant alpha, beta-unsaturated carbonyl compounds: in vitro toxicity genotoxic (mutagenic) effectiveness and reactivity towards glutathione, in: G. Eisenbrand, A.D. Dayan, P.S. Elias, W. Grunow, J. Schlatter (Eds.), Carcinogenic/Anticarcinogenic Factors in Food, Wiley VCH, 2000, pp. 469–474.
[82] B. Nanduri, J.B. Hayden, Y.C. Awasthi, P. Zimniak, Amino acid residue 104 in an a-class glutathione S-transferase is essential for the high selectivity and specificity of the enzyme for 4-hydroxynonenal, Arch. Biochem. Biophys. 335 (2002) 305–310.
[83] N.S. Waleh, J. Calaoagan, B.J. Murphy, A.M. Knapp, R.M. Sutherland, K.R. Laderoute, The redox-sensitive human antioxidant-responsive element induces gene expression under low oxygen conditions, Carcinogenesis 19 (1998) 1337.
[84] T.H. Rushmore, M.R. Morton, C.B. Pickett, The antioxidant-responsive element, J. Biol. Chem. 18 (1991) 11632–11639.
[85] T. Nguyen, P.J. Sherratt, C.B. Pickett, Regulatory mechanisms controlling gene expression mediated by the antioxidant response element, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 233–260.
[86] D.H. Chui, W. Tang, S.H. Orkin, C-DNA cloning of murine Nrf2 gene, coding for a p45 NF-E2 related transcription factor, Biochem. Biophys. Res. Commun. 209 (1995) 40–46.
[87] K. Itoh, T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, Y. Nabeshimat, An Nrf2/small Maf heterodimer mediates the induction of Phase II detoxifying enzyme genes through anti oxidant response elements, Biochem. Biophys. Res. Commun. 236 (1997) 313–322.
[88] D.D. Zhang, S.C. Lo, J.V. Cross, D.J. Templeton, M. Hannink, Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex, Mol. Cell Biol. 24 (2004) 10941–10953.
[89] S.B. Cullinan, J.D. Gordan, J. Jin, J.W. Harper, J.A. Diehl, The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase, Mol. Cell. Biol. 24 (2004) 8477–8486.
[90] M. Furukawa, Y. Xiong, BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase, Mol. Cell. Biol. 25 (2005) 162–171.
[91] D.D. Zhang, M. Hannink, Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress, Mol. Cell. Biol. 23 (2003) 8137–8151.
[92] N. Wakabayashi, A.T. Dinkova-Kostova, W.D. Holtzclaw, M.I. Kang, A. Kobayashi, M. Yamamoto, T.W. Kensler, P. Talalay, Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 2040–2045.
[93] H. Motohashi, T. O'Connor, F. Katsuoka, J.D. Engel, M. Yamamoto, Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors, Gene 294 (2002) 1–12.

[94] j. Jeyapaul, A.K. Jaiswal, Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene, Biochem. Pharmacol. 59 (2000) 1433–1439.
[95] P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation, Biochim. Biophys. Acta 1072 (1991) 129–157.
[96] B.R. Kim, R. Hu, Y.S. Keum, V. Hebbar, G. Shen, S.S. Nair, A.N. Kong, Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane, Cancer Res. 63 (2003) 7520–7525.
[97] M. Ramos-Gomez, M.K. Kwak, P.M. Dolan, K. Itoh, M. Yamamoto, P. Talalay, T.W. Kensler, Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 3410–3415.
[98] C. Chen, R. Yu, E.D. Owuor, A.N. Kong, Activation of antioxidant-response element (ARE), mitogen activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death, Arch. Pharm. Res. 23 (2000) 605–612.
[99] R. Pinkus, L.M. Weinert, V. Daniel, Role of oxidants and antioxidants in the induction of AP-1, NF-kB, and glutathione S-transferase gene expression, J. Biol. Chem. 271 (1996) 13422–13429.
[100] F. Desmots, C. Rauch, C. Henry, A. Guillouzo, F. Morel, Genomic organization, 5′-flanking region and chromosomal localization of the human glutatione transferase A4 gene, Biochem. J. 336 (1998) 437–442.
[101] R. Whalen, T. Boyer, Human glutathione S-transferases, Semin. Liver Dis. 18 (1998) 345–358.
[102] J. Cummings, Short-chain fatty acids in the human colon, GUT 22 (1981) 763–779.
[103] M.N. Ebert, G. Beyer-Sehlmeyer, U.M. Liegibel, T. Kautenburger, T.W. Becker, B.L. Pool-Zobel, Butyrate-induced activation of glutathione S-transferases protects human colon cells from genetic damage by 4-hydroxynonenal, Nutr. Cancer 41 (2001) 156–164.
[104] L.C. Boffa, J.R. Lupton, M.R. Mariani, M. Ceppi, H.L. Newmark, A. Scalmati, M. Lipkin, Modulation of colonic epithelial cell proliferation, histone acetylation and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats, Cancer Res. 52 (1992) 5906–5912.
[105] P.A. Marks, R.A. Rifkind, V.M. Richon, R. Breslow, T. Miller, W. Kelly, Histone deacetylases and cacner: causes and therapies, Nat. Rev. Cancer 1 (2001) 194–202.
[106] P.A. Marks, V.M. Richon, R.A. Rifkind, Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells, J. Natl. Cancer Inst. 92 (2000) 1210–1216.
[107] D.C. Drummond, C.O. Noble, D.B. Kirpotin, Z. Guo, G.K. Scott, C.C. Benz, Clinical development of histone deacetylase inhibitors as anticancer agents, Annu. Rev. Pharmacol. Toxicol. 28 (2004) 28.
[108] L. Kopelovich, J.A. Crowell, J.R. Fay, The epigenome as a target for cancer chemoprevention, J. Natl. Cancer Inst. 95 (2003) 1747–1757.
[109] J. Kiefer, G. Beyer-Sehlmeyer, B.L. Pool-Zobel, Wirkung von Na-Butyrat und Trichostatin A auf den Histon-Acetylierungsstatus in HT29 Kolonkarzinom-Zellen, in: Proceedings of the 19th Annual Conference of the German Society for Nutrition, Jena, 9–11 March 2002, 2002, Ref type: Abstract.
[110] P. Perrin, F. Pierre, Y. Patry, M. Champ, M. Berreur, G. Pradal, P. Bornet, K. Meflah, J. Menenteau, Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats, GUT 48 (2001) 53–61.
[111] G. Beyer-Sehlmeyer, M. Glei, F. Hartmann, R. Hughes, C. Persin, V. Böhm, I.R. Rowland, R. Schubert, G. Jahreis, B.L. Pool-Zobel, Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources, Br. J. Nutr. 90 (2003) 1057–1070.
[112] A. Klinder, E. Gietl, R. Hughes, N. Jonkers, P. Karlsson, H. McGlyn, S. Pistoli, K.M. Tuohy, J. Rafter, I.R. Rowland, J. Van Loo, B.L. Pool-Zobel, Gut fermentation products of inulin-derived prebiotics inhibit markers of tumor progression in human conon tumor cells, Int. J. Cancer Prev. 1 (2004) 19–32.
[113] M.B. Sporn, N.M. Dunlop, D.L. Newton, J.M. Smith, Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids), Fed. Proc. 35 (1976) 1332–1338.
[114] I.T. Johnson, G. Williamson, S.R.R. Musk, Anticarcinogenic factors in plant foods: a new class of nutrients, Nutr. Res. Rev. 7 (1994) 175–204.
[115] T.W. Kensler, K.J. Helzlsouer, Oltipraz: clinical opportunities for cancer chemoprevention, J. Cell Biochem. 22 (Suppl.) (1995) 101–107.
[116] E.M.M. van Lieshout, W.H.M. Peters, J.B. Jansen, Effect of oltipraz, alpha-tocopherol, betacarotene and phenethylisothiocyanate on rat oesopageal, gastric, colonic and heptatic glutathone, glutathione S-transferase and peroxidase, Carcinogenesis 17 (1996) 1439–1445.
[117] N. Knoll, C. Ruhe, M. Glei, E.P. Gallagher, J. Sauer, S. Veeriah, B.L. Pool-Zobel, Associations of butyrate-mediated glutathione S-transferase (GSTA4-4) induction, cellular glutathione depletion and genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells, Toxicol. Sci. 86 (2005) 27–35.
[118] T. Katoh, N. Nagata, Y. Kuroda, H. Itoh, A. Kawahara, N. Kuroki, R. Ookuma, D.A. Bell, Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genetic polymorphism and susceptibility to gastric and colorectal adenocarcinoma, Carcinogenesis 17 (1996) 1855–1859.
[119] N.T. Brockton, UGT1A1 polymorphisms and colorectal cancer susceptibility, GUT 50 (2002) 749.
[120] M. Ichiba, Y. Wang, H. Oishi, M. Iyadomi, N. Shono, K. Tomokuni, Smoking-related DNA adducts and genetic polymorphism for metabolic enzymes in human lymphocytes, Biomarkers 1 (1996) 211–214.
[121] L.L. Marchand, J.H. Hankin, L.R. Wilkens, L.M. Pierce, A. Franke, L.N. Kolonel, A. Seifried, L.J. Custer, W. Chang, A. Lum-Jones, T. Donlon, Combined effects of well-done red meat, smoking, and rapid N-acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk, Cancer Epidemiol. Biomarker Prev. 10 (2002) 1259–1266.

[122] J.W. Lampe, S. Peterson, *Brassica*, biotransformation and cancer risk: genetic polymorphisms alter the preventive effects of cruciferous vegetables, J. Nutr. 132 (2002) 2991–2994.
[123] H.J. Prochaska, C.L. Fernandes, Elevation of serum Phase II enzymes by anticarcinogenic enzyme inducers: markers for a chemoprotected state, Carcinogenesis 14 (1993) 2441–2445.
[124] S. Hecht, Inhibition of carcinogenesis by isothiocyanates, Drug Metab. Rev. 32 (2000) 395–411.
[125] Y. Zhang, P. Talalay, Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanism, Cancer Res. 54 (Suppl.) (1994) 1976s–19812.
[126] P. Talalay, J.W. Fahey, Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism, J. Nutr. 131 (2001) 3027S–3033S.
[127] Y. Zhang, R.H. Kolm, B. Mannervik, P. Talalay, Reversible conjugation of isothiocyanates with glutathione catalyzed by human glutathione transferases, Biochem. Biophys. Res. Commun. 206 (1995) 755.
[128] Y. Zhang, P. Talalay, Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes, Cancer Res. 58 (1998) 4632–4639.
[129] H. Adlercreutz, T. Fotsis, J.W. Lampe, et al., Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas-chromatography mass-spectrometry, Scand. J. Clin. Lab. Invest. 53 (1993) 5–18.
[130] M.L. Slattery, E. Kampman, W. Samowitz, B. Caan, J.D. Potter, Interplay between dietary inducers of GST and the GSTM-1 genotype in colon cancer, Int. J. Cancer 87 (2000) 728–733.
[131] S. Murray, B.G. Lake, S. Gray, A.J. Edwards, C. Springall, E.a. Bowey, G. Williamson, A.R. Boobis, N.J. Gooderham, Effect of cruciferous vegetable consumption on heterocyclic aromatic amine metabolism in man, Carcinogenesis 22 (2001) 1413–1420.
[132] S. Hecht, Chemoprevention of lung cancer by isothiocyanates, American Institute for Cancer Research, Dietary Phytochemicals in Cancer Prevention and Treatment, Plenum Press, New York and London, 1996, pp. 1–12.
[133] J.W. Lampe, C. Chen, S. Li, J. Prunty, M.T. Grate, D.E. Meehan, K.V. Barale, D.a. Dightman, Z.P.J.D. Feng, Modulation of human glutathione S-transferases by botanically defined vegetable diets, Cancer Epidemiol. Biomarker Prev. 9 (2000) 787–793.
[134] T. Prestera, W.D. Holtzclaw, Y. Zhang, P. Talalay, Chemical and molecular regulation of enzymes that detoxify carcinogens, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2965–2969.
[135] G. van Poppel, D.T. Verhoeven, H. Verhagen, R.A. Goldbohm, *Brassica* vegetables and cancer prevention. Epidemiology and mechanisms, Adv. Exp. Med. Biol. 472 (1999) 159–168.
[136] A. Seow, J.M. Yuan, C.L. Sun, D. Van Den Berg, H.P. Lee, M.C. Yu, Dietary isothiocyanates, glutathione S-transferase polymorphisms and colorectal cancer risk in the Singapore Chinese Health Study, Carcinogenesis 23 (2002) 2055–2061.
[137] I.T. Johnson, Glucosinolates: bioavailability and importance to health, Int. J. Vitam. Nutr. Res. 72 (2002) 26–31.
[138] C. Bonnesen, I.M. Eggleston, J.D. Hayes, Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines, Cancer Res. 61 (2001) 6120–6130.
[139] C. Xia, J. Hu, B. Ketterer, J.B. Taylor, The organization of the human GSTP1-1 gene promoter and its response to retinoic acid and cellular redox status, Biochem. J. 313 (Part 1) (1996) 155–161.
[140] F. Morceau, A. Duvoix, S. Delhalle, M. Schnekenburger, M. Dicato, M. Diederich, Regulation of glutathione S-transferase P1-1 gene expression by NF-kappaB in tumor necrosis factor alpha-treated K562 leukemia cells, Biochem. Pharmacol. 67 (2004) 1227–1238.
[141] J.D. Hayes, S.A. Chanas, C.J. Henderson, M. McMahon, C. Sun, G.J. Moffat, C.R. Wolf, M. Yamamoto, The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin, Biochem. Soc. Trans. 28 (2000) 33–41.
[142] R. Venugopal, A.K. Jaiswal, Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14960–14965.
[143] M. Lorper, A. Clairmont, C. Carlberg, H. Sies, Identification of two activating elements in the proximal promoter region of the human glutathione transferase-A1 and -A2 genes, Arch. Biochem. Biophys. 359 (1998) 122–127.
[144] K.C. Falkner, T.H. Rushmore, M.W. Linder, R.A. Prough, Negative regulation of the rat glutathione S-transferase A2 gene by glucocorticoids involves a canonical glucocorticoid consensus sequence, Mol. Pharmacol. 53 (1998) 1016–1026.
[145] E.Y. Park, I.J. Cho, S.G. Kim, Transactivation of the PPAR-responsive enhancer module in chemopreventive glutathione S-transferase gene by the peroxisome proliferator-activated receptor-gamma and retinoid X receptor heterodimer, Cancer Res. 64 (2004) 3701–3713.
[146] P.A. Bartley, R.A. Keough, J.K. Ludwyche, T.J. Gonda, Regulation of the gene encoding glutathione S-transferase M1 (GSTM1) by the Myb oncoprotein, Oncogene 22 (2003) 7570–7575.
[147] A. Kumar, E.P. Reddy, Genomic organization and characterisation of the promoter region of murine GSTM2 gene, Gene 270 (2001) 221–229.
[148] Y.V. Patskovsky, M.Q. Huang, T. Takayama, I. Listowsky, W.R. Pearson, Distinctive structure of the human GSTM3 gene-inverted orientation relative to the mu class glutathione transferase gene cluster, Arch. Biochem. Biophys. 361 (1999) 85–93.
[149] K. Ogura, T. Nishiyama, A. Hiratsuka, T. Watabe, T. Watabe, Isolation and characterization of the gene encoding rat class theta glutathione S-transferase subunit yrs, Biochem. Biophys. Res. Commun. 205 (1994) 250–256.
[150] J.E. Ladner, J.F. Parsons, C.L. Rife, G.L. Gilliland, R.N. Armstrong, Parallel evolutionary pathways for glutathione trans-

ferases:structure and mechanism of the mitochondrial class Kappa enzyme rGSTK1-1, Biochemistry 43 (2004) 352–361.
[151] A. Robinson, G.A. Huttley, H.S. Booth, P.G. Board, Modelling and bioinformatics studies of the human Kappa class glutathione transferase predict a novel third transferase family with homology to prokaryotic 2-hydroxychromene-2-carboxylate isomerases, Biochem. J. 379 (2004) 541–552.
[152] P.J. Jacobbson, R. Morgenstern, J. Mancini, A. Ford-Hutchinson, B. Persson, Common structural features of *MAPEG*–a widespread superfamily of membrane associated proteins with highly divergent functions in eicosanoid and glutathione metabolism, Protein Sci. 8 (1999) 689–692.
[153] P.J. Holm, R. Morgenstern, H. Herbert, The 3-D structure of microsomal glutathione transferase 1 at 6 Å resolution as determined by electron crystallography of p22121 crystals, Biochim. Biophys. Acta 1594 (2002) 276–285.
[154] B.C. Zhang, Y.R. Zhu, J.B. Wang, Y. Wu, Q.N. Zhang, G.S. Qian, S.Y. Kuang, Y.-F. Li, X. Fang, L.Y. Yu, S. DeFlora, L.P. Jacobson, A. Zarba, P.A. Egner, X. He, J.S. Wang, B. Chen, C.L. Enger, N.E. Davidson, G.B. Gordon, M.B. Gorman, H.J. Prochaska, J.D. Groopman, A. Munoz, K.J. Helzlsouer, T.W. Kensler, Oltipraz chemoprevention trial in Qidong, Jiangsu Province, People's Republic of China, J. Cell. Biochem. Suppl. 28/29 (1997) 166–173.
[155] S.S. Ansher, P. Dolan, E. Bueding, Biochemical effects of dithiolthiones, Food Chem. Toxicol. 24 (1986) 415.
[156] T.W. Kensler, P.A. Egner, P.M. Dolan, J.D. Groopman, B.D. Roebuck, Mechanism of protection against aflatoxin tumorigenicity in rats fed 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-ones, Cancer Res. 47 (1987) 4271–4277.
[157] N.E. Davidson, P.A. Egner, T.W. Kensler, Transcriptional control of glutathione S-transferase gene expression by the chemoprotective agent 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz) in rat liver, Cancer Res. 50 (1990) 2251–2255.
[158] P.A. Egner, T.W. Kensler, T. Prestera, P. Talalay, A.H. Libby, H.H. Joyner, T.J. Curphey, Regulation of phase 2 enzyme induction by oltipraz and other dithiolethiones, Carcinogenesis 15 (1994) 177–181.
[159] F. Morel, O. Fardel, D.J. Meyer, S. Langouet, K.S. Gilmore, B. Meunier, C.P.D. Tu, T.W. Kensler, B. Ketterer, A. Guillouzo, Preferential increase of glutathione S-transferase class a transcripts in cultured human hepatocytes by pheobarbital, 3-methylcholanthrene, and dithioethiones, Cancer Res. 53 (1993) 231–234.
[160] J.S. Wang, X. Shen, X. He, Y.R. Zhu, B.C. Zhang, J.B. Wang, G.S. Qian, S.Y. Kuang, A. Zarba, P.A. Egner, L.P. Jacobson, A. Munoz, K.J. Helzlsouer, J.D. Groopman, T.W. Kensler, Protective alterations in Phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China, J. Natl. Cancer Inst. 91 (1999) 347–354.

2.5 **Publication V:** ***Veeriah S,*** *Miene C, Habermann N, Hofmann T, Klenow S, Sauer J, Böhmer FD, Wölfl S, Pool-Zobel BL.* "Apple polyphenols modulate expression of selected genes related to toxicological defence and stress response in human colon adenoma cells". *Submitted, 2007*

An important mechanism of antigenotoxicity is the induction of phase II detoxifying enzymes. Apples contain significant amounts of polyphenols which are antigenotoxic and chemoprotective by this mechanism. The purpose of this study was to investigate whether polyphenols from apples modulate expression of genes related to colon cancer prevention in preneoplastic cells derived from colon adenoma (LT97). For this, LT97 cells were treated with apple extracts (AE). RNA was isolated and gene expression studies were performed using cDNA-arrays contains genes related to mechanisms of carcinogenesis or chemoprevention. Real-time PCR and enzyme activity assays were additionally performed to confirm selected array results. Treatment of cells with AE altered several genes including GSTs and UGTs. The enzyme activities of GSTs and UGTs were altered by treatment of LT97 cells with AE. The observed altered gene expression patterns in LT97 cells resulting from AE treatment points to a possible protection of the cells against some toxicological insults. Our approach to determine this specific profile of gene expression in preneoplastic human cells provides a relevant possibility to identify target genes and agents that could contribute to chemoprotection in colon mucosa cells.

Own contribution to the manuscript:

- Establishment of custom-made cDNA-microarray system in the lab
- Cell culture and RNA isolation, execution of the cDNA-arrays, gene expression analysis and verification of array genes by real-time PCR
- Data evaluation, interpretation and representation of the results

Apple polyphenols modulate expression of selected genes related to toxicological defence and stress response in human colon adenoma cells

Selvaraju Veeriah, Claudia Miene, Nina Habermann, Thomas Hofmann, Stefanie Klenow, Julia Sauer, Frank Böhmer[ψ], Stefan Wölfl[‡], Beatrice Louise Pool-Zobel*

Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University, Dornburger Str. 24, D-07743 Jena, Germany

[ψ]*Institute of Molecular Cell Biology, Medical Faculty, Friedrich-Schiller-University, Drackendorfer Str. 1, D-07747 Jena, Germany*

[‡]*Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany*

Address of corresponding author*

Prof. Dr. Beatrice L. Pool-Zobel

Friedrich-Schiller-University Jena

Institute for Nutritional Sciences

Department of Nutritional Toxicology

Dornburger Str. 24

07743 Jena, Germany

Tel. +49 3641 949670

Fax. +49 3641 949672

E-mail: b8bobe@uni-jena.de

ABSTRACT

Apples contain significant amounts of flavonoids that are potentially cancer risk reducing by acting antioxidative or antiproliferative and by favourably modulating gene expression. The purpose of this study was to investigate whether polyphenols from apples modulate expression of genes related to colon cancer prevention in preneoplastic cells derived from colon adenoma (LT97). For this, LT97 cells were treated with effective concentrations of apple extracts (AE). RNA was isolated and used for synthesis and labelling of cDNA which was hybridized to cDNA-arrays. Gene expression studies were performed using a commercial cDNA-array from Superarray® which contains a limited number of genes (96 genes) related to drug metabolism and a custom-made cDNA microarray which contains a higher number of genes (300 genes, including some genes from Superarray®) related to mechanisms of carcinogenesis or chemoprevention. Real-time PCR and enzyme activity assays were additionally performed to confirm selected array results. Treatment of cells with AE resulted in 30 and 46 genes expressed over cut-off values (≥1.5 or ≤0.7-fold) in Superarray® and custom array, respectively. Of 87 genes spotted on both arrays, four genes (*CYP3A7, CYP4F3, CHST7, GSTT2*) were regulated with similar directional changes. Expression of selected phase II genes (*GSTP1, GSTT2, GSTA4, UGT1A1, UGT2B7*) regulated on either array was confirmed by real-time PCR. The enzyme activities of glutathione *S*-transferases and UDP-glucuronosyltransferases were altered by treatment of LT97 cells with AE. The observed altered gene expression patterns in LT97 cells, resulting from AE treatment, points to a possible protection of the cells against some toxicological insults.

Keywords: Apple polyphenols, colon cancer chemoprevention, gene expression, LT97 cells, microarray

INTRODUCTION

Colon cancer is among the most common types of epithelial malignancies in both genders, affecting the Western world and most probably related to dietary habits [1]. Although debatable, it is thus a promising expectation that changes in diet, such as the frequent consumption of fruits and vegetables with high contents of polyphenols and flavonoids, may reduce the risk of developing carcinoma of the colon [2]. One mechanism is thought to occur through the composition of the luminal environment that may alter the expression of genes in the intestinal epithelium and thus, directly influence disease. Experimental studies have shown that selected polyphenolic compounds can affect cellular processes that are important for cancer development. For instance, *in vitro* studies with colorectal cancer cell lines have demonstrated that flavonoids (e.g. quercetin, phloretin) are able to inhibit cell proliferation, induce cell differentiation and apoptosis, and enhance anti-inflammatory responses [3;4]. The chemopreventive properties of various flavonoid compounds also have been found to include the induction of carcinogen detoxifying systems [5], interaction with cellular signalling pathways, and modulation of gene expression [6].

Apples have significant amounts of polyphenolic compounds [7] and are an important source of flavonoids in peoples' diets in the United States of America and in Europe [8]. Effective absorption of the apple polyphenols from the mammalian colon has been shown *in vivo* and *in vitro* [9;10]. Other than antioxidative effects [11] and their ability to scavenge carcinogens, the apple polyphenols may be chemoprotective by affecting molecular events in the initiation, promotion, and progression stages of carcinogenesis [12]. A variety of cellular effectors have been recognized as targets for actions of apple flavonoids as well. One recent *in vitro* study has shown that polyphenolic procyanidins from apples can increase the expression of extracellular signal-regulated kinase 1 and 2 (ERK1, 2), c-Jun N-terminal kinase (JNK), activity of caspase-3, inhibit G2/M phase cell cycle arrest and suppress PKC in SW620 cells [13]. The epidermal growth factor receptor (EGFR) signalling, that plays an important role in the regulation of cell proliferation, was substantially inhibited by a polyphenol-rich apple extract [14]. On the other hand, the *PTPRJ* gene, encoding the protein-tyrosine phosphatase DEP-1, a candidate tumour suppressor with antiproliferative activity in colon epithelial cells, was induced upon cell treatment with apple polyphenols [15]. Interestingly, AE can significantly reduce the DNA

strand breakage and the proliferation of rat colon cells isolated from animals pretreated with dimethylhydrazine (DMH) [16]. Another *in vivo* rat study showed that intervention with apple procyanidins reduced the number of aberrant crypt foci (ACF) and preneoplastic lesions initiated by azoxymethane (AOM) [13]. However, the molecular bases of the chemopreventive effects of flavonoids, in general, and of apple flavonoids, in particular, are poorly understood.

Gene expression studies using microarrays can yield important information on the mechanisms of how dietary components may influence colorectal carcinogenesis. We have reported previously that a HT29 colon carcinoma cell line treated with AE differentially modulated the expression of several genes associated with the process of chemoprevention [4]. We therefore, proposed that the upregulation of chemoprevention-related genes, especially those involved on phase II metabolism such as, glutathione *S*-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) could potentially reduce the availability of carcinogens to the healthy colonic mucosa, thereby preventing the carcinogen from acting directly on the tissue. In support of this proposal, now it was of high interest to explore the effect of AE on gene expression in preneoplastic colon cells that are not tumourigenic. Therefore in this study, we have used as target cells a recently established human colon adenoma (LT97) cell line, which represents preneoplastic lesions [17]. We sought to identify genes specifically related to mechanisms of carcinogenesis or chemoprevention using cDNA microarrays, which allow the simultaneous detection of expression of a high number of genes [18].

First, cDNA microarray analysis of AE (128 µg/ml) treated LT97 cell line was performed using a commercially available cDNA array membrane (SuperArray® Inc., Frederick, USA) which, however, contained only a limited number of genes (96 human drug metabolism genes) and the analysis of more of the relevant target genes of interest would have necessitated the use additional arrays. Therefore, a more focussed, custom-made cDNA microarray (PIQOR™, Memorec, Germany) was designed which was spotted with 300 human genes involved in distinct pathways of carcinogenesis. It was commercially produced (Miltenyi Biotec, Köln, Germany) according to our selection of the genes that was based on (a) functionality of the gene products in chemoprevention or carcinogenesis and (b) expression levels in the numerous cell systems used in our laboratory. The baseline expression levels had previously been determined with

Superarray® membranes in several types human colon [19], breast [20] and prostate [21] cells and it was the aim of this study to assess the modulation of their expression in LT97 cells treated with AE.

MATERIALS AND METHODS

Apple polyphenols extract

The apple extract (AE) was produced as has been described before [4] and was kindly provided by Dr. Frank Will and Prof. Dr. Helmut Dietrich (Institute for Oenology and Beverage Research, Geisenheim, Germany). An aliquot was used for analysis of individual polyphenol ingredients, such as chlorogenic acid, which was identified as one of the major flavonoids of the AE [4]. Other aliquots were needed for determining biological properties in the colon cells.

Cell line and culture conditions

The human colon adenoma cell line LT97 was established from colon micro adenomas of a patient with Familial Adenoma Polyposis coli [17] and were kindly provided by Brigitte Marian (Institute of Cancer Research, University of Vienna, Vienna, Austria). The LT97 cell line was cultured as previously described [19] but instead of 2.5 U/ml PenStrep, 10 mg/ml Gentamicin (Invitrogen GmbH, Karlsruhe, Germany) was used. The cells between passages 21 and 32 were used for the experiments described here.

