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Assessment of mRNA, protein levels and activities of xenobiotic metabolizing enzymes in colon and rectal mucosa of colorectal cancer patients

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To my family

Affidavit according to the §8 of the doctoral degree regulations of the Combined Faculty of Natural Sciences and Mathematics of the Ruperto-Carola University of Heidelberg, Germany

The thesis I have submitted entitled *Assessment of mRNA, protein levels and activities of xenobiotic metabolizing enzymes in colon and rectal mucosa of colorectal cancer patients* is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given. I have not yet presented this thesis or parts there of to a university as part of an examination or degree. I confirm that the declarations made above are correct. I am aware of the importance of a sworn affidavit and possible criminal prosecution in the case of a false or incomplete affidavit. I affirm that the above is the absolute truth to the best of my knowledge and that I have not concealed anything.

Heidelberg, 7th July 2016 Signature

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Abstract

Introduction: Colorectal cancer (CRC) is the most common type of gastrointestinal cancer and a major cause of morbidity and mortality throughout the world. Today we know that exposure to exogenous chemicals (xenobiotics) combined with a modified ability to detoxify carcinogens increases the risk of developing cancer. Xenobiotic metabolizing enzymes (XMEs) play a major role in the activation and detoxification of several carcinogens but their expression and activity in human colorectal tissue as well as their role in the development of CRC has not been sufficiently explored. In order to shed light on this question, this study aimed to link XME metabolism, lifestyle and risk factors with the etiology of CRC investigating two different aims. Therefore, the thesis is split into two parts:

1) Evaluation of mRNA, protein and enzyme activities of relevant phase I and phase II xenobiotic metabolizing enzymes in normal colorectal tissue of colorectal cancer patients: In this study, three different layers of XME abundance (mRNA, protein, and enzyme activity) were evaluated in cytochromes P450 (CYPs), glutathione *S*-transferases (GSTs), and UDP-glucuronosyltransferases (UGTs). Gene expression was assessed by quantitative real-time PCR (qRT-PCR), protein expression evaluated by immunoassay detection, and enzymatic activities measured by biochemical assays, in the normal tissue of 97 patients with CRC. The mean relative expression levels in normal colorectal tissue were highest for *GSTP1* [mean (± standard deviation): 7.70 (0.60)] and lowest for *GSTM1* [mean: 4.08 (1.80)]. Associations of xenobiotic metabolism-related gene expression, protein level and enzyme activities with clinical parameters in patients with CRC, were evaluated by the Mann-Whitney U-test and the Kruskal-Wallis test. Results of the univariate analysis revealed a 1.2-fold lower *UGT1A8* expression and 1.7-fold lower UGT activity in normal tissue of rectal compared to colon cancer patients (p=0.008; p_{FDR}=0.34 and p=0.002; p_{FDR}=0.17, respectively). Furthermore, lower *GSTP1* expression levels among recent nonsteroidal anti-inflammatory drug (NSAIDs) users compared to non-users (p=0.04; p_{FDR}=0.58) were detected. Associations with lifestyle and dietary factors were evaluated using linear regression models. Results of the multivariable models adjusted for relevant covariables, showed that regular consumption (>1x/week) of cooked vegetables was associated with higher CYP3A4 protein levels (β=5.62, p_n=0.009; p_{FDR}=0.65) and regular consumption of raw vegetables was associated with lower UGT activities (β=-0.56; p_n=0.03; p_{FDR}=0.72) compared to non-regular consumption of raw and cooked vegetables (≤ 1x/week) in the normal mucosa of CRC patients.

Relation of mRNA expression, protein levels and enzyme activities were assessed using Spearman correlation coefficients. No statistically significant associations were found between mRNA expression and neither protein levels, nor enzymatic activities for the CYPs and GSTs. For the UGTs, statistically significant, albeit weak, positive associations between UGT1A8 protein and mRNA abundance (r=0.20, $p \leq 0.05$) and UGT1A10 mRNA levels and UGT activity (r=0.28, $p \leq 0.01$) were observed. However, between UGT1A10 protein level and UGT activity, a statistically significant negative correlation (r= -0.27, $p \leq 0.01$) was detected.

2) Evaluation of differentially expressed XMEs in normal and tumor colorectal tissue: The expression of eight selected XMEs (*GSTP1*, *GSTA1*, *GSTM1*, *UGT1A10*, *UGT1A8*, *CYP2W1*, *CYP2C9*, and *CYP3A4*) in colorectal carcinomas and adjacent normal mucosa (n=71) was compared and associations of sociodemographic, lifestyle and dietary factors with the expression of these genes were investigated. Differences between XMEs' gene expression (IlluminaHT-12 Expression BeadChips) in tumor and normal mucosa were tested by the paired Wilcoxon-Rank-sum test. Among the genes analyzed, *GSTM1*, *GSTA1*, *UGT1A8*, *UGT1A10*, *CYP3A4*, and *CYP2C9* were down-regulated in tumor tissue as compared to normal tissue, while *GSTP1* and *CYP2W1* were up-regulated. Linear regression models were used to evaluate potential associations between sociodemographic, lifestyle and dietary factors and the relative gene expression in tumor and normal mucosa tissue. Although none of these relationships remained statistically significant after false discovery rate (FDR)-adjustment for multiple testing, a trend toward significance (β=-0.21; p_n=0.0005; p_{FDR}=0.05) with lower *CYP2C9* expression in normal tissue of rectal compared to colon cancer patients was detected. CYP2C9 plays a key role in the metabolic activation of many environmental and dietary mutagens and interactions of its expression with these factors should be considered in larger studies.

Overall conclusions: In summary, it can be concluded that in normal tissue of CRC patients:

- Correlations between XMEs' mRNA, protein and enzyme activities are moderate to poor
- Colon and rectum showed considerable differences regarding expression and activities of several XMEs
- Regular consumption of cooked vegetables was associated with CYP3A4 gene expression and protein levels

Taken together, the results of this thesis suggest some interesting differences, which should be considered in larger studies to elucidate its potential contribution to CRC etiology.

Zusammenfassung

Einleitung: Darmkrebs ist die häufigste Krebserkrankung des Magen-Darmtraktes und eine der Hauptursachen für durch Krebs verursachte Erkrankungen und Todesfälle weltweit. Heute wissen wir, dass eine Exposition gegenüber chemischen Stoffen (Fremdstoffe oder Xenobiotika) zusammen mit einer veränderten Fähigkeit diese Karzinogene zu entgiften, das Risiko erhöht an Krebs zu erkranken. Fremdstoffmetabolisierende Enzyme spielen bei der Aktivierung und Entgiftung einiger Karzinogene eine wichtige Rolle. Allerdings wurde deren Expression und Aktivität im menschlichen Darmgewebe, genauso wie deren Rolle bei der Entstehung von Darmkrebs, noch nicht ausreichend untersucht. Um dieser Frage nachzugehen, versucht diese Studie Fremdstoffmetabolismus, Lebensstil- und Risikofaktoren mit der Entstehung von Darmkrebs durch zwei verschiedene Zielsetzungen zu verbinden:

1) Evaluierung von mRNA, Protein und Enzymaktivitäten relevanter Phase I und Phase II fremdstoffmetabolisierender Enzyme im normalen Darmgewebe von Darmkrebspatienten: In dieser Studie wurden drei verschiedene Parameter (mRNA, Protein und Enzymaktivität) in den fremdstoffmetabolisierenden Enzymen Cytochrom P450 (CYP), Glutathion-S-Transferase (GST) und UDP-Glucuronosyltransferase untersucht. Genexpression wurde durch quantitative Echzeit-PCR (qRT-PCR) bestimmt, Proteinexpression durch immunologische Verfahren und die Messung der Enzymaktivität erfolgte durch biochemische Analysen. Alle Analysen fanden im Normalgewebe von 97 Darmkrebspatienten statt. Die mittleren Expressionslevel waren am höchsten für *GSTP1* [Mittelwert: 7.70 (0.60)] und am niedrigsten für *GSTM1* [Mittelwert: 4.08 (1.80)]. Zusammenhänge fremdstoffmetabolismus-bezogener Genexpression, Proteinlevel und Enzymaktivität mit klinischen Parametern, wurden unter Anwendung des Mann-Whitney-U-Testes und des Kruskal-Wallis-Testes, in Darmkrebspatienten analysiert. Ergebnisse der univariaten Analyse zeigten eine 1.2-fach niedrigere *UGT1A8* Expression und eine 1.7-fach geringere UGT Aktivität im Normalgewebe von Patienten mit Rektumkarzinom im Vergleich zu Patienten mit Kolonkarzinom(p=0.008; p_{FDR}=0.34 und p=0.002; p_{FDR}=0.17). Des Weiteren wurden bei Patienten die regelmäßig nicht-steroidale entzündungshemmende Medikamente (NSAIDs) einnahmen, niedrigere *GSTP1* Expressionslevel festgestellt als bei Patienten die keine NSAIDs einnahmen (p=0.04; p_{FDR}=0.58). Zusammenhänge mit Lebensstil- und Ernährungsfaktoren wurden mittels linearer Regressionsmodelle analysiert. Ergebnisse der multivariablen Modelle zeigten im Normalgewebe von Darmkrebspatienten einen Zusammenhang zwischen regelmäßigem Verzehr von gekochtem Gemüse (>1x/Woche) und höheren CYP3A4 Proteinlevel (β=5.62, p_n=0.009; p_{FDR}=0.65) und einen Zusammenhang zwischen regelmäßigem Verzehr von rohem Gemüse und niedrigeren UGT Aktivitäten (β=-0.56; p_n=0.03; p_{FDR}=0.72).

Das Verhältnis von mRNA-Expression, Proteinlevel und Enzymaktivität wurde mittels Spearman Korrelationskoeffizienten analysiert. Es wurden weder für die mRNA-Expression, noch für Proteinlevel oder Enyzmaktivität der CYPs und GSTs statistisch signifikante Zusammenhänge gefunden. Für die UGTs wurde ein schwach positiver, jedoch statistisch signifikanter Zusammenhang, zwischen UGT1A8 Protein und mRNA ($r=0.20$, $p \leq 0.05$) und UGT1A10 mRNA und UGT Aktivität ($r=0.28$, $p \leq 0.01$) gefunden. Allerdings wurde zwischen UGT1A10 Protein und UGT Aktivität ein statistisch signifikant negativer Zusammenhang gefunden ($r= -0.27$, $p \leq 0.01$).

2) Evaluierung unterschiedlich exprimierter XMEs im normalen Darm- und Tumorgewebe
Die Expression acht ausgewählter XMEs (*GSTP1*, *GSTA1*, *GSTM1*, *UGT1A10*, *UGT1A8*, *CYP2W1*, *CYP2C9*, and *CYP3A4*) wurde im Tumor- und angrenzendem Normalgewebe verglichen (n=71) und Zusammenhänge von soziodemografischen-, Lebensstil-, und Ernährungsfaktoren mit der Expression dieser Gene untersucht. Unterschiede im Normal- und Tumorgewebe zwischen Genexpression dieser XMEs wurden mittels des Wilcoxon-Rangsummen-Tests verglichen. Unter den untersuchten Genen waren, im Tumorgewebe im Vergleich zum Normalgewebe, *GSTM1*, *GSTA1*, *UGT1A8*, *UGT1A10*, *CYP3A4*, und *CYP2C9* herunterreguliert und *GSTP1* und *CYP2W1* hochreguliert. Um mögliche Zusammenhänge zwischen Lebensstil- und Ernährungsfaktoren mit der relativen Genexpression im Tumor- und Normalgewebe zu untersuchen, wurden lineare Regressionsmodelle verwendet. Obwohl keiner dieser Zusammenhänge nach einer „false discovery rate“ (FDR) Adjustierung für multiples Testen signifikant war, wurde ein deutlicher Trend zu statistischer Signifikanz ($\beta=-0.21$; $p_n=0.0005$; $p_{FDR}=0.05$) mit niedriger *CYP2C9* Expression im Normalgewebe von Rektum- im Vergleich zu Kolonkarzinom Patienten gefunden. CYP2C9 spielt eine wesentliche Rolle bei der metabolischen Aktivierung vieler Umwelt- und Ernährungsmutagene und Zusammenhänge dieser Faktoren mit der Expression von CYP2C9, sollten in größeren Studien berücksichtigt werden.

Allgemeine Schlussfolgerungen: Zusammengefasst wurde festgestellt, dass im Normalgewebe von Darmkrebspatienten:

- Genexpression, Proteinlevel und Enzymaktivitäten nicht oder nur schlecht korrelieren,
- Kolon und Rektum sich in Bezug auf Expression und Aktivität einiger XMEs unterscheiden,
- Regelmäßiger Verzehr von gekochtem Gemüse mit CYP3A4 mRNA- und Proteinexpression assoziiert war.

Insgesamt zeigten die Ergebnisse dieser Arbeit einige interessante Unterschiede, die in größeren Studien berücksichtigt werden sollten, um deren möglichen Beitrag bei der Darmkrebsentstehung aufzuklären.

1 Introduction

1.1 Colorectal Cancer

1.1.1 Basic- and histologic organization of the gastrointestinal tract

The term colorectal cancer (CRC) refers to cancer that arises in the lower gastrointestinal tract (GI tract), also known as the large intestine. The large intestine is divided into three main sections: cecum including the appendix, colon, and rectum with the anal canal. The digestive system removes and processes nutrients (minerals, carbohydrates, vitamins, proteins, fats, and water) from foods and helps pass waste material out of the body [1]. After food is chewed and swallowed, it travels through the esophagus to the stomach. There it is partially broken down and send to the small intestine, where digestion continues and most of the nutrients are absorbed. The small intestine joins the large intestine in the lower right abdomen. The first and longest part of the large intestine is the colon, a muscular tube about five feet long. Water and mineral nutrients are absorbed from the food matter in the colon. Waste (feces) left from this process passes into the rectum, the final six inches of the large intestine, and is then expelled via the anus. All segments of the GI tract are divided into four layers: the mucosa, the submucosa, the muscularis mucosa, and the serosa (see Figure 1). The mucosa is the innermost layer which surrounds the lumen of the GI tract and consists of three sublayers [2]. The first sublayer is made up of epithelial cells supported by a thin second sublayer of connective tissue known as the lamina propria. The third sublayer, the muscularis mucosa, is a thin layer of smooth muscle that produces local movements of the mucosa. The submucosa is a thick connective tissue layer that consists of a variety of arteries, veins, lymphatics, and nerves. The muscularis mucosa surrounds the submucosa and is composed of two muscle layers, the inner circular layer and outer longitudinal layer. These two layers move perpendicularly to one another and form the basis of peristalsis. The outermost layer is named as the serosa or, if it lacks an outer layer of mesothelial cells, the adventitia. The serosal layer forms a natural barrier from the spread of inflammatory and malignant processes.

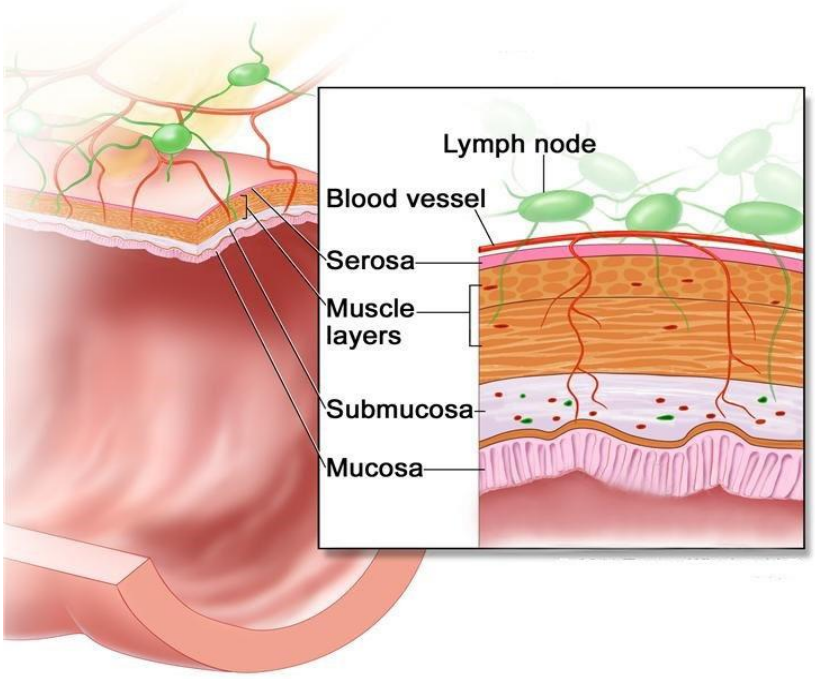

Figure 1: Layers of the gastrointestinal tract
Illustration of the four layers of the GI tract, from deep to superficial, are the mucosa, submucosa, muscularis, and serosa. Illustration modified from National Cancer Institute, March 2016. http://www.cancer.gov/images/cdr/live/CDR688427-750.jpg

1.1.2 Epidemiology of colorectal cancer

With about 62 000 newly diagnosed cases in 2010 and a lifetime risk of more than 5% among both, men and women, CRC is the second most common cancer in Germany [3]. Globally, 1.4 million people are diagnosed with the disease, and about 700 000 die from it each year [4]. The incidence rates of CRC vary widely between different geographical areas. Countries of the Western world, such as United States, Canada, United Kingdom, and Germany have a much higher incidence of CRC compared to India, Angola, Namibia, Uganda and Congo. CRC incidence rates increase with age. In general, 90% of cases are newly diagnosed and 94% of deaths occur in individuals 50 years or older. The incidence rate of CRC is more than 15 times greater in individuals 50 years or older compared to the incidence rate of those aged 20 to 49 years [5]. The risk of a German developing colon cancer during his or her lifetime is about 6% and the risk of subsequent death is about 2.5-3.0%. Of 1000 people between the ages 45 to 75 years, 300 will have benign polyps in the colon and ten will have an undetected CRC tumor [3].

1.1.3 Risk factors of colorectal cancer

CRC is traditionally divided into sporadic and familial (hereditary) cases with some overlapping of clinical features. Most commonly, CRC arises sporadically (>80%), and is influenced by environmental factors. An overview on causes for the development of CRC is shown in Figure 2 [6].

In sporadic CRC, dietary factors play an important role. Geographical differences in CRC incidence rate can primarily be explained by dietary patterns (Western style diet) [7]. Diets high in vegetables, fruits, and whole grain products as consumed in African-, Asian- and South American countries have been linked to lower risk of CRC [8]. A high fiber diet has a protective effect as shown in several studies [9-14]. Migrant studies have shown that people from a country with low prevalence of CRC emigrating to countries with higher prevalence, soon acquire the risk rates of the host country by the second or third generation post lifestyle-adoption [15]. Other important inverse associations exist with nonsteroidal anti-inflammatory drugs (NSAIDs), hormone replacement therapy, and physical activity [16-18].

Environmental factors, such as lack of sun exposure are also pertinent; vitamin D plays a key role. Insufficient vitamin D, vitamin D precursors, and UV-light are associated with colon cancer [19] and also long-term smoking and high alcohol consumption are known to increase the risk of CRC [20-24]. Additional studies revealed that being overweight or obese are additional risk factors for colon cancer [25].

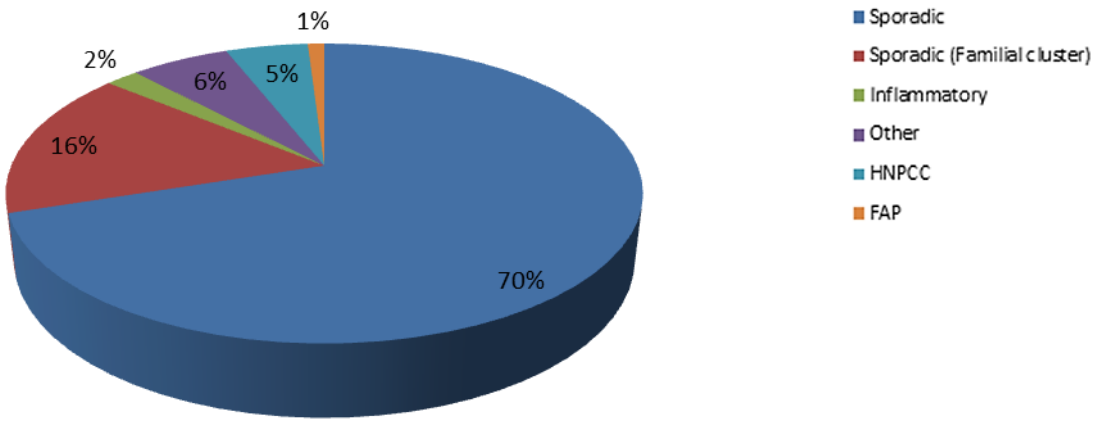

Figure 2: Causes for developing colorectal cancer
The majority of CRC arises sporadically, 16% are referred to as “familial” and may be caused by the interaction of low-penetrance genes, gene-environment interactions, or both. An additional 6% are caused by hereditary factors (FAP and HNPCC) [6]. CRC: colorectal cancer; FAP: Familial adenomatous polyposis; HNPCC: Hereditary nonpolyposis colorectal cancer.

Aggregations of cancer within kindred's are usually referred to as familial, which does not necessarily mean that the disease is genetically inherited. Familial risks calculated between family members are unable to distinguish between hereditary and environmental factors, because families share many environmental factors, such as diet and lifestyle, which may increase or decrease exposures to cancer-related factors [26]. In the third type of CRC, hereditary factors play a decisive role. Familial adenomatous polyposis (FAP) causes less than 1% of all CRC cases and affects approximately 1 in 7000 people. FAP is caused by an inherited mutation in the *APC* (adenomatous polyposis coli) gene [27]. Hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome, affects 1 in 5000 people and first occurs, on average between the ages of 40 to 50 years. This syndrome is caused by a mutation in one of the DNA mismatch repair system (MMR) genes, and accounts for 2% of CRC cases [28].

1.1.4 Colorectal cancer carcinogenesis

The stepwise progression from adenoma to carcinoma may take several decades. As a result of genetic alterations that allow growth of neoplastic cells, these alterations may lead to deregulated growth, replication and apoptosis- pathways that ultimately lead to a progressive malignant phenotype [9].