Cell treatment and RNA extraction

Cell growth assays had been previously conducted to determine effective concentration ranges of the apple polyphenol extract in LT97 cells. The studies showed that AE concentrations of 128 and 255 µg/ml reduced cell numbers to 93 % and 83 %, respectively, after treatment for 24h. Prolonged treatment resulted in pronounced loss of numbers of cells compared to the medium control after 72 hours to 82 % and 71 %, respectively [22]. In the present study these two concentrations were used to test if there were any differences in biological effects on gene expression and enzyme activities. The LT97 cells were subcultured in the T25 flask (Falcon, UK). 72 h later, cells were treated with 128 and 255 µg/ml concentrations of AE for 24 h. LT97 cells were harvested by incubation with EDTA-PBS and then resuspended in 5 ml of medium. After centrifugation, the cell pellet was resuspended and washed once with ice-cold PBS (pH 7.4). Total RNA from these cells was isolated using a RNeasy mini kit (Qiagen GmbH, Hilden, Germany). RNA was checked for stability and DNA contamination by gel electrophoresis. The purity of RNA samples was determined based on the ratio of spectrophotometric absorbance of the sample at 260 nm to that of 280 nm (A260/A280) using a NanoDrop ND-1000 photometer

(Wilmington, USA)**.** Only RNA samples with A260/A280 ratio ≥ 1.8 were used in further experiments.

Superarray® analysis

The Superarray® system (SuperArray® Inc., Frederick, USA) was utilized to compare the relative levels of mRNA expressed in LT97 cells with or without AE treatment (128 µg/ml). The Superarray® (Cat #: HS-011-N) contained 96 spotted genes probes related to drug metabolism. cDNA synthesis, probe preparation, and hybridizations to the array were done, as previously described in quadruplicate [4]. For each set of quadruplicates the mean value for each gene was determined and used to calculate the fold-changes (treatment versus medium control). Using cut-off criteria, a 1.5-fold induction or a 0.7-fold repression in expression were considered to be of biological importance.

Custom-made cDNA microarray analysis

In addition to the drug metabolism related genes it was of interest to also analyse a broader range of genes involved in additional mechanisms of carcinogenesis or chemoprevention. This was done using a customized cDNA-array system (Miltenyi Biotec, Germany) spotted with 300 genes belonging to nine functional categories, namely: 1) phase I metabolism, 2) phase II metabolism, 3) phase III metabolism, 4) phase II gene regulation pathway, 5) stress and signal transduction pathway, 6) apoptosis signalling pathway, 7) tumour suppressor genes, 8) cell cycle arrest/regulation of cell cycle and 9) miscellaneous (several functions). The total RNA prepared for Superarray® analysis was 3 years old and therefore, the new batch of total RNA was isolated from LT97 cells treated with AE (128 µg/ml) for 24 h. First-strand cDNA was synthesized from 2 µg of total RNA with a primer incorporating a T7-RNA polymerase promoter. After second-strand cDNA synthesis (Invitrogen GmbH, Karlsruhe, Germany), cRNA amplification was performed with RNA polymerase (Ambion**,** Cambridgeshire, UK). Subsequently, the cRNA was used as template to prepare labelled cDNAs with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Uppsala, Sweden). Labelled cDNAs were hybridized to the custom cDNA-arrays overnight at 65°C by following the manufacturer's instructions.

Preprocessing of custom array data

Slides ware scanned by GenePix® 4000B microarray scanner and preprocessing of the custom arrays data was carried out using GenePix Pro v6.0 software (Molecular Devices, Union City, USA). Experiments were independently reproduced four times and the average intensity of each set of probes was used for normalization based on the GAPDH gene. The differential gene expression was analysed by using the same cut-off value (≥1.5 or ≤0.7-fold change) as described for the Superarray® analysis and changes in genes of interest were confirmed by real-time PCR analysis.

Quantification of mRNA levels by real-time PCR

The relative differences in expression levels of selected genes of interest (*GSTT2, GSTP1, GSTA4, UGT1A1, UGT1A4 and UGT2B7)* were evaluated by real-time PCR using the iCycler iQ® (Bio-Rad GmbH München, Germany) system. First-strand cDNA probes were prepared as reported previously [4]. The real-time PCR experiments were performed with gene specific primers (Table 1). A standard curve was generated for each run using cDNA-templates to determine the PCR amplification efficiency of the target and of an endogenous reference. Each experiment was independently repeated three times, with in each sample was run in duplicate each time. The relative mRNA expression levels of targets genes were calculated as previously described [4].

Total GST and UGT enzyme activity assays

LT97 medium (control) and AE (128 and 255 μg/ml, 24 h) treated cells were washed with PBS, and harvested with 5 mM EDTA in PBS and the cytosol was extracted as previously described [23]. Total GST enzyme activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the procedure of Habig *et al*. [24]. Enzyme activities were normalized to total cytosolic protein measured according to the Bradford method [25]. Mean enzyme activities for AE-treated cells were calculated and the fold-induction enzyme activities determined by taking a ratio of AE-treated enzyme activities to the controls.

UGT enzyme activity was determined with 4-methylumbelliferone (4MU, 50 μM) as a substrate in 96-well microtiter plates as described previously by Collier *et al*. [26]. The rate of UGT

activity was determined by the 4MU-dependent, fluorescent reduction during 4MU-glucuronidating activity. The microsomal proteins were prepared as described by Collier *et al*., cells were resuspended with homogenisation buffer (0.1 M Tris, 2 mM Pefabloc, pH 7.8), followed by ultrasonification and centrifugation (9,000 g, 20 min, 4°C). 105 µl of 4MU, 30 µl homogenization buffer, 30 µl of microsomal proteins were added to the reaction mixture and reactions were started by the addition of 20 µl of UDPGA (0.2 M) in a final volume of 155 µl. Blank controls for each experiment were incubated in the presence of assay buffer (0.1 M Tris-HCl, 5 mM $MgCl_2$, 0.05 % BSA, pH 7.4) instead of microsomal proteins. Using a microtiter plate reader (SpectraFLUOR Plus, Tecan, Austria) readings were obtained at Ex/Em 360/465 nm for 60 min at 5 min intervals, at 37°C. Mean specific enzyme activities were calculated from the slope and the fold-inductions were calculated by taking a ratio of the values obtained for AE treated LT97 cells and for the medium controls.

Statistical Analysis

Statistical analysis was performed using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA) and Microsoft Excel 2003 (Microsoft Corporation, USA), based on the mean values of independently reproduced experiments. All experiments performed *in vitro* were repeated at least three times unless otherwise indicated. Significant differences between treatment and control values were determined by the two-tailed student *t*-test and one-way ANOVA with Bonferroni's post test. The values of $p \leq 0.05$ were taken to be statistically significant.

RESULTS

Effects of apple flavonoids on gene expression in LT97 cells (Superarray® analysis)

For Superarray® array analysis resulted in there was no statistically significant ($p \leq 0.05$, *t*-test) regulation of genes in LT97 cells in response to treatment with AE. However, on the basis of the evaluation criteria (≥1.5 or ≤0.7-fold), the analysis revealed an increased expression for 30 genes of the 96 spotted sequences after 24 h treatment with AE (Table 2). 25 of the 30 altered genes belonged the phase II genes and included major detoxifying enzyme systems of glutathione *S*-transferases family (seven genes, *GSTA3, GSTM5, GSTP1, GSTT2, MGST1, MGST2, MGST3*). Also five acetyltransferase genes (*CRAT, DLAT, HAT1, NAT1, NAT5*), five sulfotransferase genes (*CHST5, CHST6, CHST7, SULT1C1, TPST1*), three methyltransferase genes (*TPMT, HNMT, COMT*), two genes from UDP-glycosyltransferase family (*UGT1A1, UGT1A4*) and three epoxide hydrolase genes (*EPX1, EPH2, LT4H*) were upregulated. Three genes of the phase I cytochrome-p450 family (*CYP2B6, CYP3A7, CYP4F3*) and two miscellaneous function genes (*MT1E, ABCC3*) were altered as well.

Effects of apple flavonoids on gene expression in LT97 cells (custom-made cDNA microarray analysis)

Custom array results indicated that expression of 14 genes was significantly ($p \leq 0.05$, *t*-test) modulated (Table 3). Moreover, a total of 46 genes were upregulated on the basis of the evaluation criteria (≥1.5 or ≤0.7-fold) in response to AE (128 µg/ml) for 24 h. The target genes were all upregulated, and none of the genes were down regulated. Notably, a relatively higher number of carcinogen metabolism-related genes tended to be upregulated (19 genes) by AE treatment than genes belonging to the other functional categories. The mRNA of glutathione *S*-transferases (*GSTT2* [p=0.009], *GSTA4* [p=0.022]), carbohydrate sulfotransferases (*CHST3* [p=0.016], *CHST7*) and UDP-glucuronosyltransferases (*UGT1A6, UGT2B7* [p=0.056]) were markedly elevated. Treatment of LT97 cells with AE also resulted in upregulated expression of genes related to cell cycle control (*CDKN1A, NFKB2* [p=0.008], *NFKBIB* [p=0.039], *CGRRF1* [p=0.039]), apoptosis signalling (*CASP10* [p=0.009], *WNT3*), oxidative stress *(GPX2, FMO1, HSPA5, UBE2D1)* and tumour suppression (*BRCA2, PTPRJ* [p=0.019], *PTPN6* [p=0.034], *PTPRN, PTPRT*).

Comparison of results from Superarray® and custom array analysis

Of the 96 genes present on the Superarray®, 87 genes were also spotted on the custom array (see Figure 1 for detailed information). Of these 87 genes, 30 were found upregulated in the Superarray® analysis of which only 4 were similarly altered in the custom array analysis (*CYP3A7, CYP4F3, GSTT2, CHST7*) in response to AE treatment in LT97 cells. Thus 86 % of the genes detected in the superarray experiments were not identified with the custom array. This can be explained with a higher sensitivity of the Superarray® in comparison to the custom array. However, the differences obtained by custom array were statistically significant and the differences obtained from superarray were not statistically significant meaning that the custom array platform seemed to be higher accuracy. This discrepancy indicates that results from screening using cDNA-arrays must be confirmed by additional methods as real-time PCR analysis described below.

Real-time PCR analysis for mRNA enhanced expression in LT97 cells by apple flavonoids

To confirm gene expression differences observed from microarray results, real-time PCR was performed on 6 genes (*GSTA4, GSTT2, GSTP1, UGT1A1, UGT1A4, UGT2B7*). The choice of these genes had been based on their functional importance and expression levels in the human cell model. We analyzed the mRNA expression levels in response to two AE concentrations (128 and 255 µg/ml). Figure 2 shows that RNA from LT97 cells, thus treated, revealed modulation of *GSTP1* (1.7, 1.2-fold), *GSTT2* (1.9, 1.4-fold), *GSTA4* (1.8, 1.4-fold), *UGT1A1* (1.1, 1.8-fold) and *UGT2B7* (2.7, 1.9-fold) which were all similar directional changes as those revealed in the analysis of the arrays. However, *UGT1A4* (0.7, 0.5-fold, data not shown) was altered in the opposite direction as in the Superarray® (Table 2). With the exception of *UGT1A1,* which was most effectively altered at the higher AE concentration (255 µg/ml, which was cytotoxic), the 128 µg/ml was most effective in eliciting modulated responses of gene expression. The mRNA expression levels of all of these verified genes were significantly ($p \leq 0.05$) altered in response to AE treatment in LT97 cells (Figure 2).

Induction of total GST and UGT enzyme activities in colon cells by apple polyphenols

In addition to the induction of GSTs and UGTs transcript levels, also total GST and UGT enzyme activities were assessed in LT97 cells treated with AE (128 and 255 µg/ml). The

analysis revealed that GST and UGT enzyme activities seemed to be altered in LT97 cells in response to the AE treatment (Figure 3). The altered GST (3.8, 2.8-fold) enzyme activities, however, were not significantly different from the control (Figure 3a), whereas the UGT (1.2, 1.5-fold) enzyme activities were enhanced significantly ($p \leq 0.001$) in the presence of the higher concentration (255 µg/ml) of AE which was cytotoxic (Figure 3b).

DISCUSSION

This study aimed to assess the effects of complex mixtures of apple polyphenols on patterns of expression of genes related to toxicological defence and to mechanisms relevant for early stages of carcinogenesis. According to Superarray® and custom array analysis, it was observed that 30 and 46 genes were upregulated by treatment of LT97 cells with AE, respectively. Among these, 83 % (25 genes from Superarray) and 41 % (19 genes from custom array) of the genes are potentially involved in the key processes of phase II detoxification metabolism, such as GSTs and UGTs. Genes belonging to these groups were selected for more detailed studies to verify their responses to AE. In particular *GSTT2, GSTP1, GSTA4, UGT1A1, UGT2B7* were also up regulated according to real-time PCR analysis. The products of these target genes are involved in detoxification and excretion of carcinogens, including putative food carcinogens such as polycyclic aromatic hydrocarbons and heterocyclic amines [27]. Moreover, some GSTs are known to have peroxidase activity, like GSTT2 [28]. Therefore, the upregulation of *GSTT2,* as observed here, could be an important mechanism of chemoprotection. The enhanced levels of *GSTT2* products possibly result in an elevated detoxification of peroxides. For instance the enzyme has pronounced substrate specificity towards cumene hydroperoxide (Cum-OOH) [28] which however is not physiologically available. Examples of physiological peroxides, are those formed during lipid peroxidation or arachidonic acid metabolism, such as arachidonic acid 15-hydroperoxide which potentially damages DNA and is pronounced substrate for rGSTT2 [29]. The metabolic deactivation of endogenously formed peroxides may indeed be favoured by enhanced *GSTT2* levels, thus providing cellular chemoprotection. This mechanism is also of interest in the case of an upregulated *GSTP1*, although the carcinogenic substrates that may be deactivated belong to different chemical classes. One candidate of interest is benzo[a]pyrene diol epoxide (BPDE), a metabolically activated form of the polycyclic aromatic hydrocarbon benzo(a)pyrene [30]. Another target of interest is *GSTA4* which is involved in the detoxification of final lipid peroxide products, such as 4-hydroxy-2-nonenal (HNE) [31]. Thus its up regulation by AE could also be related to an antigenotoxic potential and chemoprotection of LT97 cells. UGTs such as, *UGT1A1, UGT2B7* are another group of phase II enzymes. They play a role in glucuronidation and subsequent elimination of potentially toxic xenobiotics, endogenous compounds (e.g. bilirubin) and exogenous carcinogenic compounds (e.g. heterocyclic amines, polycyclic aromatic hydrocarbons) [32;33].

In addition to upregulation of GSTs and UGTs mRNA, AE also seemed to alter GST and UGT enzyme activities in LT97 cells. This is meaningful since GSTs and UGTs potentially play important roles in cancer chemoprevention [34]. Both GST and UGT genes are regulated by the transcription factor Nrf2 [35]. Related to this mechanism is our finding from the custom array analysis that AE also resulted in an upregulation of four down stream transcription factors of Nrf2 namely, *PIK3CB, PIK3CG, FRA-2* and *AHR.* These transcription factors may result in the enhanced transcription of phase II genes such as GSTs, UGTs and NAD(P)H dehydrogenase, quinone 1 (NQO1) [36]. Moreover, the data of the Superarray® analysis showed that exposure of LT97 cells to AE triggers the transcription level of the *HAT1* gene. *HAT1* is a member of the histone gene family involved in histone acetylation, particularly of histone H4. As a consequence, histone acetylation alters nucleosomal conformation, which can increase the accessibility of transcriptional regulatory proteins to chromatin templates and subsequently increases gene expression, while deacetylation is associated with decreases in gene expression [37]. Together, these results show that activation of these transcription factors and the histone gene family by AE could be a relevant mechanism of transcriptional activation of phase II genes such as GSTs and UGTs.

In addition to the capacity of AE to modulate detoxification related genes, AE also seemed to modulate several other genes spotted on the custom array which are related to important functions such as, tumour suppression, cell cycle control, cell signalling as well as apoptosis. For instance, AE increased the expression of five established or candidate tumour suppressor genes namely, *BRCA2, PTPRT, PTPN6, PTPRN* and *PTPRJ* (*Dep-1*) in LT97 cells. Expression of *BRCA2* is reported to play a role in DNA repair and thus to maintain chromosomal stability [38]. *PTPRJ* (*Dep-1*), a newly identified candidate tumour suppressor gene, that has been reported to be involved in apoptosis, cell cycle arrest and tumour suppression. It's re-expression in *PTPRJ*-negative SW480 cells leads to inhibition of cell growth and cell migration, and induction of *PTPRJ* expression by apple polyphenols in HT29 cells [39]. These findings indicate that the upregulation of several tumour suppressor genes by AE might reduce the probability for tumour progression.

Custom array analysis also showed an increase of mRNA from genes involved in the cell cycle arrest and regulation of cell cycle, such as *CDKN1A, CGRRF1, ESR2, NFKB2, NFKBIB* and *TGFA* in response to AE treatment. Upregulation of these genes could modulate cell signalling, block progression of cell cycle and inhibit proliferation [40]. This is in accordance with previous findings showing that AE modulated growth and survival of LT97 cells with EC_{50} values of 650.9 µg/ml after 24 h treatment [41]. In addition, AE influenced genes from the stress and signal transduction pathways namely, *FMO1, GPX2, HSPA5* and *UBE2D1*, the products of which are have several functions such as peroxidase activity and ubiquitination [42]. Moreover, there was an AE-mediated upregulation of the apoptosis mediator *CASP10* which is part of the caspase cascade. Overexpression of *CASP10* (a caspase closely related to *CASP8*) induces apoptosis [43] and thus this the finding suggests that AE can modulate apoptosis signalling [13]. Indeed, induction of apoptosis in HT29 cells by treatment with relatively high concentrations of apple polyphenols has been shown [44].

Our previous study with Superarray® gene expression analysis of the human colon carcinoma cell line (HT29) treated with AE (510 µg/ml or 30 µM, Ph.E) showed 8 genes were altered and most of them were related to phase II metabolism gene family [4]. In our present study using the same Superarray® system in human colon adenoma (LT97) cell line, 30 genes were altered, even though the concentration of AE added to LT97 cells was lower (128 µg/ml) compared to the treatment used for the HT29 cells. Accordingly, apple polyphenols might have stronger biological effects in the preneoplastic colon epithelial cells than in adenoma cells or carcinoma cells. Moreover, validation of microarray results by real-time PCR with two different concentrations (128 and 255 µg/ml) of AE showed that induction was more pronounced with the lower concentration, indicating that AE can alter the gene expression in colon cells even at low concentrations.

In this study with LT97, the comparison of Superarray® to custom array data showed that of 87 genes mutually spotted on both arrays, only 4 genes (*CYP3A7, CYP4F3, GSTT2, CHST7*) were altered on both arrays which may be due to differences in sensitivity. These two genes (*CYP3A7, CYP4F3*) not involved in carcinogen activation and not yet described to be involved in colon carcinogenesis. The Venn diagram [45] was used to asses the across-platform agreement. It is

important to note that this was used to compare results from two different RNA preparation experiments, and thus we did not expect a perfect agreement. From the genes present on both arrays 11 genes were only detected as regulated on the custom array and 25 were only detected with Superarray®, respectively. Collectively, the results indicate that there were considerable differences in the responsiveness of the two arrays. Since the work up procedure was very similar, the observed differences were probably more due to the platform and to the different probes than to the processes involved in amplification and hybridisation [46]. Accordingly, additional methods were used to confirm mRNA expression data. The lower variation of spotted glass slide array is most likely due to the much lower dynamic rang of the system which is limited by the thermodynamics of the hybridisation process. A very crucial point which is often not considered when looking at data (Wölfl S *et al*., personal communication).

In conclusion, apple polyphenols are able to induce gene expression of enzymes related to tumour suppression, cell cycle arrest, regulation of cell cycle, apoptosis signalling, stress and signal transduction and, in particular, detoxification enzymes systems (GST and UGT) in LT97 adenoma cells. Statistically significant modulations were generally more pronounced in custom array than in Superarray® analyse. Based on gene expression analysis using real-time PCR, the lower concentration of AE was more effective. However, the higher concentration used here, which was also cytotoxic, was more effective in enhancing UGT enzyme activity. Such induction of detoxification enzymes in colon cells may account for the chemopreventive potential of apple polyphenols. However, one should realize that the dose of apple polyphenols used here (128 and 255 µg/ml) needs to be related to the amounts that can be reached in the human colon. Our recent studies in volunteers with ileostomy pouches have, for instance, revealed that a total of 16.0±3.5 mg polyphenols (16000 µg per bag with ~360 ml) could be retrieved from the samples of eleven subjects, who had consumed one litre of cloudy apple juice 2 hours before [47]. This may not be too far removed from the concentrations that reach the colon, but definite measurements will be needed with colon samples before coming to a conclusion. An additional consideration regards the total human diet which contains additional polyphenols from many more sources. Jointly, these might lead to a prolonged exposure and thus be able to exert effects on detoxification enzymes, such as GSTs and UGTs. The induction of these detoxification enzymes by AE or other plant foods in adenoma cells may detoxify

compounds that lead to further progression and thus contribute to chemoprotection in stages subsequent to the initiation stage. The hypothesis will need further experimental back up investigations, but the mechanism could be a promising contribution to cancer chemoprotection by diet.

Acknowledgements:

Funding for this project was provided by the Bundesministerium für Bildung und Forschung (BMBF, FKZ.01EA0103), Germany. We thank Prof. Brigitte Marian, Institute of Cancer Research, University of Vienna, Austria for the generous gift of LT97 adenoma cells. The author is deeply grateful to PD. Dr. Ferdinand von Eggeling and Dipl. Biol. Julia Diegmann at the Institute for Human genetics and Anthropology, Jena for the GenePix scanner, and our collaboration partner Dr. Frank Will and Prof. Dr. Helmut Dietrich for providing the apple extract. We are grateful to Dr. Herbert Thijs (Center for Statistics, Hasselt University, Belgium) for his statistical advice.

Reference List

1. Ferguson,L.R., Philpott,M., and Karunasinghe,N. (2004) Dietary cancer and prevention using antimutagens. *Toxicology*, **198**, 147-159.

2. Terry,P., Giovannucci,E., Michels,K.B., Bergkvist,L., Hansen,H., Holmberg,L., and Wolk,A. (2001) Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J.Natl.Cancer Inst.*, **93**, 525-533.

3. Kawaii,S., Tomono,Y., Katase,E., Ogawa,K., and Yano,M. (1999) Antiproliferative activity of flavonoids on several cancer cell lines. *Biosci.Biotechnol.Biochem.*, **63**, 896-899.

4. Veeriah,S., Kautenburger,T., Habermann,N., Sauer,J., Dietrich,H., Will,F., and Pool-Zobel,B.L. (2006) Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol.Carcinog.*, **45**, 164-174.

5. Canivenc-Lavier,M.C., Vernevaut,M.F., Totis,M., Siess,M.H., Magdalou,J., and Suschetet,M. (1996) Comparative effects of flavonoids and model inducers on drug-metabolizing enzymes in rat liver. *Toxicology*, **114**, 19-27.

6. Wenzel,U., Herzog,A., Kuntz,S., and Daniel,H. (2004) Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoid in human colon cancer cells. *Proteomics.*, **4**, 2160-2174.

7. Sun,J., Chu,Y.F., Wu,X., and Liu,R.H. (2002) Antioxidant and antiproliferative activities of common fruits. *J.Agric.Food Chem.*, **50**, 7449-7454.

8. Arts,I.C., Hollman,P.C., Feskens,E.J., Bueno de Mesquita,H.B., and Kromhout,D. (2001) Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women. *Eur.J.Clin.Nutr.*, **55**, 76-81.

9. Hollman,P.C., van Trijp,J.M., Buysman,M.N., van der Gaag,M.S., Mengelers,M.J., de Vries,J.H., and Katan,M.B. (1997) Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.*, **418**, 152-156.

10. Boyer,J., Brown,D., and Liu,R.H. (2004) Uptake of quercetin and quercetin 3-glucoside from whole onion and apple peel extracts by Caco-2 cell monolayers. *J.Agric.Food Chem.*, **52**, 7172-7179.

11. Wolfe,K., Wu,X., and Liu,R.H. (2003) Antioxidant activity of apple peels. *J.Agric.Food Chem.*, **51**, 609-614.

12. Yang,C.S., Landau,J.M., Huang,M.T., and Newmark,H.L. (2001) Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu.Rev.Nutr.*, **21**, 381-406.

13. Gosse,F., Guyot,S., Roussi,S., Lobstein,A., Fischer,B., Seiler,N., and Raul,F. (2005) Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis*, **26**, 1291-1295.

14. Kern,M., Tjaden,Z., Ngiewih,Y., Puppel,N., Will,F., Dietrich,H., Pahlke,G., and Marko,D. (2005) Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol.Nutr.Food Res.*, **49**, 317-328.

15. Balavenkatraman,K.K., Jandt,E., Friedrich,K., Kautenburger,T., Pool-Zobel,B.L., Ostman,A., and Bohmer,F.D. (2006) DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients. *Oncogene*, **25**, 6319-6324.

16. Barth,S.W., Fahndrich,C., Bub,A., Dietrich,H., Watzl,B., Will,F., Briviba,K., and Rechkemmer,G. (2005) Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis*, **26**, 1414-1421.

17. Richter,M., Jurek,D., Wrba,F., Kaserer,K., Wurzer,G., Karner-Hanusch,J., and Marian,B. (2002) Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro. *European Journal of Cancer*, **38**, 1937-1945.

18. Duggan,D.J., Bittner,M., Chen,Y., Meltzer,P., and Trent,J.M. (1999) Expression profiling using cDNA microarrays. *Nat.Genet.*, **21**, 10-14.

19. Pool-Zobel,B.L., Selvaraju,V., Sauer,J., Kautenburger,T., Kiefer,J., Richter,K.K., Soom,M., and Wolfl,S. (2005) Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, an approach in nutrigenomics. *Carcinogenesis*, **26**, 1064-1076.

20. Steiner,C., Peters,W.H., Gallagher,E.P., Magee,P., Rowland,I., and Pool-Zobel,B.L. (2006) Genistein protects human mammary epithelial cells from benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide and 4-hydroxy-2-nonenal genotoxicity by modulating the glutathione/glutathione S-transferase system. *Carcinogenesis*.

21. Raschke,M., Rowland,I.R., Magee,P.J., and Pool-Zobel,B.L. (2006) Genistein protects prostate cells against hydrogen peroxide-induced DNA damage and induces expression of genes involved in the defence against oxidative stress. *Carcinogenesis*, **27**, 2322-2330.

22. Veeriah,S., Hofmann,T., Glei,M., Dietrich,H., Will,F., Schreier,P., Knaup,B., and Pool-Zobel,B.L. (2007) Apple Polyphenols and Products Formed in the Gut Differently Inhibit Survival of Human Cell Lines Derived from Colon Adenoma (LT97) and Carcinoma (HT29). *J.Agric.Food Chem.*, **55**, 2892-2900.

23. Kautenburger,T., Beyer-Sehlmeyer,G., Festag,G., Haag,N., Kuhler,S., Kuchler,A., Weise,A., Marian,B., Peters,W.H.M., Liehr,T., Claussen,U., and Pool-Zobel,B.L. (2005)

The gut fermentation product butyrate, a chemopreventive agent, suppresses glutathione S-transferase theta (hGSTT1) and cell growth more in human colon adenoma (LT97) than tumor (HT29) cells. *Journal of Cancer Research and Clinical Oncology*, **131**, 692-700.

24. Habig,W.H., Pabst,M.J., and Jakoby,W.B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J.Biol.Chem.*, **249**, 7130-7139.

25. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.*, **72**, 248-254.

26. Collier,A.C., Tingle,M.D., Keelan,J.A., Paxton,J.W., and Mitchell,M.D. (2000) A highly sensitive fluorescent microplate method for the determination of UDP-glucuronosyl transferase activity in tissues and placental cell lines. *Drug Metab Dispos.*, **28**, 1184-1186.

27. Malfatti,M.A. and Felton,J.S. (2001) N-glucuronidation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and N_hydroxy-PhIP by specific human UDP-glucuronosyltransferases. *Carcinogenesis*, **22**, 1087-1093.

28. Hayes,J.D., Flanagan,J.U., and Jowsey,I.R. (2005) Glutathione transferases. *Annu.Rev.Pharmacol.Toxicol.*, **45**, 51-88.

29. Hiratsuka,A., Nishijima,T., Okuda,H., Ogura,K., and Watabe,T. (1997) Rat liver theta-class glutathione S-transferases T1-1 and T2-2: their chromatographic, electrophoretic, immunochemical, and functional properties. *Anal.Biochem.*, **252**, 229-237.

30. Sundberg,K., Seidel,A., Mannervik,B., and Jernstrom,B. (1998) Detoxication of carcinogenic fjord-region diol epoxides of polycyclic aromatic hydrocarbons by glutathione transferase P1-1 variants and glutathione. *FEBS Lett.*, **438**, 206-210.

31. Knoll,N., Ruhe,C., Veeriah,S., Sauer,J., Glei,M., Gallagher,E.P., and Pool-Zobel,B.L. (2005) Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione S-transferase A4 expression by butyrate. *Toxicol.Sci.*, **86**, 27-35.

32. Galijatovic,A., Otake,Y., Walle,U.K., and Walle,T. (2001) Induction of UDP-glucuronosyltransferase UGT1A1 by the flavonoid chrysin in Caco-2 cells--potential role in carcinogen bioinactivation. *Pharm.Res.*, **18**, 374-379.

33. Thibaudeau,J., Lepine,J., Tojcic,J., Duguay,Y., Pelletier,G., Plante,M., Brisson,J., Tetu,B., Jacob,S., Perusse,L., Belanger,A., and Guillemette,C. (2006) Characterization of common UGT1A8, UGT1A9, and UGT2B7 variants with different capacities to inactivate mutagenic 4-hydroxylated metabolites of estradiol and estrone. *Cancer Res.*, **66**, 125-133.