Currently, two major pathways in colorectal carcinogenesis are known. The most common genetic pathway is called the "canonical" (adenoma-carcinoma sequence) or "suppressor" pathway, which involves chromosomal instability (CIN) [10]. The second pathway is referred to as the microsatellite instability (MSI) pathway.

The "canonical" pathway is present in 80%-85% of colorectal carcinomas and it is assumed to arise from pre-existing adenomas. For this, Fearon and Vogelstein [3] proposed a model of colorectal carcinogenesis that correlates specific genetic events with evolving tissue morphology, which is described below.

As shown in Figure 3, the cancer sequence is initiated after a mutation in the *APC* gene located on chromosome 5. Damage to the *APC* gene may result in the formation of an adenoma which is a benign lesion. Loss of *APC* is necessary, but insufficient to cause cancer. The next step involves the mutation in the *KRAS* (Kirsten rat sarcoma) gene which is located on chromosome 12 and leads to a constantly activated KRAS protein. With *APC* wildtype, *KRAS* does not cause cancer but results in a condition known as aberrant crypt foci (ACF).

The third genetic mutation in this pathway is the mutation of the *SMAD2/4* gene which is located on chromosome 18q. The fourth step in this pathway is a mutation of *TP53* (tumor suppressor protein p53), a tumor suppressor gene which is deactivated in at least 50% of CRC cases. This gene is located on chromosome 17p and works by recognizing damaged DNA and then prevents the cell from replicating. If genetic repair fails, *TP53* probably will stimulate apoptosis. Ultimately, loss of *TP53* permits abnormal cells to avoid death and continue proliferating without restraint [9]

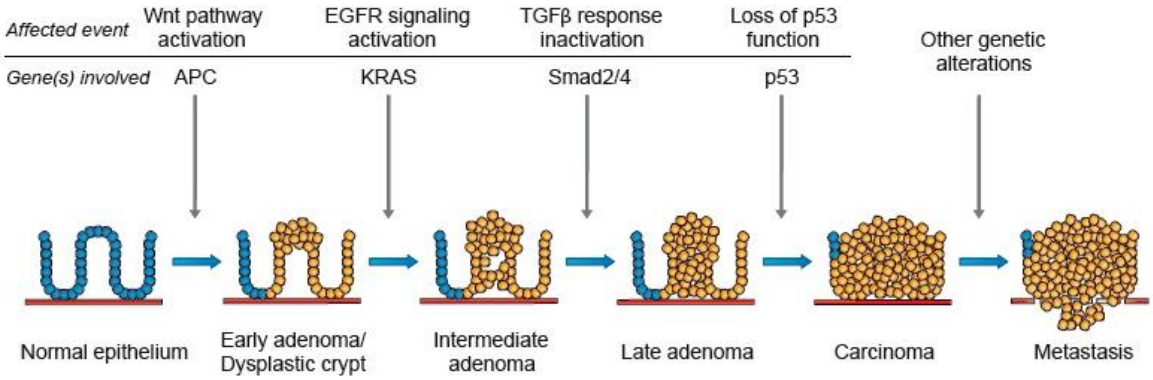

Figure 3: Adenoma-carcinoma sequence
Illustration of the stepwise progression from normal epithelium to carcinoma due to a series of genetic changes [29]. Wnt: Wingless type integration site family, EGFR: Epidermal Growth Factor Receptor; TGF*ß*: Tumor Growth Factor *ß*; APC: adenomatous polyposis coli; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; SMAD: portmanteau of SMA (small body size) and MAD (mothers against decapentaplegic). Illustration adapted from Davies, R.J *et al*. 2005 [30]

The second pathway of colorectal carcinogenesis involves MSI, and is called the "mutator" pathway. The mutator pathway is present in approximately 15-20% of sporadic CRC. Mismatch of DNA nucleotides occurs, if DNA polymerase inserts the wrong bases in newly synthesized DNA. Normally, cells respond to DNA mismatch by means of mismatch repair. Disturbances of the MMR system can result in a situation known as MSI. MSI refers to abnormal variations in short, repetitive DNA sequences. However, defects in the mismatch repair mechanism lead to MSI. There are two different mechanisms for MSI in sporadic and hereditary CRC. HNPCC is caused by a germline mutation in DNA mismatch repair genes, most frequently *MutS homologue 2* (*MSH2*) and *MutL homologue 1* (*MLH1*). Instability in microsatellite sequences in sporadic CRC exhibiting MSI is often due to loss of expression of a mismatch repair gene (most commonly *MLH1*) caused by epigenetic silencing [31].

1.2 Xenobiotic metabolism and its impact on carcinogenesis

The metabolism of xenobiotics is complex and involves multiple steps and multiple enzymes. Variable levels of expression of these enzymes as well as the level of exposure to their substrates could alter metabolism and clearance of potential carcinogens and thus alter CRC susceptibility. Recently, many studies have focused on the relationship between genetic polymorphisms and risk of CRC. However, there have been very few studies on the tissue-specific expression of xenobiotic metabolizing enzymes (XMEs) in the human colon and rectum. During my doctoral studies I have written and published a review: *Biotransformation of xenobiotics in the human colon and rectum and its association with colorectal cancer* [32], which addresses this issue in a comprehensive manner, providing a systematic overview of currently available data on the relation between the cytochrome P450 (CYP), the glutathione *S*-transferase (GST), and the UDP-glucuronosyltransferase (UGT) biotransformation system and CRC in human colon and rectum. I this section, I mention the most important findings. Table 1, Table 2 and Table 3 provide an overview on human studies on colon and rectum-specific CYP, GST and UGT expression and activities The full bibliographic information and discussion of the respective studies can be found in the original publication [32].

The human body is exposed to a great number of xenobiotics in a lifetime, including a variety of pharmaceuticals, dietary supplements, plant constituents, and food additives. Most of these compounds are lipophilic and therefore poorly excreted, because of the lipid-rich cell membranes in the GI and urinary tract. The organism can only excrete them by metabolism to more hydrophilic metabolites; otherwise they would accumulate in body fat. It is, therefore, the task of XMEs to increase the water-solubility of these lipophilic compounds, which can afterwards easily be eliminated through the bile or urine. Conversely, XMEs can by its action also convert certain chemicals to highly toxic metabolites, which is usually the initial event of chemical carcinogenesis. In general, the metabolism of xenobiotics is divided into three phases: The phase I reactions include the introduction of polar functional groups into the molecule or the modification by oxidation, reduction or hydrolysis. Phase II reactions comprise chemical conjugation to water soluble molecules. The excretion of biotransformed molecules is often referred to as phase III metabolism. The most important phase I metabolic enzymes are the CYP superfamily. UGT and GST enzymes play a crucial role in the phase II metabolism [33-35]. The primary biotransformation site is the liver, as it is the main detoxification organ. However, extra-hepatic tissues also show

metabolic activity, i.e. the GI tract, kidney or bladder. The GI tract is the first site in the body that is exposed to a vast majority of xenobiotics. Food constituents, orally administered drugs, as well as chemicals from inhaled air end up in the bowel. The ingested chemicals are spread by the peristaltic movement of the GI tract over a very large surface area which provides favorable conditions for the absorption of lipophilic compounds. Most metabolic enzymes are characterized by several genetic polymorphisms, which can affect their activities [36]. Different alleles of enzymes involved in xenobiotic metabolism contribute to different CRC susceptibility [37]. It is now widely accepted that the development of CRC is determined by a complex interaction of both genetic polymorphisms and environmental factors [38]. Recently, many studies have focused on the relationship between these genetic polymorphisms and risks of CRC [39-43]. However, there have been very few studies on the tissue-specific expression of XMEs in the human colon and rectum.

1.2.1 Human cytochromes P450

The CYP enzymes are a multi-gene superfamily of heme-dependent monooxygenases which catalyze the oxidation of a broad range of exogenous and endogenous compounds. The highest amounts of the most abundant CYPs involved in the metabolism of xenobiotics are present in the liver, although some specific forms are also expressed in extra-hepatic tissues. CYPs are mainly embedded in the membrane of the endoplasmatic reticulum (ER). However, some CYPs are also located in other subcellular compartments, including mitochondria and plasma membranes [33, 43, 44].

In humans, 18 CYP families are known, consisting of 44 subfamilies, within 57 annotated functional genes [45]. Around 30 CYP enzymes are responsible for drug metabolism and belong to families 1-3. CYPs are expressed in a cell type– and tissue-specific pattern [46]. They play a major role in the detoxification and elimination of toxic xenobiotics. However, CYPs can also activate potential pro-carcinogens into highly toxic metabolites. As a result, CYP overexpression is also linked to the development of various types of tumors. CYPs activate polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) to toxic metabolites that are implicated in the etiology CRC [47]. Because CYPs are involved in the oxidative metabolism of many anticancer drugs, they are not only important for the development of tumors but also during treatment of colonic neoplasia [48, 49].

In human colon, expression of CYP1A1, CYP1B1, CYP2C, CYP2E1, CYP3A4 and CYP3A5 has been detected [46].

1.2.1.1 The human CYP1 family

The human CYP1 family consists of CYP1A1, CYP1A2, and CYP1B1. CYP1A1 is mainly expressed in extra-hepatic tissues and is present only at low levels in the liver [50, 51]. It is one of the most important enzymes participating in the bioactivation of procarcinogens to generate reactive metabolites but it also detoxifies environmental carcinogens such as PAHs, HCAs, and industrial arylamines [52]. CYP1A1 gene expression is mediated by the aryl hydrocarbon (Ah) receptor-mediated signal pathway [53]. The Ah-receptor is a cytosolic transcription factor that is activated upon ligand binding, resulting in translocation to the Ah receptor-ligand complex to the nucleus and finally to changes in gene transcription.

Human CYP1A2 is constitutively expressed in human liver, albeit not in extra-hepatic tissues. It metabolizes several critical endogenous compounds (retinols, melatonin, steroids, uroporphyrinogen and arachidonic acids), a number of procarcinogens (PAHs, HCAs, mycotoxins), and a variety of therapeutic drugs (clozapine, tacrine, tizanidine, and theophylline) [54, 55]. Similar to CYP1A1, the CYP1A2 gene expression is also regulated by the Ah receptor [56].

CYP1B1 is a main extra-hepatic cytochrome and its activity has been implicated in carcinogenesis, drug resistance and hypertension [57].

1.2.1.2 The human CYP2 family

The human CYP2 family comprises the subfamilies CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, and CYP2J. The most important genes of the CYP2 family contributing to the metabolism of clinically relevant drugs and alcohol are CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP2F1. Unlike the CYP1 family, the members of the CYP2 family do not possess shared regulatory features. Also, the substrate and tissue-specificities of these enzymes differ markedly.

CYP2A6 is by far the best studied enzyme in its subfamily [58, 59]. It has been shown to catalyze the metabolic activation of several procarcinogens and clinically used drugs such as coumarin [60-63]. It is also a primary metabolizer of nicotine and cotinine [64, 65]. CYP2A6 is

mainly expressed in the human liver but also in extra-hepatic tissues, most notably kidney, lung, nasal mucosa and other areas of the respiratory tract [59, 66-69].

CYP2B6 is the only functional enzyme of its subfamily in humans. It is the major biotransformation enzyme for a number of clinically used drugs including chemotherapeutics such as cyclophosphamide, the antiestrogen tamoxifen and the benzodiazepine diazepam [70-72]. CYP2B6 also plays an important role in the metabolism of several pesticides and other environmental xenobiotics such as chlorpyrifos, metolachlor, butachlor, and the insect repellant N, N-diethyl-m-toluamide (DEET) [73-75]. The functional CYP2B6 gene is primarily expressed in the liver with large inter-individual variability [76-79]. Besides the liver, CYP2B6 is also expressed in the brain, skin, kidney, lung, peripheral blood lymphocytes, and in different parts of the GI- and respiratory tract [46, 76, 80].

The human CYP2C subfamily provides approximately 20% of the CYP enzyme complement in the liver, but also this subfamily is expressed in various extra-hepatic tissues (lung, kidney, gut, and cardiovascular tissues) [81-83].

CYP2C8 metabolizes the chemotherapeutic drug paclitaxel and the anti-diabetic drug rosiglitazone, among others [84]. Additionally, CYP2C8 is the principal enzyme in the in the liver and kidney that metabolizes arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs) [85, 86]. EETs induce vasodilation and exert anti-inflammatory effects in blood vessels but they may also promote cancer progression by directly inducing cancer cell proliferation [87, 88].

CYP2C9 is the most abundant CYP isoform of its subfamily and the second most expressed CYP in human liver and intestine [89]. It is involved in the metabolism of numerous substrates including the highly carcinogenic HA 2-amino-3, 4-dimethylimidazo(4,5-f)quinolone or the PAH dibenzo(a,h)anthracene [90, 91]. The enzyme also plays a key role in the metabolism of NSAIDs and individuals with several variant alleles demonstrated decreased metabolic clearance compared with individuals with the wild-type enzyme [92].

CYP2C19 metabolizes the anticonvulsant drug S-mephenytoin, the antiulcer drug omeprazole and other proton pump inhibitors, certain tricyclic antidepressants, and the β-adrenoceptor blocker propranolol. It is also responsible for the biotransformation of the antimalarial drug proguanil to its active metabolite cycloguanil [92-95].

CYP2D6 is the only functional gene in the human CYP2D subfamily and probably the most studied polymorphically expressed drug metabolizing enzyme in humans. Its activity is highly

variable ranging from no activity in poor metabolizers (PM) to ultrarapid metabolizers (UM), depending on the gene locus. To date, more than 88 different polymorphic CYP2D6 alleles are known. CYP2D6 is involved in the metabolism of at least 25% of the common drugs used today [96-99]. This includes antipsychotic agents, antidepressants, opioids, and anticancer agents. The most prominent anticancer agent metabolized by CYP2D6 is tamoxifen [100]. Tamoxifen is currently used to prevent recurrence of estrogen receptor positive breast cancer [101]. Tamoxifen is a largely inactive pro-drug, requiring metabolism by CYP2D6 into its active metabolites 4-hydroxytamoxifen and endoxifen [102, 103]. Patients with variant forms of the CYP2D6 gene may not receive full benefit from tamoxifen because of a too slow metabolism to the more potent active metabolites 4-hydroxytamoxifen and endoxifen [100, 104, 105]. Inhibition of CYP2D6 by other drugs (e.g. selective serotonin reuptake inhibitors (SSRIs)) can result in reduced endoxifen formation which can lead to failure of tamoxifen therapy [106].

CYP2E1 is implicated in several diseases such as diabetes, non-alcoholic steatohepatitis (NASH), and cancer. It is a key player in alcohol metabolism, oxidative stress, and drug metabolism. Apart from the liver, CYP2E1 is also expressed in many other tissues such as lung, brain, kidney, GI tract and breast tissues (for review see [107]).

Predominantly expressed in the lung, CYP2F1 is the only functional member of the CYP2F subfamily [108-110]. Its substrates are pneumotoxicants and styrene [111-115].

1.2.1.3 The human CYP3 family

The human CYP3 family is composed of only of one subfamily, CYP3A, which comprises the four CYP genes 3A4, 3A5, 3A7, and 3A43. The CYP3A subfamily is the most abundant CYP isoform expressed at both, the mRNA- and protein levels in the human liver and metabolizes 30-40% of all clinically used drugs [116, 117].

CYP3A7 is the major CYP3A expressed in the human fetal liver, the placenta and endometrium [118, 119]. CYP3A43 is expressed at comparatively low levels in fetal and adult liver, skeletal muscle, pancreas, kidney, prostate and testis [120, 121]. The amino acid sequence of CYPA43 is to 75% identical to CYP3A4 and CYP3A5 and to 71% identical to CYP3A7.

CYP3A4 is the major CYP3A isoform in the small intestine, whereas CYP3A5 has been reported to be the main form in the human colon [122]. CYP3A4 and CYP3A5 share approximately 85% sequence identity leading to similar substrate specificities of these isoforms and making a determination of their specific contribution to the overall CYP3A-mediated drug metabolism difficult [123, 124]. CYP3A4 and CYP3A5 metabolize drugs and many endogenous substrates, such as bile acids, steroid hormones and retinoic acid [125].

Table 1: Human studies on colon and rectum-specific cytochrome P450 expression and activities

Study	Tissue	CYP isoform	Protein expression	mRNA expression	Enzyme activity	Additional findings
Plewka *et al*., 2014*	• UC biopsies • CD biopsies • Normal colorectal biopsies	• 3A4 • 2C9 • 2E1 • 2B6 • 1A1	**CYP2C9** 1.3 fold higher in CD and 0.75 fold lower in UC vs. control tissue; **CYP2B6** higher in the disease group vs. the control group [Immunohistochemistry]	n.a.	n.a.	n.a.
Androutsopoulos *et al.*, 2013*	• Colon cancer tissue (n=20) • Adjacent normal mucosa (n=20)	• 1A1 • 1B1	n.a	***CYP1A1*** and ***CYP1B1*** higher in tumor tissues vs. adjacent normal tissues [qRT-PCR]	Higher **CYP1** activity in colon tumors vs. normal tissue	mRNA level do not correlate with enzyme activity
Stenstedt *et al.*, 2012*	• Colon cancer tissue (n=235)	• 2W1	**CYP2W1** high in 30% of the tumors [Immunohistochemistry]	n.a.	n.a.	High **CYP2W1** expression is correlated with worse outcome

Continues on next page.

Table 1 continued

Study	Tissue	CYP isoform	Protein expression	mRNA expression	Enzyme activity	Additional findings
Matsuda *et al*., 2007*	• Adenoma (n=16) • Adenocarcinoma (n=30) • Carcinoma in or with adenoma (n=7) • Adjacent normal mucosa for all colon specimens (n=53)	• 2A6	**CYP2A6** higher in adenocarcinomas, carcinomas in adenoma, and adenoma vs. control tissue. [Immunohistochemistry]	***CYP2A6*** detected in adenocarcinoma only. [in situ hybridization]	n.a.	n.a.
Thorn *et al*., 2005*	• Normal mucosa (n=27)	• 2E1 • 3A4 • 3A5	n.a.	***CYP3A5*** higher than ***CYP3A4*** [RT-PCR]	n.a.	Smoking, alcohol intake, or sex had no association with **CYP** mRNA level
Bergheim *et al*., 2005*	• Colonic adenoma (n=25) • Surrounding normal colon mucosa (n=25); • Disease-free controls (n=27)	• 2C • 2E1 • 3A4 • 3A5	**CYP2C8**, **CYP3A4**, and **CYP3A5** lower (86%, 69%, and 54%) in normal tissue of adenoma patients vs. disease-free controls [Immunohistochemistry]	***CYP3A5*** higher in adenoma vs. normal tissue of adenoma patients (48%) [RT-PCR]	n.a.	CYP protein level and mRNA expression are not related in normal colonic tissue

Continues on next page.

Table 1 continued

Study	Tissue	CYP isoform	Protein expression	mRNA expression	Enzyme activity	Additional findings
Bergheim *et al*., 2005*	Normal mucosa of • Ascending (n=10), • Descending (n=7) • Sigmoid (n=24) colon	• 2C • 2E1 • 3A4 • 3A5	**CYP2C8** higher (73%) in sigmoid colon vs. descending colon; **CYP2E1** in sigmoid colon 81% of. descending colon [Immunohistochemistry]	***CYP2C9*** higher in ascending colon vs. sigmoid colon; ***CYP2E1*** and ***CYP3A5*** lower in the ascending colon vs. descending and sigmoid colon [RT-PCR]	n.a.	n.a.
Kumarakulasingham *et al*., 2005*	• CRC tissue (n=264) • Normal colorectal samples (n=10) • Lymph node metastasis (n=91)	• 1A1/ B1 • 2A/B/ C/D6/ E1/F1/ J2/R1/ S1/U1 • 3A4/ A5/A7 /A43 • 4F11/ V2/X1 /Z1 • 24 • 26A1	CYP1B1, CYP2S21, CYP2U1, CYP3A5, and CYP51 higher in CRC tissue vs. normal colon tissue. [Immunohistochemistry]		n.a.	Higher **CYP51** and **CYP2S1** expression associated with poorer prognosis;

Continues on next page.

Table 1 continued

Study	Tissue	CYP isoform	Protein expression	mRNA expression	Enzyme activity	Additional findings
Chang *et al*., 2005*	• Colorectal adenocarcinoma (n=97) • Adenomas with low-grade dysplasia (n=53) • Adenomas with high-grade dysplasia (n=32) • Non-neoplastic colons (n=49)	• 1B1	**CYP1B1** expression rate higher in the adenoma and carcinoma groups vs. non-neoplastic colon group [Immunohistochemistry]	n.a.	n.a.	n.a.
Gibson *et al*., 2003*	• Adenocarcinoma tissue (n=61) • Adjacent normal mucosa (n=14)	• 1B1	**CYP1B1** higher in adenocarcinoma vs. normal colon mucosa [Immunohistochemistry]	n.a.	n.a.	n.a.
Gervot *et al*., 1996*	• Colon cancer tissue (n=21) • Adjacent normal mucosa (n=21) • Sigmoiditis biopsies (n=25)	• 3A4 • 3A5	No difference in control and tumor samples [Immunoblotting]	n.a.	n.a.	600 mg/day rifampicin did not induce **CYP3A** expression in control and tumor samples.

Continues on next page.

Table 1 continued

Study	Tissue	CYP isoform	Protein expression	mRNA expression	Enzyme activity	Additional findings
Mercurio *et al*., 1995*	• Normal colorectal biopsies (n=5)	• 1A1 • 1A2 • 3A3	n.a.	***CYP1A1*** higher in rectum vs. colon; ***CYP3A3*** higher in colon vs. rectum; ***CYP1A2*** inconsistent [RT-PCR]	n.a.	n.a.
de Waziers *et al*., 1991*	• Normal colon mucosa from patients with sigmoiditis (n=13) • Peritumoral and tumoral mucosa (n=15)	• 1A1 • 1A2 • 2C8-10 • 2E1 • 3A4	**CYP1A1** not detectable; **CYP3A4** lower in tumor tissue vs. normal tissue [Immunoblotting]	n.a.	n.a.	n.a.