34. Kensler,T.W. (1997) Chemoprevention by inducers of carcinogen detoxication enzymes. *Environ.Health Perspect.*, **105 Suppl 4**, 965-970.

35. Thimmulappa,R.K., Mai,K.H., Srisuma,S., Kensler,T.W., Yamamoto,M., and Biswal,S. (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res.*, **62**, 5196-5203.

36. Pool-Zobel,B., Veeriah,S., and Bohmer,F.D. (2005) Modulation of xenobiotic metabolising enzymes by anticarcinogens -- focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutat.Res.*, **591**, 74-92.

37. Marmorstein,R. (2001) Structure and function of histone acetyltransferases. *Cell Mol.Life Sci.*, **58**, 693-703.

38. Moynahan,M.E., Pierce,A.J., and Jasin,M. (2001) BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol.Cell*, **7**, 263-272.

39. Balavenkatraman,K.K., Jandt,E., Friedrich,K., Kautenburger,T., Pool-Zobel,B.L., Ostman,A., and Bohmer,F.D. (2006) DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients. *Oncogene*, **25**, 6319-6324.

40. el-Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W., and Vogelstein,B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817-825.

41. Veeriah,S., Hofmann,T., Glei,M., Dietrich,H., Will,F., Schreier,P., Knaup,B., and Pool-Zobel,B.L. (2007) Apple Polyphenols and Products Formed in the Gut Differently Inhibit Survival of Human Cell Lines Derived from Colon Adenoma (LT97) and Carcinoma (HT29). *J.Agric.Food Chem.*, **55**, 2892-2900.

42. Chu,F.F., Doroshow,J.H., and Esworthy,R.S. (1993) Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *J.Biol.Chem.*, **268**, 2571-2576.

43. Kischkel,F.C., Lawrence,D.A., Tinel,A., LeBlanc,H., Virmani,A., Schow,P., Gazdar,A., Blenis,J., Arnott,D., and Ashkenazi,A. (2001) Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *J.Biol.Chem.*, **276**, 46639-46646.

44. Kern,M., Pahlke,G., Balavenkatraman,K., Böhmer,F., and Marko,D. (2007) Apple polyphenols affect protein kinase C activity and the onset of apoptosis in human colon carcinoma cells. *submitted*.

45. Kestler,H.A., Muller,A., Gress,T.M., and Buchholz,M. (2005) Generalized Venn diagrams: a new method of visualizing complex genetic set relations. *Bioinformatics.*, **21**, 1592-1595.

46. Irizarry,R.A., Warren,D., Spencer,F., Kim,I.F., Biswal,S., Frank,B.C., Gabrielson,E., Garcia,J.G., Geoghegan,J., Germino,G., Griffin,C., Hilmer,S.C., Hoffman,E., Jedlicka,A.E., Kawasaki,E., Martinez-Murillo,F., Morsberger,L., Lee,H., Petersen,D., Quackenbush,J., Scott,A., Wilson,M., Yang,Y., Ye,S.Q., and Yu,W. (2005) Multiple-laboratory comparison of microarray platforms. *Nat.Methods*, **2**, 345-350.

47. Veeriah,S., Balavenkatraman,K.K., Böhmer,F., Kahle,K., Glei,M., Richling,E., Scheppach,W., and Pool-Zobel,B.L. (2007) Intervention with cloudy apple juice results in altered biological activities of ileostomy samples collected from individual volunteers. *submitted*.

Table 1. Primer sequences for real-time PCR, primers designed by using PerlPrimer v1.1 software. Primer pairs were verified by normal PCR with gene specific amplicons.

Locus ID	Gene name	Amplicon size (bp)	Anealing temperature	Primer name	Primer sequences 5' to 3'
NM_000854	*GSTT2*	142	60 - 63°C	iGSTT2_F	tgacactggctgatctcatggcc
				iGSTT2_R	gcctcctggcatagctcagcac
NM_000852	*GSTP1*	149	60 - 63°C	iGSTP1_F	ctgcgcatgctgctggcagatc
				iGSTP1_R	ttggactggtacagggtgaggtc
NM_001512	*GSTA4*	131	56 - 60°C	iGSTA4_F	ccggatggagtccgtgagatgg
				iGSTA4_R	ccatgggcacttgttggaacagc
NM_000463	*UGT1A1*	145	57 - 60°C	iUGT1A1_F	tcatgctgacggacccttttc
				iUGT1A1_R	ctgggcacgtaggagaatgg
NM_007120	*UGT1A4*	131	57 - 60°C	iUGT1A4_F	ccggatggagtccgtgagatgg
				iUGT1A4_R	ccatgggcacttgttggaacagc
NM_001074	*UGT2B7*	230	64.2 - 65°C	iUGT2B7_F	taattgcatcagccctggccc
				iUGT2B7_R	gttgatcggcaaacaatggaatc
NM_002046	*GAPDH*	110	62 - 65°C	iGPADH_F	acccactcctccacctttgac
				iGAPDH_R	tccaccaccctgttgctgtag

Table 2. Gene expression analysis using a cDNA microarray system (Superarray®) in LT97 cells. The table shows data for selected genes with fold changes ≥1.5 or ≤ 0.7. Note that all of these 30 genes were upregulated by AE (128 µg/ml). # indicates those genes for which mRNA levels were confirmed by real-time PCR. Significant differences to the medium controls were analysed with a two-tailed student *t*-test (*n*=4), but were not found.

Gene ID	Gene name	Descriptions	Ratio	STD	p values *t*-test
	Phase I				
	P450 gene family				
NM_000767	CYP2B6	Cytochrome P450, family2, subfamily B, polypeptide 6	1.6	0.35	0.489
NM_000765	CYP3A7	Cytochrome P450, family3, subfamily A, polypeptide 7	2.4	2.22	0.248
NM_000896	CYP4F3	Cytochrome P450, subfamily IVF, polypeptide 3	3.2	3.43	0.144
	Phase II				
	Acetyltransferases				
NM_000755	CRAT	Carnitine acetyltransferase	1.8	1.01	0.465
NM_001931	DLAT	Dihydrolipoamide *S*-acetyltransferase	2.8	2.87	0.405
NM_003642	HAT1	Histone acetyltransferase 1	2.1	1.45	0.358
NM_000662	NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	2.3	1.99	0.676
NM_016100	NAT5	N-acetyltransferase 5 (ARD1 homolog, S.cerevisiae)	2.6	2.34	0.478
	Sulfotransferases				
NM_012126	CHST5	Carbohydrate (Nacetylglucosamine 6-O) sulfotransferase 5	3.1	3.66	0.296
NM_021615	CHST6	Carbohydrate (Nacetylglucosamine 6-O) sulfotransferase 6	2.9	3.50	0.809
NM_019886	CHST7	Carbohydrate (Nacetylglucosamine 6-O) sulfotransferase 7	3.9	4.48	0.387
NM_001056	SULT1C1	Sulfotransferase family, cytosolic, 1C, member 1	1.9	1.11	0.440
NM_003596	TPST1	Tyrosylprotein sulfotransferase 1	2.6	1.64	0.475
	Glutathione *S*-transferases				
NM_000847	GSTA3	Glutathione *S*-transferase A3	1.6	0.51	0.220
NM_000851	GSTM5	Glutathione *S*-transferase M5	1.8	0.42	0.221
NM_000852	GSTP1#	Glutathione *S*-transferase P1	2.2	0.85	0.105
NM_000854	GSTT2#	Glutathione *S*-transferase T2	3.0	3.15	0.149
NM_020300	MGST1	Microsomal glutathione *S*-transferase 1	2.2	1.32	0.086
NM_002413	MGST2	Microsomal glutathione *S*-transferase 2	2.7	1.82	0.058
NM_004528	MGST3	Microsomal glutathione *S*-transferase 3	1.9	1.09	0.598
	Methyltransferases				
NM_000367	TPMT	Thiopurine methyltransferase	3.3	4.39	0.287
NM_001024074	HNMT	Histamine N-methyltransferase	2.5	1.19	0.326
NM_000754	COMT	Catechol-O-methyltransferase	1.8	1.08	0.458
	UDP-Glycosyltransferases				
NM_000463	UGT1A1	UDP glycosyltransferase 1 family, polypeptide A1	1.9	0.94	0.501
NM_007120	UGT2B	UDP glycosyltransferase 1 family, polypeptide A10	1.8	0.50	0.434
	Epoxide hydrolases				
NM_000120	EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)	2.4	2.55	0.519
NM_001979	EPHX2	Epoxide hydrolase 2, cytoplasmic	2.1	1.45	0.358
NM_000895	LTA4H	Leukotriene A4 hydrolase	2.3	2.17	0.428
	Miscellaneous (several functions)				
NM_175617	MT1E	Metallothionein 1E (functional)	3.5	4.32	0.286
NM_003786	ABCC3	ATP-binding cassette, subfamily C (MDR/TAP), member 3	4.1	6.05	0.439

Table 3. Gene expression analysis of AE (128 µg/ml) treated LT97 cells using a custom-made cDNA microarray: The table includes genes that have increased expression levels of ≥1.5 or ≤0.7-fold the medium control cells. Altogether 46 of 300 genes were modulated using this custom array. # indicates those genes for which mRNA levels were confirmed by real-time PCR. Numbers in bold are significantly modulated genes. Significant differences to the medium controls were calculated with a two-tailed student *t*-test ($p \leq 0.05$, $n=4$).

Gene ID	Gene symbol	Gene description	Ratio	STD	*p values t-test*
Phase I metabolism					
NM_000761	CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	1.5	1.0	0.366
NM_000782	CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	**4.5**	2.9	**0.025**
NM_000771	CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9	4.6	3.8	0.344
NM_000765	CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	1.6	0.5	0.062
NM_000896	CYP4F3	Cytochrome P450, subfamily 4 F, polypeptide 3	2.2	2.2	0.777
NM_000941	POR	P450 (cytochrome) oxidoreductase	1.7	1.5	0.519
Phase II metabolism					
NM_005891	ACAT2	Acetyl-Coenzyme A acetyltransferase 2	1.8	1.7	0.874
NM_001512	GSTA4#	Glutathione *S*-transferase A4	**1.7**	0.2	**0.022**
NM_000854	GSTT2#	Glutathione *S*-transferase theta 2	**1.5**	0.3	**0.009**
NM_004273	CHST3	Carbohydrate sulfotransferase 3	**2.2**	0.5	**0.016**
NM_005769	CHST4	Carbohydrate sulfotransferase 4	1.6	0.7	0.134
NM_019886	CHST7	Carbohydrate sulfotransferase 7	1.6	0.3	0.094
NM_004861	CST	Cerebroside sulfotransferase	2.5	1.5	0.258
NM_003595	TPST2	Tyrosylprotein sulfotransferase 2	1.5	0.7	0.160
NM_001072	UGT1A6	UDP-glycosyltransferase 1 family, polypeptide A6	2.1	1.8	0.660
NM_019077	UGT1A7	UDP-glycosyltransferase 1 family, polypeptide A7	1.5	0.5	0.109
NM_001074	UGT2B7#	UDP-glycosyltransferase 2 family, polypeptide B7	**2.1**	1.2	**0.056**
Phase III drug transporters					
NM_004996	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	**2.2**	1.0	**0.007**
NM_000392	ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	**1.6**	0.5	**0.057**
Phase II gene regulation pathway					
NM_006219	PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide	1.6	0.8	0.176
NM_002649	PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	1.6	0.6	0.068
NM_005253	FRA-2	FOS-like antigen 2	4.3	7.8	0.894
NM_001621	AHR	Arylhydrocarbon	1.8	1.9	0.721
Stress and signal transduction					
NM_002021	FMO1	Flavin containing monooxygenase 1	1.6	0.7	0.074
NM_002083	GPX2	Glutathione peroxidase 2 (gastrointestinal)	1.9	2.3	0.730
NM_005347	HSPA5	Heat shock 70kDa protein 5	1.6	1.3	0.611
NM_003338	UBE2D1	Ubiquitin-conjugating enzyme E2D 1	1.9	0.8	0.071
Apoptosis signaling					
NM_001230	CASP10	Caspase 10, apoptosis-related cysteine protease	**1.7**	0.4	**0.009**
NM_030753	WNT3	Wingless-type MMTV integration site family, member 3	1.7	0.7	0.245
Tumour suppressor					
NM_000059	BRCA2	Breast cancer 2, early onset	2.7	2.6	0.217
NM_002843	PTPRJ	Protein tyrosine phosphatase, receptor type, J	**1.6**	0.3	**0.019**
NM_002846	PTPRN	Protein tyrosine phosphatase, receptor type, N	1.5	0.8	0.330
NM_007050	PTPRT	Protein tyrosine phosphatase, receptor type, T	1.7	1.6	0.475
NM_002831	PTPN6	Protein tyrosine phosphatase, non-receptor type 6	**1.6**	0.3	**0.034**
Cell cycle arrest-regulation of cell cycle					
NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.6	0.9	0.180
NM_006568	CGRRF1	Cell growth regulator with ring finger domain 1	2.8	1.5	0.039
NM_001437	ESR2	Estrogen receptor 2 (ER beta)	1.6	0.6	0.108
NM_002502	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	**2.2**	0.6	**0.008**
NM_002503	NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	**3.8**	4.2	**0.039**
NM_003236	TGFA	Transforming growth factor, alpha	**1.5**	0.2	**0.015**
Miscellaneous (several functions)					
NM_000799	EPO	Erythropoietin	**1.6**	0.4	**0.022**
NM_000146	FTL	Ferritin, light polypeptide	1.6	1.0	0.426
NM_000578	SLC11A1	Solute carrier family 11, member 1	2.3	2.4	0.529
NM_000617	SLC11A2	Solute carrier family 11, member 2	1.6	1.2	0.586
NM_014585	SLC40A1	Solute carrier family 40, member 1	1.9	1.7	0.528
NM_003234	TFRC	Transferrin receptor (p90, CD71)	1.7	1.4	0.452

Figure 1. The Venn diagram for array platform comparison

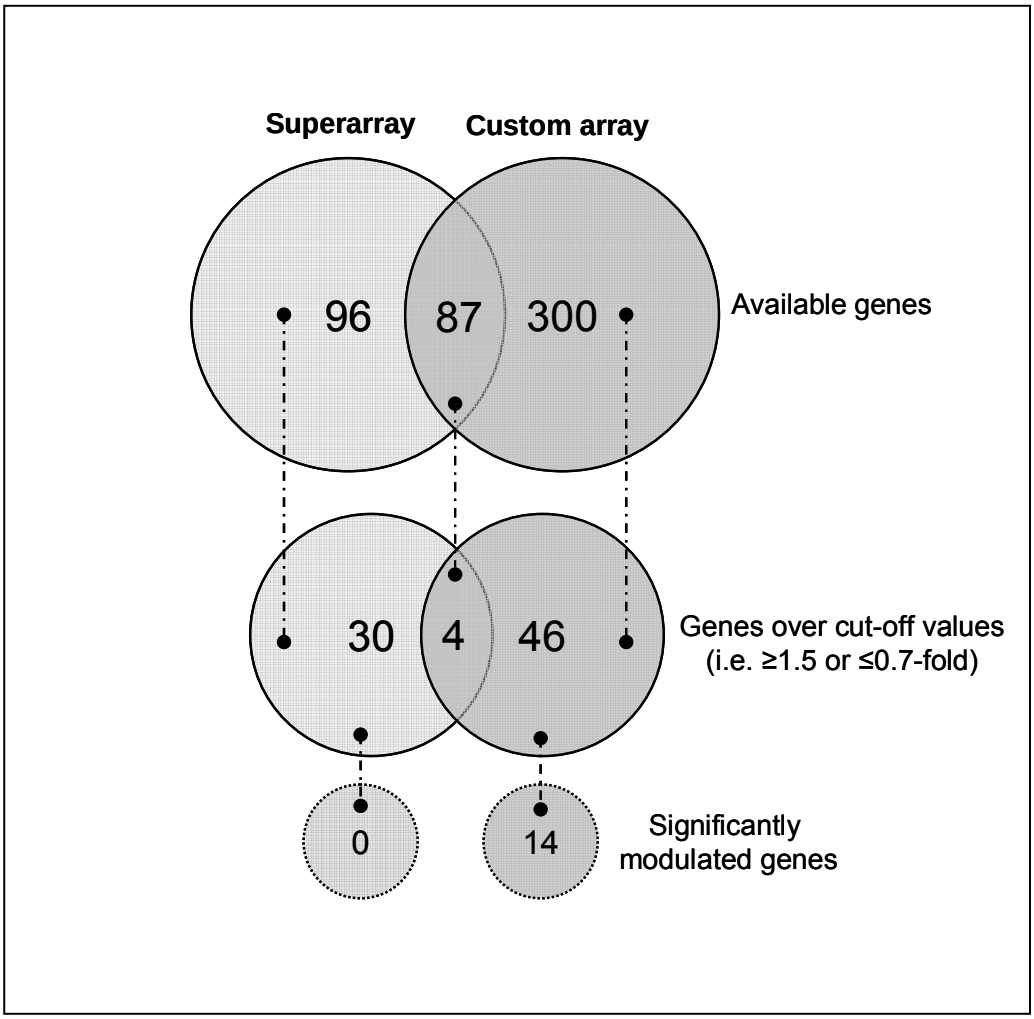


Figure 2. Real-time PCR analysis for microarray data validation

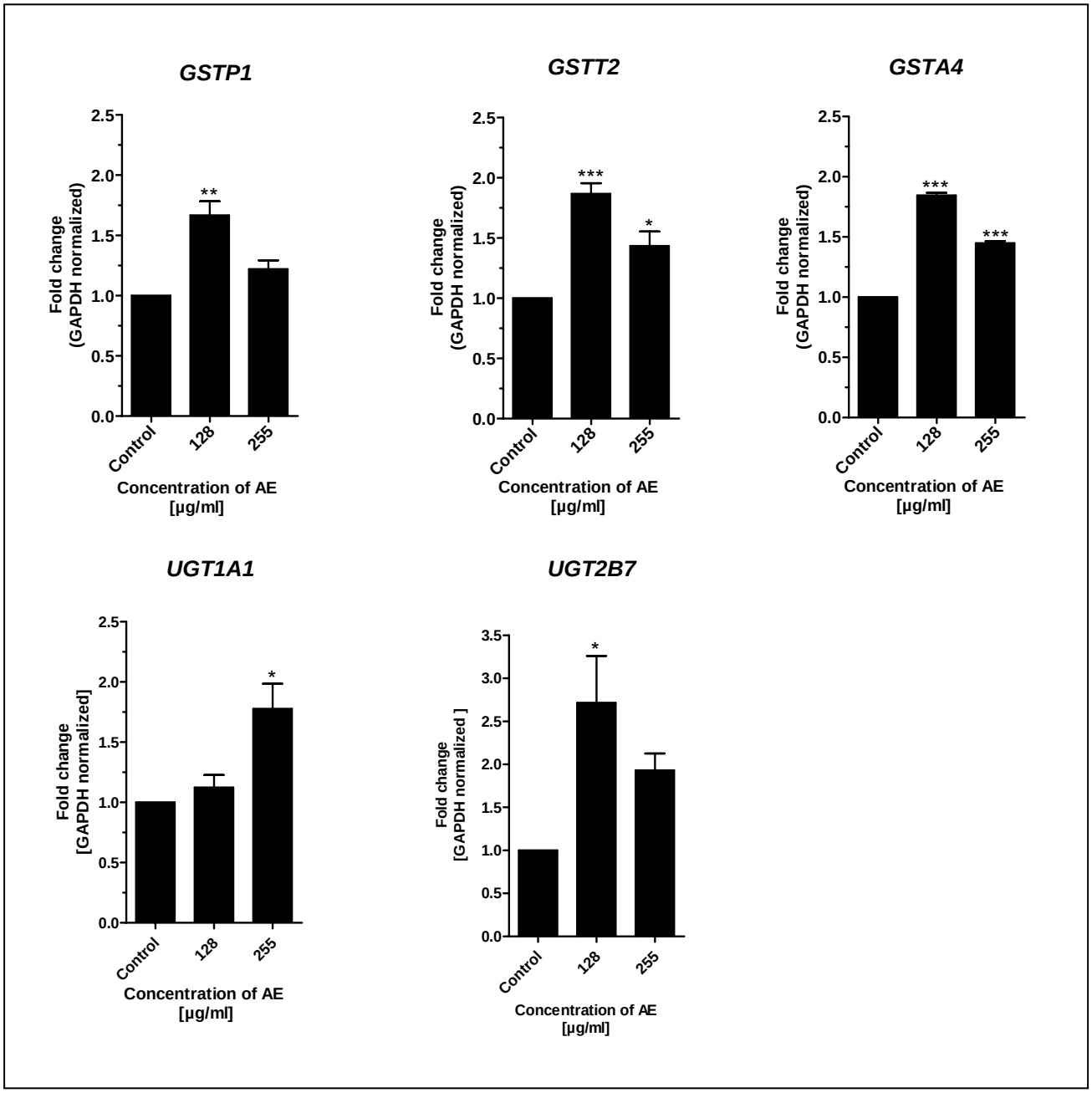
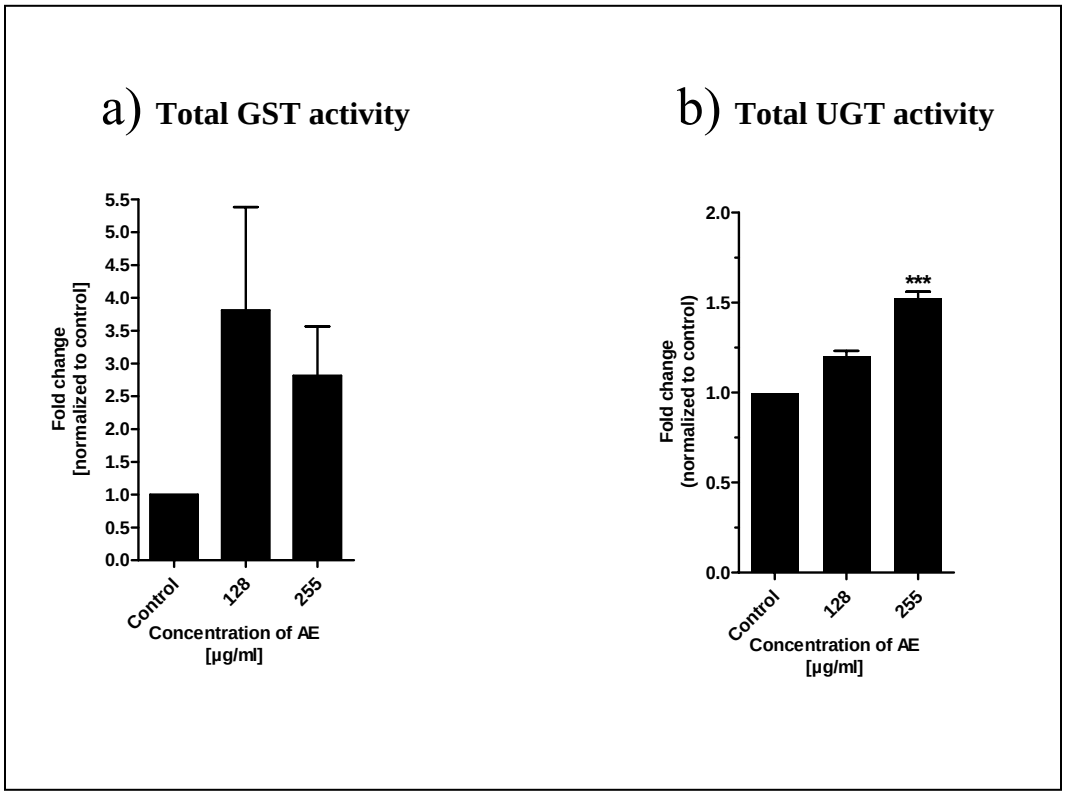


Figure 3. Enzyme activity analysis



2.6 **Publication VI:** ***Veeriah S,*** *Böhmer FD, Kamal K, Kahle K, Glei M, Rickling E, Schreyer P, Pool-Zobel BL*. "Intervention with cloudy apple juice results in altered biological activities of ileostomy samples collected from individual volunteers". *Manuscript in preparation, 2007*

Apple juice is considered to be an important component of the healthy diet, which has recently been shown to have numerous types of chemoprotective activities in experiments with colon cancer animal models and in human colon cells *in vitro*. Since only little is known on comparable activities in the human colon *in vivo*, here a pilot study was performed to assess related mechanisms in ileostomy samples from volunteers that had consumed apple juice. Ileostomy samples were collected at different time points after intervention (0 - 8 h) and were characterized analytically for major apple polyphenols and in HT29 colon cells for their potential to cause genotoxic damage, protect from the genotoxic insult by hydrogen peroxide (H_2O_2) and modulate the expression of *GSTT2*, an enzyme related to antioxidative defence of other peroxides. After the intervention, some ileostomy samples were less genotoxic and also better protected HT29 cells from genotoxic damage by H_2O_2, resulted in an increased *GSTT2* expression and an enhanced *GSTT2* promotor activity. It appears as if ileostomy samples after intervention with apple juice cause a number of biological effects related to chemoprotection and that these effects have also been shown to be mediated by the apple extracts and/or individual phenolic components or gut flora mediated fermentation products

Own contribution to the manuscript:

- Planning and organising the work
- Data evaluation, interpretation and representation of the results

Intervention with cloudy apple juice results in altered biological activities of ileostomy samples collected from individual volunteers

S. Veeriah, K. K. Balavenkatraman[ψ], F. D. Böhmer[ψ], K. Kahle[Δ], M. Glei, E. Richling[#], W. Scheppach[Δ], B. L. Pool-Zobel*

Department of Nutritional Toxicology, Institute for Nutrition, Friedrich-Schiller-University, Dornburger Str.24, D-07743 Jena, Germany

[ψ]*Center for Molecular Biomedicine, Institute of Molecular Cell Biology, Friedrich-Schiller-University, Drackendorfer Str.1, D-07747 Jena, Germany*

[Δ]*Department of Medicine II, Division of Gastroenterology, University of Wurzburg, Josef-Schneider-Str.2, D-97080 Wurzburg, Germany*

[#]*Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Erwin-Schroedinger Str.52, D-67663 Kaiserslautern, Germany*

Address of corresponding author*

Prof. Dr. rer. nat. Beatrice L. Pool-Zobel
Friedrich-Schiller-University Jena
Biological-Pharmaceutical Faculty
Institute for Nutritional Sciences
Department of Nutritional Toxicology
Dornburger Str. 24
07743 Jena, Germany
Tel. +49 3641 949670
Fax. +49 3641 949672
E-mail: b8bobe@uni-jena.de
Homepage: www.uni-jena.de/biologie/ieu/et

ABSTRACT

Apple juice is considered to be an important component of the healthy diet, which has recently been shown to have numerous types of chemoprotective activities in experiments with colon cancer animal models and in human colon cells *in vitro*. Since only little is known on comparable activities in the human colon *in vivo*, here a pilot study was performed to assess related mechanisms in ileostomy samples from volunteers that had consumed apple juice. Ileostomy samples were collected at different time points after intervention (0 - 8 h) and were characterized analytically for major apple polyphenols and in HT29 colon cells for their potential to cause genotoxic damage, protect from the genotoxic insult by hydrogen peroxide (H_2O_2) and modulate the expression of *GSTT2*, an enzyme related to antioxidative defence against other peroxides. The analytical determination of polyphenols in the ileostomy samples revealed that the majority of the compounds were recovered in the samples collected 2 h after intervention. The comparison of genotoxic effects of samples before intervention and 2 h after intervention revealed a considerable variation of genotoxic response, but there was a trend for reduced genotoxicity in 3 of 8 persons (P) after intervention. Samples collected at 2 h protected HT29 cells from genotoxic damage by H_2O_2 (for 3 of 7 persons), resulted in an increased *GSTT2* expression (for 2 of 6 persons) and of *GSTT2* promotor activity (2 of 6 persons). The intervention with apple juice results in bioavailable concentrations of related polyphenols in the gut lumen, which could contribute to reduced genotoxicity, enhanced antigenotoxicity and favourable modulation of *GSTT2* gene expression in some individuals. The pilot study for the first time used this combination of faecal biomarkers which in larger cohorts may reveal significant alterations that contribute to reduced genotoxic exposure and thus to chemoprotection of colon cells.

Keywords: Ileostomy, colon cancer chemoprevention, Comet assay, gene expression, HT29 cells

INTRODUCTION

Population based studies, including case control and cohort studies, have indicated that eating sufficient portions of fruit and vegetables may reduce the risk of developing cancer, especially cancers of the digestive tract (Steinmetz and Potter, 1991; Terry *et al*., 2001). Therefore, this dietary habit, together with other lifestyle factors, such as increasing the intensity and duration of physical activity, or reducing consumption of meat especially red meat, has been recommended by health agencies for a better protection against the aforementioned types of cancer (Cerhan *et al*., 2004). Still, the link between dietary factors and cancer protection is difficult to establish, and the protective role of fruits and vegetables is somewhat controversial (Hung *et al*., 2004; Schatzkin and Kipnis, 2004a). It is therefore, important to continue exploring possible interactions between dietary and potential cancer risk factors, and to appropriately stratify epidemiological studies (Schatzkin and Kipnis, 2004b). Furthermore, possible protective effects should be substantiated with more detailed studies of the mechanisms of protective dietary components, combined with measuring effects in human intervention studies (Branca *et al*., 2001).