*Full bibliographic information and discussion of the respective studies can be found in the original publication [32].
Abbreviations: UC: ulcerative colitis; CD: Crohn's disease; CRC: colorectal cancer; qRT-PCR: real-time quantitative PCR; RT-PCR: real-time PCR.

1.2.2 Human glutathione *S*-transferases

Human GSTs play a major role in the detoxification of numerous endo- and xenobiotics including carcinogens, environmental pollutants and cancer chemotherapeutic drugs [126, 127]. Aside from detoxifications, GSTs carry out a wide range of functions in cells, such as the removal of reactive oxygen species (ROS), apoptosis, steroid and prostaglandin biosynthesis, and tyrosine catabolism [128]. GSTs occur in three cellular compartments and can be thus distinguished into: cytosolic GSTs, mitochondrial GSTs, and microsomal GSTs, also referred to as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). The cytosolic GSTs are represented by the classes alpha, mu, pi, theta, zeta, omega, and sigma [129]. The mitochondrial GSTs belong to the class of kappa GSTs [126, 128-131]. Members of the same class share greater than 40% sequence identity, between classes the identity is less than 25%. The most abundant mammalian GSTs belong to the cytosolic classes alpha, mu, and pi [34]. Class alpha GSTs are basic proteins with intermediate subunit molecular masses, class mu GSTs are neutral proteins with high molecular masses, and class pi GSTs are acidic proteins of low molecular mass [132]. GSTs show a high tissue-specificity although the liver is regarded the most important organ with respect to biotransformation. However, GSTs are also active in the kidneys, esophagus, stomach, small intestine, and colon [133]. Specific isoenzymes of GST are known to be expressed in preneoplastic as well as neoplastic cells and are involved in the development of drug resistance [134]. Recent studies have shown decreased protein levels and activities of GST in different tumors than in the corresponding normal tissues, including colorectal carcinoma [135-138]. Therefore, interventions which increase the glutathione detoxification capacity may reduce cancer incidence. In vivo and in vitro studies revealed variety of dietary compounds to induce GSTs [139-141]. These include several non-nutrient compounds occurring in fruit and vegetables such as limonoids and flavonoids from citrus fruits, glucosinolate in brassica vegetables (e.g. broccoli, brussel sprouts), diallyl sulfides in allium vegetables (e.g. onion, garlic), and butyrate produced by colonic fermentation of fiber [34, 132, 142-146].

1.2.2.1 The human alpha class GSTs

The alpha class GSTs consists of four proteins. GSTA1 and GSTA2 catalyze the conjugation of glutathione with electrophiles [147]. They exhibit glutathione dependent steroid isomerase activity, and glutathione-dependent peroxidase activity [147]. GSTA3 catalyzes glutathione-dependent Δ^5-Δ^4 isomerization of steroids [148]. GSTA4 is involved in tissue and cell defense against oxidative stress by its high activity for the glutathione-dependent detoxification of alkenyl products of lipid peroxidation [149, 150].

Alpha class GSTs are widely expressed in human tissues. GSTA1 and GSTA2 are present at high protein and mRNA levels in liver, kidney, pancreas, testis, small intestine and adrenal gland and at low levels in a wide range of tissues including the GI tract [34, 151-155]. GSTA3 expression at the mRNA level was reported in the placenta, testis, mammary gland, adrenal gland, ovary, lung, stomach, and trachea [148, 154]. GSTA4 is present in almost all tissues at the mRNA level and its protein has been found in several cell types of the liver, kidney, skin, muscle, and brain [156]. Diets high in cruciferous vegetables, therapeutic drugs, and other xenobiotics induce GSTA1/A2 mRNA- and protein expression but also several diseases are known to affect its expression [157]. GSTA1/A2 expression in the stomach of Helicobacter pylori infected individuals is decreased in comparison to healthy individuals [158]. Also a decrease in GSTA expression has been observed in the small intestine of celiac disease patients, as well as in stomach and liver cancers [159]. In contrast, an increased expression has been observed in lung cancer and in CRC [133].

1.2.2.2 The human mu class GSTs

The human mu class GSTs are encoded by a 100-kb gene cluster on chromosome 1p13.3 and is highly polymorphic [130, 160-163]. About 50% of the human population carry a deletion for GSTM1 gene and this null polymorphism has been widely investigated as a risk biomarker for various cancers, resistance to chemotherapy treatment, drug response, and disease susceptibility and poorer outcome [164-166]. In smokers, absence of GSTM1 has been associated with an increased risk of developing urinary bladder or larynx cancer [167, 168]. The genes of four other members of the GST mu subfamily (GSTM2-GSTM5) exhibit high levels of sequence homology and substrate-specificity with GSTM1 [159].

1.2.2.3 The human pi class GSTs (GSTP)

GSTP is found in a wide range of normal and neoplastic tissues and is the predominant subclass of the GST family in colonic epithelial cells. Increased protein- and mRNA expression of GSTP1 has been shown to be associated with poor prognosis and reduced survival in many cancers, including head and neck, breast, lung, neurological, hematological and gastrointestinal malignancies [169-174]. GSTP1 is a major enzyme participating in the inactivation of toxic and carcinogenic compounds. It directly detoxifies a variety of anticancer drugs such as chlorambucil, cisplatin or melphalan and can modify also the effect of radiotherapy by conjugation of glutathione to ROS [174-176].

Table 2: Human studies on colon and rectum-specific expression and activities of Glutathione *S*-transferases

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
Hoensch *et al*., 2013*	• Adenoma biopsies (n=28) • CRC biopsies (n=20)	• GST	n.a.	Lower in CRC (241 nmol/min/mg protein) vs. adenoma patients (268 nmol/min/mg) [Spectrophotometry / CDNB]	n.a.
Bedford *et al*., 2012*	• Rectal tumor mucosa (n=92) • Adjacent normal mucosa (n=92)	• P1	GSTP1 higher in rectal adenocarcinoma vs. matched normal mucosa [6.59 µg / mg vs 4.57 µg / mg; $P < 0.001$]; Tumor GSTP1 lower in the neoadjuvant treated vs. non-treated [4.47 µg / mg vs 7.76 µg / mg; $P < 0.001$] [ELISA]	n.a.	n.a.
Tan *et al*., 2011*	• Colon tumor tissue (n=449)	• P1	[Immunohistochemistry]	n.a.	High GSTP1 expression is associated with features of tumor aggressiveness and with reduced overall survival

Continues on next page.

Table 2 continued

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
Gaitanarou *et al*., 2008*	All polyp tissue were adenoma of different stage of dysplasia: • low-grade (n=3) • mild-grad (n=9) • high-grade (n=4)	• P1	Increase from low-grade adenoma to high-grade adenoma [Immunohistochemistry]	n.a.	n.a.
Grubben *et al.*, 2006	Tissue from patients with • Adenoma (n=64) • HNPCC-Adenoma (n=33) • HNPCC+Adenoma (n=34) • FAP (n=19), • Carcinoma (n=37), • earlier data from 10 healthy controls	• GST	n.a.	GST activity lower in CRC or FAP vs. adenoma patients or healthy controls [Spectrophotometry / CDNB]	n.a.

Continues on next page.

Table 2 continued

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
Hoensch *et al.*, 2006*	• Biopsies of macroscopically normal mucosa from both proximal and distal colon (n=208)	• GST • P1 • M1	GSTP1 level decreased sig. from proximal to distal colon (2.25 vs. 2.10 µg/mg protein) GSTP1 increase in females from the age of under 50 years to over 70 years [Immunoblotting]	Decrease from proximal to distal colon (264 vs. 244 nmol/min/mg protein); GST activities increase in females from the age of under 50 years to over 70 years [Spectrophotometry / CDNB]	n.a.
Wark *et al.*, 2004*	• Rectal biopsies of HNPCC and non-HNPCC subjects (n=94)	• GST • A1 • M1 • P1	Cancer-free members of HNPCC families had lower rectal GSTA1 protein levels than those who did not belong to a HNPCC family [Immunoblotting]	[Spectrophotometry / CDNB] n.a.	Smoking is associated with higher GST activity and higher GSTA1 and GSTP protein levels; Consumption of fruits, in particular citrus fruits, is associated with higher rectal GST activity
Naidu *et al.*, 2003*	• Colorectal cancer tissue (n=13) • Adenoma (n=13) • Adjacent normal mucosa (n=13)	• GST • P1	GSTP1 protein higher in adenocarcinomas vs. normal colonic mucosa [Immunoblotting]	GST levels increase with the stage of neoplastic progression from normal colonic mucosa, to adenoma, to invasive adenocarcinoma [Spectrophotometry / CDNB]	n.a.

Continues on next page.

Table 2 continued

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
Coles *et al.*, 2002*	• Normal tissue of the GI tract (n=16)	• P1 • A1 • A2 • M1 • M3	**GSTP1** expression throughout the GI tract and decrease of expression from stomach to colon; **GSTA1** expression 20- to 800-fold lower in colon vs. corresponding small intestine [HPLC]	n.a.	n.a.
Grubben *et al.*, 2000*	• Colorectal biopsies from healthy volunteers (n=64)	• GST	n.a.	[Spectrophotometry / CDNB]	No effects of unfiltered coffee on colorectal **GST activity**
Sutoh *et al.*, 2000*	• Colorectal tumor tissue (n=130)	• P1	[Immunohistochemistry]	n.a.	High **GST Pi expressio**n was associated with reduced overall survival
Hengstler *et al*., 1998*	• Colon cancer tissue (n=23) • Adjacent healthy tissue (n=23)	• A1 • P1	**GSTP1** 1.9-fold higher in colon cancer tissue vs. normal colon tissue [15.1 µg / mg vs 8.1 µg / mg; $p = 0.035$]; **GSTA**1 higher in colon cancer tissue vs. normal tissue [0.4 µg / mg vs 0.0 µg/ mg; $p = 0.019$]. [Immunoblotting]	n.a.	n.a.

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Table 2 continued

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
O'Dwyer *et al*., 1995*	• Mucosal biopsies from patients at high risk for CRC (n=24)	• GST	n.a.	[Spectrophotometry / CDNB]	21% **GST activity** increase after a single dose of 250 mg/m^2 Oltipraz
Nijhoff *et al.*, 1995*	• Rectum biopsies from healthy volunteers (n=10)	• GST • A1 • M1 • P1	[Immunoblotting]	**GST enzyme activity** not affected by Brussels sprouts consumption [Spectrophotometry / CDNB]	300 g /d Brussel sprouts (1 week) → 30% rectal **GSTA** increase and 15% rectal **GSTP1** increase in protein expression
Mulder *et al.*, 1995*	• Colorectal tumor tissue (n=100) • Adjacent normal mucosa (n=100)	• GST • A1 • M1 P1	High **GSTA1** (>0.14 µg/mg; HR, 2.40; *p*= 0.008) levels in the carcinoma associated with poorer overall survival; Low **GSTM1** (<0.18 µg/mg; HR, 1.95; *p* = 0.004) levels in the carcinoma associated with poorer overall survival; High **GSTP1** (>5.30 µg/mg; HR, 1.92, *p* = 0.0054) associated with poorer prognosis, (borderline significant) [Immunoblotting]	High level (>243 nmol/min/mg protein; HR, 1.66, *p* = 0.0058) associated with poorer prognosis (borderline significant) [Spectrophotometry / CDNB]	significant linear correlation between **GSTP1** level and **GST enzyme activity** in the tumors

Continues on next page.

Table 2 continued

Study	Tissue	GST isoforms	Protein expression	Enzyme activity	Additional findings
Howie *et al.*, 1990*	• Colon tumor tissue (n=27) • Adjacent normal mucosa (n=27)	• GST • P1	**GSTP1** elevated in tumor tissue vs. normal colon [Immunohistochemistry and RIA]	**GST activity** increase in tumor vs. normal colon [Spectrophotometry / CDNB]	Strong correlation between **GST activity** and **GSTP1** levels in tumor tissue.
Peters *et al.*, 1989*	• Stage C colon tumor tissue (n=449)	• P1	High **GSTP1** expression associated with features of tumor aggressiveness and with reduced overall survival [Immunohistochemistry]	n.a.	n.a.

*Full bibliographic information and discussion of the respective studies can be found in the original publication [32].
Abbreviations: CRC: colorectal cancer; RIA: radioimmunoassay; sig: significantly; FAP: familial adenomatous polyposis; HNPCC: Hereditary nonpolyposis colorectal cancer; n.a.: not available; CDNB: 1-Chloro-2,4-dinitrobenzene.

1.2.3 Human UDP-glucuronosyltransferases

UGTs are a superfamily of enzymes that catalyze the glucuronidation of numerous endogenous compounds including bile acids, steroid hormones and bilirubin as well as exogenous substrates such as carcinogens, therapeutic drugs, and environmental pollutants. Currently, 21 different UGT proteins have been identified in humans and they are grouped into three subfamilies: UGT1A, UGT2A and UGT2B [177, 178]. The human UGT1A gene is located on chromosome 2q37 and encodes nine functional proteins (UGT1A1, UGT1A3-UGT1A10) and four pseudo genes (UGT1A2, UGT1A11-UGT1A13) [179]. The UGT2A and UGT2B genes are located on chromosome 4q13 and are comprised of nine individual structural genes [177, 180]. In general, UGT1A enzymes conjugate both endogenous and exogenous substrates, whereas UGT2B enzymes more often have endogenous compounds as substrates [35]. Each enzyme encoded by a UGT gene exhibits a unique, but usually overlapping substrate-specificity, tissue localization and regulation. Although the liver is recognized as the major site of glucuronidation, it is now clear, that extra-hepatic tissues such as the GI tract, kidney, breast or brain also plays an important role in glucuronidation reactions [35, 181, 182]. In terms of drug metabolism, the kidneys and GI tract are considered the most important sites of extra-hepatic metabolism. There are emerging data which suggests that different UGT isoforms might be important in the process of carcinogenesis. For example, down-regulation of several UGT1A enzymes has been shown to be an early event in hepatic and bilary cancers [183].

The GI tract has a unique complement of UGTs, with mRNA and protein expression of all isoforms present in liver and additionally UGT1A8 and UGT1A10 [184]. However, their tissue distribution along this organ varies significantly.

Table 3: Human studies on colon and rectum-specific expression of UDP-glucuronosyltransferases

Study	Tissue	UGT isoforms	Protein expression	mRNA expression	Enzyme activity	Additional findings
Wang *et al.*, 2013*	• CRC tissue and surrounding healthy tissue (n=150) • Healthy colon mucosa samples (n=120)	• 1A1 • 1A3-1A10	n.a.	***UGT1A*** reduced in cancer vs. normal tissues from the same patient; ***UGT1A*** in healthy tissue in study patients lower than control; [RT-PCR]	n.a.	n.a.
Hoensch *et al.*, 2013*	• Colorectal adenoma biopsies (n=28) • Colorectal carcinoma (20) biopsies	• UGT	n.a.	n.a.	UGT enzyme activities lower in cancer patients (150 pmol/min/mg protein) vs. adenoma patients (197 pmol/min/mg) [LC-MS with 4-nitrophenol as substrate]	n.a.

Continues on next page.

Table 3 continued

Study	Tissue	UGT isoforms	Protein expression	mRNA expression	Enzyme activity	Additional findings
Court *et al*., 2012*	• Pooled RNA from human normal tissue donors (colon: purchased from BD-Clontech)	• 1A1-1A10 • 2A1-2A3 • 2B4 • 2B7 • 2B10 • 2B11 • 2B15 • 2B17 • 2B28	n.a.	In colon highly expressed (mRNA content >100 copies per 10^9 copies of 18rRNA): ***UGT1A4, 1A6, 1A10, 2A3, 2B4;*** Moderately expressed 50-100*): **UGT 1A3, 1A7, 1A9*** Weakly expressed: (<50): ***UGT1A1, 1A5, 1A8, 2B7, 2B15, 2B17*** Not expressed: ***UGT2A1, 2A2, 2B10, 2B11, 2B28*** [qRT-PCR]	n.a.	n.a.
Wang *et al*., 2012*	• Normal colonic mucosa (n=24) • Adenoma tissue (n=30) • Adenocarcinoma tissue (n=77)	• 1A	**UGT1A** is highly expressed in normal colonic mucosa vs. low or no expression in the adenoma and adenocarcinoma tissues [Immunohistochemistry]	n.a.	n.a.	n.a.

Continues on next page.

Table 3 continued

Study	Tissue	UGT isoforms	Protein expression	mRNA expression	Enzyme activity	Additional findings
Nakamura *et al*., 2008*	• Total RNA from human normal tissues (purchased from Stratagene) Tissues: liver, colon, kidney, bladder, breast, ovary, uterus, stomach, small intestine, adrenal gland and testis) • Total RNA from cell lines	• 1A1-1A10 • 2B4 • 2B7 • 2B10 • 2B11 • 2B15 • 2B17 • 2B28	n.a.	In colon highly expressed: ***UGT1A1, 1A3, 1A8, 1A10, 2B7, 2B17*** Moderately expressed: ***UGT1A4, 1A5, 1A6, 1A7, 2B15*** Weakly expressed: ***UGT1A9, 2B4, 2B11*** No expression: ***UGT2B10, 2B28*** [RT-PCR]	n.a.	n.a.

Continues on next page.

Table 3 continued

Study	Tissue	UGT isoforms	Protein expression	mRNA expression	Enzyme activity	Additional findings
Giuliani *et al.,* 2005*	• Sections of urothelial carcinoma, lymph node metastasis and liver metastasis • Sections of low grade adenoma (n=5), high grade adenoma (n=5), and colon carcinoma (n=11)	• 1A	**UGT1A** positive only in adenomas with low grade dysplasia (5/5) and in carcinomas (2/11); High grade dysplasia adenoma (5/5) and carcinomas (9/11), are **UGT1A** negative [Immunohistochemistry]	n.a.	n.a.	n.a.
Strassburg *et al.,* 1999*	• Normal colon mucosa from CRC patients (n=5) • Normal liver samples from patients undergoing hemihepatectomy (n=5)	• 1A1 • 1A3 • 1A4-1A10	**UGT1A** diversity greater in colon than in liver microsomes [Immunoblotting]	**UGT1A1, 1A3, 1A4, 1A6,** and **1A9** are expressed in human liver; Human colon additionally expresses **UGT1A8** and **1A10** [RT-PCR]	Maximum in liver: 3637 +/- 1202 pmol/min/mg; Maximum in colon: 100 pmol/min/mg [Spectrophotometry:54 different substrates]	96-fold higher UGT activity in liver vs. colon

*Full bibliographic information and discussion of the respective studies can be found in the original publication [32].
Abbreviations: CRC: colorectal cancer; RIA: radioimmunoassay; sig: significantly; FAP: Familial adenomatous polyposis; HNPCC: Hereditary nonpolyposis colorectal cancer; GI tract: gastrointestinal tract; n.a. not available; qRT-PCR: real-time quantitative PCR; RT-PCR: real-time PCR; LC-MS: liquid chromatography-mass spectrometry.

1.3 The central dogma of molecular biology and its implication in xenobiotic metabolism

The central dogma of molecular biology was first coined by Francis Crick in 1958 [185] and re-stated in 1970 [186]. It proposes an unidirectional flux of information from genes to mRNA (transcription) to proteins (translation) and explains the flow of genetic information within a biological system. With modern research it is becoming clear that some aspects of the central dogma are not entirely accurate and numerous findings suggest that the control mechanisms in a living cell are more complex than what is presented by the central dogma [187] (see Figure 4). One of the most notable exceptions of this is reverse transcription, in which RNA encodes DNA. Because genetic, environmental and dietary factors influence the expression of XMEs, their inter-individual variability is large, and the susceptibility of humans to the pharmacological and toxicological actions of drugs and other chemical varies considerably [188]. Therefore, expression level and activities of XMEs, especially for CYPs, have been a focus of interest for a long time. Despite advances in genome sequencing and high throughput methods, the relationship between XMEs' gene expression, protein abundance, and enzyme activity is still unclear. Although gene expression levels have been thoroughly analyzed, mRNA levels do not necessarily reflect protein expression or activity of the corresponding enzymes [189]. For example, a study by Rodriguez-Antona *et al*. determined activity and mRNA contents of 10 CYPs in human liver samples and demonstrated high correlation coefficients for CYP1A1, CYP1A2, CYP3A4, CYP2D6, and CYP2B6, but no significant correlations for CYP2C9, CYP2A6, and CYP2E1 were found [190]. Several other studies also reported poor correlations between mRNA expression and activity of various CYPs and UGTs [74, 188, 191, 192]. Since biological processes are typically driven by proteins, protein expression levels are considered to be a more suitable parameter of the functional activity of enzymes. Some studies have demonstrated that, in fact, CYP, GST, and UGT protein expression levels were better correlated with enzyme activities than mRNA expression levels [74, 192, 193]. For example, Hayashi *et al*. reported that protein expression of CYP2E1 correlated well with activity but not with mRNA expression in human intestinal tissue samples [192]. However, high-throughput quantification of protein expression has been shown to be more difficult and less straightforward than transcriptome quantification and high-throughput methods for most enzyme activity measurements are not available.

Thus, it has become common practice to carry out microarray and qRT-PCR measurements and use the obtained transcript data to infer functions without information about how the protein levels and their activities change. To increase their usefulness, the relationship of such genomic and proteomic data to gene product activity should be evaluated in future studies.