Apple juice is an important component of fruit intake in Europe. Recent animal experiments have shown a protective activity of cloudy apple juice with respect to carcinogenesis in the distal colon of rats induced by 1,2-dimethylhydrazine (DMH) treatment (Barth *et al*., 2005b). The underlying mechanisms are not yet understood and warrant further investigation. Polyphenols extracted from apple juice contain a number of different flavonoids with known antioxidative effects (Boyer and Liu, 2004). Apple polyphenol extracts, as well as some of their major ingredients like chlorogenic acid, phloretin and quercetin were shown to inhibit the growth of the colon tumour-derived cell line HT29 (Veeriah *et al*., 2006d), which may partially be related to inhibition of different growth factor signalling events (Balavenkatraman *et al*., 2006; Kern *et al*., 2005). In HT29 cells treated with the apple polyphenol extract, a modulation of gene expression patterns occurred, including an up regulation of several genes involved in drug detoxification, notably *GSTP1* and *GSTT2* (Veeriah *et al*., 2006c). Induction of *GSTT2* could also be recapitulated using a corresponding reporter construct (K. Palige *et al*., unpublished data). In an LT97 human colon adenoma cell line also *GSTT2* was transcriptionally elevated, in addition to other target genes (Veeriah *et al*., 2007). Together, the results imply that apple compounds are protecting human colon cells from oxidative stress by causing an up regulation of enzymes involved in detoxification of specific peroxides.

To link the above described findings better to the *in vivo* situation, it was the objective of this study to determine whether apple juice intervention in humans could affect genotoxin levels in the gut lumen. Furthermore, the capacity of those apple juice components which passed the small intestine for modulation of *GSTT2* expression and for prevention of oxidative genotoxic stress was studied in HT29 cells using ileostomy samples from volunteers who had consumed cloudy apple juice (Kahle *et al*., 2005b). The samples were collected at different time points after intervention and were characterized analytically and biologically for various parameters associated with chemoprotection.

MATERIALS AND METHODS

Design and procedure of intervention study

Information on the ileostomy study performed, and data on the preparation of ileostomy bags were published previously (Kahle *et al*., 2005a). Quantification of polyphenols in the apple juices under study and the ileostomy samples were performed using HPLC-DAD and HPLC-MS/MS methods as described before (Kahle *et al*., 2005c; Kahle *et al*., 2006).

The study was approved by the Ethics Commission of the University of Wuerzburg. The 11 volunteers gave their informed consent to participate in the study and agreed to avoid all foods contain polyphenols the day before the study started. After an overnight fasting period, all volunteers drank one litre of cloudy apple juice within 15 minutes. A light meal which did not contain polyphenols was served four hours later. The ileostomy bag was removed before (= control value) and 1, 2, 4 and 6 h after the start of the apple juice intake (person 1 and 2). When we recognized, that after 6 h, polyphenols had not completely passed the small intestine, samples were taken from the remaining persons at 8 h (persons 3 to 11) as well. All ileostomy samples were immediately frozen at -20°C for storage, freeze-dried and homogenized. For cell culture experiments, ileostomy samples were thawed and reconstituted with phosphate buffered saline (PBS). The samples were then stored at -80°C.

Cell line and cell culture

HT29 colon carcinoma cells were obtained from the American Type Culture Collection (*ATCC*, Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Life Technology, USA) supplemented with 10 % foetal calf serum (FCS) and antibiotics (1 % penicillin/streptomycin (v/v); Roche Molecular Biochemicals, Meylan, France) according to our laboratory standard culture conditions. Confluent cultures were passaged three or four days after trypsinization. HT29 cells were maintained under sterile conditions at 37°C in a 95 % humidified incubator (5 % CO_2). Every batch of HT29 cells was routinely checked for mycoplasma contaminations using highly sensitive PCR analysis (Minerva Biolabs GmbH, Germany). In the experiments described here, cell passages 30 – 41 were used.

Detection of genotoxicity and antigenotoxicity

DNA damage was measured using single cell micro gel electrophoresis (Comet assay). The alkaline version of this assay was performed to detect single strand breaks and alkali-labile sites, such as apyrimidinic and apurinic (AP)-sites that are formed when bases are lost. HT29 cells were incubated with the ileostomy samples (5 %, v/v) for 24 h. H_2O_2 (obtained as a 30 % aqueous solution from Merck, Darmstadt, Germany) was used as the genotoxic reference compound (positive control) and to induce DNA damage (challenge) after preincubation of the cells with the ileostomy samples to measure their antigenotoxic capacity. The cell suspensions were treated with H_2O_2 at 37°C for 5 min. After this, the suspensions were diluted by adding DMEM and were centrifuged (2,000 g, 5 min) to recover the cells. The cell pellets were resuspended in DMEM and stored on ice.

Finally, cell suspensions were centrifuged (2,000 g, 5 min), and cells were embedded into agarose on microscopical slides, lysed (10 mM Tris–HCl, 100 mM Na_2EDTA, 2.5 M NaCl, 10 % dimethylsulfoxide, 1 % Triton X-100, pH 10.0) for at least 60 min and subjected to electrophoresis. For this, slides were placed into an electrophoresis chamber containing alkaline (1 mM Na_2EDTA, 300 mM NaOH, pH 13.0) buffer for DNA unwinding. After 20 min, the electrophoresis was carried out at 1.25 V/cm, 300 mA for 20 min. The slides were removed from the electrophoresis chamber and washed three times for 5 min each with neutralisation buffer (4.2 M Tris-HCl, 0.08 M Tris–base, pH 7.2). Slides were stained with the DNA-specific fluorescence dye SYBR-Green (Sigma-Aldrich Chemie GmbH, Steinheim, 1 µl/ml; 30 µl per slide).

All steps of the Comet assay were conducted under red light. The extent of DNA migration was determined for 150 DNA spots per treatment using the image analysing system of Perceptive Instruments (Suffolk, UK, www.perceptive.co.uk). The intensity of fluorescence in the comet tail, expressed as "tail intensity", was the evaluation criterion presented in the table and graphs. Each experiment was performed independently at least three times.

GSTT2 - gene expression with real-time PCR

HT29 cells were seeded into 6 well culture dishes (Falcon, UK) at 0.4×10^6 cells/well and cultured for 48 h. Ileostomy samples were added at a concentration of 5 % (v/v) and incubation was done for 24 h. RNA was isolated using RNeasy mini plus kit (QIAGEN,

Hilden, Germany). Total RNA was checked for purity and stability by gel electrophoresis. First-strand cDNA was synthesized using the SuperScript II reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany) with 3 µg of DNA free total RNA according to the manufacturer's protocol. Real-time PCR was performed on the iCycler iQ® instrument (Bio-Rad GmbH München, Germany) with the iQ™ SYBR® Green Supermix (Bio-Rad GmbH München, Germany). PCR was performed with a set of specific targets (*GSTT2*) and reference (*GAPDH*) gene primers as follows: *GSTT2*(forward) 5'-tgacactggctgatctcatggcc-3', *GSTT2*(reverse) 5'-gcctcctggcatagctcagcac-3' and *GAPDH* (forward) 5'-acccactcctccacctttgac-3', *GAPDH* (reverse) 5'-tccaccaccctgttgctgtag-3'. Melting curve analyses were conducted after completion of the cycling process to confirm the gene specific amplicons. Average threshold cycle (Ct) values were used to determine the relative differences between control and treated groups and the REST® tool program (Pfaffl *et al*., 2002) was utilized for the data normalization.

Reporter gene assay for GSTT2 promotor activity

Promoter constructs of human *GSTT2*, driving expression of Firefly luciferase in the vector pGL3 were kindly provided by Dr. Paul R. Buckland (Cardiff, UK) (Guy *et al*., 2004b). Among the polymorphic variants, the most prevalent A form was used. 2.5×10^6 HT29 cells were seeded in a 6 cm dish. The next day, the cells were transfected with 0.8 µg *GSTT2* in pGL3 (or empty pGL3 for control) and 0.04 µg of the Renilla luciferase expressing construct pRL-TK (Promega GmbH, Mannheim, Germany) using lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the recommendations of the manufacturer. A ratio of lipofectamine (µl) /DNA (µg) of 6:1 was used. After 24 h, the cells were trypsinized and seeded into 96-well plates (2×10^4 cells per well). The next day, ileostomy samples and corresponding controls were added, and incubation continued for 24 h. Thereafter, the cells were washed with PBS and processed for measurement of luciferase activities with the Dual Glo™ assay kit (Promega GmbH, Mannheim, Germany) according to the method recommended by the manufacturer. Luminiscence was read in a LumiStar Galaxy reader (BMG Labtechnologies, Durham, NC, U.S.A.)

Statistical analysis

Results represent the mean value ± SD of triplicate experiments unless stated otherwise. Data were analysed using the one- and two-way ANOVA with Bonferroni's post test in order to determine the presence of statistically significant differences.

RESULTS

The kinetic of excretion of total polyphenols (flavonoids and phenolic acids such as chlorogenic acid) were determined following ingestion of cloudy apple juice. From Figure 1, it is apparent that the majority of the compounds were recovered in the samples collected 2 h after the intervention. At an average a total of 16.0±3.5 mg polyphenols could be retrieved from the samples of eleven subjects. The average volume of the samples was 360 ± 143. Chlorogenic acid concentrations were also at their maximum in the 2 h samples and the absolute mean values of the eleven subjects were 6.0±1.0 mg. Interestingly, person number 3 seemed to have a delayed response, in that the total polyphenol concentrations were higher in the 4 h sample than in the 2 h sample.

The samples before intervention (0 h) and after intervention (2 h) were compared for genotoxic activity in HT29 colon tumour cells. As shown in Figure 2, there is a considerable variation of genotoxic response. In comparison to the medium control only the ileostomy sample of subject 1 (P1, 0 h) was significantly genotoxic ($p \leq 0.01$). But there was a trend of increased genotoxicity as compared with the medium control also in the 0 h samples of subjects 8, and 11. For subject 1, the sample collected 2 h after cloudy apple juice intervention was significantly ($p \leq 0.01$) less genotoxic than before the intervention. A trend in the same direction of reduced genotoxicity after the intervention (sample 2 h) was also observed in person 8 and 11. There was no indication for an increased level of genotoxicity after intervention.

Table 1 compares the dose related genotoxic effects of H_2O_2 in cells pretreated with medium or with ileostomy samples colleted before (0 h) or after invention (2 h) with apple juice. Only two of the three antigenotoxic samples (P8, P11) also protected from H_2O_2-mediated DNA damage. In addition, the 2 h sample from person 9 (Table 1) and from person 3 (Figure 3) significantly protected more from H_2O_2-mediated damage than did the corresponding 0 h samples from the same subjects. The results obtained with samples from person 3 were an interesting exception since the 2 h sample of this person was the most protective, without exhibiting a reduced baseline genotoxic potential and are therefore plotted in Figure 3. Pretreatment of cells with three other 2 h-samples (P1, P4, P7), did not result in reduced genotoxic effects by H_2O_2, compared to the corresponding samples collected at 0 h (Table 1)

Since apple extracts have been shown to increase expression of *GSTT2* (Veeriah *et al*., 2006b), we next addressed the question of whether the ileostomy samples might also induce this enzyme that may deactivate other peroxides than H_2O_2. Figure 4 shows that the 2 h samples of P8 and P11 tended to enhance expression of the *GSTT2* mRNA in comparison with cells treated with the corresponding sample at 0 h, as determined by real-time PCR. The results did not reach statistical significance after 3 independent replications possibly on account of the experimental variability. The samples sizes were too limited in quantity and it was therefore not possible to perform additional determinations. 2 h samples from P1, P4, P7 and P9 in contrast had no apparent capacity to elevate *GSTT2* mRNA levels. Samples of subject 3 could not be investigated also due to limited amounts available.

The samples were also investigated using a newly established reporter assay measuring *GSTT2* promoter activity. HT29 cells were transfected with a luciferase expression construct under control of the most prevalent form of a *GSTT2* promoter sequence (Guy *et al*., 2004a). Apple polyphenol extracts have the capacity to activate this reporter, with detectable induction upon treatment for 24 h and optimal induction at 48 h treatment (K. Palige *et al*., unpublished data). Cells were treated for 24 h with ileostomy samples taken at 0 h and 2 h after the intervention with apple juice. The obtained reporter activity was compared. The 2 h sample of person 11 induced *GSTT2* promoter activities, relative to the cells treated with the 0 h sample, a very weak induction was detected with the sample from patient 1 (Figure 5). No induction was seen with samples from persons 4, 7, 8, or 9. These data, although obtained under not optimal conditions for reporter stimulation, are in support of a regulatory activity of apple constituents which have passed the ileum with respect to *GSTT2* expression.

DISCUSSION

This study deals with the investigation of possible chemoprotective effects in the gut lumen of humans resulting from apple juice consumption. In this context the reported pilot study with 11 ileostomy subjects is a first exploratory effort to assess whether different faecal biomarkers can be used to determine the reduction of specific risk parameters related to exposure. Foremost, the analytical determination of polyphenols in the ileostomy samples revealed that the majority of the apple phenolic compounds were recovered in the ileostomy samples collected 2 h after intervention, and chlorogenic acid was one of the predominant detected polyphenols. Such a compound could be responsible for reducing exposure to genotoxins and oxidants in the gut lumen, thus reducing the likelihood of damage to the DNA of colon cells, as has been demonstrated recently (Glei *et al*., 2006). To analyse this we determined the genotoxicity of the ileostomy samples before and after intervention of 8 volunteers, for whom samples were still available in sufficient quantities. The method was used according to procedures established to study genotoxic activities of faecal water. Faecal water genotoxicity is a biomarker method being developed to indicate whether dietary intervention with e.g. pro- and prebiotics may result in a reduced exposure to genotoxic agents (Glei *et al*., 2005; Oberreuther-Moschner *et al*., 2004). In rats there seem to be an direct association between development of tumours and degree of faecal water genotoxicity (Klinder *et al*., 2004). The studies have however shown that there is a high inter- and intraindividual variability of faecal water genotoxicity, even in subjects consuming identical diets (Osswald *et al*., 2000). Nevertheless, the impact of dietary intervention still yields significant results when analysing each subject as his own control, as we have also seen in this study with ileostomy samples from 3 of the 8 investigated subjects. In rats, there was a reduction of DNA damage in the colon cells of animals receiving cloudy apple juice (Barth *et al*., 2005a). Since apple juice contains polyphenols, typical antioxidants, we next addressed the question as to whether this reduction of genotoxicity is associated with protection against oxidants, like H_2O_2 that is also available in the colon (Babbs, 1992). Using a challenge assay, which is performed by first treating model cells with biological samples obtained before or after intervention and then challenging them with genotoxic agents, here it was found that several samples obtained 2 h after intervention indeed reduced the genotoxic response toward H_2O_2. This antigenotoxicity of the ileostomy samples could be due to a direct antioxidative effect by the polyphenols excreted in the 2 h samples. Among others, especially chlorogenic acid could be responsible for this effect, since it also reduced H_2O_2 genotoxicity in the challenge assay (Glei *et al*., 2006). The comparison of genotoxic and antigenotoxic effects of samples before and 2 h after

intervention revealed a considerable variation of responses and only two of the subjects showed significant differences for both endpoints. Self evidently the numbers of these subjects is too small to find significant associations, but the lack of association also could relate to completely different mechanisms. In the context of a reduced basal genotoxicity, apple ingredients may be scavenging or inactivating genotoxic and toxic components naturally available in the gut lumen. In the context of the challenge assay, antigenotoxicity could also mean that the apple phenols are enhancing stress response or antioxidant defence in HT29 cells, thus leading to an enhanced deactivation of H_2O_2. Enzymes involved could be catalase (CAT) and glutathione peroxidases (GPX) of which *GPX2* was shown to be transcriptionally activated by AE in LT97 colon adenoma cells using gene expression studies with Affymetrix® arrays (Veeriah *et al*., 2007). Next to these target genes, a number of other genes which produce products related to defence against other factors of oxidative stress, such as superoxide dismutase 2 (*SOD2*), glutathione reductase (*GSR*), metallothionein 1X (*MT1X*), and glutathione *S*-transferase theta 2 (*GSTT2*) according to the array analyses. Of particular interest in this context is *GSTT2*, the expression of which is altered by apple polyphenols not only in HT29 cells (Veeriah *et al*., 2006a) but also in LT97 colon adenoma cells (Veeriah *et al*., 2007). This is why we chose to investigate more closely, the effects of ileostomy samples on *GSTT2* expression, using two fully new biomarker approaches. One was to assess the transcriptional induction of *GSTT2* by the samples using real-time PCR and the other was to study effects on *GSTT2* promoter activity using a novel reporter gene assay. The main objective of the present study was to use these identified "apple-juice senitive parameters", to compare the effects of ileostomy samples obtained before and after intervention with apple juice and to assess how this relates to the excreted apple products. For person 11 there was an increase of *GSTT2* promotor activity and also an increase of mRNA in the 2 h sample in comparison to the control at 0 h. It is not known which compounds in the ileostomy sample are responsible for the observed *GSTT2* gene regulation. Chlorogenic acid may be a candidate, since it has been found to weakly induce the *GSTT2* promoter (K.Palige *et al*., unpublished data). It is efficiently excreted, and reaches the relatively highest levels in the ileostomy sample of person 11 at 2 h after intervention. However, the other ileostomy samples of this study containing nearly similar amounts of chlorogenic acid did not respond to these parameters. Also the compounds responsible for the genotoxicity before intervention, reduced genotoxicity after intervention and responses in the challenge assay were different in different individuals. This finding is not only interesting but also to be expected based on the known pharmacological, genomic and dietary variability in humans (Ordovas and Corella, 2004).

The differences of susceptibility deserve more in depth investigations, as it may be possible to identify different individuals who may more or less profit from the habit of consuming apple juice in the basis of their gut luminal contents.

Altogether, we may conclude that intervention with apple juice results in bioavailable concentrations of related polyphenols in the gut lumen. These could contribute to reduced genotoxicity, enhanced antigenotoxicity and favourable modulation of *GSTT2* gene expression, possibly together with other ingredients of the gut lumen content. The effects were not significant on a group level and the number of subjects that participated in the study as well as the amount of samples were both too small to show an intervention effect or to disprove the possibility that apple juice could lead to chemoprotection in the gut lumen. The pilot study, however, for the first time used this new combination of faecal biomarkers which in larger cohorts may reveal significant alterations that contribute to reduced genotoxic exposure and thus to chemoprotection of colon cells. Taken together, it appears as if ileostomy samples, especially 2 h after intervention with cloudy apple juice, causes a number of biological effects related to chemoprotection, and that these effects have also been shown to be mediated by the apple extracts and/or individual phenolic components.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the excellent technical assistance of Mrs. Esther Woschee at the Institute for Nutrition, Friedrich-Schiller-University of Jena. *GSTT2* reporter assays were performed by Ms. Katja Palige, and Ms. Gabriele Schulz-Raffelt. We thank Antje Volk and Gerda Dusel for taking care of the study at the Division of Gastroenterology, University of Wuerzburg and we appreciate all volunteers for their participation. This project was supported by a grant from the Bundesministerium für Bildung und Forschung (*BMBF,* FKZ.01EA0103), Germany.

References

Babbs, C. F., Oxygen radicals in ulcerative colitis, *Free Radical Biology and Medicine, 13*, 169-181, 1992.

Balavenkatraman, K. K., E. Jandt, K. Friedrich, T. Kautenburger, B. L. Pool-Zobel, A. Ostman and F. D. Bohmer, DEP-1 protein tyrosine phosphatase inhibits proliferation and migration of colon carcinoma cells and is upregulated by protective nutrients, *Oncogene, 25*(47), 6319-6324, 2006.

Barth, S. W., C. Fahndrich, A. Bub, H. Dietrich, B. Watzl, F. Will, K. Briviba and G. Rechkemmer, Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats, *Carcinogenesis, 26*(8), 1414-1421, 2005b.

Barth, S. W., C. Fahndrich, A. Bub, H. Dietrich, B. Watzl, F. Will, K. Briviba and G. Rechkemmer, Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats, *Carcinogenesis, 26*(8), 1414-1421, 2005a.

Boyer, J. and R. H. Liu, Apple phytochemicals and their health benefits, *Nutrition Journal, 3*(1), 5, 2004.

Branca, F., A. B. Hanley, B. L. Pool-Zobel and H. Verhagen, Biomarkers in Disease and Health, *Br J Nutr, 86*(Supplement 1), S55-S92, 2001.

Cerhan, J. R., J. D. Potter, J. M. E. Gilmore, C. A. Janney, L. H. Kushi, D. Lazovich, K. E. Anderson, T. A. Sellers and A. R. Folsom, Adherence to the AICR Cancer Prevention Recommendations and Subsequent Morbidity and Mortality in the Iowa Women's Health Study Cohort, *Cancer Epidemiol Biomarkers Prev, 13*(7), 1114-1120, 2004.

Glei, M., N. Habermann, K. Osswald, C. Seidel, V. Böhm, C. Persin, G. Jahreis and B. L. Pool-Zobel, Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the comet assay: a biomarker model, *Biomarkers, 10*, 203-217, 2005.

Glei, M., A. Kirmse, N. Habermann, C. Persin and B. L. Pool-Zobel, Bread enriched with green coffee extract has chemoprotective and antigenotoxic activities in human cells, *Nutrition and Cancer-An International Journal, 56*(2), 182-192, 2006.

Guy, C. A., B. Hoogendoorn, S. K. Smith, S. Coleman, M. C. O'Donovan and P. R. Buckland, Promoter polymorphisms in glutathione-S-transferase genes affect transcription, *Pharmacogenetics, 14*(1), 45-51, 2004b.

Guy, C. A., B. Hoogendoorn, S. K. Smith, S. Coleman, M. C. O'Donovan and P. R. Buckland, Promoter polymorphisms in glutathione-S-transferase genes affect transcription, *Pharmacogenetics, 14*(1), 45-51, 2004a.

Hung, H. C., K. J. Joshipura, R. Jiang, F. B. Hu, D. Hunter, S. A. Smith-Warner, G. A. Colditz, B. Rosner, D. Spiegelman and W. C. Willett, Fruit and vegetable intake and risk of major chronic disease, *JNCI Cancer Spectrum, 96*(21), 1577-1584, 2004.

Kahle, K., M. Kraus, W. Scheppach, M. Ackermann, F. Ridder and E. Richling, Studies on apple and blueberry fruit constituents: Do the polyphenols reach the colon after ingestion?, *Mol. Nutr. Food Res., 50*(4-5), 418-423, 2006.

Kahle, K., M. Kraus, W. Scheppach and E. Richling, Colonic availability of apple polyphenols--a study in ileostomy subjects, *Mol. Nutr. Food Res., 49*(12), 1143-1150, 2005a.

Kahle, K., M. Kraus, W. Scheppach and E. Richling, Colonic availability of apple polyphenols--a study in ileostomy subjects, *Mol. Nutr. Food Res., 49*(12), 1143-1150, 2005c.

Kahle, K., M. Kraus, W. Scheppach and E. Richling, Colonic availability of apple polyphenols--a study in ileostomy subjects, *Mol. Nutr. Food Res., 49*(12), 1143-1150, 2005b.

Kern, M., Z. Tjaden, Y. Ngiewih, N. Puppel, F. Will, H. Dietrich, G. Pahlke and D. Marko, Inhibitors of the epidermal growth factor receptor in apple juice extract, *Mol. Nutr. Food Res., 49*(4), 317-328, 2005.

Klenow, S., S. Veeriah, Y. Knöbel and B. L. Pool-Zobel, Apple polyphenols modulate the genotoxic effects of different DNA damaging compounds, *in preparation,* 2007.

Klinder, A., A. Förster, G. Caderni, A. P. Femia and B. L. Pool-Zobel, Fecal water genotoxicity is predictive of tumor preventive activities by inulin-like oligofructoses, probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium lactis)* and their synbiotic combination , *Nutr Canc, 49*, 144-155, 2004.

Oberreuther-Moschner, D., G. Jahreis, G. Rechkemmer and B. L. Pool-Zobel, Dietary intervention with the probiotics *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913 modulates DNA-damage inducing potential of human faecal water in HT29clone19A cells, *Br J Nutr, 91*, 1-9, 2004.

Ordovas, J. M. and D. Corella, Nutritional genomics, *Annu. Rev. Genomics Hum. Genet., 5*, 71-118, 2004.

Osswald, K., T. W. Becker, M. Grimm, G. Jahreis and B. L. Pool-Zobel, Inter- and Intra-individual variation of faecal water – genotoxicity in human colon cells, *Mutation Res, 472*, 59-70, 2000.

Pfaffl, M. W., G. W. Horgan and L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, *Nucleic Acids Res., 30*(9), e36, 2002.

Schatzkin, A. and V. Kipnis, Could exposure assessment problems give us wrong answers to nutrition and cancer questions?, *J. Natl. Cancer Inst., 96*(21), 1564-1565, 2004b.

Schatzkin, A. and V. Kipnis, Could exposure assessment problems give us wrong answers to nutrition and cancer questions?, *J. Natl. Cancer Inst., 96*(21), 1564-1565, 2004a.

Steinmetz, K. A. and J. D. Potter, Vegetables, fruit, and cancer. I. Epidemiology, *Cancer Causes and Control, 2*, 325-357, 1991.

Terry, P., E. Giovannucci, K. B. Michels, L. Bergvist, H. Hansen, L. Holmberb and A. Wolk, Fruit, Vegetables, Dietary Fiber, and Risk of Colorectal Cancer , *J Natl Cancer Inst, 93*, 525-533, 2001.

Veeriah, S., C. Miene, N. Habermann, T. Hofmann, S. Klenow, S. Julia, F. Böhmer, S. Wölfl and B. L. Pool-Zobel, Apple polyphenols modulate expression of selected genes related to toxicological defense and stress response in human colon adenoma cells, *submitted for publication,* 2007.

Veeriah, S., T. Kautenburger, N. Habermann, J. Sauer, H. Dietrich, F. Will and B. L. Pool-Zobel, Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, *Mol. Carcinog., 45*(3), 164-174, 2006d.

Veeriah, S., T. Kautenburger, N. Habermann, J. Sauer, H. Dietrich, F. Will and B. L. Pool-Zobel, Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, *Mol. Carcinog., 45*(3), 164-174, 2006c.

Veeriah, S., T. Kautenburger, N. Habermann, J. Sauer, H. Dietrich, F. Will and B. L. Pool-Zobel, Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, *Mol. Carcinog., 45*(3), 164-174, 2006b.

Veeriah, S., T. Kautenburger, N. Habermann, J. Sauer, H. Dietrich, F. Will and B. L. Pool-Zobel, Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics, *Mol. Carcinog., 45*(3), 164-174, 2006a.

Table 1: Genotoxic effects of H_2O_2 in human colon cells (HT29) treated (24 h) with ileostomy samples (5 %, v/v) obtained before and after intervention with apple juice. Shown are tail intensities (%) (means ± SD) of 3 independent comet assay experiments. Values in bold print indicate significant ($^{\S}p\leq0.05$, $^{\S\S}p\leq0.01$, $^{\S\S\S}p\leq0.001$) differences between the ileostomy samples obtained before (0 h) and after 2 h intervention. *p≤0.05, **p≤0.01, ***p≤0.01 indicate significant differences to the untreated controls (cell culture medium with PBS) or H_2O_2 treatments.