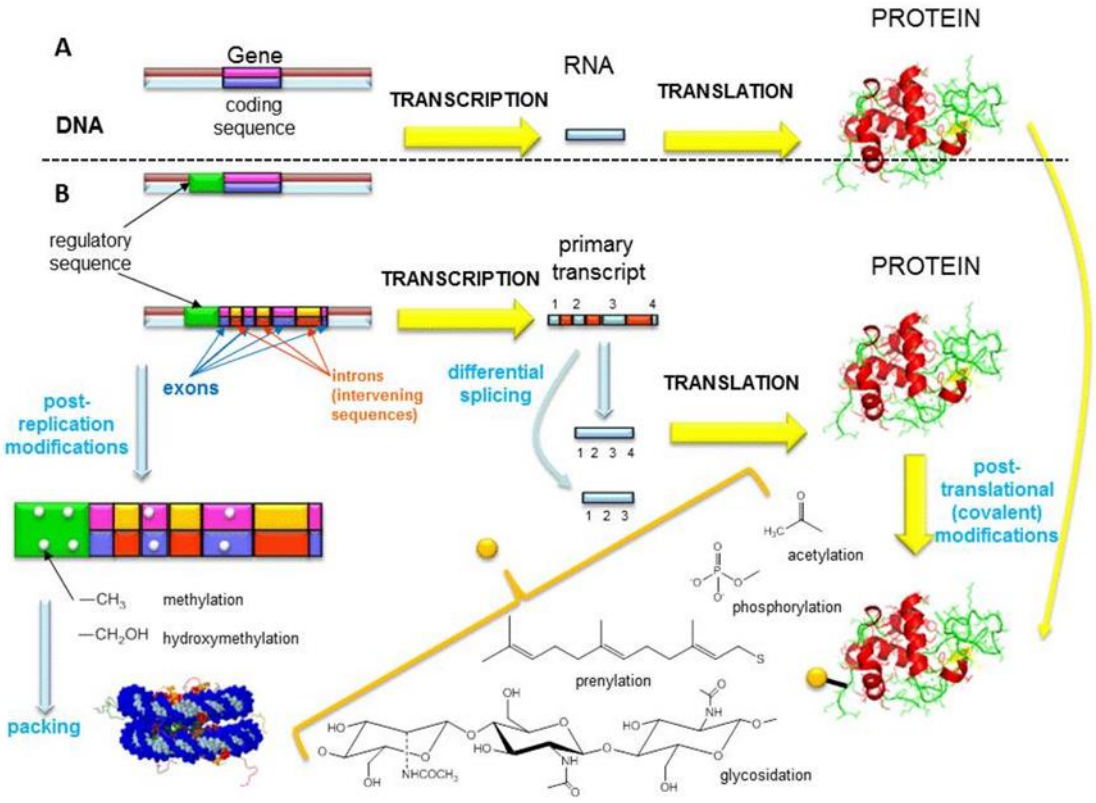

Figure 4: The central dogma of molecular biology
(A) Classical view. The central dogma of molecular biology explains that DNA codes for RNA, which codes for proteins. (B) Expanded view showing post-translational and post-transcriptional modifications.
Illustration modified from http://biochem-vivek.tripod.com/id32.html, March 2016.

2 Aims of the project

2.1 Aim 1: Evaluation of mRNA, protein and enzyme activities of relevant phase I and phase II xenobiotic metabolizing enzymes in normal colorectal tissue of colorectal cancer patients

The central aim of this thesis was an attempt to link molecular data on enzymes, important in the metabolism of foreign compounds to which humans are exposed through their food and environment to the etiology of CRC. Data on activities in colon mucosa of enzyme activating and detoxicating carcinogens are scarce. Expression at the mRNA and protein level show great inter-individual variability and the correlation between mRNA and protein levels are poor. The reasons for these variations may be genetic, or caused by food ingredients inducing or inhibiting expression. Because the etiology of CRC is not well understood due to its complexity, this study on molecular mechanisms of expression and activities of enzymes involved in the metabolism of foreign compounds aimed to shed further light on this situation. The specific aims were:

- To quantify mRNA, protein and enzyme activities of the phase I enzymes CYPs known or hypothesized to be expressed in normal human colonic mucosa, including CYP2C9 and CYP3A4/5, and the phase II enzymes GSTM1, GSTP1, UGT1A8 and UGT1A10 in mucosa of 97 CRC patients
- To compare mRNA, protein and enzyme activity levels among all patients included in this project
- To investigate associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolism-related genes

2.2 Aim 2: Evaluation of differentially expressed xenobiotic metabolizing enzymes in normal and tumor colorectal tissue

XMEs are involved in the activation and detoxification of diverse chemical carcinogens. Inter-individual variations in the metabolism of carcinogens may occur from various activities of metabolizing enzymes in the human colon and rectum. These variations may result in different susceptibilities to CRC development. XMEs may also be targets for chemoprevention. Up-regulation of detoxifying enzymes and/or down-regulation of

activating enzymes may reduce the activity of carcinogenic compounds at the target sites. Thus, primary aims included:

- To compare mRNA expression of eight different XMEs in colorectal carcinomas and adjacent normal mucosa in 71 CRC patients
- To investigate associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolism-related genes

3 Material and Methods

A detailed listing of the materials used for this project is set out in Appendix A.

3.1 Study design and study population

This thesis was based on CRC patients who participate in the ColoCare study, Heidelberg, Germany. ColoCare is an international prospective cohort study of newly-diagnosed stage I-IV CRC patients (ICD-10 C18-C20). The ColoCare Consortium is a multicenter initiative establishing an international cohort of CRC patients for interdisciplinary studies of CRC prognosis and outcomes, with sites at the Fred Hutchinson Cancer Research Center, Seattle (Washington, USA), H. Lee Moffitt Cancer Center and Research Institute, Tampa (Florida, USA), and the National Center for Tumor Diseases (NCT), Heidelberg (Germany). Eligible for the study were CRC patients, at least 18 years of age, with a first diagnosis of colon or rectal cancer (stages I-IV), and availability of normal mucosal tissue and tumor tissue. Subjects meeting the inclusion criteria are recruited to the ColoCare study prior to tumor surgery. Baseline examination includes anthropometric measurements, biospecimen collection (fresh frozen tissue), and self-administered questionnaires on symptoms and health-related quality-of-life. Participants are followed-up (1) passively by retrieving medical data from hospital records, and (2) actively at 3, 6, 12, 24 and 36 months post-surgery. The study was approved by the Institutional Review Board and all participants provided written informed consent. ColoCare uses standard operating procedures that are highly standardized across all participating centers to ensure data comparability. Data presented in this thesis were exclusively based on ColoCare-Heidelberg.

3.1.1 Study sub-populations for different parts of this thesis

This thesis is subdivided into two different main aims (see section 2). For each aim, a different sub-population of the ColoCare study was analyzed (see Figure 5).

Study population 1

Aim 1: Evaluation of mRNA, protein and enzyme activities of relevant phase I and phase II xenobiotic metabolizing enzymes in normal colorectal tissue of colorectal cancer patients

This pilot study involved 97 CRC patients selected from the ColoCare study and recruited between June 2013 and July 2015 at the Division of Preventive Oncology in Heidelberg, Germany. CRC patients were eligible for selection if they were at least 18 years of age, with a first diagnosis of colon or rectal cancer (stages I-IV), and availability of at least 0.5 g normal mucosal tissue adjacent to the tumor tissue.

Study population 2

Aim 2: Evaluation of differentially expressed xenobiotic metabolizing enzymes in normal and tumor colorectal tissue of colorectal cancer patients

This pilot study involved 71 CRC patients selected from the ColoCare study and recruited between November 2010 and May 2014 at the Division of Preventive Oncology in Heidelberg, Germany. Eligible for the study were CRC patients, at least 18 years of age, with a first diagnosis of colon or rectal cancer (stages I-IV), and availability of both tumor and normal mucosal tissue (at least 0.5 g tissue).

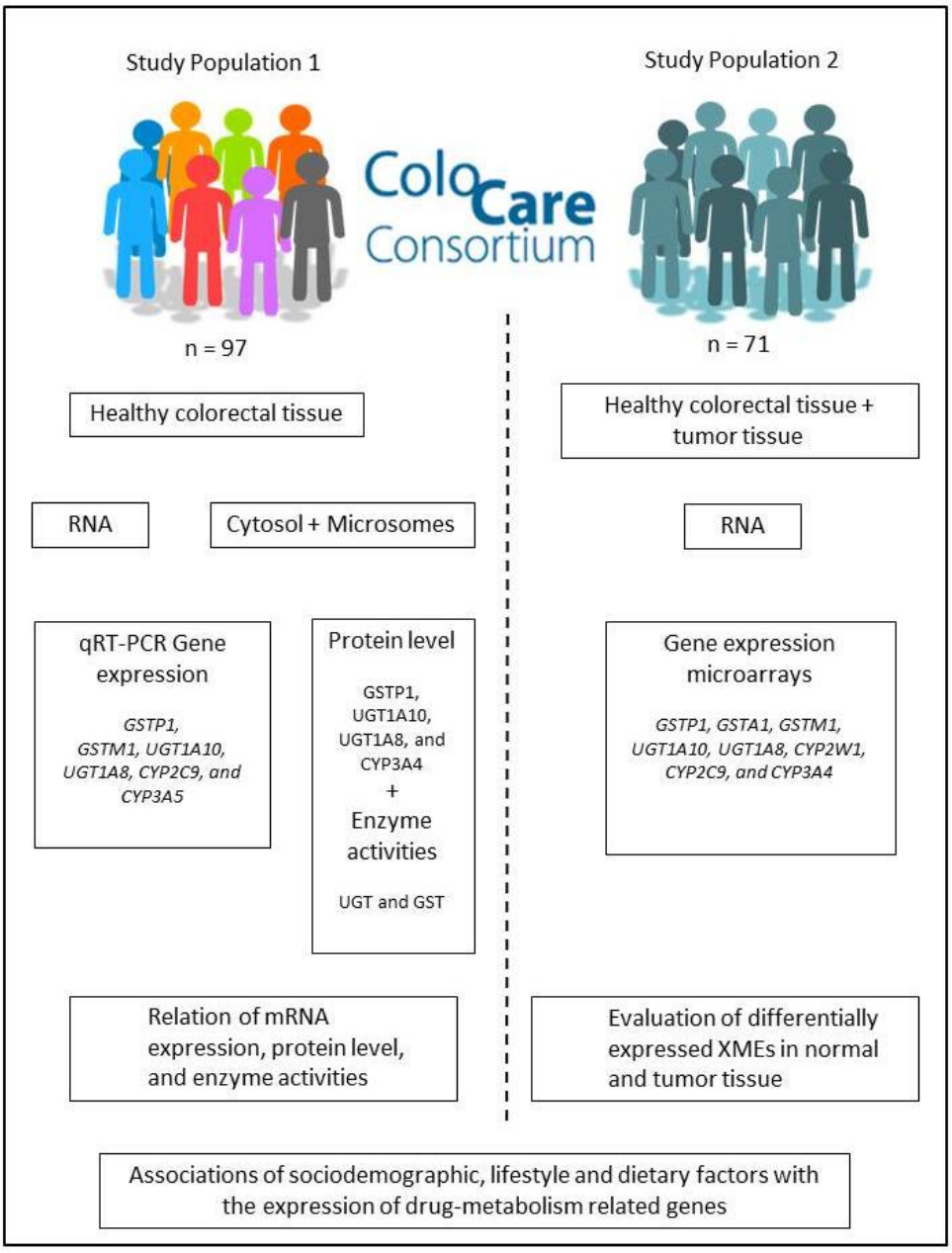

Figure 5: Study sub-populations
Two different sub-populations within the ColoCare study were identified, and are characterized by different availability of biospecimen. Study population 1 consists of n = 97 CRC patients recruited between June 2013 and July 2015. Study population 2 involves n = 71 patients recruited between November 2010 and May 2014. Participants in study population 1 provided colorectal tissue (normal mucosa) adjacent to the tumor, whereas study population 2 provided tumor tissue in addition to the normal mucosa. Following RNA isolation, gene expression profiles were analyzed following two different approaches: Gene expression profiles were determined by qRT-PCR analysis (for study population 1) and by gene expression Illumina HT-12v4 beadchips (for study population 2). Afterwards, associations of sociodemographic, lifestyle, and dietary factors with the expression of xenobiotic metabolism-related genes were determined in both study populations. Additionally, cytosol and microsomes were isolated from normal colorectal tissue (study population 1) and protein levels and enzyme activities were determined and correlated with each other and with mRNA expression.

3.1.2 Data collection

Demographic, clinical and treatment data were abstracted from patients' charts and records from the University Clinic of Heidelberg and the NCT tumor registry. Self-administered ColoCare and interview-based DACHS (Darmkrebs: Chancen der Verhütung durch Screening) questionnaires were used to consider lifestyle and dietary data. Variables derived from the DACHS questionnaire were marked by an asterisk*. The following variables were used within this thesis:

Demographic data

- Patients' age at surgery and gender was collected using a self-administered ColoCare questionnaire.

Clinical data

- Patients' height and weight at the time point of surgery were abstracted from anesthesia documentation sheets. Patients' weight was further self-reported at follow-up time points. Body mass index (BMI) was computed as the ratio of weight in kilograms to height in meters squared.
- Patients were staged according to the American Joint Committee on Cancer (AJCC) based on histopathological findings and stratified into two groups for analysis; 'early stage CRC patients' (stages I and II) and 'late stage CRC patients' (stages III and IV).
- Cancer site was defined according to the tenth revision of the International Classification of Diseases, Injuries and Causes of Death (ICD-10) as colon cancer (C18.0-C18.7) and rectal cancer (C19-C20).
- Patients' treatment was stratified into 'neoadjuvant therapy' and 'no neoadjuvant therapy' groups. The 'therapy' group included patients who had undergone neoadjuvant therapy (chemotherapy or radiotherapy) prior to surgery and the 'no therapy' group included those patients who had not undergone neoadjuvant therapy prior to surgery.

Lifestyle data

- Cigarette smoking was categorized as 'non- smoker' (never and former smoker who stopped smoking more than 2 years ago) and 'smoker' (current smoker).
- Use of NSAIDs (reported as acetylsalicylic acid (aspirin), ibuprofen, naproxen or celecoxib/ etoricoxib) was categorized as either being a user or a non-user. NSAID user took at least one pill per month in the past month before surgery (baseline).
- Mean daily alcohol intake* (in grams) was calculated assuming an ethanol content of 4 g in 100 ml of beer, 8.6 g of ethanol in 100 ml of wine and 33 g ethanol in 100 ml of spirit. Alcohol consumption was categorized as: '0-<4.7 g/day' (nondrinker and light drinker) and '>4.7 g/day' (moderate and heavy drinker).

Dietary data

Consumption of red meat*, processed meat*, and raw- and cooked vegetables* were categorized into two groups based on the empirical distributions in the population. The 'low intake' group included patients who consumed less than one serving of meat or vegetables per week and the 'high intake group' consisted of patients who consumed at least one serving per week during the period of 12 months prior to surgery.

3.1.3 Tissue collection

Normal mucosal tissue with a distance of at least 10 cm from the primary tumor (Study population 1) and adjacent colorectal tumor tissue (Study population 2) from the same patient were collected by a pathologist (Tissue Bank of the NCT Heidelberg) from a surgical specimen in collaboration with the University Clinic Section for Surgical Oncology following standard protocols by the surgeon. Normal tissue samples were placed into vials containing Hank`s Balanced Salt Solution (HBSS), in order to stabilize the samples during the transport process for subsequent isolation of cytosol and microsomes. Normal and tumor tissue samples were aliquoted, then snap-frozen in liquid nitrogen and stored at -80°C until use. One part of the sample was placed in 4% formalin fixative and, sent to the Department of Pathology for histopathologic tissue diagnosis in each case.

3.2 Methods Aim 1

Evaluation of mRNA, protein and enzyme activities of relevant phase I and phase II xenobiotic metabolizing enzymes in normal colorectal tissue of colorectal cancer patients

3.2.1 Isolation of cytosol and microsomes

Microsomal and sub-cellular fractions were isolated from an aliquot of fresh-frozen tissue, pulverized under liquid nitrogen and suspended in 1/15 M Na/K phosphate buffer with 0.5% KCl (pH 7.4) followed by centrifugation at 18 000 g (4°C) for 30 min. The resulting supernatant fraction was spun at 100 000 g (4°C) for 60 min, resulting in sedimentation of the microsomes. The microsomal pellet was suspended in 1/4 vol. of the organ weight of 1/15 M Na/K phosphate buffer. Microsomal and cytosolic protein fractions were stored as aliquots at -80°C until further use. Protein concentrations were estimated in duplicate by the method of Lowry *et al.* [194] using bovine serum albumin (BSA) as the standard.

3.2.2 Gene expression experiments

3.2.2.1 RNA isolation and quantification

RNA isolation was performed by an RNeasy protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To this end, 0.15-0.5 g colon or rectal mucosa frozen in liquid nitrogen was homogenized using a micro-dismembrator S (Sartorius, Göttingen, Germany) in a frozen Teflon container (1 min, 2500 rpm). The powder was transferred into a 15 ml tube and 7.5 ml of 1% β-mercaptoethanol (β-ME) lysis buffer (Qiagen, Hilden, Germany) was added to each sample and mixed until complete homogenization was achieved. After homogenization, the tissue lysate was spun for 10 min at 4300 rpm. The supernatant was then transferred to a new 15 ml tube and 7.5 ml of 70% ethanol added. The lysate was mixed and applied to an RNeasy maxi column placed in a 50 ml tube and spun for 5 min at 4300 rpm. The column was washed with 15 ml of wash buffer-1 (RW1 Buffer, RNeasy kit) and spun for 5 min. at 4300 rpm. A second wash followed using 10 ml of RPE Buffer (RNeasy Kit) and the samples were spun again. The eluates were in both cases discarded. Another 10 ml RPE Buffer was added to the column and spun for 10 min at 4300 rpm to dry the RNeasy silica-gel membrane. The eluate was again discarded.

Finally, the RNA elution was completed with RNAse free water (RNeasy kit). A first elution with 800 µl was followed by another 600 µl. Each elution was spun for 1 min at 4300 rpm. The RNA eluates were aliquoted and stored at - 80°C until further use. Total RNA was quantified using micro-spectrophotometry (Nano-Drop Technologies, Inc.).

3.2.2.2 Reverse transcription

Single-stranded cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. In short: 10 µl of 2X Reverse transcription (RT) Master Mix (see Table 4) and 10 µl of RNA sample (1 µg/10 µl) were added into a 96-well reaction plate, mixed and loaded into the thermal cycler. Thermal cycler conditions are listed in Table 5.

Table 4: Components of the 2X RT Master Mix used for the reverse transcription

Component	Volume/Reaction
10X RT Buffer	2.0 µl
25X dNTP Mix (100 mM)	0.8 µl
10X RT Random Primers	2.0 µl
MultiScribe™Reverse Transcriptase	1.0 µl
RNase Inhibitor	1.0 µl
Nuclease-free H_2O	3.2 µl
Total per Reaction	10.0 µl

Table 5: Thermal cycler conditions for the reverse transcription

Temperature	Time
25°C	10 min
37°C	120 min
85°C	5 min
4°C	∞

3.2.2.3 Real-time quantitative PCR (qRT-PCR)

cDNA was amplified using predesigned TaqMan® gene expression assays (Applied Biosystems, Foster, USA) *for GSTP1, GSTM1, CYP2C9, CYP3A5, UGT1A8*, *UGT1A10*, and the endogenous controls *GAPDH* and β-*Actin* (see Table 6).

For the real-time PCR reaction, 2X TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster, USA) were used. The reaction mixtures were composed as described in Table 7.

Table 6: TaqMan® MGB probes used in this study

Gene	Amplicon Length (bp)	Assay ID
GAPDH	93	Hs02758991_g1
ACTB (β-Actin)	63	Hs01060665_g1
GSTP1	54	Hs00168310_m1
GSTM1	80	Hs01683722_gH
UGT1A8	137	Hs01592482_m1
UGT1A10	119	Hs02516990_s1
CYP2C9	104	Hs01682803_mH
CYP3A5	82	Hs00241417_m1

Table 7: Reaction mixture for the real-time PCR reaction

Component	Volume/Reaction
2X TaqMan® Gene Expression Master Mix	10.0 µl
20X TaqMan® Gene Expression Assay	1.0 µl
cDNA template	2.5 µl
Nuclease-free H_2O	6.5 µl
Total per Reaction	20.0 µl

TaqMan® PCR amplification was performed using an Applied Biosystem 7900HT Fast Real-Time PCR system. The cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles: 15 sec at 95°C, 1 min at 60°C. Reactions were performed in duplicate. Changes in *GSTP1*, *GSTM1*, *CYP2C9*, *CYP3A5*, *UGT1A8*, and *UGT1A10* expression levels, normalized to *GAPDH* and β-*Actin* mRNA levels, were calculated by the $2^{-\Delta CT}$ method [195].

3.2.3 Protein expression analyses

3.2.3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

25 µg of either microsomal (for CYP and UGT analyses) or cytosolic (for GST analyses) protein fractions were mixed with 4x Laemmli Sample Buffer (Bio-Rad Laboratories, München), heated for 5 min at 95°C and separated by SDS-PAGE. Known amounts of purified proteins were run in parallel with the experimental samples and, served as standards for the calculation of the protein levels. A prestained protein molecular weight marker (Precision Plus Protein WesternC Standards in conjunction with Precision Protein Streptactin HRP Conjugate; Bio-Rad Laboratories, München) was used for protein molecular weight estimation. An upper 4% polyacrylamide stacking gel concentrates the samples in the gel. Proteins were separated on a 12% polyacrylamide gel (see Table 8). The electrophoresis was run at 110 V in 1x running buffer.

Table 8: Gel casting reagents used for SDS-polyacrylamide gel electrophoresis

	Running Gel 12%	**Stacking Gel 4%**
dH_2O	8.4 ml	7.6 ml
3 M Tris-HCl, pH 8.8	1.88 ml	---
1 M Tris-HCl, pH 6.8	---	1.3 ml
40% Acrylamide-Solution*	4.5 ml	1 ml
10% (w/v) SDS	200 µl	100 µl
10% (w/v) APS	150 µl	75 µl
TEMED	15 µl	10 µl
	15 ml	**10 ml**

* Ratio of acrylamide:bisacrylamide = 19:1.
Abbreviations: SDS: sodium dodecyl sulfate; APS: ammonium persulfate; TEMED: tetramethylethylenediamine.

3.2.3.2 Immunoblot analysis

Following electrophoretic separation, proteins were transferred onto a nitrocellulose membrane (pore size 0.45 µm) (GE Healthcare, München, Germany) using a Mini Trans-Blot electrophoretic transfer cell system (Bio-Rad Laboratories, München, Germany). Gels and nitrocellulose membranes were equilibrated for 20 minutes in ice cold transfer buffer.

The sandwich was prepared with a pre-wetted fiber pad, a sheet of filter paper, the equilibrated gel, the pre-wetted membrane, a sheet of filter paper and a fiber pad. This sandwich was placed in the anode part of the cassette and assembled into the transfer tank filled with 1x transfer buffer. Proteins were transferred at 4°C at 125 V for 2.30 h. To confirm a successful transfer, membranes were stained with Ponceau-S staining solution (AppliChem, Darmstadt, Germany) for 2 min, followed by a brief rinse in distilled water, so that the lanes and bands were clearly visible. Subsequently, the staining solution was removed by washing three times with phosphate buffered saline (PBS) for 5 min, and non-specific binding sites were blocked with 5% (w/v) non-fat dry milk in PBS (blocking buffer) for 45 min at room temperature (RT). The primary antibodies were diluted in blocking buffer to the appropriate concentrations (1:200-1:1000) and incubated overnight at 4°C. After washing three times with wash buffer (PBS + Tween 20), membranes were incubated for 1 h at RT with the appropriate HRP-conjugated secondary antibody and, diluted to a final concentration of 0.12 µg/ml in the same media as the primary antibody. After removing excess antibody by three washes with wash buffer, the enhanced chemiluminescence reagent (ECL) (Thermo Scientific, Rockford, USA) was added in a 1:1 (v/v) ratio, and chemiluminescence was detected by conventional standard radiography (Protec, Oberstenfeld, Germany). After the detection of the proteins, the blots were stripped and re-probed with anti-β-Actin as a loading control.

Table 9: Primary- and secondary antibodies used for Western blot analyses

Antibody	Host	Dilution	Company	Article number
Primary antibodies				
anti-GSTP1	Rabbit polyclonal IgG	1:200	Sigma-Aldrich, Germany	HPA019869
anti-β-Actin	Rabbit monoclonal IgG	1:200	Cell Signaling, Danvers, USA	4970
anti-UGT1A10	Mouse polyclonal IgG	1:500	Abnova, Taipei, Taiwan	H00054575-A01
anti-CYP3A4	Rabbit polyclonal IgG	1:200	Sigma-Aldrich, Germany	SAB1400065
Secondary antibodies				
anti-rabbit IgG (whole molecule)-Peroxidase	Goat	1:1000	Sigma-Aldrich, Germany	A0545
anti-mouse IgG (whole molecule)-Peroxidase	Rabbit	1:1000	Sigma-Aldrich, Germany	A9044

Abbreviations: GST: glutathione *S*-transferase; UGT: UDP-glucuronosyltransferase; CYP: cytochrome P450; IgG: immunoglobulin G.

Table 10: Purified protein standards used for Western blot analyses

Protein	Host	Mw (kDa)	Company	Article number
GSTP1 (Human) Recombinant Protein	Wheat Germ (*in vitro*)	48.84	Abnova, Taipei, Taiwan	H00002950-P01
CYP3A4 (Human) Recombinant Protein	Wheat Germ *(in vitro*)	83.70	Abnova, Taipei, Taiwan	H00001576-P01
UGT1A10 (Human) Recombinant Protein	Wheat Germ (*in vitro*)	37.07	Abnova, Taipei, Taiwan	H00054575-Q01

Abbreviations: GST: glutathione *S*-transferase; UGT: UDP-glucuronosyltransferase; CYP: cytochrome P450.

3.2.3.3 Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

High-binding ELISA plates (Alpha Diagnostics, Texas, USA) were coated with 0.1 µg/well antigen (microsomal protein fraction; all samples were loaded in duplicate) in 50 µl of coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and incubated for 2 h at 37°C. Plates were blocked with 5% casein in PBS overnight, followed by the addition of 100 µl anti-UGT1A8 antibody (2 mg/ml) (Abcam, Cambridge, US) for an additional 2 h at room temperature. Horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (1:1000) in blocking buffer was added (2 h at room temperature) and the reaction was visualized by the addition of 50 µl of TMB (3,3',5,5'-tetramethylbenzidine) ELISA substrate. After incubation for 20 min (previously determined as the optimum time for the determination of the absorption, data not shown), 50 µl of stop solution (Abcam, Cambridge, US) was added and the plates were read on a µQuant™ microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany) at 450 nm. Plates were washed once with washing buffer (PBS, pH 7.4, containing 0.1% (v/v) Tween 20) and five times with PBS, pH 7.4 after each step. Due to the lack of an appropriate reference, it was not possible to quantify the relative amount of UGT1A8 in the samples. Binding specificity of the antibody was verified by Immunoblot analysis using human UGT1A8 Supersomes (Corning, NY, USA) as a control.

3.2.4 Enzyme activities

3.2.4.1 Establishment of a fluorescence-based assay for the determination of Cytochrome P450 activity in human colorectal microsomes

Crespi *et al.* reported a high-throughput microtiter plate fluorescence assay for CYP1A1/2, activity measurements [196]. Assays were based on using the substrate 3-cyano-7-ethoxycoumarin (CEC), which is deethylated to 3-cyano-7-hydroxycoumarin (CHC), a fluorescent metabolite. Although the assay is well-established for *in vitro* models, such as human liver microsomes, where CYP1A1/2 activity is high, it is questionable whether it is also suitable for the measurement of CYP activities in human colon and/or rectal microsomes, especially in low amounts of tissue To test whether the assay is working in general, CYP activities in Sudan I induced rat liver microsomes and control microsomes without Sudan I were assessed in different concentrations. In a 96-well plate the incubation mixture (200 µL) contained 67 mM potassium phosphate buffer (pH 7.4),

3 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, 10 mM $MgCl_2$, 1 mM NADP, 5 µM CEC (dissolved in DMSO) and 50 µg, 20 µg and 10 µg of microsomal fractions from Sudan I induced microsomes and 200 µg, 100 µg and 50 µg of control microsomes. The reaction was initiated by the addition of 20 µl CEC and the formation of 3-cyano-7-hydroxycoumarin was measured every 2 min for 20 min at 37°C in a microplate fluorescence reader (Biotek Synergy™ 2) (excitation wavelength 400/30 nm, emission wavelenght 460/40 nm).

A similar test with human colorectal microsomes and human liver microsmes (as control) was subsequently performed in the same way as described above. Concentrations of the human liver microsomes were: 50 µg, 20µg and 10 µg and the concentration of the colorectal microsomes was 40 µg.

3.2.4.2 GSH assay for the determination of colorectal glutathione levels

The spectrophotometric/microplate reader assay method for glutathione (GSH) is a modification of the method first described by Tietze [197]. The assay is based on the reaction of GSH with DTNB (5,5'-dithio-*bis*-2-nitrobenzoic acid, Ellman's reagent) that produces a yellow 5-thio-2-nitrobenzoic acid (TNB) (see Figure 6). The disulfide product, GS-TNB, is reduced by glutathione reductase (GR) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), to recycle the GSH back into reaction and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is also directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of GSH in the sample.

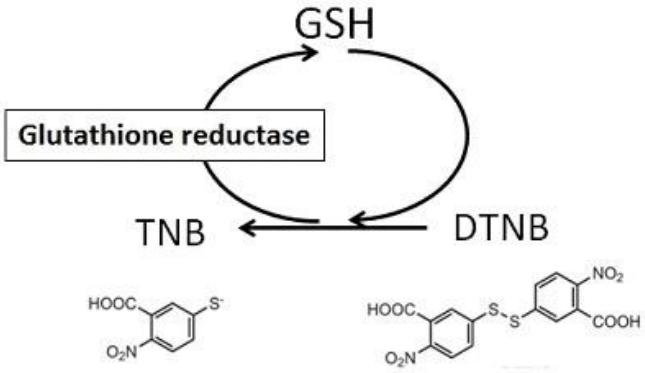

Figure 6: Mechanism of total glutathione quantification
The reaction of GSH with Ellman's reagent (DTNB) gives rise to a product that can be quantified spectrophotometrically at 412 nm. This reaction is used to measure the reduction of glutathione disulfide (GSSG) to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration. GSH: Glutathione; DTNB: (5, 5'-dithio-*bis*-2-nitrobenzoic acid; TNB: 5-thio-2-nitrobenzoic acid

Total colon or rectal GSH was determined using the above mentioned spectrophotometric/microplate reader assay method for glutathione. All cytosolic samples were deproteinated using 5% sulfosalicylic acid (SSA). One cytosol volume was mixed with 17 volumes of 5% SSA, incubated for 10 min and spun for 10 min at 10 000 rpm. After deproteination, cytosol was diluted with 5% SSA to the appropriate concentration (5.25 µg cytosolic protein/10 µl SSA). GSH standards (0.2 µM to 4 µM) were prepared from 84 µM stock solution. The assay was set up under the following conditions:

Twenty µl of deproteinized sample, standard and blank were added to different columns of a 96-well-microplate. Equal volumes of freshly prepared DTNB and GR solutions were mixed together and 150 µl were added to each well. After 5 min for the conversion of glutathione disulfide (GSSG) to GSH, 50 µl of β-NADPH was added. The absorbance at 412 nm was immediately read in a µQuant™ microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany) and measurements were taken every minute for 15 min. All reactions were performed in triplicates. The actual total GSH concentration in the sample was determined by using linear regression to calculate the values obtained from the standard curve.

3.2.4.3 Glutathione *S*-transferase activity measurement

Cytosolic GST activity was measured by the conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced GSH [198]. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample (see Figure 7).

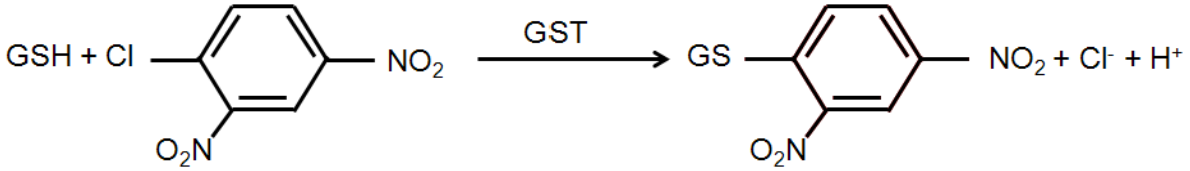

Figure 7: The reaction of CDNB with GSH
GST catalyzes the conjugation of L-glutathione to CDNB through the thiol group of the glutathione. The reaction product, GS-CDNB conjugate, absorbs at 340 nm. GSH: glutathione

The reaction mixture was prepared by mixing 16 ml of 0.1 M KH_2PO_4 buffer, pH 6.5, (prewarmed to 37°C) with 1 ml of 20 mM GSH and 1 ml of 20 mM CDNB. 20 µl (5 µg/20 µl) of the cytosolic protein fraction was added to each well of a 96-well-plate. Each sample was set up in triplicate and 20 µl 0.1 M KH_2PO_4 buffer was used as a blank control. Then, 180 µl of the above reaction mixture was added to each well in the microplate and, the plate was immediately scanned at 340 nm every minute up to 5 min in a spectrophotometer. The rate of conjugated CDNB formation was calculated using the extinction coefficient (**ε**) 9.6 mM x 1000 cm^2 and a light path of 0.549 cm. The GST activity was calculated as follows:

$$c = \frac{\Delta ABS/min}{\varepsilon \times d}$$

- ΔABS/min = absorptions per min.
- ε = Extinction coefficient of CDNB: 9.6mM x 1000 cm^2
- d = 0.591 cm

Data were normalized to protein concentration.

3.2.4.4 UDP-glucuronosyltransferase activity

A stock solution of 0.01 mM for 4-nitrophenyl β-D-glucuronide (4-NPG) was prepared. For calibration curves and quality control samples, serial dilutions of 0.05 – 1 µM were prepared. UGT activities toward 4-nitrophenol (4-NP) were determined by quantification of the 4-NPG production from glucuronidation by colon microsomes. Incubations were performed in 2 ml test tubes. The standard incubation mixture contained 4-NP (0.5 mM), colon microsomal proteins from humans (250 µg), 10 mM $MgCl_2$ and, 2 mM uridine-diphosphate-glucuronic acid (UDPGA), in a final volume of 400 µl of 50 mM Tris–HCl buffer (pH 7.4). After pre-incubation at 37°C for 2 min, the reaction was initiated by the addition of 40 µl UDPGA. The mixture was incubated at 37°C for 60 min and the reaction terminated with 50 µl of ice-cold 15% (w/v) perchloric acid and 50 µl of 1% (w/v) bovine serum albumin with vortexing. Deproteinated samples were placed on ice for 30 min. The contents of the tubes were transferred to 1.5-ml polypropylene test tubes and spun at 12 000 g for 10 min at 4°C. The supernatant was filtered with a polytetrafluorethylene (PTFE) membrane filter of 0.45 µm pore size (Millipore, Bedford, MA) and analyzed by high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) within 8 h. Blank samples containing all components except the UDPGA were added after termination of the reaction.

HPLC-ESI-MS-MS was conducted on an Agilent 1100 HPLC coupled to an Agilent single-quadrupole mass-selective detector (HP 1101; Agilent Technologies, Waldbronn, Germany) fitted with a reverse-phase C18 Gemini column (250 mm, 4.6 mm i.d., 5 µm; Phenomenex, Aschaffenburg, Germany). The mobile phase comprised 2% acetic acid in doubly distilled water (solvent A) and acetonitrile (solvent B), with the following gradient profile: initially 80% A for 20 min; to 0% A over 1 min and continuing at 0% A until completion of the run (30 min). The flow rate of the mobile phase was 1.0 ml/minute, the samples were cooled at 4°C and 20 µl were injected on column. 4-NP and its glucuronidated product (4-NPG) were detected at 305 nm, with a diode-array UV detector (HP 1040M) at 40°C.

Negative-ion mass spectra, in selected ion monitoring (SIM) mode were generated under the following conditions: drying gas (9 l/ min), drying gas temperature 350°C, fragmentor voltage, 50; capillary voltage, 2500 V; nebulizer pressure, 30 psi; *m/z* scan range, 100-1500 D. The pseudomolecular ions $[M-H]^-$ of 4-NP and 4-NPG were monitored at *m/z* 138.2 and 314.2 respectively. Calibration curves for 4-NPG were prepared from stock standard solutions at concentrations of 0.05, 0.1, 0.25, 0,5, and 1 µM, as described above. Instrument control and data handling were performed with the HP Chemstation software.

3.2.5 Statistical methods

Standard descriptive methods were used to assess sociodemographic, lifestyle and dietary factors of the study population. Continuous variables are reported as mean with standard deviation (SD) and categorical variables as percentages.

For qRT-PCR analyses, SDS 2.4, RQ manager 1.2 (Life Technologies) and Microsoft Excel were used with automatic baseline and threshold settings for all targets. Changes in *GSTP1, GSTM1, CYP2C9, CYP3A5, UGT1A8*, and *UGT1A10* expression levels, normalized to GAPDH and β-Actin mRNA levels, were calculated by the $2^{-\Delta CT}$ method. All normalized expression levels were multiplied by a factor 100 000 and log2 transformed to improve legibility and comparability. The 97 samples were processed in 20 batches and ComBat, an Empirical Bayes method [199], was subsequently used to remove batch effects.

Expression of genes linked to drug metabolism in normal mucosa of CRC patients was visually evaluated using box plots.

To evaluate potential associations between lifestyle and dietary factors and the relative gene expression, protein level and enzyme activities in normal mucosa, linear regression models were used. After evaluating univariate associations in unadjusted linear regression models, multivariable models were used to adjust for potential confounding variables, including age (continuous), sex (male, female), smoking status (yes, no), tumor site (colon, rectum), neoadjuvant therapy (yes, no), alcohol consumption (0-4.7g/day, >4.7 g/day), consumption of red meat, -processed meat, -raw vegetables, and –cooked vegetables (≤ 1x/week, > 1x/week).

The Mann-Whitney U-test and the Kruskal-Wallis test were used to compare associations of xenobiotic metabolism-related gene expression, protein level and enzyme activities with clinical parameters in patients with CRC.

To address the multiple comparison issue, the Benjamini-Hochberg method to adjust *p*-values to the false discovery rate (FDR) was applied (25). FDR-adjusted p-values < 0.05 were considered statistically significant.

Correlations of mRNA expression, protein level and enzyme activities of XMEs in normal colorectal mucosa of CRC patients were tested using Spearman correlation coefficients.

Statistical tests were two-sided, and an alpha level of 0.05 was used to determine statistical significance. Analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary, NC) and R, version 3. 2. 3.

3.3 Methods Aim 2

Evaluation of differentially expressed xenobiotic metabolizing enzymes in normal and tumor colorectal tissue of colorectal cancer patients

3.3.1 RNA extraction and gene expression measurements

RNA was isolated from 50 mg of tumor and adjacent normal colorectal tissue of 71 CRC patients using QIAGEN AllPrep DNA/RNA Mini Kit according to the manufacturer's instructions. Total RNA integrity was assessed on an Agilent Bioanalyzer and the RNA Integrity Number (RIN) was calculated; all RNA samples had a RIN > 6.0. Gene expression patterns were measured using Illumina HumanHT-12 Expression BeadChips that target more than 47 000 transcripts including *GSTP1, GSTA1, GSTM1, UGT1A10, UGT1A8, CYP2W1, CYP2C9*, and *CYP3A4*. Raw gene expression data was transformed using variance stabilization transformation and normalized using robust spline normalization (lumi package) [200]. Data was adjusted for possible batch effects using ComBat (sva package) [199, 201]. All preprocessing steps were conducted using the statistical software R 3.1.0 (www.r-project.org).

3.3.2 Statistical methods

Standard descriptive methods were used to assess sociodemographic, lifestyle and dietary factors of the study population. Continuous variables are reported as mean with SD and categorical variables as percentages.

Expression of genes linked to drug metabolism in tumor and normal mucosa of CRC patients was visually evaluated using box plots.

Differences between tumor and normal mucosa were compared by the paired Wilcoxon-Rank-sum test.

For each gene (*GSTP1, GSTA1, GSTM1, UGT1A10, UGT1A8, CYP2W1, CYP2C9, and CYP3A4*), linear regression models were used to evaluate potential associations between sociodemographic, lifestyle and dietary factors and relative gene expression in tumor and normal mucosa tissue, respectively. After evaluating univariate associations in unadjusted linear regression models, multivariable models were used to adjust for potential confounding variables, including age (continuous), sex (male, female), smoking status (yes, no), tumor site (colon, rectum), and neoadjuvant therapy (yes, no).

Further potential confounding variables were selected separately for each gene, by backward elimination using an alpha-value of 0.1. The following covariates were included in the backward elimination: BMI (continuous), alcohol (g/day, 0-4.7, >4.7), stage (I/II, III/IV), regular NSAID use (yes, no), and consumption of red meat, processed meat-, cooked vegetables-, and raw vegetables (≤ 1x/week, > 1x week). Because candidate genes were investigated and specifically hypothesized, in parts previously reported associations [202, 203], nominal *p*-values (p_n) are presented. However, to address also the potential impact of multiple comparisons, the Benjamini-Hochberg method was applied to adjust *p*-values to the false discovery rate (FDR) [204] and adjusted *p*-values were reported as well. FDR-adjusted *p*-values < 0.05 (p_{FDR}) were considered statistically significant.

Statistical tests were two-sided, and an alpha level of 0.05 was used to determine statistical significance. Analyses were performed using SAS, version 9.3 (SAS Institute, Inc., Cary, NC).

4 Results Aim 1

Evaluation of mRNA, protein and enzyme activities of relevant phase I and phase II xenobiotic metabolizing enzymes in normal colorectal tissue of colorectal cancer patients

4.1 Characteristics of the ColoCare study sub-population 1

Of the 103 patients who were selected for the study, six met exclusion criteria or had no carcinoma. Finally, the remaining 97 patients were included, constituting the population of the present study.

The demographic characteristics of the patients entered into the study are shown in Table 11. The average age of the participants was 62.4 ± 12.2 years, of which 64% were men. Half of the participants were diagnosed with rectal cancer and 58% of the patients had later stage disease (stage III or IV). Neoadjuvant radiotherapy was received by 22% of the patients and 24% of the patients took NSAIDs regularly in the past month (baseline). Of all patients, 81% were non-smoker and over 60% had a BMI higher than 25. Evaluation of the dietary characteristics showed that a high percentage (≥ 75%) of people ate red and processed meat, as well as raw and cooked vegetables several times a week.

Table 11: Characteristics of the ColoCare study sub-population 1

Characteristic	n (97)	%
Age (years)[1]	62.4 ± 12.2	
< 60	38	39%
60-70	33	34%
>70	26	27%
Sex		
Women	35	36%
Men	62	64%
BMI (kg/m^2)[1]	26.9 ± 4.5	
<18.5	1	1%
18.5-25	34	35%
25-30	42	43%
>30	20	21%
Tumor site		
Colon	48	50%
Rectum	49	50%
Smoking status[2]		
No smoker	78	81%
Active smoker	18	19%
Alcohol consumption (g/day)		
0-<4.7	38	40%
>4.7	56	60%
NSAID use (past month)[3]		
No	73	76%
Yes	23	24%
Tumor stage		
I/II	38	42%
III/IV	53	58%
Neoadjuvant therapy		
No	74	78%
Yes	21	22%

Table continues on next page.

Table 11 continued

Characteristic	n(97)	%
Red meat consumption		
>1x/week	70	80%
≤1x/week	18	20%
Consumption of processed meat		
>1x/week	70	80%
≤1x/week	18	20%
Consumption of raw vegetables		
>1x/week	66	75%
≤1x/week	22	25%
Consumption of cooked vegetables		
>1x/week	77	88%
≤1x/week	11	12%

[1] Mean ± SD.
[2] Cigarette smoking was categorized as 'non- smoker' (never and former smoker who stopped smoking more than 2 years ago) and 'smoker' (current smoker).
[3] NSAID user took at least one pill per month in the past month before surgery (baseline).
Abbreviations: NSAIDs: nonsteroidal anti-inflammatory drugs; BMI: body mass index
Missing values: smoking status: n=1; alcohol consumption: n=4; NSAID use: n=1; tumor stage: n=6; consumption of red meat, processed meat, cooked vegetables, and raw vegetables n=9.