Challenge for 5 min with	Person 1					Person 8					Person 11				
	Samples were from before (0 h) and 2 h after intervention														
	0 h		2 h		Difference	0 h		2 h		Difference	0 h		2 h		Difference
H_2O_2 (µM)	Mean	SD	Mean	SD	0 - 2 h	Mean	SD	Mean	SD	0 - 2 h	Mean	SD	Mean	SD	0 - 2 h
Control	2.9	0.5	2.3	0.7	0.6	2.0	0.5	1.4	0.2	0.7	2.7	0.7	3.3	0.8	-0.5
4.7	5.7	2.0	3.9	1.2	1.8	2.2	0.9	1.2	0.5	0.9	2.0	0.5	3.0	0.5	-1.0
9.4	3.7	0.9	4.0	1.5	-0.3	2.4	0.2	2.1	0.9	0.4	4.2	1.7	3.2	1.1	0.9
18.8	4.4	2.4	4.1	2.0	0.3	7.8	4.6	3.1	0.4	4.7	*11.0	3.6	3.5	0.9	§§**7.5**
37.5	5.9	1.0	5.6	2.1	0.3	**11.8	2.3	2.7	1.1	§§§**9.1**	**13.4	0.9	***10.3	2.2	3.2
75.0	*9.8	1.8	***9.5	2.3	0.3	***21.4	4.1	***7.6	1.7	§§§**13.7**	***24.7	2.8	***18.3	1.8	§**6.4**
150.0	***28.3	5.0	***25.2	0.9	3.2	***41.6	4.2	***14.2	0.9	§§§**27.4**	***44.1	7.0	***30.8	2.3	§§§**13.3**

Challenge for 5 min with	Person 4					Person 7					Person 9				
	Samples were from before (0 h) and 2 h after intervention														
	0 h		2 h		Difference	0 h		2 h		Difference	0 h		2 h		Difference
H_2O_2 (µM)	Mean	SD	Mean	SD	0 - 2 h	Mean	SD	Mean	SD	0 - 2 h	Mean	SD	Mean	SD	0 - 2 h
Control	3.4	1.1	3.3	0.9	0.1	1.4	0.5	4.7	1.7	-3.3	2.5	0.5	3.4	0.8	-0.9
4.7	4.3	1.0	3.8	1.0	0.5	5.0	1.8	4.3	2.1	0.7	*12.5	0.9	10.6	5.1	1.9
9.4	8.8	0.6	**14.4	2.2	-5.6	10.1	1.0	7.7	1.0	2.4	***16.8	2.3	*14.6	4.8	2.1
18.8	***20.1	3.8	***29.1	1.4	§§**-8.9**	6.3	0.3	*10.6	1.5	-4.4	***28.6	3.5	***22.2	2.5	6.4
37.5	***37.8	5.8	***42.0	6.1	-4.1	***21.5	8.9	***24.1	2.3	-2.6	***30.6	1.1	***40.2	5.9	§**-9.6**
75.0	***41.9	3.5	***35.2	1.2	6.7	***35.5	3.3	***51.1	2.6	§§§**-15.6**	***47.9	2.5	***34.7	4.4	§§§**13.2**
150.0	***39.9	0.5	***41.0	2.8	-1.0	***53.4	4.5	***56.8	3.0	-3.4	***45.8	5.8	***42.4	0.2	3.4

Legends to the figures:

Figure 1:

Kinetic of excretion of (a) the total polyphenol and (b) chlorogenic acid in the ileostomy samples (P=8) obtained before (0 h) and after (1- 8 h) intervention with apple juice.

Figure 2:

Effect of apple juice on genotoxic activity assessed by the Comet assay in HT29 cells treated with ileostomy samples (5 % v/v, n = 8) obtained before (0 h) and after 2 h intervention with apple juice. Induction of oxidative damage in HT29 cells with H_2O_2 was used as a positive control. Data are expressed as mean ± SD. Two-way ANOVA with Bonferroni's post-test was used to compare the statistical differences between 0 h and 2 h ileostomy samples and one-way ANOVA with Bonferroni's post-test was used to compare each subject to untreated control; **$p \leq 0.01$.

Figure 3:

Effects of ileostomy samples derived from subject 3 on DNA damage induced by H_2O_2 (37°C, 5 min) in HT29 cells. Cells were pretreated for 24 h with the ileostomy samples obtained before (0 h) and after 2 h intervention with apple juice. The figure shows tail intensities (%) (mean ±SD, n=3). Statistical differences were determined by one-way ANOVA with Bonferroni's post-test.

Figure 4:

Transcriptional expression of *GSTT2* gene was measured by SYBR green I real-time PCR in HT29 cells. Cells were treated with ileostomy samples (5 %, v/v) before (0 h) and after intervention (2 h) with apple juice for up to 24 h. The differences in the average threshold cycle (Ct) values were

determined and normalized to the expression of *GAPDH* mRNA. The data reflect the effect of the 2 h sample against the 0 h sample (average of 3 separate experiments, mean ± SD).

Figure 5:

Effect of ileostomy samples on *GSTT2* promotor activity. HT29 cells were transfected with a *GSTT2* promoter construct driving expression of firefly luciferase and treated with the indicated ileostomy samples for 24 h. Thereafter, reporter activity was assayed and normalized to a cotransfected Renilla luciferase reporter. The change of reporter activity in presence of the 2 h ileostomy samples of the indicated subjects in comparison with the 0 h (100 %) sample is depicted.

Figure 1. Total polyphenol and chlorogenic acid excretion

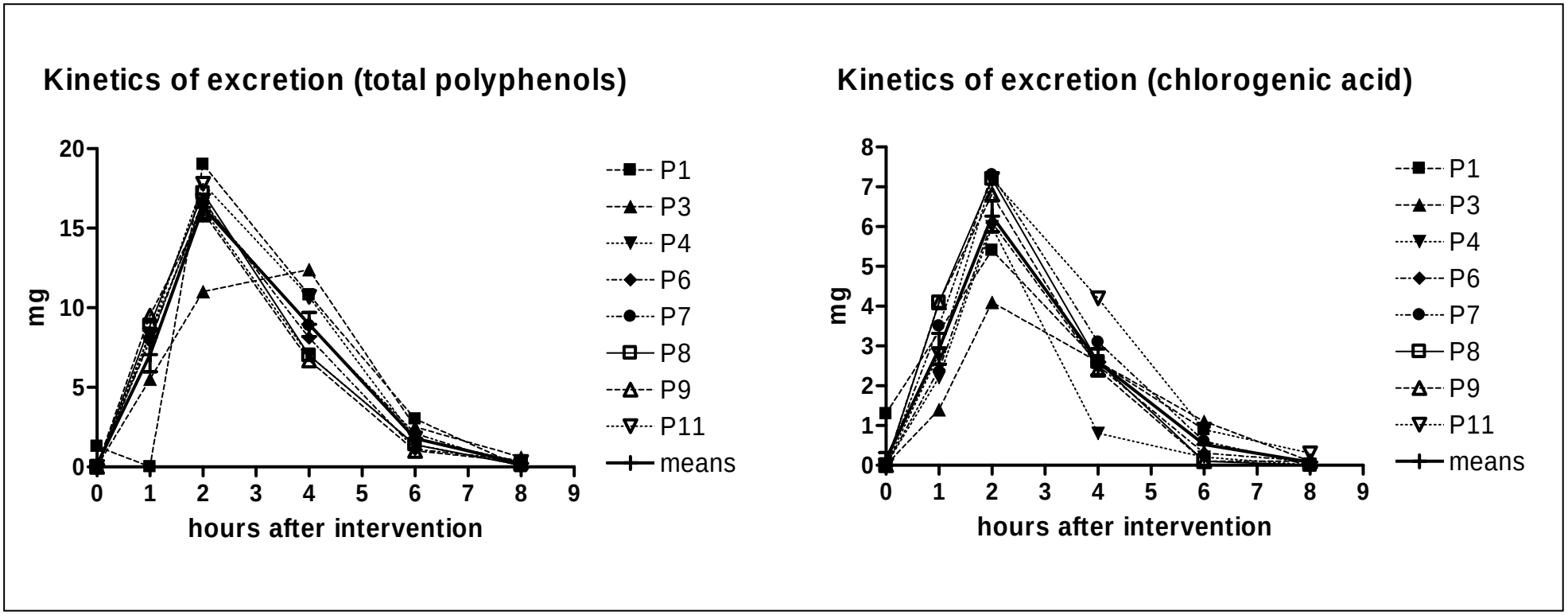


Figure 2: Genotoxicity before and after intervention

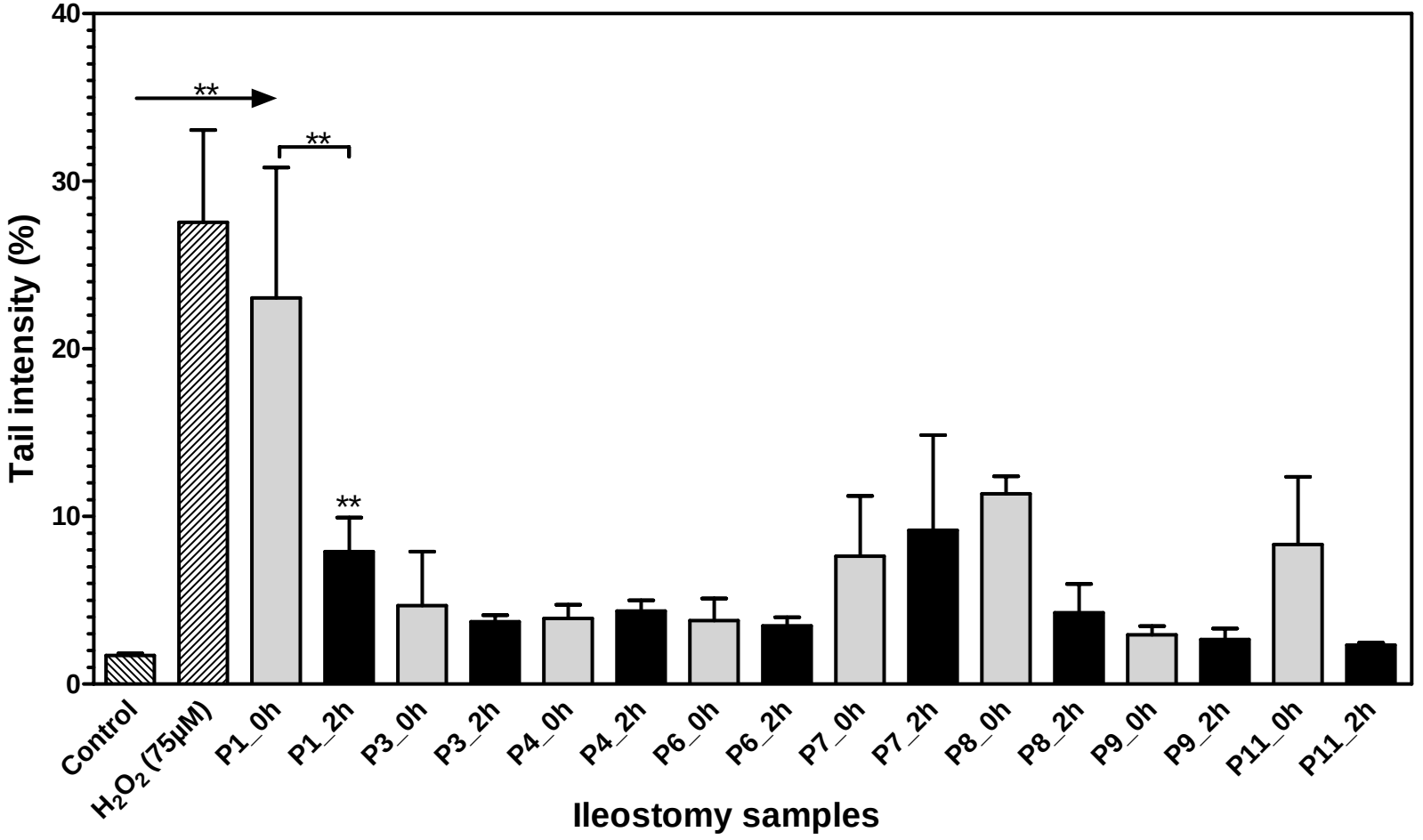


Figure 3: Effects of ileostomy sample 3 on genotoxicity

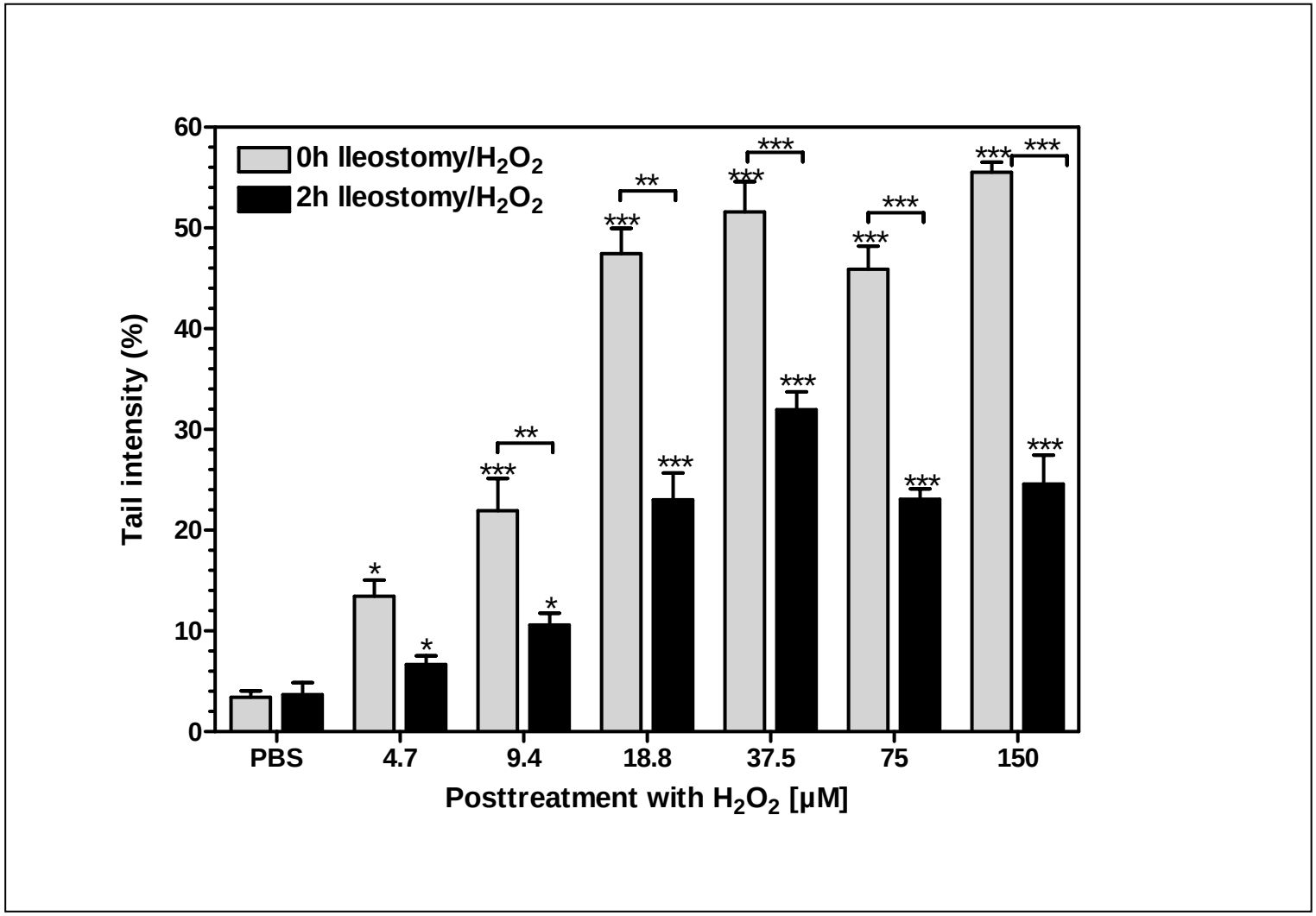


Figure 4: *GSTT2* mRNA induction in HT29 cells by ileostomy samples

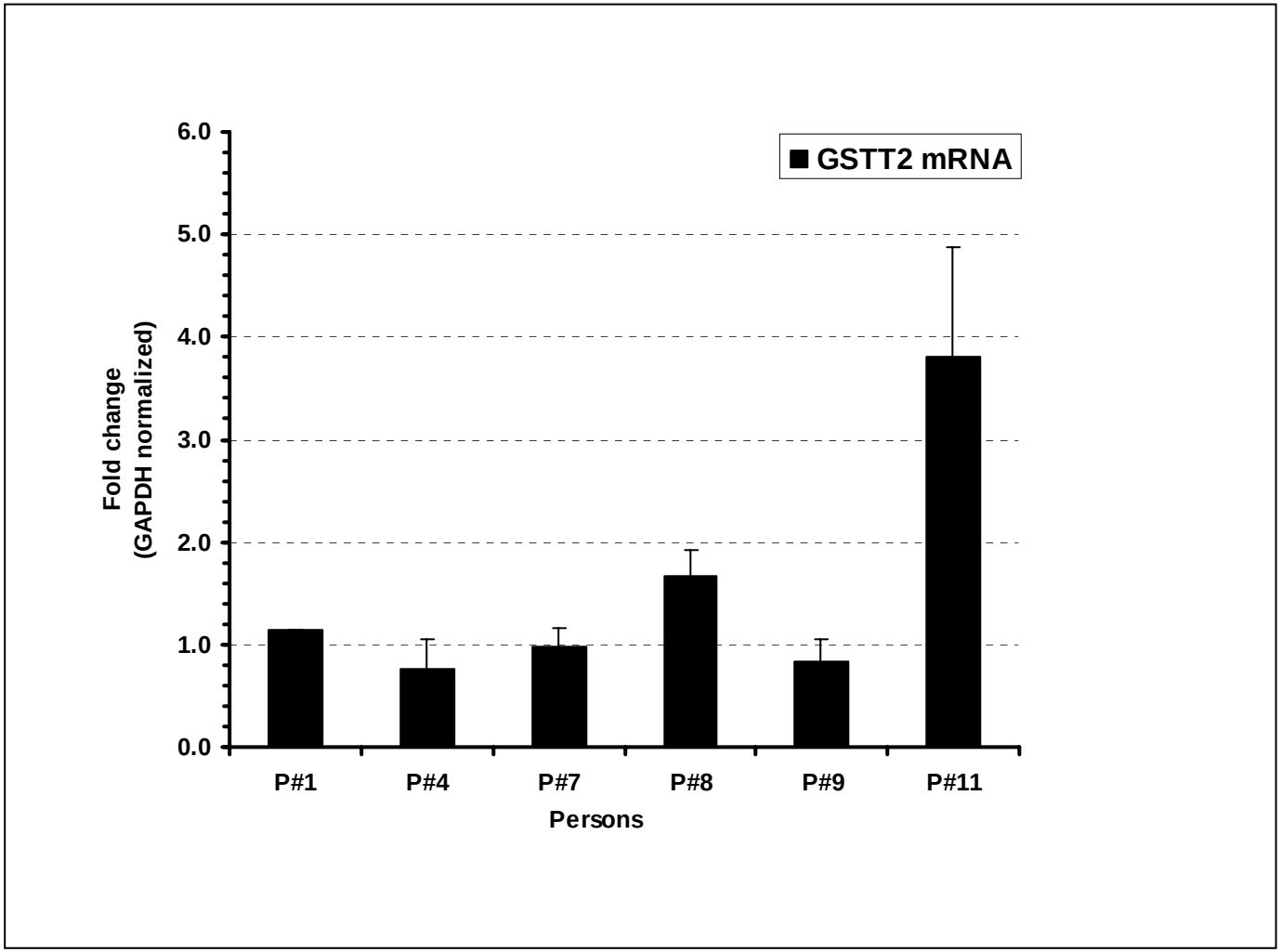
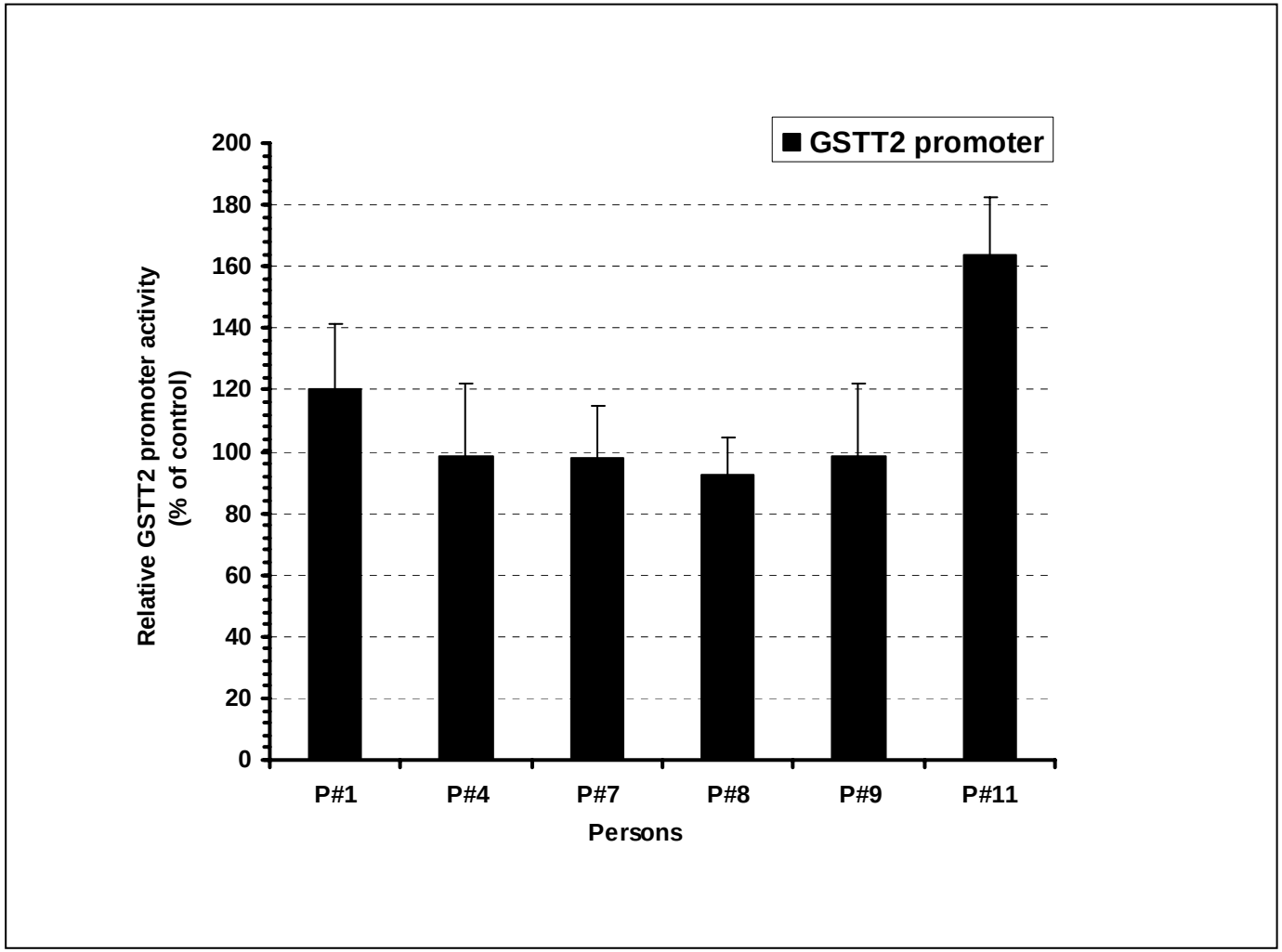


Figure 5: Effects of ileostomy on *GSTT2* promoter activity



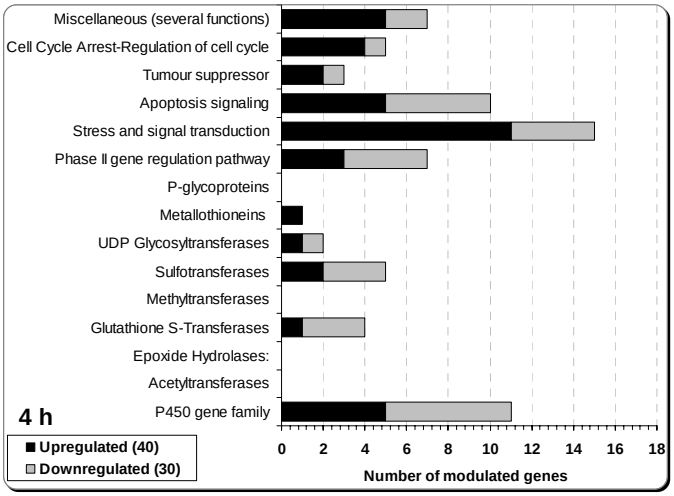
3 Additional Results

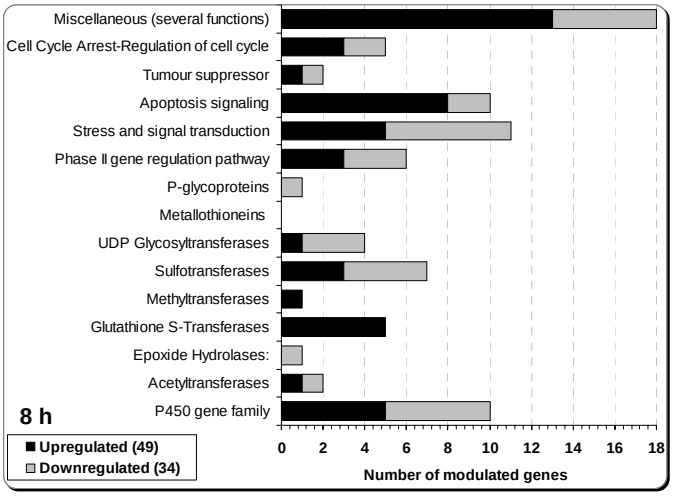
3.1 Affymetrix arrays for global gene expression analysis in time series

Previous studies have demonstrated the effects of polyphenols in cultured human colon epithelial cells after a 24 h exposure period (Noe *et al.*, 2004). In our study a similar exposure time was chosen to determine the effects of apple polyphenols on gene expression in colon cells. Now, it would be interesting to study the expression changes at earlier time points because gene expression changes can occur already after short-time exposure (Guo *et al.*, 2005). The effects of AE on global gene expression in human colon cells have not been reported before. Therefore, the aim of this work was to study the molecular effects of AE on LT97 cells, by gene expression analysis in time series using the Affymetrix GeneChip™.

We performed global gene expression analysis using the Human Genome U133A chip (Affymetrix GeneChip™, Mercury Park, UK), which contains approximately 34,000 sequences. For this the LT97 cells were treated with AE (128 μg/ml) for 4, 8, 12 and 24 h. Total RNA was isolated from control (cell culture medium only) and AE treated cells using Qiagen RNAeasy plus mini kit (QIAGEN, Hilden, Germany). cRNA probes were synthesised according to the Affymetrix GeneChip expression analysis manual and hybridised with Human Genome U133A array (Affymetrix). Hybridisation data were normalised and analysed. The treated samples were compared to the corresponding untreated culture at the same time point. Genes that showed changes ≥1.5 or ≤0.7-fold in experiments were chosen for further analysis. The labelling and hybridisation was done in a single experiment.

Only these 300 genes that are spotted on the custom array (**Publication V**) were chosen for the analysis of the affymetrix array results. Based on gene functions the altered genes were grouped and lists of up- and down-regulated genes at any of the four time points were created (Figure 9). Affymetrix analysis showed that the 8 h treatment was most effective in terms of number of upregulated genes and showed a total of 49 upregulated genes and 34 downregulated genes. 24 h treatment showed a total of 44 upregulated genes and 39 downregulated genes. 12 h treatment had the second most effective (46-up/28-down) and 4 h treatment was least effective (40-up/30-down) in terms of upregulated genes. AE effectively upregulates higher numbers of genes at early time (8 and 12 h) points than 24 h nevertheless, the total number modulated genes were similar for 12 and 24 h treatment time points. Thus, these 8 or 12 h incubations would be preferred to study the effects of AE on gene expression in LT97 cells. Moreover, it was observed that more genes were induced than repressed at all time points except for 24 h time points, suggesting a common mechanism of AE induced differentiation than repression in LT97 cells.





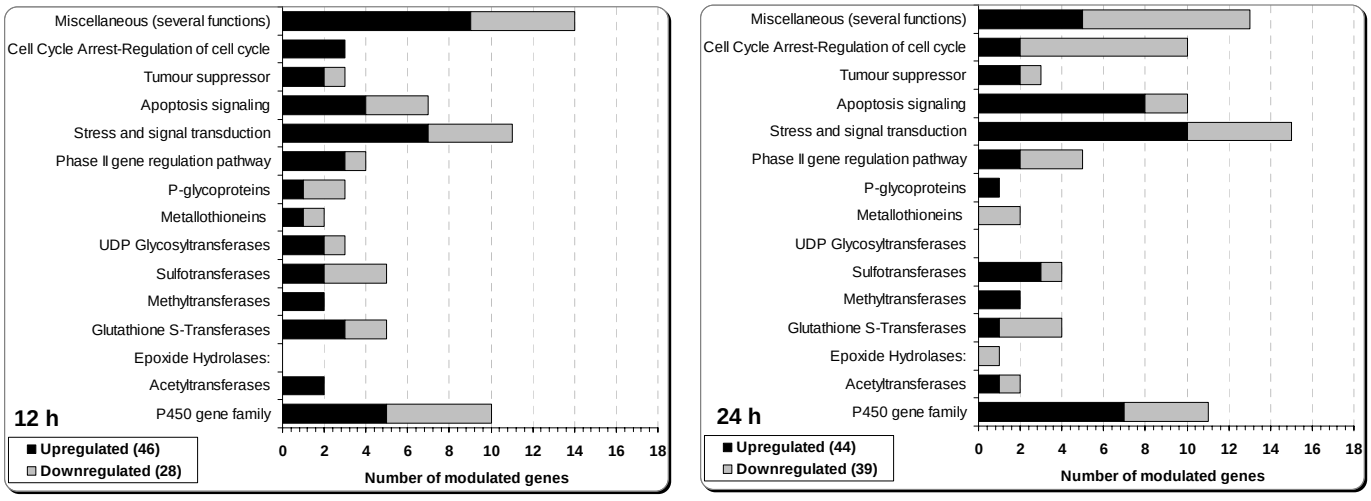


Figure 9. Effects of AE on global gene expression in LT97 cells analysed by affymetrix arrays in time series (4 - 24 h).