4.2 Expression of xenobiotic metabolism-related genes, protein levels, enzyme activities, and glutathione levels in normal colorectal tissue of colorectal cancer patients

4.2.1 Expression of xenobiotic metabolism-related genes

For this thesis, gene expression of six candidate genes (*GSTP1, UGT1A8, UGT1A10, CYP3A5,* and *GSTM1*) involved in drug metabolism was successfully detected in normal colorectal tissue of CRC patients. Figure 8 shows the transcript levels in normal colorectal tissues for the six genes analyzed. *GSTP1, UGT1A8, UGT1A10,* and *CYP3A5* expression was detectable in all the samples analyzed, whereas *GSTM1* expression was detectable in only 65 out of 97 samples (67%). The mean expression levels in normal colorectal tissue were highest for *GSTP1* [mean 7.70 (0.60)] and lowest for *GSTM1* [mean: 4.08 (1.80)] (Supplementary Table 4).

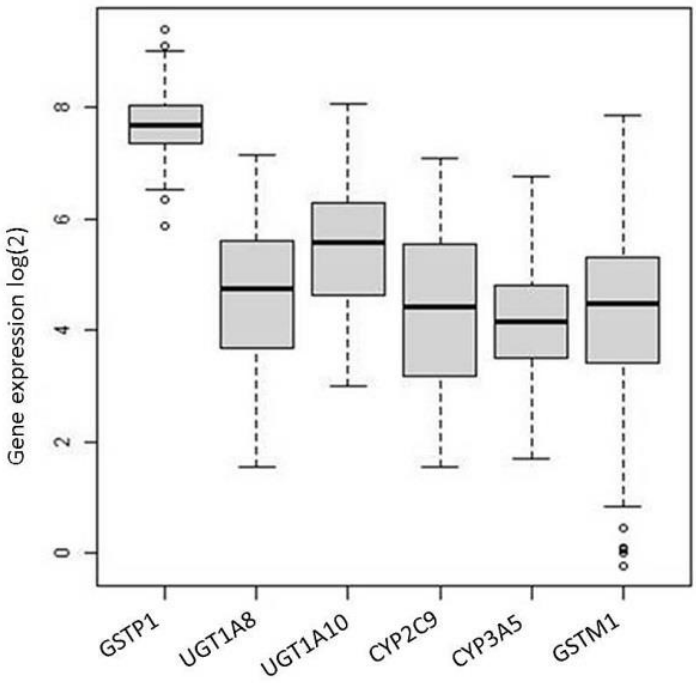

Figure 8: Expression of genes linked to drug metabolism in normal mucosa samples of CRC patients
Box plot presenting the expression of *UGT1A10, UGT1A8, GSTP1, GSTM1, CYP2C9,* and *CYP3A5* in normal mucosa samples of CRC patients. The boxes represent the interquartile range, which contains 50% of the values. The whiskers extend from the box to the highest and lowest values. Outliers are presented as dots. A line across the box indicates the median value

4.2.2 Levels of xenobiotic metabolism-related proteins

Western blot analyses of GSTP1 and CYP3A4 were performed on the microsomal and cytosolic fractions in normal colorectal mucosa of 95 and 92 CRC patients, respectively. A representative Western blot is depicted in Supplementary Figure 1.

Protein levels of UGT1A10 and UGT1A8 were determined by ELISA in the microsomal fractions of 96 normal mucosa samples. Due to the lack of an appropriate purified protein standard, it was not possible to quantify the relative amount of UGT1A8 in the samples and protein values are based upon the use of photometric measurements at an OD of 450 nm. However, binding specificity of the antibody was verified by Immunoblot analysis using human UGT1A8 supersomes as control.

As shown in Table 12, UGT enzymes could be quantified in all of the samples analyzed, whereas GSTP1 and CYP3A4 protein levels were quantified in 95 and 92 samples, respectively.

UGT1A10 exhibited the greatest inter-individual differences in protein expression level, with a 28-fold difference between the highest and lowest levels measured.

Table 12: Protein expression levels of GSTP1, CYP3A4, UGT1A10, and UGT1A8 in normal mucosa of colorectal cancer patients

Enzyme	Fraction	n	Mean activity	Max	Min	Method	Substrate
GST	Cytosol	94	95.62 ± 36.18 nmol/min x mg protein	175.33	27.32	Photometry	CDNB
UGT	Microsomes	96	16.69 ± 18.21 pmol/min x mg protein	103.97	0.61	HPLC	*p*-nitro-phenol

* Due to the lack of an appropriate reference it was not possible to quantify the relative amount of UGT1A8 in the samples. Values listed are based upon the use of photometric measurements at an OD of 450 nm.

4.2.3 Cytochrome P450 enzyme activities

Results of the CYP1A1/2 activity measurements in Sudan I induced rat liver microsomes and control microsomes without Sudan I can be found in Supplementary Figure 2. However, in human colorectal microsomes no CYP1A1/2 activity could be detected.

4.2.4 Glutathione *S*-transferase and UDP-glucuronosyltransferase enzyme activities

Cytosolic GST activity was measured spectrophotometrically in 94 normal mucosa samples of CRC patients using CDNB as substrate and UGT activity was determined in the microsomal fraction of 96 normal mucosa samples by HPLC-ESI-MS using *p*-nitrophenol as substrate. Table 13 shows the mean GST and UGT activities in normal mucosa samples of CRC patients. UGT enzymes showed high inter-individual variability in their activities, with 170-fold difference between the highest and lowest levels measured. A representative chromatogram showing the detection of UGT activity toward 4-NP is depicted in Supplementary Figure 3.

Table 13: GST and UGT enzyme activities in normal colorectal tissue of CRC patients

Protein	Fraction	n (97)	Mean amount (µg/mg protein)	Max	Min	Method
GSTP1	Cytosol	95	12.93 ± 4.17	22.11	3.05	Western blot
CYP3A4	Microsomes	92	16.60 ± 6.02	36.65	3.06	Western blot
UGT1A10	Microsomes	96	28.03 ± 13.19	63.29	2.25	ELISA
UGT1A8*	Microsomes	96	0.23 ± 0.15	0.75	0.04	ELISA

4.2.5 Cytosolic glutathione level

Cytosolic GSH levels were measured in normal mucosa samples of 93 CRC patients and values are reported as mean with SD (see Table 14). GSH level varied greatly among CRC patients with more than a 20-fold difference between the highest and lowest levels measured.

Table 14: Cytosolic GSH level in normal colorectal tissue of CRC patients

	Fraction	n	Mean level	Max	Min	Method
GSH	Cytosol	93	19.02 ± 7.86 nmol/mg	42.64	1.81	Photometry

4.3 Association of xenobiotic metabolism-related gene expression, protein levels and enzyme activities with clinical parameters

Associations of xenobiotic metabolism-related gene expression, protein levels and enzyme activities with clinical parameters, including age at diagnosis, gender, BMI, and tumor site, tumor stage, neoadjuvant therapy, and NSAID use are summarized in Table 15. Results of the univariate analysis revealed a 1.2-fold lower *UGT1A8* expression and a 1.7-fold lower UGT activity in normal tissue of rectal compared to colon cancer patients (p=0.008; p_{FDR}=0.34 and p=0.002; p_{FDR}=0.17, respectively). Furthermore, a lower GSTP1 expression level among recent NSAID users compared to non-users (p=0.04; p_{FDR}=0.58) was detected. However, after FDR-adjustment for multiple testing, none of the associations tested remained significant.

Table 15: Association of xenobiotic metabolism-related gene expression, protein levels and enzyme activities with clinical parameters in patients with colorectal can

	***UGT1A8* gene expression**			***UGT1A10* gene expression**			***GSTP1* gene expression**			***GSTM1* gene expression**		
	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}
Age (years)												
< 60	4.23 ± 1.33			5.37 ± 1.09			7.69 ± 0.54			4.53 ± 0.91		
60-70	4.95 ± 1.13	0.051	0.58	5.80 ± 1.10	0.14	0.60	7.77 ± 0.76	0.57	0.91	3.63 ± 2.14	0.58	0.91
>70	4.81 ± 1.01			5.24 ± 1.00			7.62 ± 0.45			4.02 ± 2.13		
Sex												
Women	4.60 ± 1.19	0.81	0.92	5.63 ± 0.96	0.23	0.60	7.74 ± 0.55	0.55	0.91	4.40 ± 1.70	0.26	0.66
Men	4.64 ± 1.24			5.38 ± 1.15			7.67 ± 0.62			3.85 ± 1.85		
BMI (kg/m^2)												
18.5-25	4.34 ± 1.20			5.26 ± 1.24			7.65 ± 0.59			4.42 ± 1.61		
25-30	4.91 ± 1.20	0.09	0.58	5.63 ± 1.02	0.56	0.91	7.75 ± 0.63	0.79	0.92	4.09 ± 1.68	0.47	0.91
>30	4.38 ± 1.17			5.51 ± 0.98			7.62 ± 0.54			3.44 ± 2.37		
Tumor site												
Colon	4.98 ± 1.10	**0.008**	0.34	5.65 ± 1.02	0.11	0.58	7.59 ± 0.64	0.11	0.58	3.98 ± 1.65	0.27	0.67
Rectum	4.27 ± 1.23			5.29 ± 1.13			7.80 ± 0.54			4.19 ± 1.98		
Tumor stage												
I/II	4.50 ± 1.21	0.32	0.73	5.50 ± 1.22	0.87	0.96	7.67 ± 0.58	0.54	0.91	4.23 ± 1.37	0.82	0.92
III/IV	4.82 ± 1.19			5.44 ± 1.01			7.72 ± 0.62			4.01 ± 2.09		
Neoadjuvant therapy												
No	4.80 ± 1.16	0.051	0.58	5.53 ± 1.10	0.69	0.91	7.70 ± 0.63	0.94	0.99	3.92 ± 2.03	0.49	0.91
Yes	4.17 ± 1.29			5.33 ± 1.07			7.69 ± 0.53			4.57 ± 0.87		
NSAIDs (past month)[1]												
No	4.51 ± 1.29	0.12	0.59	5.45 ± 1.13	0.63	0.91	7.79 ± 0.58	**0.04**	0.58	4.09 ± 1.78	0.63	0.91
Yes	4.97 ± 0.89			5.59 ± 0.99			7.44 ± 0.58			4.03 ± 1.92		

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Table 15 continued

	CYP2C9 gene expression			*CYP3A5* gene expression			UGT1A8 protein level			UGT1A10 protein level		
	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}
Age (years)												
< 60	4.56 ± 1.30			4.38 ± 0.75			0.19 ± 0.11			25.90 ± 12.44		
60-70	4.11 ± 1.48	0.17	0.60	4.23 ± 1.26	0.08	0.58	0.22 ± 0.10	0.17	0.60	26.39 ± 12.12	0.20	0.60
>70	4.75 ± 1.33			3.88 ± 0.95			0.22 ± 0.11			31.57 ± 11.26		
Sex												
Women	4.75 ± 1.35	0.13	0.60	4.39 ± 0.91	0.19	0.60	0.18 ± 0.08	0.05	0.58	24.85 ± 11.64	0.09	0.58
Men	4.29 ± 1.38			4.08 ± 1.05			0.23 ± 0.12	2		29.23 ± 12.24		
BMI (kg/m^2)												
18.5-25	4.54 ± 1.38			4.36 ± 1.07			0.19 ± 0.08			27.91 ± 12.37		
25-30	4.67 ± 1.46	0.15	0.60	4.03 ± 1.04	0.21	0.60	0.23 ± 0.14	0.52	0.91	28.44 ± 12.09	0.59	0.91
>30	3.98 ± 1.09			4.36 ± 0.75			0.18 ± 0.08			24.96 ± 12.30		
Tumor site												
Colon	4.70 ± 1.35	0.10	0.58	4.19 ± 1.03	0.92	0.98	0.22 ± 0.13	0.72	0.91	26.62 ± 13.17	0.29	0.70
Rectum	4.21 ± 1.37			4.20 ± 0.99			0.19 ± 0.09			28.52 ± 11.10		
Tumor stage												
I/II	4.21 ± 1.51	0.18	0.60	4.09 ± 1.12	0.66	0.91	0.21 ± 0.12	0.76	0.91	28.11 ± 11.24	0.66	0.91
III/IV	4.59 ± 1.28			4.22 ± 0.92			0.20 ± 0.10			28.00 ± 12.79		
Neoadjuvant therapy												
No	4.42 ± 1.45	0.37	0.78	4.13 ± 1.05	0.17	0.60	0.20 ± 0.11	0.33	0.73	26.76 ± 12.04	0.33	0.73
Yes	4.71 ± 1.10			4.45 ± 0.85			0.22 ± 0.09			30.27 ± 12.66		
NSAIDs (past month)[1]												
No	4.50 ± 1.46	0.81	0.92	4.26 ± 0.99	0.69	0.91	0.20 ± 0.10	0.50	0.91	27.43 ± 12.21	0.74	0.91
Yes	4.38 ± 1.14			4.06 ± 1.07			0.23 ± 0.12			28.41 ± 12.36		

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Table 15 continued

	GSTP1 protein level			CYP3A4 protein level			GST activity			UGT activity		
	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}
Age (years)												
< 60	13.70 ± 4.24			15.74 ± 5.72			95.05 ± 38.62			15.74 ± 13.98		
60-70	13.04 ± 3.82	0.22	0.60	18.38 ± 4.82	0.07	0.58	96.13 ± 28.50	0.75	0.91	14.47 ± 13.50	0.23	0.60
>70	11.68 ± 4.35			15.29 ± 5.65			89.62 ± 37.17			19.55 ± 14.63		
Sex												
Women	13.32 ± 3.08	0.69	0.91	15.66 ± 4.67	0.39	0.80	86.58 ± 28.20	0.22	0.60	16.89 ± 13.83	0.53	0.91
Men	12.69 ± 4.70			17.01 ± 5.92			97.89 ± 37.36			15.99 ± 14.18		
BMI (kg/m^2)												
18.5-25	13.20 ± 3.34			16.51 ± 5.28			91.01 ± 32.69			14.31 ± 11.40		
25-30	12.81 ± 4.85	0.99	1.00	16.36 ± 5.67	0.99	1.00	99.00 ± 36.79	0.63	0.91	17.41 ± 15.90	0.67	0.91
>30	12.61 ± 4.20			16.90 ± 5.87			89.68 ± 35.03			17.63 ± 14.57		
Tumor site												
Colon	12.92 ± 4.48	0.92	0.98	16.18 ± 6.13	0.52	0.91	101.29 ± 33.43	0.06	0.58	20.69 ± 15.25	**0.002**	0.17
Rectum	12.93 ± 3.89			16.90 ± 4.84			87.22 ± 34.85			12.13 ± 11.31		

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Table 15 continued

	GSTP1 protein level			CYP3A4 protein level			GST activity			UGT activity		
	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}	Mean ± SD	*p*	p_{FDR}
Tumor stage												
I/II	12.96 ± 3.98	0.92	0.98	16.29 ± 5.36	1.00	1.00	86.34 ± 31.02	0.15	0.60	16.07 ± 15.14	0.95	0.99
III/IV	13.13 ± 4.35			16.43 ± 5.80			98.81 ± 35.61			15.52 ± 12.60		
Neoadjuvant therapy												
No	12.87 ± 4.10	0.80	0.92	15.75 ± 5.45	0.06	0.58	93.62 ± 35.10	0.74	0.91	16.74 ± 13.74	0.62	0.91
Yes	12.93 ± 4.66			18.74 ± 5.29			89.36 ± 30.62			15.76 ± 15.52		
NSAIDs (past month)[1]												
No	13.05 ± 4.04	0.74	0.91	16.40 ± 5.90	0.46	0.91	90.59 ± 34.60	0.10	0.58	15.58 ± 14.09	0.37	0.78
Yes	12.79 ± 4.54			17.15 ± 4.25			103.99 ± 34.65			17.20 ± 12.19		

[1] NSAID user took at least one pill per month in the past month before surgery (baseline).

Note: *p*-values correspond to Mann-Whitney U test (two-sample group comparisons) and Kruskal-Wallis test (multiple group comparisons).

Abbreviations: p_{FDR}: false-discovery-rate-adjusted *p*-value; BMI: body mass index; SD: standard deviation; NSAIDs: nonsteroidal anti-inflammatory drugs.

Units: Protein level (µg/mg) except of UGT1A8 (OD 450nm); GST activity (nmol/min x mg protein); UGT activity (pmol/min x mg protein).

4.4 Glutathione levels are not associated with clinical parameters in normal colorectal tissue of colorectal cancer patients

Patient's normal tissue GSH levels are not associated with clinical parameters including age at diagnosis, gender, BMI, tumor site, tumor stage, neoadjuvant therapy, and NSAID use (see Table 16).

Table 16: Association of patient's GSH level with clinical parameters

	GSH level nmol/mg Mean ± SD	*p*
Age (years)		
< 60	19.00 ± 7.77	
60-70	20.27 ± 9.21	0.52
>70	17.55 ± 6.07	
Sex		
Women	19.69 ± 6.98	0.31
Men	18.61 ± 8.37	
BMI (kg/m^2)		
18.5-25	18.41 ± 7.23	
25-30	18.19 ± 6.33	0.62
>30	21.86 ± 11.05	
Tumor site		
Colon	19.25 ± 5.88	0.47
Rectum	18.80 ± 9.46	
Tumor stage		
I/II	18.00 ± 8.00	0.33
III/IV	19.97 ± 7.87	
Neoadjuvant therapy		
No	19.51 ± 7.99	0.47
Yes	13.91 ± 7.62	
NSAID use (past month)[1]		
No	19.27 ± 8.66	0.87
Yes	18.02 ± 4.87	

[1] NSAID user took at least one pill per month in the past month before surgery (baseline).
Note: *p*-values correspond to Mann-Whitney U test (two-sample group comparisons) and Kruskal-Wallis test (multiple group comparisons).
Abbreviations: BMI: body mass index; SD: standard deviation; NSAIDs: nonsteroidal anti-inflammatory drugs.

4.5 Association of xenobiotic metabolism-related gene expression, protein levels and enzyme activities with diet and lifestyle

Regression estimates of the univariate analyses and multiple adjusted regression models separated for each variable are shown in Table 17. Results of the univariate analyses showed statistically significant differences in UGT1A8, UGT1A10 and CYP3A4 protein level and UGT and GST enzymatic activities between high and low categories of lifestyle and dietary variables, but these differences were not shown for gene expression data. Multivariable analyses of the eight significant variables determined by univariate analysis identified two prognostic factors. Regular consumption of cooked vegetables (>1x/week) was associated with higher CYP3A4 protein level (β=5.62; p_n=0.009; p_{FDR}=0.65) and regular consumption of raw vegetables was associated with lower UGT activities (β=-0.56; p_n=0.03; p_{FDR}=0.72) compared to non-regular consumption of raw and cooked vegetables (≤ 1x/week) in the normal mucosa of CRC patients. However, after FDR-adjustment for multiple testing, none of the associations tested remained significant.