3.2 Comparison of affymetrix vs. custom array vs. superarray gene expression

Comparing different microarray data across different experiments may provide the basis for further choice of array platform and development of array methodologies. Therefore, in addition to the analysis of time kinetic gene expression pattern in LT97 cells after AE treatment, we have also compared the gene expression pattern between three major types of technology platforms, namely Affymetrix GeneChip™, cDNA spotted on glass array (custom array) and cDNA spotted on membrane array (superarray®). The effects of AE on gene expression pattern in LT97 cells were obtained from superarray and custom array analysis (**Publication V**). These array results were produced from 24 h treated cells. Therefore, only the results of 24 h treatment obtained from affymetrix analysis were used in order to compare between the different array platforms and the results are presented in Figure 10. Affymetrix

array analysis revealed a total of 44 upregulated (≥1.5 or ≤0.7-fold) genes and showed that 39 genes were downregulated after 24 h treatment. Superarray contains 96 genes related to drug metabolism and a custom array which contains 300 genes (including some genes from superarray) related to mechanisms of carcinogenesis or chemoprevention. Treatment of LT97 cells with AE resulted in 30 and 46 genes over cut-off values (≥1.5 or ≤0.7-fold) in superarray and custom array, respectively. Superarray array analysis resulted in statistically insignificant regulation of genes. Custom array results indicated that 14 genes were significantly ($p \leq 0.05$, *t*-test) modulated. Indicating, the custom array platform seems to attain better accuracy than superarray platform. Comparison of affymetrix vs. custom array reveals 16 genes were similarly altered. In terms of similarly expressed genes between affymetrix and custom array are higher number (16 genes) than custom array vs superarray (4 genes) and thus, affymetrix and custom array matches well. However, since the affymetrix experiment was produced from a single attempt, statistical analysis was not possible. Analysis of affymetrix vs superarray showed 5 genes were similarly altered. Analysis of custom array vs. superarray showed 4 genes were similarly regulated. Moreover, comparisons of affymetrix vs. custom array vs. superarray showed that the responses were indeed very different. Only 2 genes (CYP3A7, CYP4F3) were similarly altered in all three arrays (Figure 10).

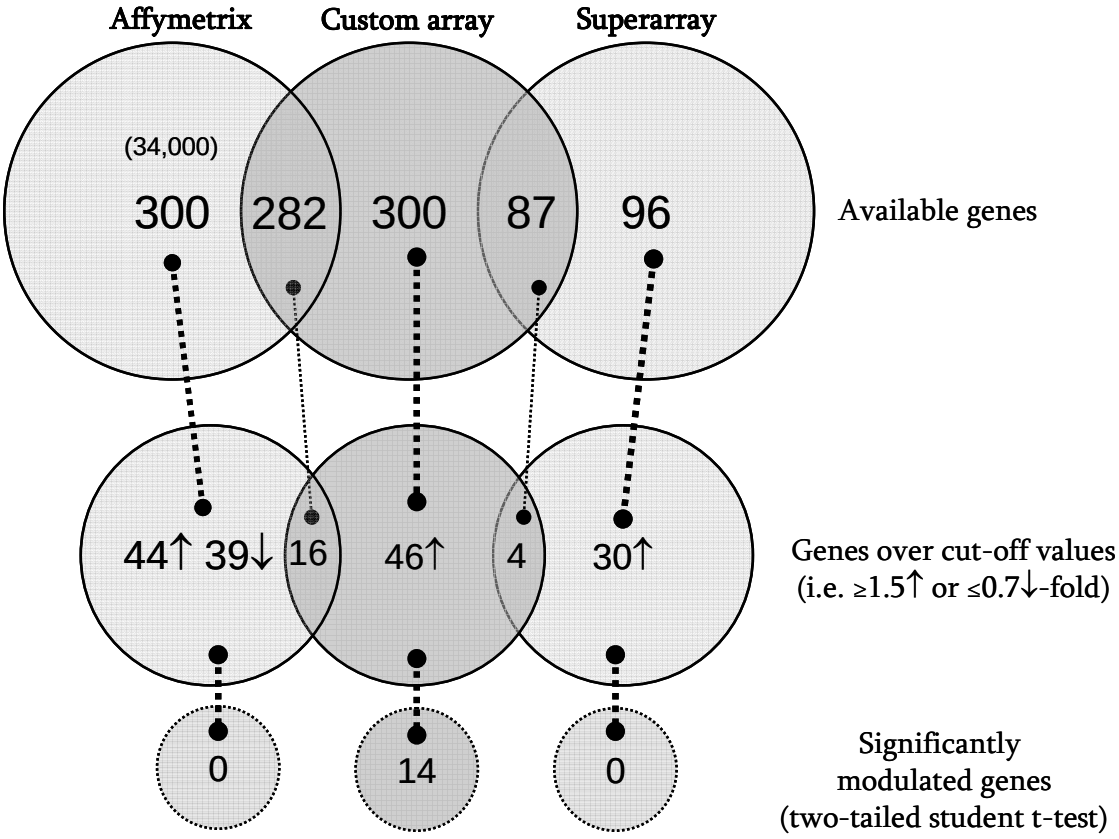


Figure 10. Venn diagram illustrates the comparison of gene expression pattern between three array (Affymetrix vs Custom array vs Superarray) platforms. *For each mapping the data were obtained from affymetrix (n=1), custom array (n=4) and superarray (n=4) experiments. The numbers that are shown in big grey circles are the total number of genes spotted on either array. The numbers that are shown in small grey circles are chosen as the number of regulated (≥1.5 or ≤0.7-fold change) genes. The numbers in small dotted grey circles refer to the number of genes that are detected as significantly differentially expressed (two-tailed student t-test). Statistical analysis was not possible for affymetrix data, since there were no treatment replicates.*

3.3 Apple flavonoids modulate the genotoxic effects of different DNA damaging compounds

Apple polyphenols are possibly chemoprotective, since they enhance gene expression of detoxifying glutathione *S*-transferases (e.g. *GSTT2*, *GSTP1*) in human colon cells. Aim of this study was to elucidate whether pretreatment of the cells with an apple extract (AE) also reduces DNA-damage caused by compounds that are deactivated by induced GSTs. HT29 cells were incubated with the AE for 24 h. Concentration capable of modulating xenobiotic enzymes gene expression (510 µg/ml) was used. The treated cells were then challenged with genotoxic compounds and DNA damage was determined with the Comet assay. The Comet assay was carried out under the conditions described by Tice et al. (Tice *et al.*, 2000). Cumene hydroperoxide (Cum-OOH, 60-360 µM) and hydrogen peroxide (H_2O_2, 4.7-150 µM) were used to challenge the pretreated cells, both for 5 minutes at 4°C, since they may be conjugated and deactivated by *GSTT2*. In addition, hydrogen peroxide formation in the cell free culture media in the presence of the AE was analysed using the ferrous oxidation in xylenol orange (FOX, version 2) assay (Jaeger *et al.*, 1994).

The synthetic hydroperoxide Cum-OOH was significantly genotoxic in HT29 cell line (grey bars in Figure 11A). Preincubation of HT29 cells with AE reduced viability of HT29 cells significantly after the challenge (84±4% in medium control to 56±5% in AE treated cells, $p \leq 0.001$, t-test). Moreover, preincubation with AE reduced the genotoxic effects of Cum-OOH (black bars in Figure 11A). H_2O_2 was investigated as a model for endogenously, relevant compounds. H_2O_2 was also significantly genotoxic at 37.5 µM and higher (grey bars in Figure 11B). Again, viability was strongly reduced in AE treated cells after the challenge (81±6% in medium control to 41±7% in AE treated cells, $p \leq 0.001$, t-test) and genotoxicity of H_2O_2 was significantly lowered (black bars in Figure 11B).

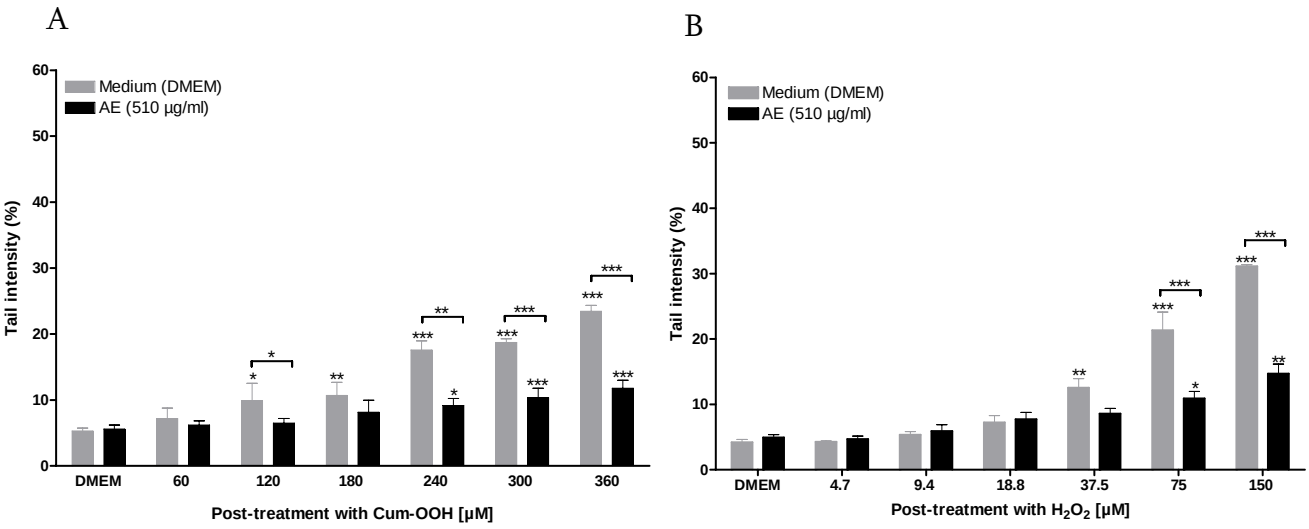


Figure 11: Levels of DNA damage induced by (A) Cum-OOH (B) H_2O_2 after preincubation of HT29 cells with AE measured with the Comet assay (mean ± SEM, n=3)*. The significant differences of the genotoxines were calculated to the untreated control by one-way ANOVA, including Bonferroni's multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). The effect of the apple extract preincubation was calculated using two-way ANOVA, including Bonferroni's multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).*

Incubation of the AE in HT29 cell culture medium (DMEM with 10 % FCS) resulted in a significant production of hydrogen peroxide already at 170 µg (Figure 14). In a parallel study after addition catalase to the incubation mixture, H_2O_2 was not detectable any longer, confirming formation of H_2O_2 (not shown).

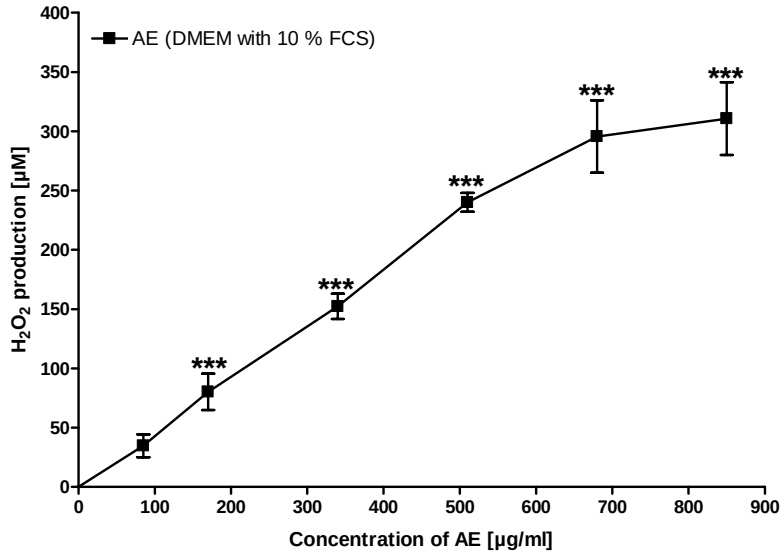


Figure 12: Hydrogen peroxide formation in the culture media (DMEM with 10 % FCS) in the presence of the apple extract AE (30 min, mean ± SEM, n=3). *The significant differences to the untreated medium control were calculated by one-way ANOVA, including Bonferroni's multiple comparison test (*** $p \leq 0.001$).*

Additionally, we will continue to analyse the antigenotoxic activity of AE also in colorectal adenoma cell line (LT97) which represents an early stage of tumour development. Even less concentration of AE induced gene expression of phase II enzymes to a greater extent in LT97 cells. Thus, we would expect that more pronounced antigenotoxic effect of AE in LT97 than HT29 cells with the applied genotoxins. In detail, we could show that one of the most important intestinal GSTs *(GSTP1)* was induced by AE (Publication I). Benzo(a)pyrene diolepoxide (BPDE), as a substrate for *GSTP1*, plays also role in colon carcinogenesis thus it will be also the of interest whether apple preincubation reduced BPDE-induced DNA damage.

4 Discussion

4.1 Colon adenoma (LT97) and carcinoma (HT29) cell lines as a model system

Identifying potential anticancer properties of phytochemicals using animal models is time consuming and expensive. *In vitro* methods can provide a more practical alternative. *In vitro* methods can and should play a much more important role in the risk assessment process (e.g. DNA damage, reduction in genotoxicity) and, in fact, with the appropriate data *in vitro* methods might completely bypass animal use (Fearon and Vogelstein, 1990). Cell culture techniques have been used extensively as an *in vitro* method to assess the effects of polyphenols on humans. HT29, a human colon carcinoma cell line, have numerous morphological and biochemical characteristics of enterocytes (Fogh, 1975). This cell model has been used in a wide variety of nutritional studies, particularly in the study of mechanisms and in the regulation of gene expression (Pool-Zobel *et al.*, 2005b). Although many studies have utilised this model (HT29) to investigate the effects of polyphenols only very few studies compared the same effects with such induced in human colon adenoma cells. The present study was carried out to evaluate the beneficial health effects of apple polyphenols and we compared the effects on HT29 cells and LT97 cells (**Publication II**). LT97 is another human colon cell line but of adenomatous origin which is representative of preneoplastic leasons of human colon cells (Richter *et al.*, 2002). The results of our study will strengthen the use of this LT97 cell model to study the effects of different food components.

4.2 Inhibition of proliferation of colon cancer cell lines by apple polyphenols

Epidemiological findings suggest that plant foods decrease colorectal tumour risks (Glade, 1999). This could be due to a number of different phenolic phytoprotectants, which act chemopreventive by inhibiting the growth of tumour cells (Boyer and Liu, 2004; Terry *et al.*, 2001). Apples contain significant amounts of flavonoids that have antioxidative or antiproliferative activities, and thus can possibly reduce the cancer risk. Previous studies have shown that apple flavonoids can inhibit liver cancer cell growth *in vitro* (Eberhardt *et al.*, 2000). In the present study the growth of colon carcinoma cells (HT29) was significantly inhibited by the complex apple extract (**Publication I**). Two groups reported that quercetin aglycones arrested growth in cell lines derived from gastric, colonic and leukemic cancers (Hosokawa *et al.*, 1990; Yoshida *et al.*, 1992). Some of these compounds are also ingredients of apple flavonoid mixtures, such as quercetin aglycones and phloridzin aglycones that we investigated in our cellular system. We observed that the aglycones quercetin and phloretin significantly inhibited HT29 tumour cell growth, suggesting that these components also contributed to the growth inhibitory properties of the complete apple extract. This is in line with other studies showing that the individual apple flavonoid aglycones possess strong cell growth inhibitory activities and are biologically more active than the glycoside derivatives (Kuo, 1996; Shen *et al.*, 2003). An important, and so far unique, finding of our study was the observation that the individually tested apple flavonoids and their glycosides were hardly inhibitory on their own, but that equimolar concentrations applied as mixtures (mimicking the complete apple extract) were biologically active, resulting in an impairment of cell growth and survival (**Publication I**).

In another part of the study, the effects of apple polyphenols on survival of colon adenoma (LT97) and carcinoma-derived (HT29) cell lines were investigated. Three apple extracts (AEs) from harvest years 2002-2004 were isolated (AE02, AE03, AE04) and fermented *in vitro* with human faecal flora. Extracts and fermentation products were analysed for polyphenols with HPLC. The cells were treated with AEs or fermented AEs (F-AE02, F-AE03, F-AE04) and survival was measured by DNA staining (**Publications II**). The analyses of polyphenols showed that each AE contained different concentrations and types of polyphenols and provided evidence for remarkable differences depending on cultivars, varieties, and harvest years. In addition, the fermentation process resulted in formation of short chain fatty acids (SCFA), and the polyphenols were degraded (99.9 %). Thus, by the fermentation of apple polyphenols through the gut flora, SCFA can be produced in the human colon. AEs were consistently about 3 fold more growth inhibitory than F-AEs in both LT97 and HT29 cells. Thus, fermentation reduced the effectiveness of AEs. The antiproliferative activity of AE03 was higher than that of AE04 and AE02 in both LT97 and HT29 cells. The pronounced antiproliferative activities of AE03 could be a result of its higher quercetin concentration which was about 10 and 13 fold higher than the respective concentrations in AE04 and AE02. Moreover, F-AE03 inhibited cell growth more efficiently than F-AE04 and F-AE02 in both LT97 and HT29 cells. An explanation for this finding is that F-AE03 contained higher concentrations of metabolites (e.g. 3,4-dihydroxyphenylpropionic acid, phloroglucin) compared to other F-AEs, indicating that the adenoma and carcinoma cell proliferation is significantly inhibited by a specific combination of apple polyphenols/flavonoids. The growth inhibition of adenoma-derived LT97 was more pronounced than of carcinoma-derived HT29 cells after treatment with both AEs and F-AEs. Thus, apple polyphenols might have higher antiproliferative efficacy in the preneoplastic lesion than in carcinoma cells.

4.3 Efficacy of apple polyphenols to modulate gene expression in colon cells

Understanding the chemical inducibility of phase II enzymes in colon cells is of importance for development of chemoprotective approaches for the management of colon cancer disease. It has been previously demonstrated that polyphenols are capable of inducing several phase II enzymes in cultured human colon cells as well as in mouse colon tissue *in vivo* (Breinholt *et al.*, 1999; Galijatovic *et al.*, 2000). Since colon epithelium is a critical target of oxidative and electrophilic stress during colon carcinogenesis, investigation of the inducibility of endogenous phase II enzymes by apple polyphenols in colon cells is warranted. Therefore, this study aimed to assess the effects of AE on patterns of expression of genes related to toxicological defence and to mechanisms relevant for early stages of carcinogenesis. Gene expression studies were performed using cDNA-arrays which contain genes related to mechanisms of carcinogenesis or chemoprevention. The results of the present study demonstrated that incubation of human adenoma (LT97) and colon carcinoma (HT29) cells with AE resulted in upregulation of many phase II genes, including GSTs, UGTs and GPXs (**Publication I and V**). This could be possibly related to chemoprevention (Massaad *et al.*, 1992), since the induction of phase II genes has been suggested to serve as biomarker of reduced cancer risk and of chemopreventive response (Clapper and Szarka, 1998; Talalay, 2000). Furthermore, inducers of GSTs and UGTs are generally considered to be protective compounds against cancer, acting as "blocking agents" (Graziani *et al.*, 2005; Kensler, 1997; Khan *et al.*, 1992). Apple polyphenols have been reported to be anticarcinogenic in several animal models (Barth *et al.*, 2005; Gosse *et al.*, 2005). However, induction of phase II enzymes such as, GSTs and UGTs by apple polyphenols has not been reported before. Furthermore, our study showed that the treatment of LT97 cells with AE altered the GST and UGT enzyme activity levels. These data provide the first examination of the modulation of the phase II enzymes

by AE and indicating a unique aspect of preventing cellular damage from carcinogens (**Publication I and V**).

The signaling pathway(s) underlying AE-mediated upregulation of the several phase II enzymes in colon cells remain to be investigated. Moreover, the nuclear factor E2-related factor 2 (Nrf2) has been demonstrated to be an essential regulator of phase II gene expression in various tissues and cell types (Lee and Surh, 2005). Nrf2 is a transcription factor important for the stress-dependent expression of a set of chemoprotective genes, such as those for glutathione *S*-transferase (GST), NAD(P)H-quinone oxidoreductase 1 (NQO1) and glutamate cysteine ligase (Surh *et al.*, 2005). Nrf2 activates the expression of these genes through a *cis*-acting element called the antioxidant responsive element (ARE) (**Publication IV**). Studies are currently underway in our laboratory to investigate if Nrf2 signalling is also involved in the AE-mediated upregulation of phase II genes in HT29 cells.

In addition, AE fermentation with human gut flora produces several SCFA including butyrate and it may play an important role in the human colon. Colon crypts use butyrate as an energy source, whereas in tumour cells butyrate stimulate pathways of growth arrest, differentiation, and apoptosis (Heerdt *et al.*, 1994; Mariadason *et al.*, 2000; Singh *et al.*, 1997). Although the present study showed that the treatment of different human cells such as primary, adenoma and tumour colon cells treated with butyrate modulated several detoxifying enzyme systems and thus, may enhance toxicological defence in human colon cells (**Publication III**).

Apple polyphenols have been shown to inhibit G2/M phase cell cycle and suppress protein kinase C (PKC) and can increase the expression of extracellular signal-regulated kinase 1 and 2 (ERK1, 2), c-Jun N-terminal kinases (JNK) and activity of

caspase-3 in SW620 cells (Gosse *et al.*, 2005). These actions would inhibit cell growth and transformation, induce apoptosis, and inhibit angiogenesis. Moreover, our study has shown that after AE exposure (128 µg/ml) to LT97 cells expression of genes related to several functions such as tumour suppression, cell cycle arrest, cell signalling and apoptosis was significantly enhanced. It is possible that differential modulation of certain genes, such as *PTPRJ*, *PTPRN*, *MAPK* and *CASP10* may cause differential effects of AE on the growth arrest and induction apoptosis of cancer cells (**Publication V**).

4.4 Effects of apple polyphenols on global gene expression in colon cells analysed by affymetrix arrays in time series

Affymetrix array analysis of gene expression in time kinetics (4, 8, 12 and 24 h) showed, AE modulates a higher number of transcriptional changes rather at the early time points (8 and 12 h) than after 24 h, indicating that a large part of early events occur at the level of transcription in LT97 cells after addition of AE. Thus, further analysis of AE mediated gene expression in LT97 cells at earlier time points provide better insights in the complex molecular mechanisms of AE effects and potential targets for the development of new biomarker for chemoprevention. Interestingly, most of the altered genes were shown to be transcriptionaly upregulated, suggesting a common mechanism of AE induced differentiation than repression in LT97 cells.

Comparison of multiple microarray platforms for gene expression is not easy because of many ambiguities, e.g., the genes spotted on affymetrix array are oligo nucleotides and each target gene has at least 10 different oligo probes. In contrast, superarray and custom array contain genes that are spotted as cDNA fragments (200-400 bp). In practice, gene expression comparison between custom array and superarray are

possible since both platforms have higher similarity such as length of cDNA nucleotide sequence (200-400 bp) and array processing. However, we have compared all three platforms to see if the genes were similarly expressed by chance. Only two genes *(CYP3A7, CYP4F3)* were consistently found to be altered across all platforms. These two genes (*CYP3A7, CYP4F3*) not involved in carcinogen activation and not yet described to be involved in colon carcinogenesis. Comparison of the gene expression from three different array platform (cDNA and oligonucleotide) showed that the responses are indeed very different indicates that difficulties in platform comparisons.

4.5 Apple polyphenols protect against genotoxic carcinogens *in vitro* and *ex vivo*

It has been proposed that polyphenols exert their chemoprotective effects by inducing several phase II detoxifying enzymes which results in modification and rapid excretion of carcinogens (Lin and Liang, 2000). The upregulation of GSTs can protect against DNA-damaging effects of 4-hydroxy-2-nonenal (HNE) in colon cells (Ebert *et al.*, 2001). In this study we investigated in human colon cell line (HT29) *in vitro* whether an apple juice extract contains polyphenols has chemoprotective effects. In particular, the apple extract was tested for its ability to reduce DNA-damage induced by different genotoxic agents or oxidants. Furthermore, production of H_2O_2 by AE was studied to understand additional mechanisms of chemoprotective effects. Present data provided evidence that polyphenol-rich apple extracts reduce DNA damage in colon carcinoma (HT29) cells initiated by relevant risk factors (Cum-OOH, H_2O_2). Obviously, an increased expression of *GSTT2* (pronounced substrate for Cum-OOH) gene was also noticed in colon cells by AE (**Publication I, V**). Therefore, the coordinated actions of the above cellular phase II enzymes ensure effective detoxification of genotoxines. H_2O_2 production by polyphenols is normal process

(Akagawa *et al.*, 2003) however; further investigations are necessary to clarify the H_2O_2-producing property of polyphenols and their prooxidative and on the other hand protective effects *in vitro*. Altogether, the reduction of DNA damage in human colon cells by apple polyphenols could be a new target for colon cancer chemoprotection.

Apple juice is considered to be an important component of a healthy diet, which has recently been shown to have numerous types of chemoprotective activities in experiments with colon cancer animal models (Barth *et al.*, 2005) and in human colon cells *in vitro* (Gosse *et al.*, 2005). Since only little is known on comparable effects in human colon from *in vivo* studies, here a pilot study was performed to assess related mechanisms in ileostomy samples from volunteers that had consumed apple juice. Eight ileostomy samples were collected at different time points after intervention (0 - 8 h) and were characterised analytically for major apple polyphenols (Kahle *et al.*, 2005) and in HT29 colon cells for their potential to cause genotoxic damage, protect from the genotoxic insult by H_2O_2 and modulate the expression of *GSTT2*, an enzyme related to antioxidative defence of other peroxides. The analytical determination of polyphenols in the ileostomy samples revealed that the majority of the compounds were recovered in the samples collected 2 h after intervention, and chlorogenic acid was one of the predominant detected polyphenols (**Publication VI**). Such a compound could be responsible for reducing exposure to genotoxins and oxidants in the gut lumen, thus reducing the probability of damage to DNA of colon cells (Glei *et al.*, 2006).

The comparison of genotoxic effects of ileostomy samples before intervention and 2 h after intervention revealed a considerable variation of genotoxic response, but there was a trend for reduced genotoxicity potential in 3 of 8 persons after intervention

(**Publication VI**). In the context of a reduced basal genotoxicity, apple ingredients may be scavenging or inactivating genotoxic and toxic components naturally available in the gut lumen (Barth *et al.*, 2005). Samples collected at 2 h protected HT29 cells from genotoxic damage by H_2O_2 (for 3 of 7 persons) and increased *GSTT2* expression and of *GSTT2* promotor activity. This antigenotoxicity of the ileostomy samples could be due to a direct antioxidative effect by the polyphenols excreted in the 2 h samples. Among others, especially chlorogenic acid could be responsible for this effect, since it also reduced H_2O_2 genotoxicity in the challenge assay (Glei *et al.*, 2006). However, the other ileostomy samples of this study containing nearly similar amounts of chlorogenic acid did not respond to these parameters. This interesting finding deserves more in depth investigations, as it may be possible to identify different individuals which may more or less profit from the habit of consuming apple juice on the basis of their gut luminal contents. The effects were not significant on a group level and the number of subjects that participated in the study was too small to show an intervention effect and to prove the possibility that apple juice could lead to chemoprotection in the gut lumen. The pilot study, however, for the first time used this combination of faecal biomarkers which in larger cohorts may reveal significant alterations that contribute to reduced genotoxic exposure and thus to chemoprotection of colon cells. Taken together, it appears as if ileostomy samples, especially 2 h after intervention with cloudy apple juice, causes a number of biological effects related to chemoprotection, and that these effects have also been shown to be mediated by the apple extracts and/or individual phenolic components.

5 Conclusions

The effects of apple polyphenols on modulation of chemoprotective enzyme systems in human colon cells were studied in this work. Based on the results of this study, the following conclusions can be drawn:

- Different types of AEs (AE02, AE03, AE04), each containing different concentrations and types of polyphenols, significantly inhibit the growth of carcinoma (HT29) and colon adenoma (LT97) cells which represent late and an early premalignant stage of tumour development. Thus, evidence for antiproliferative activity of apple polyphenols is provided.

- Apple flavonoid aglycones potently inhibit the colon carcinoma cell growth whereas the individual glycosides are not effective. This indicates that aglycones may enter the cells easier than their glycosides.

- A synthetic mixture of polyphenols (mimicking the major apple polyphenols constituents) has a potent growth suppressing effect on colon carcinoma cells. Thus, growth inhibition may be due to the synergistic effects between the phytochemicals of the AE. Even though the synthetic mixture was more efficient than the single compounds, it did not reach the efficiency of the natural apple extract. Thus, the natural AE possibly contain additional compounds that contribute the higher chemoprotective potential.

- Fermentation of AEs resulted in an increase of SCFA and degradation of polyphenols. Thus, by the fermentation of apple polyphenols through the gut flora, SCFA can be produced in the human colon.

- Fermented AEs significantly inhibit the growth of LT97 and HT29 cells. However, the F-AEs were approximately 3 fold less bioactive (in terms of cell growth inhibition) than the corresponding AEs, indicating lower chemoprotective properties, this is possibly due to degradation of polyphenols.