Table 17: Association of xenobiotic metabolism-related gene expression, protein level and enzyme activities with diet and lifestyle in patients with colorectal cancer

		***UGT1A8* gene expression**					***UGT1A10* gene expression**				
		Unadjusted Model (n=97)		Adjusted Model (n=80)			Unadjusted Model (n=97)		Adjusted Model (n=82)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.45 [-1.10, 0.19]	0.17	-0.28 [-0.95, 0.39]	0.40	0.98	0.16 [-0.43, 0.74]	0.59	0.18 [-0.48, 0.83]	0.60	0.98
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.02 [-0.56, 0.52]	0.95	0.11 [-0.43, 0.65]	0.70	0.98	-0.15 [-0.62, 0.32]	0.52	-0.17 [-0.70, 0.35]	0.23	0.87
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.22 [-0.43, 0.86]	0.50	0.04 [-0.67, 0.75]	0.92	0.98	0.21 [-0.38, 0.81]	0.47	0.43 [-0.27, 1.13]	0.52	0.98
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	-0.18 [-0.82, 0.47]	0.58	0.07 [-0.63, 0.77]	0.85	0.98	-0.21 [-0.80, 0.37]	0.47	-0.01 [-0.70, 0.67]	0.97	0.98
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	0.17 [-0.44, 0.78]	0.59	0.54 [-1.58, 0.41]	0.17	0.72	-0.38 [-0.93, 0.18]	0.18	-0.35 [-1.09, 0.38]	0.34	0.93
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-0.25 [-1.05, 0.55]	0.54	-0.58 [-1.58, 0.41]	0.25	0.89	-0.28 [-0.99, 0.43]	0.43	-0.10 [-1.01, 0.81]	0.83	0.98

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Table 17 continued

		GSTP1 gene expression					*GSTM1* gene expression				
		Unadjusted Model (n=97)		Adjusted Model (n=82)			Unadjusted Model (n=61)		Adjusted Model (n=55)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.02 [-0.29, 0.34]	0.90	0.02 [-0.35, 0.38]	0.92	0.98	0.25 [0.69, -1.00]	0.69	-0.13 [-1.61, 1.35]	0.86	0.98
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.21 [-0.05, 0.46]	0.11	0.24 [-0.05, 0.53]	0.11	0.72	0.19 [-0.77, 1.15]	0.70	0.32 [-0.79, 1.42]	0.57	0.98
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.07 [-0.26, 0.40]	0.68	0.08 [-0.30, 0.47]	0.67	0.98	0.52 [-0.64, 1.68]	0.38	0.76 [-0.76, 2.28]	0.32	0.93
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	0.06 [-0.26, 0.39]	0.69	-0.004 [-0.38, 0.37]	0.98	0.98	-0.24 [-1.38, 0.90]	0.68	-0.53 [-1.91, 0.85]	0.45	0.98
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	0.20 [-0.11, 0.50]	0.21	0.31 [-0.10, 0.72]	0.14	0.72	-0.29 [-1.37, 0.78]	0.58	-0.11 [-1.56, 1.33]	0.87	0.98
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-0.15 [-0.54, 0.24]	0.45	-0.37 [-0.87, 0.13]	0.15	0.72	-0.65 [-1.99, 0.68]	0.33	-0.86 [-2.51, 0.79]	0.30	0.93

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Table 17 continued

		CYP2C9 gene expression					*CYP3A5* gene expression				
		Unadjusted Model (n=97)		Adjusted Model (n=82)			Unadjusted Model (n=97)		Adjusted Model (n=83)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.16 [-0.90, 0.58]	0.67	-0.11 [-0.90, 0.68]	0.78	0.98	-0.07 [-0.61, 0.47]	0.80	-0.22 [-0.81, 0.36]	0.45	0.98
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.08 [-0.68, 0.52]	0.79	-0.15 [-0.78, 0.49]	0.64	0.98	-0.13 [-0.56, 0.30]	0.55	-0.20 [-0.67, 0.27]	0.39	0.98
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.54 [-0.22, 1.29]	0.17	0.86 [0.01, 1.71]	0.046	0.72	0.20 [-0.34, 0.74]	0.48	0.44 [-0.18, 1.07]	0.16	0.72
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	-0.22 [-0.97, 0.52]	0.56	-0.14 [-0.96, 0.69]	0.74	0.98	-0.02 [-0.55, 0.51]	0.94	0.01 [-0.59, 0.62]	0.96	0.98
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-0.36 [-1.08, 0.35]	0.32	-0.47 [-1.37, 0.42]	0.30	0.93	-0.29 [-0.80, 0.22]	0.26	-0.31 [-0.97, 0.35]	0.35	0.93
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-0.50 [-1.40, 0.41]	0.28	-0.13 [-1.23, 0.97]	0.81	0.98	-0.23 [-0.87, 0.42]	0.49	-0.06 [-0.87, 0.75]	0.89	0.98

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Table 17 continued

		UGT1A8 protein expression					UGT1A10 protein expression				
		Unadjusted Model (n=97)		Adjusted Model (n=78)			Unadjusted Model (n=97)		Adjusted Model (n=81)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.32 [-0.62, -0.03]	**0.03**	-0.23 [-0.55, 0.09]	0.16	0.72	0.81 [-5.58, 7.21]	0.80	-0.37 [-7.08, 6.33]	0.92	0.98
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.003 [-0.25, 0.25]	1.00	0.07 [-0.20, 0.35]	0.58	0.98	-0.12 [-5.42, 5.17]	0.96	-0.24 [-5.84, 5.35]	0.93	0.98
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.41 [0.12, 0.70]	**0.006**	0.31 [-0.03, 0.64]	0.07	0.72	7.45 [1.20, 13.70]	**0.02**	6.06 [-1.12, 13.25]	0.10	0.72
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	0.17 [-0.13, 0.47]	0.25	0.13 [-0.20, 0.47]	0.43	0.98	8.74 [2.43, 15.06]	**0.007**	6.36 [-0.94, 13.66]	0.09	0.72
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	0.19 [-0.09, 0.48]	0.18	0.13 [-0.22, 0.47]	0.47	0.98	-1.37 [-7.38, 4.65]	0.65	-1.30 [-8.50, 5.90]	0.72	0.98
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	0.11 [-0.27, 0.50]	0.55	0.09 [-0.36, 0.54]	0.68	0.98	-1.86 [-9.71, 5.99]	0.64	-1.84 [-11.00, 7.33]	0.69	0.98

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Table 17 continued

		GSTP1 protein level					CYP3A4 protein level				
		Unadjusted Model (n=97)		Adjusted Model (n=82)			Unadjusted Model (n=97)		Adjusted Model (n=78)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.12 [-2.05, 2.29]	0.92	-0.68 [-3.07, 1.72]	0.58	0.98	-0.86 [-3.83, 2.11]	0.57	-0.73 [-3.84, 2.38]	0.64	0.98
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.41 [-2.17, 1.35]	0.64	-0.21 [-2.20, 1.78]	0.83	0.98	-0.55 [-3.01, 1.91]	0.66	-0.86 [-3.49, 1.77]	0.52	0.98
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	1.49 [-0.66, 3.63]	0.17	2.01 [-0.55, 4.58]	0.12	0.72	1.36 [-1.59, 4.31]	0.36	-0.12 [-3.44, 3.20]	0.94	0.98
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	-1.67 [-3.81, 0.47]	0.13	-1.93 [-4.48, 0.62]	0.14	0.72	1.14 [-1.88, 4.16]	0.45	0.23 [-3.10, 3.56]	0.89	0.98
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-1.13 [-3.14, 0.88]	0.27	-1.30 [-3.94, 1.34]	0.33	0.93	2.23 [-0.48, 4.94]	0.11	0.84 [-2.49, 4.18]	0.61	0.98
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-0.83 [-3.58, 1.92]	0.55	-0.62 [-4.15, 2.91]	0.73	0.98	4.47 [1.09, 7.85]	**0.01**	5.62 [1.43, 9.80]	**0.009**	0.65

Continues on next page.

Table 17 continued

		GST activity					UGT activity				
		Unadjusted Model (n=97)		Adjusted Model (n=79)			Unadjusted Model (n=97)		Adjusted Model (n=79)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-4.92 [-23.25, 13.41]	0.60	1.53 [-15.88, 18.95]	0.86	0.98	-0.35 [-0.81, 0.10]	0.13	-0.28 [-0.73, 0.17]	0.22	0.87
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-20.55 [-34.94, -6.16]	**0.006**	-10.12 [-24.78, 4.54]	0.17	0.72	-0.19 [-0.57, 0.19]	0.32	-0.22 [-0.60, 0.16]	0.26	0.89
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	20.91 [3.07, 38.75]	**0.02**	16.84 [-1.77, 35.45]	0.08	0.72	-0.18 [-0.66, 0.30]	0.46	-0.04 [-0.56, 0.47]	0.87	0.98
Processed	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
meat	**> 1x/week**	0.66 [-17.39, 18.70]	0.94	5.10 [-13.79, 24.00]	0.59	0.98	-0.37 [-0.82, 0.08]	0.10	-0.21 [-0.70, 0.28]	0.40	0.98
Raw	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-5.31 [-22.66, 12.04]	0.54	-2.19 [-21.06, 16.67]	0.82	0.98	-0.52 [-0.94, -0.11]	**0.01**	-0.56 [-1.06, -0.06]	**0.03**	0.72
Cooked	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
vegetables	**> 1x/week**	-6.22 [-31.38, 18.95]	0.62	7.46 [-20.76, 35.67]	0.60	0.98	-0.51 [-1.05, 0.04]	0.07	-0.12 [-0.74, 0.50]	0.71	0.98

Note: Uni- and multivariable linear regression models were used for the estimation of the b-coefficients. All multivariable models adjusted for age (continuous), gender (men, women), current smoking status (yes, no), neoadjuvant therapy (yes, no), tumor site (colon, rectum), alcohol consumption (0-4.7 g/day, >4.7 g/day), consumption of red meat, processed meat, raw vegetables, and cooked vegetables (≤ 1x/week, > 1x/week).

Abbreviations: p_n: nominal *p*-value; p_{FDR}: false-discovery-rate-adjusted *p*-value.

4.6 Association of glutathione level with diet and lifestyle

No statistically significant associations of GSH level with diet and lifestyle in normal tissue of CRC patients was determined by uni- and multivariable analyses (see Table 18).

Table 18: Association of GSH level with diet and lifestyle in normal colorectal tissue of CRC patients

		GSH level			
		Unadjusted Model (n=97)		Adjusted Model (n=83)	
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n
Smoking	**No**	Ref.		Ref.	
	Yes	2.26 [-1.85, 6.37]	0.28	1.24 [-3.19, 5.67]	0.58
Alcohol	**0-4.7 g/day**	Ref.		Ref.	
	>4.7 g/day	2.48 [-0.93, 5.89]	0.15	1.93 [-1.83, 5.69]	0.31
Red meat	**≤ 1x/week**	Ref.		Ref.	
	> 1x/week	1.17 [-3.01, 5.35]	0.58	2.24 [-2.37, 6.84]	0.34
Processed meat	**≤ 1x/week**	Ref.		Ref.	
	> 1x/week	2.25 [-1.90, 6.41]	0.28	3.74 [-1.07, 8.55]	0.13
Raw vegetables	**≤ 1x/week**	Ref.		Ref.	
	> 1x/week	-0.33 [-4.23, 3.58]	0.88	-0.05 [-4.83, 4.73]	0.98
Cooked vegetables	**≤ 1x/week**	Ref.		Ref.	
	> 1x/week	-2.30 [-7.58, 3.00]	0.39	-2.61 [-8.84, 3.62]	0.41

Note: Uni- and multivariable linear regression models were used for the estimation of the b-coefficients. All multivariable models adjusted for age (continuous), gender (men, women), current smoking status (yes, no = never and former smoker who stopped smoking more than 2 years ago), neoadjuvant therapy (yes, no), tumor site (colon, rectum), alcohol consumption (0-4.7 g/day, >4.7 g/day), consumption of red meat, processed meat, raw vegetables, and cooked vegetables.

4.7 Relation of mRNA expression, protein levels and enzyme activities of xenobiotic metabolizing enzymes

Relation of mRNA expression with protein levels

Gene expression levels detected by qRT-PCR of two *UGTs*, one *GST*, and one *CYP* were compared with protein expression using Spearman correlation coefficients (see Table 19). For UGT1A8, a weak statistically significant association between protein and mRNA abundance was observed (r=0.20, $p \leq 0.05$) (see Figure 9). Among the CYPs and GST, no significant correlations were detected.

Relation of mRNA expression with enzyme activities

Gene expression levels of *UGT1A8* and *UGT1A10* were compared with UGT activities and *GSTP1* and *GSTM1* expression were compared with GST activities (see Table 19). Specific activities were determined spectrophotometrically (GST) or by HPLC-ESI-MS (UGT) using *p*-nitrophenol and CDNB as UGT and GST substrates, respectively. Spearman correlation analysis revealed a weak, but statistically significant association of *UGT1A10* mRNA levels with UGT activities (r=0.28, $p \leq 0.01$) (see Figure 9). No further correlations were found.

Relation of protein levels with enzyme activities

UGT1A8 and UGT1A10 protein levels measured by ELISA and GSTP1 protein levels determined by Western blot were compared with the respective enzyme activities. As shown in Table 20, there was a statistically significant negative correlation between UGT1A10 protein level and UGT activity (r= -0.27, $p \leq 0.01$). UGT1A8 and GSTP1 protein levels showed no significant correlations with enzyme activities (see Figure 9).

Table 19: Correlation of mRNA with protein level and enzyme activities

	Gene expression log(2)				
	UGT1A10	***UGT1A8***	***GSTP1***	***GSTM1***	***CYP3A5***
Protein level (µg/mg)					
UGT1A10	-0.05				
UGT1A8		0.20*			
GSTP1			-0.08		
CYP3A4					-0.10
Enzyme activity					
UGT (pmol/min x mg protein)	0.28**	0.14			
GST (nmol/min x mg protein)			-0.03	-0.04	

* $p \leq 0.05$,
** $p \leq 0.01$.
Note: Association of relative gene expression level with protein level and enzyme activities (with rho values) in normal colorectal tissue.

Table 20: Correlation of protein level with enzyme activities

	Protein level (µg/mg)		
	GSTP1	**UGT1A10**	**UGT1A8**
Enzyme activity			
GST (nmol/min x mg protein)	0.02		
UGT (pmol/min x mg protein)		-0.28**	-0.13

* $p \leq 0.05$,

** $p \leq 0.01$.

Note: Association of relative gene expression level with protein level and enzyme activities (with rho values) in normal colorectal tissue.

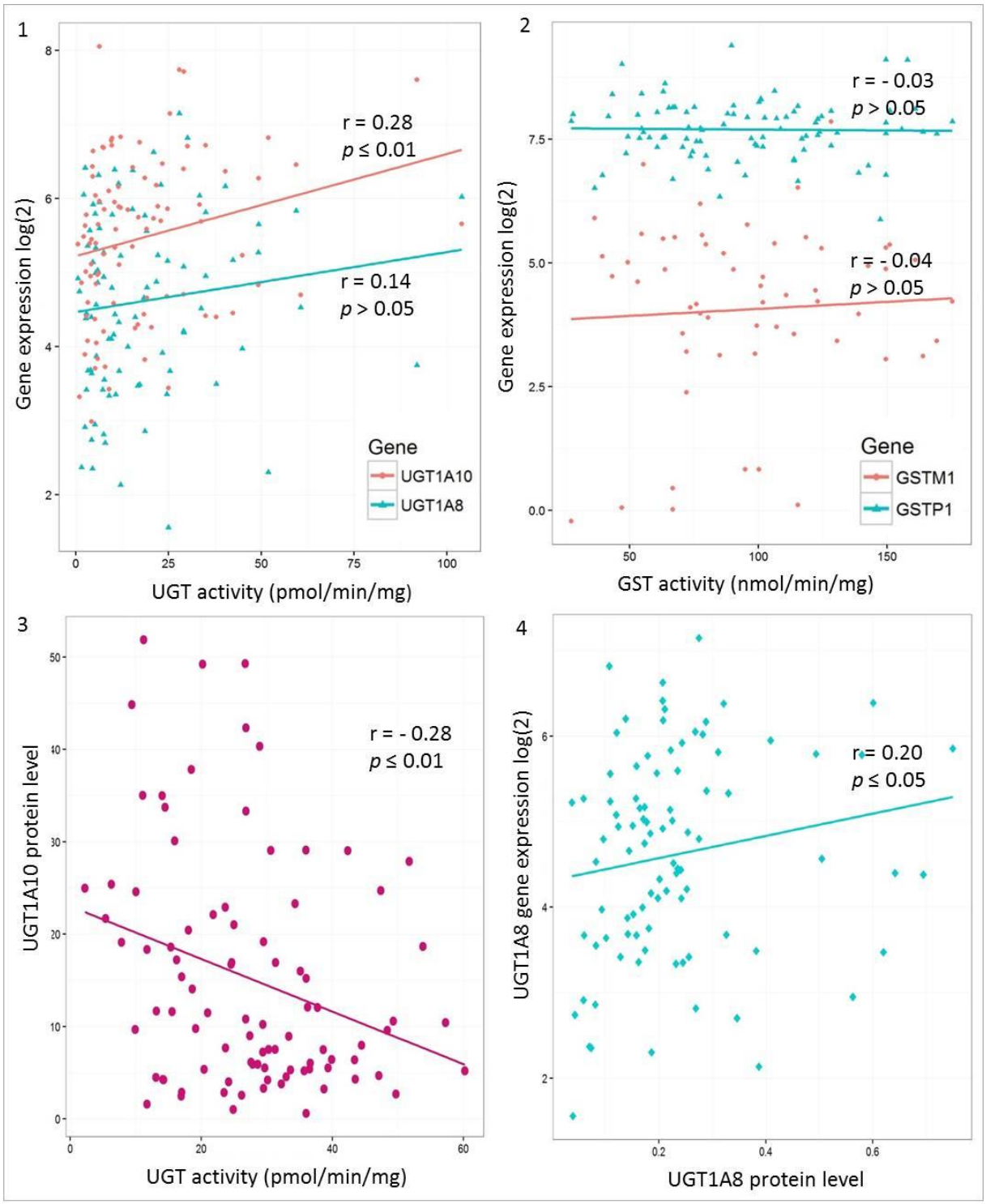

Figure 9: Scatter plots showing Spearman correlations
of 1) UGT activity (pmol/min x mg protein) with *UGT1A10* and *UGT1A8* gene expression level (log2), 2) GST activity (nmol/min x mg protein) with *GSTM1* and *GSTP1* gene expression level (log2), 3) UGT activity (pmol/min x mg protein) with UGT1A10 protein level (µg/mg), and 4) UGT1A8 protein levels with *UGT1A8* gene expression (log2). Circles and triangles show individual samples.

4.8 GST activities in *GSTM1* negative and -positive groups

The most widely used method for genotyping null polymorphisms has been either conventional multiplex PCR followed by gel electrophoresis analysis, or Taqman based qRT-PCR assays to discriminate between the wild-type, hemizygous deletion, and homozygous deletion of the *GSTM1* gene. These methods involve the use of DNA which was not available in this study. For the purpose of this analysis, however, RNA was used to compare the "null" genotype with the "non-null" genotype, but do not distinguish between one and two copy numbers of the genes. Quantitative RT-PCR analysis was used to detect *GSTM1* expression in 94 normal colorectal tissue samples adjacent to the tumor. Among these 94 individuals, 32 (34%) were found to have no detectable *GSTM1* expression and were therefore categorized as '*GSTM1-negative*'. The remaining 62 (66%) individuals were positive for *GSTM1* (detectable *GSTM1* expression). GST activity using CDNB as a substrate was determined in all of the 94 samples. No significant difference was observed in the overall GST activity between the *GSTM1-negative* and –positive individuals (see Figure 10).

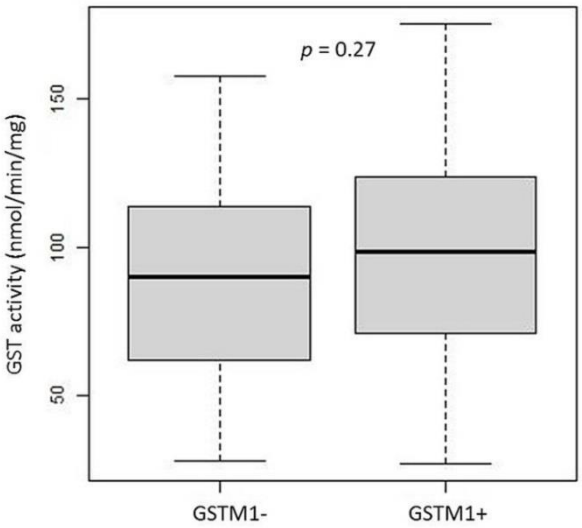

Figure 10: Total GST activity in *GSTM1*-positive and- negative groups
Total enzyme activity (nmol/min x mg protein) using CDNB as substrate was found to be similar in both groups.

5 Discussion Aim 1

Cancer, by its nature, is a biologically complex disease and has been associated with several environmental and dietary risk factors [205]. Phase I and phase II-dependent drug metabolism is mainly carried out by polymorphic enzymes which are responsible for the metabolism and disposition of these compounds. These enzymes can either inactivate carcinogens or, in some cases, generate reactive species that lead to higher reactivity compared to the substrate [206]. A number of studies have shown that CYPs, GSTs and UGTs are commonly expressed in both normal and tumor tissue in a variety of organs [207]. Although the liver is the main detoxification organ in mammalian species, virtually all tissues possess the ability to metabolize foreign compounds, including the GI tract, kidney or bladder [208]. However, most data in this field of research originate from animal or in vitro studies, whereas human studies are limited. The GI-tract is the major path of entry for a wide variety of compounds including food, and orally administered drugs, but also compounds – with neither nutrient nor other functional value – such as carcinogens. The inter-individual variation in the activity of XMEs has been shown to be associated with substantial differences in toxicity or cancer risk, even in response to the same amount of exposure. Thus, individuals with a diminished capacity to detoxify carcinogens due to reduced enzymatic activity undergo more DNA and cell damage and are at greater risk of developing toxicity or cancer. For example, the GSTM1 enzyme plays a significant role in the detoxification of PAHs found in tobacco smoke. A null genotype which results in no GSTM1 activity may, in the presence of PAHs, increase the risk of CRC through an inability to deactivate carcinogens. A large number of studies have been conducted on the effect of *GST* genes and CRC that show Caucasian *GSTM1* null allele carrier's exhibit increased CRC risk. Associations between colon cancer, *GSTM1* null genotype and use of tobacco have been identified in a few studies, although most studies did not show such associations [209-211]. In order to shed light on those questions, mRNA, protein and enzyme activities of the phase I enzymes CYPs known or hypothesized to be expressed in normal colonic mucosa, including CYP2C9 and CYP3A4/5, and of the phase II enzymes GSTM1 and GSTP1 and UGT1A8 and UGT1A10 were quantified. Thereafter, associations of sociodemographic, lifestyle and dietary factors with the expression and activity of those XMEs were investigated.

Association of cytochrome P450 gene expression and protein levels with clinical parameters, diet and lifestyle in normal colorectal tissue of colorectal cancer patients

Relative expression levels of *CYP2C9* and *CYP3A5* and protein levels of CYP3A4 were quantified in the microsomal fraction of normal mucosa samples from CRC patients by qRT-PCR and Western blot, respectively. Higher levels of *CYP2C9* compared to *CYP3A5* expression were detected.

Results of the multiple adjusted regression models showed that regular consumption of cooked vegetables (>1x/week) was associated with increased CYP3A4 protein level in the normal mucosa of CRC patients. When evaluating associations of cooked vegetables with *CYP3A4* mRNA expression in study population 2 in the second aim of this thesis (page 74), a regular consumption of cooked vegetables was associated with decreased mRNA expression of *CYP3A4* in the normal mucosa of CRC patients. As mRNA expression does not necessarily reflect expression at the protein level (see introduction page 24), and I did not quantify for *CYP3A4* mRNA expression in this study population (Study population 1), a direct comparison between CYP3A4 mRNA and protein could not be made. However, this could be the subject of future studies.

From a biological point of view, one would expect rather decreased CYP3A4 protein levels among regular consumers of cooked vegetables. This can be explained by the fact that dietary isothiocyanates are formed by the hydrolysis of glucosinolates of ingested cruciferous vegetables (which are mostly eaten cooked). The anticarcinogenic activities of isothiocyanates are conferred to a variety of target organs including the lungs, liver, stomach, mammary gland, esophagus, small intestine, colon and bladder [212, 213].

Isothiocyanates are direct and very potent inhibitors of members of the CYP family, including CYP3A4 and had been shown to down-regulate *CYP3A4* mRNA expression in human intestinal cells and human hepatocytes [214].