- Apple extract AE03 and the fermented counter part (F-AE03) contain more quercetin compounds as well as the related metabolites and have the most pronounced effect on cell growth inhibition. The pronounced effect on cell growth inhibition might be triggered by higher concentrations of bioactive quercetin and their metabolites. Thus, the mixtures of major apple flavonoids as well as the amount of specific bioactive flavonoids are important factors for growth arrest in human colon cell lines.

- LT97 cells are more sensitive than HT29 cells towards growth inhibitory activities of AEs and F-AEs. This reflects higher antiproliferative potential of apple polyphenols in the preneoplastic lesions than in carcinoma. LT97 and HT29 cells were grown in different cell culture media. Thus, the higher antiproliferative potential of AEs and F-AEs in LT97 cells may also depend on the culture media used.

- Treatment of HT29 and LT97 cells with AE markedly influences the expression of genes encoding phase II enzymes, such as GSTs and UGTs. Moreover, AE increases the expression of several transcription factors related to ARE activation and histone family genes. This could be an important mechanism of transcriptional activation of phase II genes.

- AE modulates several genes which are related to important functions such as tumour suppression, cell cycle control, cell signalling as well as apoptosis in LT97 cells. Thus, the apple polyphenols serve as integrators of numerous signal-dependent pathways that control a multitude of genes.

- Confirming array results by real-time PCR shows that phase II genes such as *GSTT2, GSTP1, GSTA4, UGT1A1, UGT2B7* are indeed target genes. They are upregulated and thus point to induction of carcinogen detoxification by AE.

- AE effectively upregulates higher numbers of genes at early time points (8 and 12 h) than 24 h. Furthermore, these 8 or 12 h incubations would be preferred to study the effects of AE in LT97 cells.

- Comparison of different array platforms may not be possible unless the gene probes sets and array processing method matched.

- AE protects colon cells against DNA damage induced by relevant risk factors like Cum-OOH and H_2O_2 genotoxins by modulating the phase II gene such as *GSTT2* (pronounced substrate for Cum-OOH).

- Ileostomy samples obtained after apple juice interventions are less genotoxic than before the intervention. Pretreatment of HT29 cells with ileostomy samples protects HT29 cells from genotoxic damage by H_2O_2 and this treatment results in an increased *GSTT2* expression and *GSTT2* promotor activity. The intervention with apple juice results in bioavailable concentrations of related polyphenols in the gut lumen, which could contribute to reduced genotoxicity, enhanced antigenotoxicity and favourable

modulation of *GSTT2* gene expression, possibly together with other ingredients of the gut lumen content. The pilot study for the first time used this combination of faecal biomarkers which in larger cohorts may reveal significant alterations that contribute to reduced genotoxic exposure and thus to chemoprotection of colon cells.

- Altogether, these findings clearly underline the hypothesis that overexpression of multiple GST isoforms participate in the metabolism and elimination of potential human carcinogens by apple polyphenols. Chemoprophylaxis by apple polyphenols may, thus, continue to be a possible method of prevention of colon cancer since risks a hypothesis possibility that need to be verified in further human studies.

6 Outlook

In this work, the complex mixture of apple polyphenols on expression of chemoprotection related genes were assessed in cultured human colon cells. Now, it would be important to examine the effects of apple polyphenols and their metabolites on the expression of these gene products in primary human colon cells *(ex vivo)*, to improve chemoprotective strategies.

Apple polyphenols are indeed potential mediators for the transcriptional activation of several target genes that are related to colon cancer chemoprevention. However, the mechanism of signal transduction for the induction of these genes by apple polyphenols is not clear, but it may be related to the activation of the transcriptional factor Nrf2. Future mechanism-based *in vitro* or animal studies may facilitate understanding of the potential health benefits of apple polyphenols.

Although considerable research has been carried out on apple polyphenols and their chemopreventive role against carcinogens in cell culture and in animal model, it is still not fully clear how these compounds exert their action in human. Therefore, further experiments, carefully designed, are required to verify how apple polyphenols protect DNA from interaction with activated electrophilic metabolites.

7 Abstract

Introduction: Colorectal cancer is one of the most common cancers in the developed-world with Western style diets. Flavonoids from fruits and vegetables probably reduce colorectal tumour risks. Apples contain significant amounts of polyphenols that are potentially cancer risk reducing, possibly by acting antioxidative or antiproliferative and by favourably modulating gene expression.

Purpose: The objectives of this study were to investigate the effect of apple polyphenols (a) on survival of colon carcinoma (HT29) and adenoma-derived (LT97) cell lines, (b) on modulation of expression of genes related to colon cancer chemoprevention, (c) on the defence of cells against DNA-damage caused by genotoxic compounds *in vitro*, (d) to determine whether apple juice intervention could result in a decrease of genotoxins in the gut lumen *ex vivo* in humans.

Methods: HT29 and LT97 cells were treated with apple extracts (AE) or fermented AEs (F-AEs). HT29 cells were also treated with a synthetic flavonoid mixture mimicking the composition of the AE or with individual flavonoids and cell growth was measured by DAPI assay. Cells were treated with effective concentrations of AE and RNA was isolated to elucidate patterns of gene expression using human cDNA microarrays containing genes related to mechanisms of carcinogenesis or chemoprevention. Global gene expression measurements in time series (4 - 24 h) are additionally performed using affymetrix arrays and the results were compared to other array platforms. Real-time PCR and enzyme activity assays were additionally performed to confirm selected array results. Furthermore, AE treated cells were challenged with genotoxic compounds and DNA damage was determined with the Comet assay *in vitro*. Human ileostomy samples before (0 h) and after (2 h)

interventions with apple juice were compared for genotoxic activity in HT29 cells. HT29 cells pretreated (*ex vivo*) with the ileostomy samples were then also challenged with H_2O_2 and DNA damage was determined with the Comet assay. Moreover, HT29 cells pretreated with the ileostomy samples were assessed for modulation of the expression of *GSTT2 mRNA* level and *GSTT2* promoter activity using real-time PCR and reporter gene assay, respectively.

Results: The growth of LT97 and HT29 cell lines was significantly inhibited by the AE, and by the mixture of mimicking the major apple polyphenols constituents. Different AEs contained varying amounts of quercetin and relevant metabolite, which was associated with a different potential to cell growth inhibition. Fermentation of AEs resulted in an increase of short chain fatty acids, but polyphenols were degraded. The F-AEs were ~3 fold less bioactive (in terms of cell growth inhibition) than the corresponding AEs, pointing to reduced chemoprotective properties through fermentation. The growth inhibition of LT97 was more pronounced than of HT29 cells, indicating a higher effectiveness of AE in preneoplastic lesions of the human colon. Treatment of cells with AE resulted in an upregulation of several genes related to drugmetabolism and other genes belonging to several functions such as, tumour suppression, cell cycle control, cell signalling as well as apoptosis. Time kinetics gene expression analysis revealed most of the genes were upregulated at 8 and 12 h time points. Expression of selected genes *(Glutathione S-transferases [GST] P1, GSTT2, GSTA4, UDP-glucuronosyltransferases [UGT] 1A1, UGT2B7)* regulated on cDNA-array was confirmed by real-time PCR. In addition, AE also altered the total enzyme activities of GST and UGT. AE reduced DNA-damage by genotoxins in colon cells indicating might be due to higher GST activity. Ileostomy samples after interventions were less genotoxic than before the intervention. Pretreatment of HT29 cells with

ileostomy samples protected HT29 cells from genotoxic damage by H_2O_2 and this treatment results in an increased *GSTT2* expression and *GSTT2* promotor activity.

Conclusions: The inhibition of tumour cell proliferation could be a one mechanism of cancer risk reduction by AE. Furthermore, AE can alter transcriptional changes in colon cells rather at the early time points (8 and 12 h) than after 24 h. The observed altered gene expression patterns in colon cells resulting from AE treatment parts to a protection of the cells against toxicological insult. Our approach to determine this specific profile of gene expression in preneoplastic human cells provides a relevant possibility to identify target genes and agents that could contribute to chemoprotection in colonic mucosa cells. The present study also reveals that apple polyphenols have antigenotoxic activities *in vitro* and *ex vivo* and the consequences of which need to be resolved for the *in vivo*. Taken together, this study demonstrates that a scope of key endogenous phase II enzymes in cultured colon cells can be upregulated by apple polyphenols and that cellular defences rendered cells more resistant to genotoxic insults. The results of this study, thus, suggested a new mechanism which might contribute to the colon cancer protective effects of apple polyphenols.

8 Zusammenfassung

Einleitung: Zu den häufigsten Krebsarten in den durch die „western style diet" geprägten Industrieländern, gehört der Dickdarmkrebs. Flavonoide aus Früchten und Gemüse können möglicherweise das Risiko an kolorektalen Tumoren zu erkranken, minimieren. Vor allem Äpfel enthalten signifikante Mengen an Polyphenolen, welche potentiell das Krebsrisiko senken können. Dies kann auf die antioxidativen oder antiproliferativen Effekte sowie den Einfluss auf die Genexpression zurückzuführen sein.

Ziel: Im Rahmen dieser Arbeiten wurden Untersuchungen zum Effekt von Apfelpolyphenolen und deren Metabolite (a) auf das Überleben der Kolonadenokarzinom- (HT29) und Kolonadenom- (LT97) Zelllinien, (b) auf die Modulation der Expression von Genen, welche mit der Prävention von Kolonkrebs in Zusammenhang gebracht werden, (c) und auf das Potential die durch genotoxische Substanzen verursachten DNA-Schäden in den Zellen (*in vitro*) zu reduzieren, durchgeführt. Des Weiteren wurde bestimmt, ob eine Apfelsaftintervention zur Senkung der Genotoxine im humanen Darmlumen (*ex vivo*) führen kann (d).

Methoden: HT29 und LT97 Zellen wurden mit Apfelextrakt (AE) oder fermentiertem Apfelextrakt (F-AE) behandelt. Außerdem wurden die HT29 Zellen mit einer synthetischen Mischung aus Flavonoiden, die die Zusammensetzung des AE widerspiegelten, oder mit ausgesuchten Einzelkomponenten inkubiert, um den Einfluss auf das Zellwachstum anschließend mittels DAPI-Assay zu untersuchen. Die Zellen wurden mit den ermittelten effektiven Konzentrationen an AE behandelt und die RNA isoliert, um mit Hilfe von humanen cDNA-Microarrays, welche Gene der Karzinogenese oder der Chemoprävention beinhalteten, Muster der Genexpression

aufzuzeigen. Globale Genexpressionsanalysen wurden in Zeitabhängigkeit zusätzlich mittels Affimetrix-Arrays durchgeführt und mit anderen Array-Plattformen verglichen. Real-time PCR und Enzymaktivitätsassays wurden zur Verifizierung ausgewählter Array-Ergebnisse genutzt. Die mit AE behandelten Zellen wurden anschließend mit genotoxischen Substanzen inkubiert und DNA-Schäden mit dem Comet Assay bestimmt. Ileostomieproben von humanen Probanden vor (0 h) und nach (2 h) Apfelsaftintervention wurden genutzt, um deren genotoxisches Potential in HT29 Zellen zu vergleichen. Die mit den Ileostomieproben *(ex vivo)* vorbehandelten HT29 Zellen wurden ebenfalls mit Genotoxinen geschädigt und die DNA-Schäden mittels Comet Assay untersucht.

Ergebnisse: Das Wachstum von LT97 und HT29 Zellen wurde durch die AE und die synthetische Mischung signifikant inhibiert. Die Fermentation der AEs führte zu einem Anstieg der kurzkettigen Fettsäuren und der Degradierung der Polyphenole. Die F-AEs waren 3-fach weniger wirksam und demnach weniger chemoprotektiv verglichen mit den unfermentierten Testsubstanzen. Es zeigte sich im Gegensatz zu den HT29 Zellen eine stärkere Wachstumsinhibierung in den LT97 Zellen. Die Behandlung der Zellen mit AE resultiert in einer Hochregulierung von Genen des Fremdstoffmetabolismus und Genen, die der Zellzykluskontrolle, den Zellsignalwegen wie auch der Apoptose zuzuordnen sind. Den stärksten Effekt auf die Genexpression wurde nach 8 h und 12 h beobachtet. Die Expression ausgewählter Gene *(Glutathion S-Transferasen [GST] P1, GSTT2, GSTA4, UDP-Glucuronosyltransferasen [UGT] 1A1, UGT2B7)*, welche im Array reguliert wurden, konnten mittels Real-time PCR bestätigt werden. Außerdem beeinflusste der AE auch die Gesamtenzymaktivitäten der GST und der UGT. Der AE reduzierte durch Genotoxine verursachte DNA-Schäden in Kolonzellen, was unter anderem auf die gesteigerte GST-Aktivität zurückzuführen sein könnte. Die Ileostomieproben nach

Apfelsaftintervention waren verglichen mit denen vor der Intervention weniger genotoxisch. Die Vorinkubation von HT29 Zellen mit Ileostomieproben nach Intervention resultierte in einer geringeren Sensitivität gegenüber dem Genotoxin H_2O_2, einer erhöhten GSTT2-Expression und einer gesteigerten GSTT2 Promotor Aktivität.

Schlussfolgerungen: Die Inhibierung der Tumorzellproliferation durch AE könnte ein Mechanismus zur Reduzierung des Krebsrisikos darstellen. AE kann transkriptionelle Veränderungen in Kolonzellen nach 8 h sowie nach 24 h hervorrufen. Die durch AE-Behandlung beobachteten veränderten Genexpressionsmuster in Kolonzellen resultieren in einen Schutz der Zellen gegenüber toxischen Einflüssen. Unser Ansatz zur Bestimmung dieser spezifischen Genexpressionsprofile in präneoplastischen humanen Zellen bieten eine bedeutende Möglichkeit um Zielgene und Faktoren, die die Chemoprotektion bedingen, zu identifizieren. Die vorliegende Arbeit zeigt, dass Apfelpolyphenole antigenotoxische Fähigkeiten *in vitro* und *ex vivo* besitzen. Zusammenfassend macht diese Arbeit deutlich, dass Phase II-Enzyme in kultivierten Kolonzellen durch Apfelpolyphenole hochreguliert werden können und dass Zellen mit erhöhtem zellulären Schutz resistenter gegenüber genotoxischen Einträgen sind. Die Ergebnisse dieser Arbeit zeigen neue Wirkungen von Apfelpolyphenolen auf, welche mögliche Mechanismen hinsichtlich der Dickdarmkrebsprotektion erklären.

9 References

References

Akagawa, M., T. Shigemitsu and K. Suyama, Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions, *Biosci. Biotechnol. Biochem., 67*(12), 2632-2640, 2003.

Alazzouzi, H., P. Alhopuro, R. Salovaara, H. Sammalkorpi, H. Jarvinen, J. P. Mecklin, A. Hemminki, Schwartz S Jr, L. A. Aaltonen and D. Arango, SMAD4 as a prognostic marker in colorectal cancer, *Clin. Cancer Res., 11*(7), 2606-2611, 2005.

American Cancer Society, Cancer Facts and Figures, 2006. Atlanta,GA: American Cancer Society; 2006, 2006.

Arts, I. C. W., P. C. H. Hollman, E. J. M. Feskens, H. B. B. de Mesquita and D. Kromhout, Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women, *European Journal of Clinical Nutrition, 55*(2), 76-81, 2001.

Ates, N. A., L. Tamer, C. Ates, B. Ercan, T. Elipek, K. Ocal and H. Camdeviren, Glutathione S-transferase M1, T1, P1 genotypes and risk for development of colorectal cancer, *Biochem. Genet., 43*(3-4), 149-163, 2005.

Back, W., S. Loff, D. Jenne and U. Bleyl, Immunolocalization of beta catenin in intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes, *J. Clin. Pathol., 52*(5), 345-349, 1999.

Barth, S. W., C. Fahndrich, A. Bub, H. Dietrich, B. Watzl, F. Will, K. Briviba and G. Rechkemmer, Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats, *Carcinogenesis, 26*(8), 1414-1421, 2005.

Beecher, G. R., Overview of dietary flavonoids: nomenclature, occurrence and intake, *J. Nutr., 133*(10), 3248S-3254S, 2003.

Behrens, J., The role of the Wnt signalling pathway in colorectal tumorigenesis, *Biochem. Soc. Trans., 33*(Pt 4), 672-675, 2005.

Bingham, S. and E. Riboli, Diet and cancer--the European Prospective Investigation into Cancer and Nutrition, *Nat. Rev. Cancer, 4*(3), 206-215, 2004.

Blobe, G. C., W. P. Schiemann and H. F. Lodish, Role of transforming growth factor beta in human disease, *N. Engl. J. Med., 342*(18), 1350-1358, 2000.

Block, G., B. Patterson and A. Subar, Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, *Nutr. Cancer, 18*(1), 1-29, 1992.

Bock, K. W., Vertebrate UDP-glucuronosyltransferases: functional and evolutionary aspects, *Biochem. Pharmacol., 66*(5), 691-696, 2003.

Bodmer, W. F., Cancer genetics: colorectal cancer as a model, *J. Hum. Genet., 51*(5), 391-396, 2006.

Boyer, J. and R. H. Liu, Apple phytochemicals and their health benefits, *Nutr. J., 3*(1), 5, 2004.

Boyle, P. and J. Ferlay, Cancer incidence and mortality in Europe, 2004, *Ann. Oncol., 16*(3), 481-488, 2005.

Bray, F., R. Sankila, J. Ferlay and D. M. Parkin, Estimates of cancer incidence and mortality in Europe in 1995, *Eur. J. Cancer, 38*(1), 99-166, 2002.

Breinholt, V., S. T. Lauridsen and L. O. Dragsted, Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes in female rat, *Xenobiotica, 29*(12), 1227-1240, 1999.

Brosens, L. A., W. A. van Hattem, M. Jansen, W. W. de Leng, F. M. Giardiello and G. J. Offerhaus, Gastrointestinal polyposis syndromes, *Curr. Mol. Med., 7*(1), 29-46, 2007.

Burchell, B., C. H. Brierley and D. Rance, Specificity of human UDP-glucuronosyltransferases and xenobiotic glucuronidation, *Life Sci., 57*(20), 1819-1831, 1995.

Burchell, B. and R. Hume, Molecular genetic basis of Gilbert's syndrome, *Journal of Gastroenterology and Hepatology, 14*(10), 960-966, 1999.

Chin, K. V., I. Pastan and M. M. Gottesman, Function and regulation of the human multidrug resistance gene, *Adv. Cancer Res., 60*, 157-180, 1993.

Chung, D. C., The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis, *Gastroenterology, 119*(3), 854-865, 2000.

Clapper, M. L. and C. E. Szarka, Glutathione S-transferases--biomarkers of cancer risk and chemopreventive response, *Chem. Biol. Interact., 111-112*, 377-388, 1998.

Colditz, G. A., T. A. Sellers and E. Trapido, Epidemiology - identifying the causes and preventability of cancer?, *Nature Reviews Cancer, 6*(1), 75-83, 2006.

Croce, C. M., How can we prevent cancer?, *Proc. Natl. Acad. Sci. U. S. A, 98*(20), 10986-10988, 2001.

Dang, D. T., F. Chen, M. Kohli, C. Rago, J. M. Cummins and L. H. Dang, Glutathione S-transferase pi1 promotes tumorigenicity in HCT116 human colon cancer cells, *Cancer Res., 65*(20), 9485-9494, 2005.

de, S. and R. Fernando, Familial adenomatous polyposis, *Ceylon Med. J., 43*(2), 99-105, 1998.

Doll, R. and R. Peto, The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today, *J. Natl. Cancer Inst., 66*(6), 1191-1308, 1981.

Doucas, H., G. Garcea, C. P. Neal, M. M. Manson and D. P. Berry, Chemoprevention of pancreatic cancer: a review of the molecular pathways involved, and evidence for the potential for chemoprevention, *Pancreatology., 6*(5), 429-439, 2006.

DuPont, M. S., R. N. Bennett, F. A. Mellon and G. Williamson, Polyphenols from alcoholic apple cider are absorbed, metabolized and excreted by humans, *J. Nutr., 132*(2), 172-175, 2002.

Eberhardt, M. V., C. Y. Lee and R. H. Liu, Antioxidant activity of fresh apples, *Nature, 405*(6789), 903-904, 2000.

Ebert, M. N., G. Beyer-Sehlmeyer, U. M. Liegibel, T. Kautenburger, T. W. Becker and B. L. Pool-Zobel, Butyrate induces glutathione S-transferase in human colon cells and protects from genetic damage by 4-hydroxy-2-nonenal, *Nutr. Cancer, 41*(1-2), 156-164, 2001.

Fang, J. L., F. A. Beland, D. R. Doerge, D. Wiener, C. Guillemette, M. M. Marques and P. Lazarus, Characterization of benzo(a)pyrene-trans-7,8-dihydrodiol glucuronidation by human tissue microsomes and overexpressed UDP-glucuronosyltransferase enzymes, *Cancer Res., 62*(7), 1978-1986, 2002.

Fearon, E. R. and B. Vogelstein, A genetic model for colorectal tumorigenesis, *Cell, 61*(5), 759-767, 1990.

Fernandez, E., S. Gallus and V. C. La, Nutrition and cancer risk: an overview, *J. Br. Menopause. Soc., 12*(4), 139-142, 2006.

Fields, W. R., C. S. Morrow, A. J. Doss, K. Sundberg, B. Jernstrom and A. J. Townsend, Overexpression of stably transfected human glutathione S-transferase P1-1 protects against DNA damage by benzo[a]pyrene diol-epoxide in human T47D cells, *Mol. Pharmacol., 54*(2), 298-304, 1998.

Fodde, R., The APC gene in colorectal cancer, *Eur. J. Cancer, 38*(7), 867-871, 2002.

Fodde, R., J. Kuipers, C. Rosenberg, R. Smits, M. Kielman, C. Gaspar, J. H. van Es, C. Breukel, J. Wiegant, R. H. Giles and H. Clevers, Mutations in the APC tumour suppressor gene cause chromosomal instability, *Nat. Cell Biol., 3*(4), 433-438, 2001a.

Fodde, R., R. Smits and H. Clevers, APC, signal transduction and genetic instability in colorectal cancer, *Nat. Rev. Cancer, 1*(1), 55-67, 2001b.

Fogh, J. a. G. T., Fogh, J. and Trempe X. Human Tumor Cells in Vitro.In Fogh,J. (ed.). Plenum Press, New York, pp 115-59,1975, 1975.

Frei, B. and J. V. Higdon, Antioxidant activity of tea polyphenols in vivo: evidence from animal studies, *J. Nutr., 133*(10), 3275S-3284S, 2003.

Galati, G. and P. J. O'Brien, Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties, *Free Radic. Biol. Med., 37*(3), 287-303, 2004.

Galijatovic, A., U. K. Walle and T. Walle, Induction of UDP-glucuronosyltransferase by the flavonoids chrysin and quercetin in Caco-2 cells, *Pharm. Res., 17*(1), 21-26, 2000.

Glade, M. J., Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/World Cancer Research Fund, American Institute for Cancer Research, 1997, *Nutrition, 15*(6), 523-526, 1999.

Glei, M., A. Kirmse, N. Habermann, C. Persin and B. L. Pool-Zobel, Bread enriched with green coffee extract has chemoprotective and antigenotoxic activities in human cells, *Nutrition and Cancer-An International Journal, 56*(2), 182-192, 2006.

Gonzalez, C. A. and E. Riboli, Diet and cancer prevention: where we are, where we are going, *Nutr. Cancer, 56*(2), 225-231, 2006.

Gosse, F., S. Guyot, S. Roussi, A. Lobstein, B. Fischer, N. Seiler and F. Raul, Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, *Carcinogenesis, 26*(7), 1291-1295, 2005.

Grady, W. M., Genetic testing for high-risk colon cancer patients, *Gastroenterology, 124*(6), 1574-1594, 2003.

Grant, D. M., Detoxification pathways in the liver, *J. Inherit. Metab Dis., 14*(4), 421-430, 1991.

Graziani, G., G. D'Argenio, C. Tuccillo, C. Loguercio, A. Ritieni, F. Morisco, B. C. Del Vecchio, V. Fogliano and M. Romano, Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo, *GUT, 54*(2), 193-200, 2005.

Greenwald, P., Cancer chemoprevention, *BMJ, 324*(7339), 714-718, 2002.

Grubben, M. J., F. M. Nagengast, M. B. Katan and W. H. Peters, The glutathione biotransformation system and colorectal cancer risk in humans, *Scand. J. Gastroenterol. Suppl,*(234), 68-76, 2001.

Gruber, S. B., M. M. Entius, G. M. Petersen, S. J. Laken, P. A. Longo, R. Boyer, A. M. Levin, U. J. Mujumdar, J. M. Trent, K. W. Kinzler, B. Vogelstein, S. R. Hamilton, M. H. Polymeropoulos, G. J. Offerhaus and F. M. Giardiello, Pathogenesis of adenocarcinoma in Peutz-Jeghers syndrome, *Cancer Res., 58*(23), 5267-5270, 1998.

Guo, S., S. Yang, C. Taylor and G. E. Sonenshein, Green tea polyphenol epigallocatechin-3 gallate (EGCG) affects gene expression of breast cancer cells transformed by the carcinogen 7,12-dimethylbenz[a]anthracene, *J. Nutr., 135*(12 Suppl), 2978S-2986S, 2005.

Halliwell, B., Dietary polyphenols: good, bad, or indifferent for your health?, *Cardiovasc. Res., 73*(2), 341-347, 2007.

Hayes, J. D., J. U. Flanagan and I. R. Jowsey, Glutathione transferases, *Annu. Rev. Pharmacol. Toxicol., 45*, 51-88, 2005.

Hayes, J. D. and D. J. Pulford, The glutathione S-Transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance, *Critical Reviews in Biochemistry and Molecular Biology, 30*(6), 445-600, 1995.

Hayes, J. D. and R. C. Strange, Glutathione S-transferase polymorphisms and their biological consequences, *Pharmacology, 61*(3), 154-166, 2000.

Heavey, P. M., D. McKenna and I. R. Rowland, Colorectal cancer and the relationship between genes and the environment, *Nutr. Cancer, 48*(2), 124-141, 2004.

Heerdt, B. G., M. A. Houston and L. H. Augenlicht, Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines, *Cancer Res., 54*(12), 3288-3293, 1994.

Hemmingsen, A., A. A. Fryer, M. Hepple, R. C. Strange and M. A. Spiteri, Simultaneous identification of GSTP1 Ile105-->Val105 and Ala114-->Val114 substitutions using an amplification refractory mutation system polymerase chain reaction assay: studies in patients with asthma, *Respir. Res., 2*(4), 255-260, 2001.

Hendriks, Y. M., S. Jagmohan-Changur, H. M. van der Klift, H. Morreau, P. M. van, C. Tops, O. T. van, A. Wagner, M. G. Ausems, E. Gomez, M. H. Breuning, A. H. Brocker-Vriends, H. F. Vasen and J. T. Wijnen, Heterozygous mutations in PMS2 cause hereditary nonpolyposis colorectal carcinoma (Lynch syndrome), *Gastroenterology, 130*(2), 312-322, 2006.

Hisamuddin, I. M. and V. W. Yang, Genetics of colorectal cancer, *MedGenMed., 6*(3), 13, 2004.

Hofmann, T., U. Liegibel, P. Winterhalter, A. Bub, G. Rechkemmer and B. L. Pool-Zobel, Intervention with polyphenol-rich fruit juices results in an elevation of glutathione S-transferase P1 (hGSTP1) protein expression in human leucocytes of healthy volunteers, *Mol. Nutr. Food Res., 50*(12), 1191-1200, 2006.

Hollman, P. C., M. N. Bijsman, G. Y. van, E. P. Cnossen, J. H. de Vries and M. B. Katan, The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man, *Free Radic. Res., 31*(6), 569-573, 1999.

Hollman, P. C., J. H. de Vries, S. D. van Leeuwen, M. J. Mengelers and M. B. Katan, Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers, *Am. J. Clin. Nutr., 62*(6), 1276-1282, 1995.

Hollman, P. C. H. and I. C. W. Arts, Flavonols, flavones and flavanols - nature, occurrence and dietary burden, *Journal of the Science of Food and Agriculture, 80*(7), 1081-1093, 2000.

Hollman, P. C. H., J. M. P. vanTrijp, M. N. C. P. Buysman, M. S. VanderGaag, M. J. B. Mengelers, J. H. M. deVries and M. B. Katan, Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man, *Febs Letters, 418*(1-2), 152-156, 1997.

Hosokawa, N., Y. Hosokawa, T. Sakai, M. Yoshida, N. Marui, H. Nishino, K. Kawai and A. Aoike, Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells, *Int. J Cancer, 45*(6), 1119-1124, 1990.

Hu, Z. H. and P. G. Wells, Modulation of Benzo[A]Pyrene Bioactivation by Glucuronidation in Lymphocytes and Hepatic Microsomes from Rats with A Hereditary-Deficiency in Bilirubin Udp-Glucuronosyltransferase, *Toxicology and Applied Pharmacology, 127*(2), 306-313, 1994.

Hung, H. C., K. J. Joshipura, R. Jiang, F. B. Hu, D. Hunter, S. A. Smith-Warner, G. A. Colditz, B. Rosner, D. Spiegelman and W. C. Willett, Fruit and vegetable intake and risk of major chronic disease, *JNCI Cancer Spectrum, 96*(21), 1577-1584, 2004a.

Hung, H. C., K. J. Joshipura, R. Jiang, F. B. Hu, D. Hunter, S. A. Smith-Warner, G. A. Colditz, B. Rosner, D. Spiegelman and W. C. Willett, Fruit and vegetable intake and risk of major chronic disease, *JNCI Cancer Spectrum, 96*(21), 1577-1584, 2004b.

Hursting, S. D., T. J. Slaga, S. M. Fischer, J. DiGiovanni and J. M. Phang, Mechanism-based cancer prevention approaches: targets, examples, and the use of transgenic mice, *J. Natl. Cancer Inst., 91*(3), 215-225, 1999.

Ionov, Y., H. Yamamoto, S. Krajewski, J. C. Reed and M. Perucho, Mutational inactivation of the proapoptotic gene BAX confers selective advantage during tumor clonal evolution, *Proc. Natl. Acad. Sci. U. S. A, 97*(20), 10872-10877, 2000.

Jaeger, J., K. Sorensen and S. P. Wolff, Peroxide accumulation in detergents., *J Biochem Biophys Metho, 29*(1), 77-81, 1994.

Jass, J. R., T. C. Smyrk, S. M. Stewart, M. R. Lane, S. J. Lanspa and H. T. Lynch, Pathology of hereditary non-polyposis colorectal cancer, *Anticancer Res., 14*(4B), 1631-1634, 1994.

Jeter, J. M., W. Kohlmann and S. B. Gruber, Genetics of colorectal cancer, *Oncology (Williston. Park), 20*(3), 269-276, 2006.

Kahle, K., M. Kraus, W. Scheppach and E. Richling, Colonic availability of apple polyphenols--a study in ileostomy subjects, *Mol. Nutr. Food Res., 49*(12), 1143-1150, 2005.

Kelloff, G. J., C. C. Sigman and P. Greenwald, Cancer chemoprevention: progress and promise, *Eur. J. Cancer, 35*(14), 2031-2038, 1999.

Kensler, T. W., Chemoprevention by inducers of carcinogen detoxication enzymes, *Environ. Health Perspect., 105 Suppl 4*, 965-970, 1997.

Kern, M., Z. Tjaden, Y. Ngiewih, N. Puppel, F. Will, H. Dietrich, G. Pahlke and D. Marko, Inhibitors of the epidermal growth factor receptor in apple juice extract, *Mol. Nutr. Food Res., 49*(4), 317-328, 2005.

Khan, S. G., S. K. Katiyar, R. Agarwal and H. Mukhtar, Enhancement of antioxidant and phase II enzymes by oral feeding of green tea polyphenols in drinking water to SKH-1 hairless mice: possible role in cancer chemoprevention, *Cancer Res., 52*(14), 4050-4052, 1992.

Kinzler, K. W. and B. Vogelstein, Lessons from hereditary colorectal cancer, *Cell, 87*(2), 159-170, 1996.

Knoll, N., C. Ruhe, S. Veeriah, J. Sauer, M. Glei, E. P. Gallagher and B. L. Pool-Zobel, Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione S-transferase A4 expression by butyrate, *Toxicol. Sci., 86*(1), 27-35, 2005.

Kuhnau, J., The flavonoids. A class of semi-essential food components: their role in human nutrition, *World Rev. Nutr. Diet., 24*, 117-191, 1976.

Kuo, S. M., Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells, *Cancer Lett., 110*(1-2), 41-48, 1996.

Kuo, S. M., Flavonoids and gene expression in mammalian cells, *Adv. Exp. Med. Biol., 505*, 191-200, 2002.

Kwak, M. K., P. A. Egner, P. M. Dolan, M. Ramos-Gomez, J. D. Groopman, K. Itoh, M. Yamamoto and T. W. Kensler, Role of phase 2 enzyme induction in chemoprotection by dithiolethiones, *Mutat. Res., 480-481*, 305-315, 2001.

Lambert, J. D., J. Hong, G. Y. Yang, J. Liao and C. S. Yang, Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations, *Am. J. Clin. Nutr., 81*(1 Suppl), 284S-291S, 2005.

Lee, J. S. and Y. J. Surh, Nrf2 as a novel molecular target for chemoprevention, *Cancer Lett., 224*(2), 171-184, 2005.

Lengauer, C., K. W. Kinzler and B. Vogelstein, Genetic instabilities in human cancers, *Nature, 396*(6712), 643-649, 1998.

Lin, J. K. and Y. C. Liang, Cancer chemoprevention by tea polyphenols, *Proc. Natl. Sci. Counc. Repub. China B, 24*(1), 1-13, 2000.

Liska, D. J., The detoxification enzyme systems, *Altern. Med. Rev., 3*(3), 187-198, 1998.

Lister, C. E., J. E. Lancaster, K. H. Sutton and J. R. L. Walker, Developmental-Changes in the Concentration and Composition of Flavonoids in Skin of A Red and A Green Apple Cultivar, *Journal of the Science of Food and Agriculture, 64*(2), 155-161, 1994.

Liu RH, Eberhardt M and Lee C, Antioxidant and antiproliferative activities of selected New York apple cultivars, *New York Fruit Quarterly, 9*, 15-17, 2001.

Liu, R. H., J. Liu and B. Chen, Apples prevent mammary tumors in rats, *J. Agric. Food Chem., 53*(6), 2341-2343, 2005.

Lotito, S. B. and B. Frei, The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose on urate, not apple-derived antioxidant flavonoids, *Free Radical Biology and Medicine, 37*(2), 251-258, 2004.

Lynch, H. T. and J. Lynch, Lynch syndrome: genetics, natural history, genetic counseling, and prevention, *J. Clin. Oncol., 18*(21 Suppl), 19S-31S, 2000.

Mackenzie, P. I., K. W. Bock, B. Burchell, C. Guillemette, S. Ikushiro, T. Iyanagi, J. O. Miners, I. S. Owens and D. W. Nebert, Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily, *Pharmacogenetics and Genomics, 15*(10), 677-685, 2005.

Mariadason, J. M., K. L. Rickard, D. H. Barkla, L. H. Augenlicht and P. R. Gibson, Divergent phenotypic patterns and commitment to apoptosis of Caco-2 cells

during spontaneous and butyrate-induced differentiation, *J. Cell Physiol, 183*(3), 347-354, 2000.

Massaad, L., W. de, I, V. Ribrag, F. Janot, P. H. Beaune, J. Morizet, A. Gouyette and G. G. Chabot, Comparison of mouse and human colon tumors with regard to phase I and phase II drug-metabolizing enzyme systems, *Cancer Res., 52*(23), 6567-6575, 1992.

Meyer, U. A., Overview of enzymes of drug metabolism, *Journal of Pharmacokinetics and Biopharmaceutics, 24*(5), 449-459, 1996.

Moghrabi, N., M. Boxer and B. Burchell, Three MspI polymorphisms at the UGT1 locus, *Hum. Mol. Genet., 2*(8), 1324, 1993.

Netzel, M., E. Carle, B. Kesenheimer, G. Strass, I. Bitsch and R. Bitsch, Effect of apple juice intake on the antioxidant status in humans, 1999.

Nguyen, T., P. J. Sherratt and C. B. Pickett, Regulatory mechanisms controlling gene expression mediated by the antioxidant response element, *Annu Rev Pharmacol Toxicol, 43*, 233-60, 2003.

Noe, V., S. Penuelas, R. M. Lamuela-Raventos, J. Permanyer, C. J. Ciudad and M. Izquierdo-Pulido, Epicatechin and a cocoa polyphenolic extract modulate gene expression in human Caco-2 cells, *J. Nutr., 134*(10), 2509-2516, 2004.

Paganga, G., N. Miller and C. A. Rice-Evans, The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute?, *Free Radic. Res., 30*(2), 153-162, 1999.

Parkin, D. M., Global cancer statistics in the year 2000, *Lancet Oncol., 2*(9), 533-543, 2001.

Parl, F. F., Glutathione S-transferase genotypes and cancer risk, *Cancer Lett., 221*(2), 123-129, 2005.

Petri, N., C. Tannergren, B. Holst, F. A. Mellon, Y. Bao, G. W. Plumb, J. Bacon, K. A. O'Leary, P. A. Kroon, L. Knutson, P. Forsell, T. Eriksson, H. Lennernas and G. Williamson, Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo, *Drug Metab Dispos., 31*(6), 805-813, 2003.

Pinkus, R., L. M. Weiner and V. Daniel, Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression, *J. Biol. Chem., 271*(23), 13422-13429, 1996.

Pisani, P., D. M. Parkin, F. Bray and J. Ferlay, Estimates of the worldwide mortality from 25 cancers in 1990, *Int. J. Cancer, 83*(1), 18-29, 1999.

Podsedek, A., J. Wilska-Jeszka, B. Anders and J. Markowski, Compositional characterisation of some apple varieties, *European Food Research and Technology, 210*(4), 268-272, 2000.

Pool-Zobel, B., S. Veeriah and F. D. Bohmer, Modulation of xenobiotic metabolising enzymes by anticarcinogens -- focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis, *Mutat. Res., 591*(1-2), 74-92, 2005a.

Pool-Zobel, B. L., V. Selvaraju, J. Sauer, T. Kautenburger, J. Kiefer, K. K. Richter, M. Soom and S. Wolfl, Butyrate may enhance toxicological defence in primary, adenoma and tumor human colon cells by favourably modulating expression of glutathione S-transferases genes, an approach in nutrigenomics, *Carcinogenesis, 26*(6), 1064-1076, 2005b.

Potter, J. D., Colorectal cancer: molecules and populations, *J. Natl. Cancer Inst., 91*(11), 916-932, 1999.

Powell, S. M., N. Zilz, Y. Beazer-Barclay, T. M. Bryan, S. R. Hamilton, S. N. Thibodeau, B. Vogelstein and K. W. Kinzler, APC mutations occur early during colorectal tumorigenesis, *Nature, 359*(6392), 235-237, 1992.

Puiggros, F., N. Llopiz, A. Ardevol, C. Blade, L. Arola and M. J. Salvado, Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems, *J. Agric. Food Chem., 53*(15), 6080-6086, 2005.

Rajagopalan, H., A. Bardelli, C. Lengauer, K. W. Kinzler, B. Vogelstein and V. E. Velculescu, Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status, *Nature, 418*(6901), 934, 2002.

Richter, M., D. Jurek, F. Wrba, K. Kaserer, G. Wurzer, J. Karner-Hanusch and B. Marian, Cells obtained from colorectal microadenomas mirror early premalignant growth patterns in vitro, *European Journal of Cancer, 38*(14), 1937-1945, 2002.

Rowe, J. D., E. Nieves and I. Listowsky, Subunit diversity and tissue distribution of human glutathione S-transferases: Interpretations based on electrospray ionization MS and peptide sequence-specific antisera, *Biochemical Journal, 325*, 481-486, 1997.

Rushmore, T. H. and A. N. Kong, Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes, *Curr. Drug Metab, 3*(5), 481-490, 2002.

Rushmore, T. H., M. r. Morton and C. B. Pickett, The antioxidant responsive element, *The Journal of Biological Chemistry, 18*, 11632-11639, 1991.

Satia, J. A., M. K. Campbell, J. A. Galanko, A. James, C. Carr and R. S. Sandler, Longitudinal changes in lifestyle behaviors and health status in colon cancer survivors, *Cancer Epidemiol. Biomarkers Prev., 13*(6), 1022-1031, 2004.

Scalbert, A., I. T. Johnson and M. Saltmarsh, Polyphenols: antioxidants and beyond, *Am. J. Clin. Nutr., 81*(1 Suppl), 215S-217S, 2005.

Scalbert, A. and G. Williamson, Dietary intake and bioavailability of polyphenols, *J. Nutr., 130*(8S Suppl), 2073S-2085S, 2000.

Schatzkin, A. and V. Kipnis, Could exposure assessment problems give us wrong answers to nutrition and cancer questions?, *J. Natl. Cancer Inst., 96*(21), 1564-1565, 2004.

Shen, S. C., Y. C. Chen, F. L. Hsu and W. R. Lee, Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade, *J Cell Biochem., 89*(5), 1044-1055, 2003.

Singh, B., A. P. Halestrap and C. Paraskeva, Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources, *Carcinogenesis, 18*(6), 1265-1270, 1997.

Soerjomataram, I., V. E. de, E. Pukkala and J. W. Coebergh, Excess of cancers in Europe: a study of eleven major cancers amenable to lifestyle change, *Int. J. Cancer, 120*(6), 1336-1343, 2007.

Soreide, K., E. A. Janssen, H. Soiland, H. Korner and J. P. Baak, Microsatellite instability in colorectal cancer, *Br. J. Surg., 93*(4), 395-406, 2006.

Stark, J. R., E. R. Bertone-Johnson, M. E. Costanza and A. M. Stoddard, Factors associated with colorectal cancer risk perception: the role of polyps and family history, *Health Educ. Res., 21*(5), 740-749, 2006.

Steele, V. E., G. J. Kelloff, D. Balentine, C. W. Boone, R. Mehta, D. Bagheri, C. C. Sigman, S. Zhu and S. Sharma, Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays, *Carcinogenesis, 21*(1), 63-67, 2000.

Steiner, C., W. H. Peters, E. P. Gallagher, P. Magee, I. Rowland and B. L. Pool-Zobel, Genistein protects human mammary epithelial cells from benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide and 4-hydroxy-2-nonenal genotoxicity by modulating the glutathione/glutathione S-transferase system, *Carcinogenesis, 28*(3), 738-748, 2007.

Steward BW and Kleihues P, Lyon: WHO International Agency for Research on Cancer, *World Cancer Report 2003,* 2003.

Strassburg, C. P., S. Kneip, J. Topp, P. Obermayer-Straub, A. Barut, R. H. Tukey and M. P. Manns, Polymorphic gene regulation and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine, *J. Biol. Chem., 275*(46), 36164-36171, 2000.

Strassburg, C. P., A. Strassburg, N. Nguyen, Q. Li, M. P. Manns and R. H. Tukey, Regulation and function of family 1 and family 2 UDP-glucuronosyltransferase genes (UGT1A, UGT2B) in human oesophagus, *Biochem. J., 338 (Pt 2)*, 489-498, 1999.

Strassburg, C. P., A. Vogel, S. Kneip, R. H. Tukey and M. P. Manns, Polymorphisms of the human UDP-glucuronosyltransferase (UGT) 1A7 gene in colorectal cancer, *GUT, 50*, 851-856, 2002.

Sugatani, J., K. Yamakawa, E. Tonda, S. Nishitani, K. Yoshinari, M. Degawa, I. Abe, H. Noguchi and M. Miwa, The induction of human UDP-glucuronosyltransferase 1A1 mediated through a distal enhancer module by flavonoids and xenobiotics, *Biochem. Pharmacol, 67*(5), 989-1000, 2004.

Surh, Y. J., J. K. Kundu, H. K. Na and J. S. Lee, Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals, *J. Nutr., 135*(12 Suppl), 2993S-3001S, 2005.

Svehlikova, V., S. Wang, J. Jakubikova, G. Williamson, R. Mithen and Y. Bao, Interactions between sulforaphane and apigenin in the induction of UGT1A1 and GSTA1 in CaCo-2 cells, *Carcinogenesis, 25*(9), 1629-1637, 2004.

Talalay, P., Chemoprotection against cancer by induction of phase 2 enzymes, *Biofactors, 12*(1-4), 5-11, 2000.

Talalay, P., A. T. Dinkova-Kostova and W. D. Holtzclaw, Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis, *Adv. Enzyme Regul., 43*, 121-134, 2003.

Tanaka, S. and Y. Imamura, International comparisons of cumulative risk of colorectal cancer, from cancer incidence in five continents vol. VIII, *Jpn. J. Clin. Oncol., 36*(3), 186-187, 2006.

Terry, P., E. Giovannucci, K. B. Michels, L. Bergkvist, H. Hansen, L. Holmberg and A. Wolk, Fruit, vegetables, dietary fiber, and risk of colorectal cancer, *J. Natl. Cancer Inst., 93*(7), 525-533, 2001.

Tice, R. R., E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. C. Ryu and Y. F. Sasaki, Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing., *Environ Mol Mutagen., 35*(3), 206-221, 2000.

Townsend, D. M. and K. D. Tew, The role of glutathione-S-transferase in anti-cancer drug resistance, *Oncogene, 22*(47), 7369-7375, 2003.

Tukey, R. H. and C. P. Strassburg, Human UDP-glucuronosyltransferases: metabolism, expression, and disease, *Annu. Rev. Pharmacol. Toxicol., 40*, 581-616, 2000.

Turgeon, D., S. Chouinard, P. Belanger, S. Picard, J. F. Labbe, P. Borgeat and A. Belanger, Glucuronidation of arachidonic and linoleic acid metabolites by human UDP-glucuronosyltransferases, *J. Lipid Res., 44*(6), 1182-1191, 2003.

Waleh, N. S., J. Calaoagan, B. J. Murphy, A. M. Knapp, R. M. Sutherland and K. R. Laderoute, The redox-sensitive human antioxidant responsive element induces gene expression under low oxygen conditions, *Carcinogenesis, 19*(1333), 1337, 1998.

Walle, T. and U. K. Walle, The beta-D-glucoside and sodium-dependent glucose transporter 1 (SGLT1)-inhibitor phloridzin is transported by both SGLT1 and multidrug resistance-associated proteins 1/2, *Drug Metabolism and Disposition, 31*(11), 1288-1291, 2003.

Wattenberg, L. W., Chemoprevention of cancer, *Cancer Res., 45*(1), 1-8, 1985.

Wells, P. G., P. I. Mackenzie, J. R. Chowdhury, C. Guillemette, P. A. Gregory, Y. Ishii, A. J. Hansen, F. K. Kessler, P. M. Kim, N. R. Chowdhury and J. K. Ritter, Glucuronidation and the UDP-glucuronosyltransferases in health and disease, *Drug Metab Dispos., 32*(3), 281-290, 2004.

Westerman, A. M., M. M. Entius, B. E. de, P. P. Boor, R. Koole, M. L. van Velthuysen, G. J. Offerhaus, D. Lindhout, F. W. de Rooij and J. H. Wilson, Peutz-Jeghers syndrome: 78-year follow-up of the original family, *Lancet, 353*(9160), 1211-1215, 1999.

Williamson, G., A. J. Day, G. W. Plumb and D. Couteau, Human metabolic pathways of dietary flavonoids and cinnamates, *Biochem. Soc. Trans., 28*(2), 16-22, 2000.

World Health Organization, Global cancer rates to rise by 50% by 2020, *Bulletin of the World Health Organization, 81*, 385-386, 2003.

Xu, C., C. Y. Li and A. N. Kong, Induction of phase I, II and III drug metabolism/transport by xenobiotics, *Arch. Pharm. Res., 28*(3), 249-268, 2005.

Yang, C. S., J. M. Landau, M. T. Huang and H. L. Newmark, Inhibition of carcinogenesis by dietary polyphenolic compounds, *Annu. Rev. Nutr., 21*, 381-406, 2001.

Yoshida, M., M. Yamamoto and T. Nikaido, Quercetin arrests human leukemic T-cells in late G1 phase of the cell cycle, *Cancer Res., 52*(23), 6676-6681, 1992.

Zheng, Z., J. L. Fang and P. Lazarus, Glucuronidation: an important mechanism for detoxification of benzo[a]pyrene metabolites in aerodigestive tract tissues, *Drug Metab Dispos., 30*(4), 397-403, 2002.

10 Acknowledgements

Professor Beatrice L. Pool-Zobel. She is my “supervisor” and she introduced me to the world of perfect simulation. She is the reason why I became a nutrition scientist. She always wanted me to fully develop my potential. I thank for her supervision and for very frequent and intense discussion with her made it all possible.

PD Dr. Michael Glei. He is more than just an advisor and eventually became a very good friend. Often, my words would confuse him and he’d illustrate his confusion with a joke or wisecrack. I really appreciate this because it often made me laugh at myself. I also appreciate his patience and willingness to work with me over the last four years. He was always available to answer my questions which I hadn’t carefully considered. I will never be able to thank him enough for all the advice and guidance.

I give hearty thank to my collaborators: Professor Dr. Frank Böhmer and Professor Dr. Stefan Wölfl. They have been vital part of this study throughout and always kept me focused on the bigger picture. They showed up much interest in my research and always approached me with the question, “What’s new and exciting?” When ever I found some spectacular new signature genes, their interest and enthusiasm was almost greater than my own.

Life in Jena has been fruitful with my friends, in particular: Marian, Thomas (Tomy), >Nina< (Neens), Steffi (STK), Julia (Juli), Daniel (Walter), Christoph (the Dude) and Claudia (CLM). Of course friends played a major role in my life especially “The Costly Quintet”, they are the “catalyst” who made me realize how much potential I had and always challenged and pushed me to the limit in order to make me improve.

To my Indian friends in Jena Kamal, Krishna, Anand, Pradeep and there are too many important friends to mention but they know who they are. I thank you all for your continuous support and encouragement throughout this study.

I would like to deeply thank Mrs. Esther Woschee, Ms. Claudia Lüdtke, Ms. Edda Lösch and Ms. Anke Partschefeld in our lab, provided me with useful and helpful assistance during the several years of this study in which this endeavour lasted. Without their care and consideration, this Ph.D would likely not have matured.

Many thanks to my parents, for their best qualities, such as are my father's vision and my mother's wisdom. But it was their unconditional love and support that allowed me to start this journey and be at where I am now. Most important of all, they provided the means for me to become the person I am today and made my life the most enjoyable life anyone could ask for. Also many thanks to my sister, brothers and relatives without their love and support I would not have been able to reach this point.

Finally, I would like to acknowledge that my research was funded under the Bundesministerium für Bildung und Forschung (BMBF FKZ.01EA0103), Germany.

Résumé

Selvaraju Veeriah

Indian, Unmarried, Date of birth: 10th May 1974

Junior research fellow (Jan.2001 - Oct.2002)

Department of Human Genetics, Indian Institute of Science, Bangalore, India

Education

M.Sc, (Master of Science in Biochemistry) (May.1998 - Apr.2000)

Bharathidasan University, Trichy, Tamil Nadu, India

B.Sc, (Bachelor of Science in Biochemistry) (May.1994 - Apr.1998)

Bharathidasan University, Trichy, Tamil Nadu, India

Professional associations

Member of ***GUM,-Gesellschaft für Umwelt-Mutationsforschung e.V., Germany***

Member of ***APFEL e.V.-Alumni and Partner der Friedrich-Schiller-Universität, Jena, Ernährungswissenschaften und life sciences***

List of original publications

- ***Veeriah S,*** *Hofmann T, Glei M, Dietrich H, Will F, Richling E, Pool-Zobel BL.* Apple polyphenols and products formed in the gut differentially inhibit survival of human colon cell lines derived from adenoma (LT97) and carcinoma (HT29). *J Agric Food Chem. 2007 Apr 18;55(8):2892-900.*

- ***Veeriah S,*** *Kautenburger T, Sauer J, Habermann N, Dietrich H, Will F, Pool-Zobel BL.* Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol Carcinog. 2006 Mar;45(3):164-74*

- *Pool-Zobel BL,* ***Veeriah S,*** *Böhmer FD.* Modulation of xenobiotic metabolising enzymes by anticarcinogens - focus on glutathione *S*-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutat Res. 2005 Dec 11; 591(1-2):74-92.*

- *Knoll N, Ruhe C,* ***Veeriah S,*** *Sauer J, Glei M, Gallagher EP, Pool-Zobel BL.* Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione S-transferase A4 expression by butyrate. *Toxicol Sci. 2005 Jul;86(1):27-35.*

- *Pool-Zobel BL,* ***Selvaraju V,*** *Sauer J, Kautenburger T, Kiefer J, Richter KK, Soom M, Wölfl S.* Butyrate may enhance toxicological defence in primary, adenoma and tumour human colon cells by favourably modulating expression of glutathione *S*-transferases genes, an approach in nutrigenomics. *Carcinogenesis. 2005 Jun;26(6):1064-76.*

- *Markandaya M, Ramesh TK,* ***Selvaraju V,*** *Dorairaj SK, Prakash R, Shetty J, Kumar A.* Genetic analysis of an Indian family with members affected with juvenile-onset primary open-angle glaucoma. *Ophthalmic Genet. 2004 Mar;25(1):11-23.*

- ***Selvaraju V,*** *Markandaya M, Prasad PV, Sathyan P, Sethuraman G, Srivastava SC, Thakker N, Kumar A.* Mutation analysis of the cathepsin C gene in Indian families with Papillon-Lefevre syndrome. *BMC Med Genet. 2003 Jul 12; 4:5.*

- ***Veeriah S,*** *Miene C, Habermann N, Hofmann T, Klenow S, Sauer J, Böhmer FD, Wölfl S, Pool-Zobel BL.* "Apple polyphenols modulate expression of selected genes related to toxicological defense and stress response in human colon adenoma cells". *Submitted to Int J Cancer, 2007*

- ***Veeriah S****, Böhmer FD, Kamal K, Kahle K, Glei M, Rickling E, Schreyer P, Pool-Zobel BL.* "Intervention with cloudy apple juice results in altered biological activities of ileostomy samples collected from individual volunteers". *Manuscript in preparation, 2007*

- *Klenow S,* ***Veeriah S,*** *Knöbel Y, Pool-Zobel BL.* "Apple flavonoids modulate the genotoxic effects of different DNA damaging compounds". *Manuscript in preparation, 2007*

Poster presentation

- ***Veeriah S**, Miene C, Pool-Zobel BL* "Assessment of UDP-glucuronosyltransferase (UGT) induction by apple polyphenols in the human colon adenoma cell line LT97" ***10th Karlsruhe Nutrition Congress, October 15 - 17, 2006, Karlsruhe, Germany***

- *Bellion P, Glei M,* ***Veeriah S**, Pool-Zobel BL, Dietrich H, Will F, Baum M, Eisenbrand G and Janzowski C* "Fermented apple juice extracts reduce oxidative stress in human colon carcinoma cell line Caco-2" ***10th Karlsruhe Nutrition Congress, October 15 - 17, 2006, Karlsruhe, Germany***

- *Kautenburger T, Daumann H, Waldecker M,* ***Veeriah S**, Pool-Zobel BL, will F, Dietrich H, Schrenk D* "Modulation of cell growth and HDAC activity by colonic fermentation products of dietary fibre and apple juice polyphenols" ***10th Karlsruhe Nutrition Congress, October 15 - 17, 2006, Karlsruhe, Germany***

- ***Veeriah S**, Habermann N, Hofmann T, Klenow S, Sauer J, Böhmer FD, Wölfl S, Pool-Zobel BL* "Antigenotoxic apple polyphenols modulate gene expression in human colon adenoma cells as determined with a custom-made cDNA microarray for toxicological defense and stress response" ***36th Annual Meeting of the European Environmental Mutagen Society, From Genes to Molecular Epidemiology, July 2 - 6, 2006, Prague, Czech Republic***

- *Knöbel Y, Glei M,* ***Veeriah S**, Pool-Zobel BL* "Investigations on DNA damage in the human colon carcinoma cell line HT 29 – modification of toxic effects by an apple extract" ***22.GUM Tagung, February 21-24, 2006, Darmstadt, Germany***

- ***Veeriah S**, Monika A, Helmut D, Frank W, Pool-Zobel BL* "The effect of apple polyphenol extracts on proliferation of colon adenoma (LT97) and carcinoma (HT29) cells" ***10th Symposium** ,**Vitamins and Additives in the Nutrition of Man and Animal, September 28 and 29, 2005, Jena/Thuringia, Germany***

- ***Veeriah S**, Habermann N, Dietrich H, Will F, Pool-Zobel BL* "Apple flavonoids modulate expression of genes encoding xenobiotic metabolizing enzymes in LT97 human colon adenoma cell", ***13th International AEK/AIO Cancer Congress of the German Cancer Society, March 13 - 16, 2005, Würzburg, Germany***

- *Pool-Zobel BL,* ***Veeriah S****, Böhmer FD, Balavenkatraman K.K, Wölfl S, Thijs H, Richter K.K* "Studies on parameters of detoxification and tumour suppression in human colon cells as biomarkers of chemoprotection", ***BMBF network meeting October 20, 2004, Berlin, Germany***

- ***Veeriah S****, Kautenburger T, Dietrich H, Will F, Pool-Zobel BL* "Apple flavonoids inhibit growth of the human colon cancer cell line HT-29 and modulate expression of genes involved in biotransformation of xenobiotics" ***ICMAA–VIII Eighth international conference on mechanisms of antimutagenesis and anticarcinogenesis, 4–8 October 2003. Pisa, Italy***

- ***Veeriah S*** "The Global Alliance for TB Drug Development and WHO's Special Programme for Research and Training in Tropical Diseases co-hosted with AstraZeneca" ***International Symposium on Current Developments in Drug Discovery for Tuberculosis****, AstraZeneca – Delegate, January 14th - 17th, 2002, AstraZeneca, Bangalore, India*

Certification of Originality

To the best of my knowledge and belief, this thesis does not contain any material previously submitted for a degree or diploma in any university or any material previously written or published by any other person, except where due acknowledgment is made in the text.

Jena, 2007-06-14 (Selvaraju Veeriah)