This unexpected finding should, however, be carefully interpreted, because the distribution of the patients in the high and low vegetable groups varied considerably. About two thirds of the patients ate cooked vegetables several times a week, while only one third of the patients ate cooked vegetables less than once a week. Because this study is limited in size, chance findings with regard to the effect of diet on *CYP* protein expression cannot be ruled out and, after FDR-adjustment for multiple testing, this association did not remain significant.

Association of glutathione S-transferase gene expression, protein levels, enzyme activities and glutathione level with clinical parameters, diet and lifestyle in normal colorectal tissue of colorectal cancer patients

GSTP1 and *GSTM1* mRNA expression, GSTP1 protein levels, GSH levels and GST activity in the cytosolic fraction of normal mucosa samples from CRC patients were quantified. *GSTM1* expression was detectable in only 67% of the samples and the mean expression levels in normal colorectal tissue were highest for *GSTP1* and lowest for *GSTM1.* Associations of clinical parameters with *GSTP1* expression demonstrated decreased *GSTP1* expression among recent NSAID users compared to non-users. The metabolism leading to the inactivation and elimination of NSAIDs primarily involves oxidation by CYP enzymes (CYP2C9) and glucuronide conjugation by UGT enzymes (UGT1A6) but no conjugation with glutathione. However, after FDR-adjustment for multiple testing; this association did not remain significant. With respect to the small sample size, this could be a chance finding. No significant associations were found between GST expression, protein levels, enzyme activities or GSH level with diet and lifestyle.

Association of UDP-glucuronosyltransferase gene expression, protein levels and enzyme activities with clinical parameters, diet and lifestyle in normal colorectal tissue of colorectal cancer patients

Relative expression levels, protein levels of UGT1A8 and UGT1A10 and UGT enzyme activities were measured in the microsomal fraction of normal mucosa samples from CRC patients. Associations of sociodemographic, lifestyle and dietary factors with the expression and activities of UGTs were investigated. Although the overall statistical analysis did not reveal significant differences after correction for multiple testing, we detected a trend toward significance for 1.2-fold lower *UGT1A8* expression and 1.7-fold lower UGT activity in normal tissue of rectal compared to colon cancer patients. Studies on mRNA expression profiles of UGTs in human colorectal tissue are limited and only one study thus far investigated expression profiles of genes encoding proteins that are involved in the metabolism and the disposition of xenobiotics in intestinal mucosa from five different segments (ileum, ascending colon, transverse colon, descending colon, and rectum).

They also detected lower *UGT1A8* expression in rectal biopsy samples compared to transverse colon biopsy samples [215]. The *UGT1A8* gene is expressed exclusively in extrahepatic tissues of the GI tract and has been shown to participate in the metabolism of benzo (α) pyrene and 2-acetylaminofluorene as well as in the glucuronidation of flavonoids, phenolic compounds, coumarins, anthraquinones, and certain steroids [35, 216, 217]. Therefore, UGT1A8 may play important roles in the first step of inactivation and detoxification of carcinogenic compounds (e.g. benzo (α) pyrene). Due to its high activity towards many naturally occurring compounds (e.g. coumarin), UGT1A8 can also limit the bioavailability of these potential chemopreventive agents to the body. However, additional studies are needed to investigate the regulation of UGT1A8 in the GI tract.

UGT activities in normal tissue of rectal compared to colon cancer patients until now have not been investigated. Although organ-specific patterns of UGT activities had been previously determined in different parts of the GI-tract, including the esophagus, stomach, duodenum, ileum, and colon. The highest level of UGT activity has been shown in duodenum, followed by the ileum whereas glucuronidation activity in the most distal locations was clearly reduced [218-220]. Furthermore, in animal studies it has been reported that the specific activities of GSTs and UGTs in rats gradually decrease down the small intestine, by analysis of mucosal scrapings or isolated cells [221]. In addition, the activity of most drug-metabolizing enzymes has been shown to decrease slightly from the proximal to the distal small intestine, whereas in the mucosa of the large intestine a sharp fall in activity was observed distally [222].

On evaluation of dietary factors likely to influence the expression and activities of UGTs, regular consumption of raw vegetables was associated with lower UGT activities in the normal mucosa of CRC patients, although the significant differences were lost after correction for multiple testing. Consumption of vegetables and fruits is associated with a lower risk of CRC and previous studies have shown that high dietary vegetable intake up-regulates gastrointestinal UGT activities (for review see [223]). Thus, it is possible that this association could be a chance finding and should be further evaluated in future studies.

Relation of mRNA expression, protein level and enzyme activities of xenobiotic metabolizing enzymes in normal colorectal mucosa of colorectal cancer patients

A key assumption in studying mRNA expression is that it accurately predicts protein expression levels and enzymatic activities, thus leading to the phenotype. Because the Central Dogma of molecular biology states that DNA codes for RNA, which codes for proteins, one would expect a direct relationship between mRNA, protein levels and enzyme activities. Despite the well-established DNA microarray technology for mRNA profiling, a number of studies have shown poor [193] correlations between mRNA and the corresponding protein products. Several biological factors are known to influence this correlation (posttranslational modifications, protein and mRNA half-lives…), but also methodological constraints play a role when comparing mRNA to protein levels. XMEs are highly polymorphic and inter-individual variability may influence protein level and enzyme activity. Polymorphic enzymes may lose their enzymatic activity but still be detectable by Western blot or RT-PCR, depending on the antibody or primer used. So far, only a few studies have investigated the correlation of mRNA and protein expression levels in human tissues and, the results have demonstrated moderate to poor correlation levels for multiple CYPs, UGTs, and GSTs with only a handful of exceptions [218, 220-223]. However, CYP and UGT protein levels were better correlated with enzyme activities than with mRNA expression levels [221].

In this study, three different parameters (mRNA, protein and enzyme) were compared by evaluating CYP, GST, and UGT levels. Gene expression was assessed by qRT-PCR, protein expression evaluated by immunoassay detection, and enzymatic activities measured by biochemical assays, in the normal tissue of 97 patients with CRC. No statistically significant relation was found between mRNA expression, protein levels or enzymatic activities for the CYPs and GSTs. For the UGTs, a weak, but statistically significant positive association was observed between UGT1A8 protein and mRNA abundance and UGT1A10 mRNA levels and UGT activity. However, between UGT1A10 protein level and UGT activity, a statistically significant negative correlation was found. One limitation of our study was that we measured total UGT activity which includes UGT1A8 and UGT1A10, but also the activity of other UGT isoforms. Like other XMEs, the majority of UGTs display broad and often overlapping substrate-specificities and isoform-selective substrates have not yet been identified for UGT1A8 and 10 [224].

Relation of GST activities in GSTM1 negative and -positive groups

GSTs are important phase II enzymes involved in the detoxification of a wide range of chemicals, including possible carcinogens. For two of the *GST* genes null polymorphisms (*GSTM1*0* and *GSTT1*0*) are described which are of particular interest and result in complete absence of the respective GSTM enzyme activity [130]. About 50% of the Caucasian population carries a deletion of the *GSTM1* gene and a decrease in GST enzyme activity could result in inefficient detoxification of various carcinogens, which could lead to genetic damage and increased cancer risk.

Numerous studies have attempted to identify associations of *GSTM1* null with various types of cancer, including breast, lung, prostate and CRC but the results are inconsistent [193, 225-230]. The reason could be that genes of four other members of the GST mu subfamily (*GSTM2–GSTM5*) exhibit high levels of sequence homology and substrate specificity with *GSTM1* [160].

An aim was to analyze the influence of *GSTM1* expression on the enzymatic activity of total GST in normal colorectal mucosa of CRC patients and similar activities in individuals, irrespective of the presence or absence of GSTM1 were detected. These results are supported by Bhattacharjee *et al*., who also showed similar GST enzymatic activities in *GSTM1* null and non-null groups in plasma samples of 275 healthy individuals [231]. The authors employed expression profiling and *GSTM2* over-expression following transient knockdown of *GSTM1* in HeLa cells and, confirmed that the absence of GSTM1 activity can be compensated for by overexpression of GSTM2.

Strengths and limitations

A major strength of this study is that it is the first study that comprehensively evaluated three layers of XME abundance (gene, protein and enzyme) in one and the same patient in human colorectal tissue, an important target organ of human carcinogenesis. Such a multifactorial approach enabled the correlation of these three different parameters, minimized patient-specific variance, and illustrates the power of integrated analysis of mRNA, protein and enzyme activities. The data demonstrate that protein abundance and enzyme activities cannot be reliably predicted from gene expression measurements. The mRNA, protein and enzyme activities were modestly to poor correlated, as earlier cell and animal model studies suggested [232].

A further advantage of the study is the detailed assessment of sociodemographic-, lifestyle-factors and dietary data and the standardized recruitment of patients at the time of surgery, enabling the collection of fresh-frozen tissue specimens.

Nonetheless, this study has some potential limitations. The sample size of 97 patients was very small, which causes a lack of power to detect statistically significant results, especially when performing a large number of tests. However, lack of significant findings could also be due to technical reasons. Currently available techniques are not perfectly accurate in mRNA, protein or enzyme activity quantification. Western blots and ELISAs are very sensitive methods but highly dependent on the specificity of the antibody used and this specificity can be confounded by the high degree of sequence homology between members of the same subfamily. Enzyme activity measurements are dependent on the substrate used. Many XMEs show broad and overlapping substrate specificities which makes it difficult to obtain isoform-specific substrates.

6 Results Aim 2

Evaluation of differentially expressed xenobiotic metabolizing enzymes in normal and tumor colorectal tissue

6.1 Characteristics of the ColoCare study sub-population 2

The distribution of selected population characteristics is shown in Table 21. The ColoCare study sub-population 2 involved analyses of 71 (48 men, 23 women) colorectal tumor and corresponding adjacent normal mucosal tissues from patients aged between 27 years and 85 years at the time of surgery (mean age = 64 years). Twenty-six were younger than 60 years and 45 patients were 60 years, or older. 53.5% of the patients were diagnosed with colon cancer and 46.5% with rectal cancer. More than half of the patients (n=40) suffered from advanced CRC (stage III/IV) compared to 31 patients with early disease (stage I/II). Among the study population, 10 patients received neoadjuvant therapy and 17 patients took NSAIDs regularly in the month prior to surgery.

Table 21: Characteristics of the ColoCare study sub-population 2

Characteristic	n (71)	%
Age (years)[1]	63.9 ± 12.7	
< 60	26	37%
60-70	21	30%
>70	24	33%
Sex		
Women	23	32%
Men	48	68%
BMI (kg/m^2)[1]	27.4 ± 4.2	
<18.5	0	0
18.5-25	22	30%
25-30	31	44%
>30	18	26%
Tumor site		
Colon	38	54%
Rectum	33	46%
Current smoking status[2]		
No smoker	54	83%
Active smoker	11	17%
Alcohol consumption (g/day)		
0-<4.7	22	35%
>4.7	41	65%
NSAID use (past month)		
No	48	74%
Yes	17	26%
Tumor stage		
I/II	31	44%
III/IV	40	56%

Table continues on next page.

Table 21 continued

Characteristic	n (71)	%
Neoadjuvant therapy		
No	61	86%
Yes	10	14%
Red meat consumption		
>1x/week	47	75%
≤1x/week	16	25%
Consumption of processed meat		
>1x/week	52	83%
≤1x/week	11	18%
Consumption of raw vegetables		
>1x/week	51	81%
≤1x/week	12	19%
Consumption of cooked vegetables		
>1x/week	56	89%
≤1x/week	7	11%

[1] Mean ± SD, NSAIDs: nonsteroidal anti-inflammatory drugs.
[2] Cigarette smoking was categorized as 'non- smoker' (never and former smoker who stopped smoking more than 2 years ago) and 'smoker' (current smoker).
[3] NSAID user took at least one pill per month in the past month before surgery (baseline).
Missing values: current smoking status: n=6; alcohol consumption: n=8; consumption of red meat, processed meat, raw vegetables, and cooked vegetables, respectively: n=8.

6.2 Expression of xenobiotic metabolism-related genes in normal and tumor colorectal tissues

The mRNA expression of eight xenobiotic metabolism-related enzymes was measured in both normal and tumor tissue. For each of the 71 patients, the relative expression of each gene was compared in normal and tumor tissue. As shown in Figure 11 and Table 22, all of these genes except *CYP2C9* showed statistically significant differential expression between normal and tumor tissue. Amongst the genes analyzed, *GSTM1, GSTA1, UGT1A8, UGT1A10, CYP3A4*, and *CYP2C9* were down-regulated in tumor tissue as compared to normal tissue, while *GSTP1* and *CYP2W1* were up-regulated in tumor tissue. The largest difference in the expression between tumor and normal tissue was detected for *GSTP1* (mean fold change: 0.60; $p < 0.001$) whereas the smallest difference was apparent in *CYP3A4* gene expression (mean fold change: 1.09; $p = 0.03$).

Table 22: Expression of genes linked to drug metabolism in normal mucosa and tumor tissue of CRC patients

Illumina ID	Gene	Reference ID	Tumor tissue	Normal mucosa	FC	*p*-value
1070088	*UGT1A10*	NM_019075.2	7.53 ± 0.27	8.23 ± 0.38	+1.62	< 0.0001
2070092	*UGT1A8*	NM_019076.4	7.35 ± 0.13	7.61 ± 0.21	+1.20	< 0.0001
830047	*GSTP1*	NM_000852.2	12.47 ± 0.37	11.74 ± 0.26	-0.60	< 0.0001
5260176	*GSTA1*	NM_145740.2	7.45 ± 0.21	7.93 ± 0.69	+1.39	< 0.0001
50672	*GSTM1*	NM_000561.2	7.68 ± 0.48	7.83 ± 0.54	+1.11	0.004
630743	*CYP3A4*	NM_017460.2	7.35 ± 0.12	7.48 ± 0.42	+1.09	0.03
3390767	*CYP2C9*	NM_000771.2	7.43 ± 0.12	7.49 ± 0.22	+1.04	0.06
1230152	*CYP2W1*	NM_017781.2	7.52 ± 0.33	7.31 ± 0.09	-0.86	< 0.0001

Abbreviations: FC: Fold change.

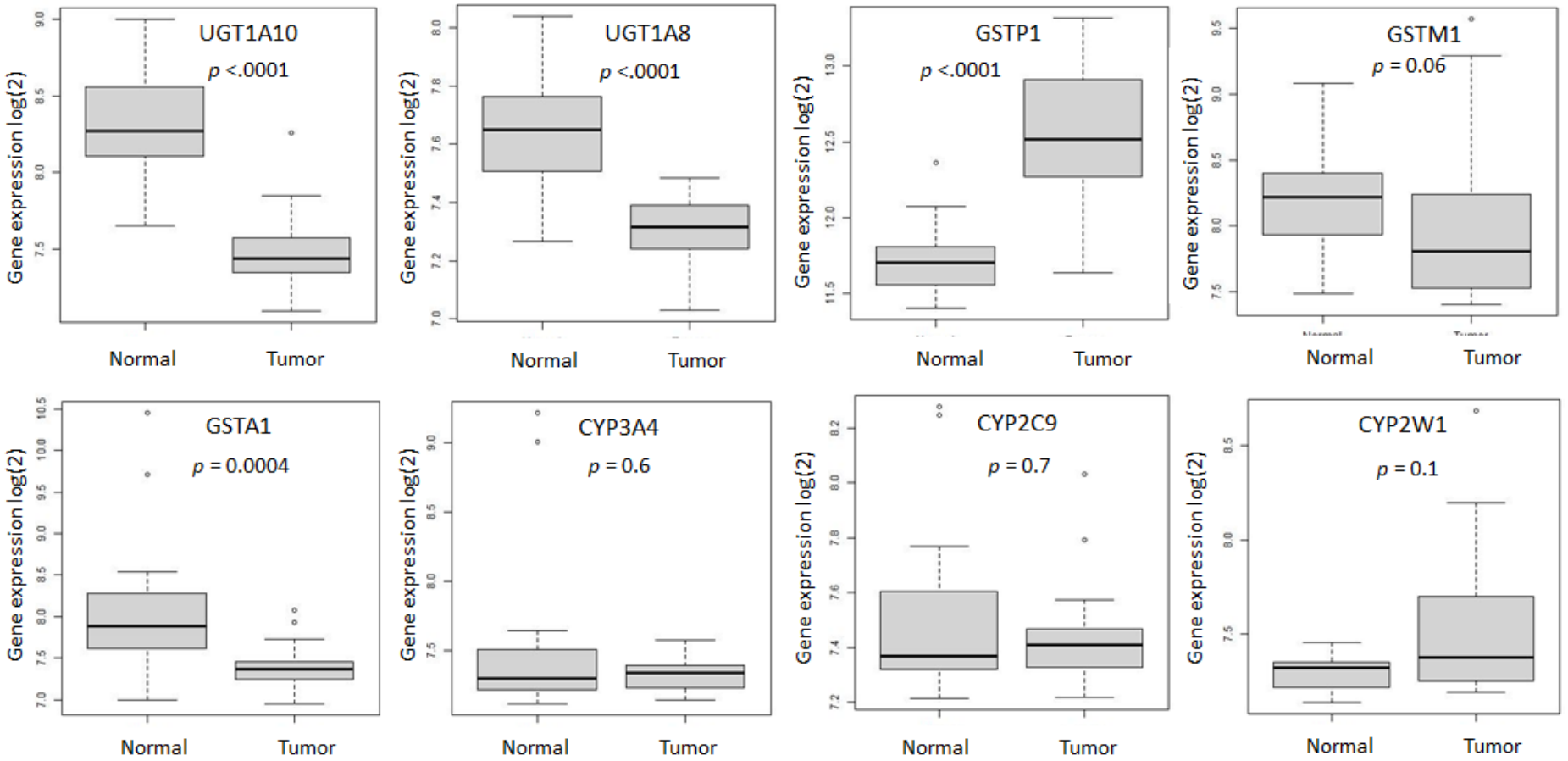

Figure 11: Expression of genes linked to drug metabolism in tumor and normal mucosa of CRC patients

Box plot diagrams presenting the expression of *UGT1A10, UGT1A8, GSTP1, GSTA1, GSTM1, CYP2W1, CYP2C9,* and *CYP3A4* in tumor and normal mucosa of CRC patients. The boxes represent the interquartile range, which contains 50% of the values. The whiskers extend from the box to the highest and lowest values. Outliers are presented as dots. A line across the box indicates the median value for each patient cohort (tumor, normal mucosa). *p*-values in each diagram refer to Wilcoxon-Rank-sum test.

6.3 Associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolism-related genes

The statistically significant estimates of the multiple adjusted regression models separated for each variable are shown in Figure 12. After adjusting *p*-values for multiple testing using the Benjamini-Hochberg method, the most salient finding is a lower *CYP2C9* expression in the normal tissue (β=-0.21; p_n=0.0005; p_{FDR}=0.05) of rectal cancer patients compared to colon cancer patients. Similar associations were observed between sociodemographic, lifestyle and dietary factors and the relative gene expression in tumor and normal mucosa tissue, albeit none remained significant after multiple testing corrections (Supplementary Table 5): Women had a higher *GSTM1* expression in the normal tissue compared to men (β=0.37; p_n=0.02; p_{FDR}=0.12). Among rectal cancer patients, a lower *UGT1A10*, and *UGT1A8*, expression in normal tissue (β=-0.32; p_n=0.003; p_{FDR}=0.10, and β=-0.13; p_n=0.03; p_{FDR}=0.15, respectively) and a higher *CYP2W1* expression in tumor tissue (β=0.24; p_n=0.007; p_{FDR}=0.10) was observed compared to colon cancer patients. It was also observed that rectal cancer patients treated with neoadjuvant therapy had a lower *GSTA1* and *UGT1A8* expression in tumor tissue (β=-0.20; p_n=0.01; p_{FDR}=0.10 and β=-0.11; p_n=0.02; p_{FDR}=0.12) compared to rectal cancer patients that were untreated. Late stage cancer patients (stage III/IV) had a lower *CYP2W1* expression (β=-0.07; p_n=0.005; p_{FDR}=0.10) in the normal tissue compared to early stage cancer patients (stage I/II). A higher *CYP2W1* expression was detected in the tumor tissue of smokers compared to non-smokers (β=0.30; p_n=0.01; p_{FDR}=0.10) and it was shown that smoking was associated with a higher *CYP2C9* expression in normal tissue (β=0.17; p_n=0.02; p_{FDR}=0.12). Moreover, a higher *GSTA1* and *UGT1A8* expression in tumor tissue of patients drinking more than 4.7 g alcohol per day compared to those drinking less than 4.7 g alcohol per day was observed (β=0.15; p_n=0.02; p_{FDR}=0.12 and β=0.09; p_n=0.01; p_{FDR}=0.10).

Assessment of associations of dietary factors with the expression of xenobiotic metabolism-related genes showed that frequent consumption of red meat (>1x/week) was statistically significantly associated with lower *CYP2W1* expression in normal tissue (β=-0.06; p_n=0.04; p_{FDR}=0.18). Frequent consumption of processed meat was statistically significantly associated with higher *CYP3A4* expression in the tumor tissue (β=0.09; p_n=0.03; p_{FDR}=0.15) and higher UGT1A10 expression in normal tissue (β=0.25; p_n=0.04; p_{FDR}=0.18). The stronger associations between dietary factors and XMEs' gene expression were seen with the

consumption of cooked vegetables. It was observed that a regular consumption of cooked vegetables (>1x/week) was associated with lower *CYP3A4*, and *GSTA1* expression in the normal tissue (β=-0.43; p_n=0.02; p_{FDR}=0.12 and β=-0.72; p_n=0.02; p_{FDR}=0.12). In tumor tissue, however, a regular consumption of cooked vegetables was associated with higher *CYP3A4* expression (β=0.14; p_n=0.007; p_{FDR}=0.10). There was also a statistically significant association between high consumption of raw vegetables and higher *GSTP1* expression in tumor tissue (β=0.32; p_n=0.008; p_{FDR}=0.10) and lower *CYP2W1* expression in normal tissue (β=-0.06; p_n=0.04; p_{FDR}= 0.18).

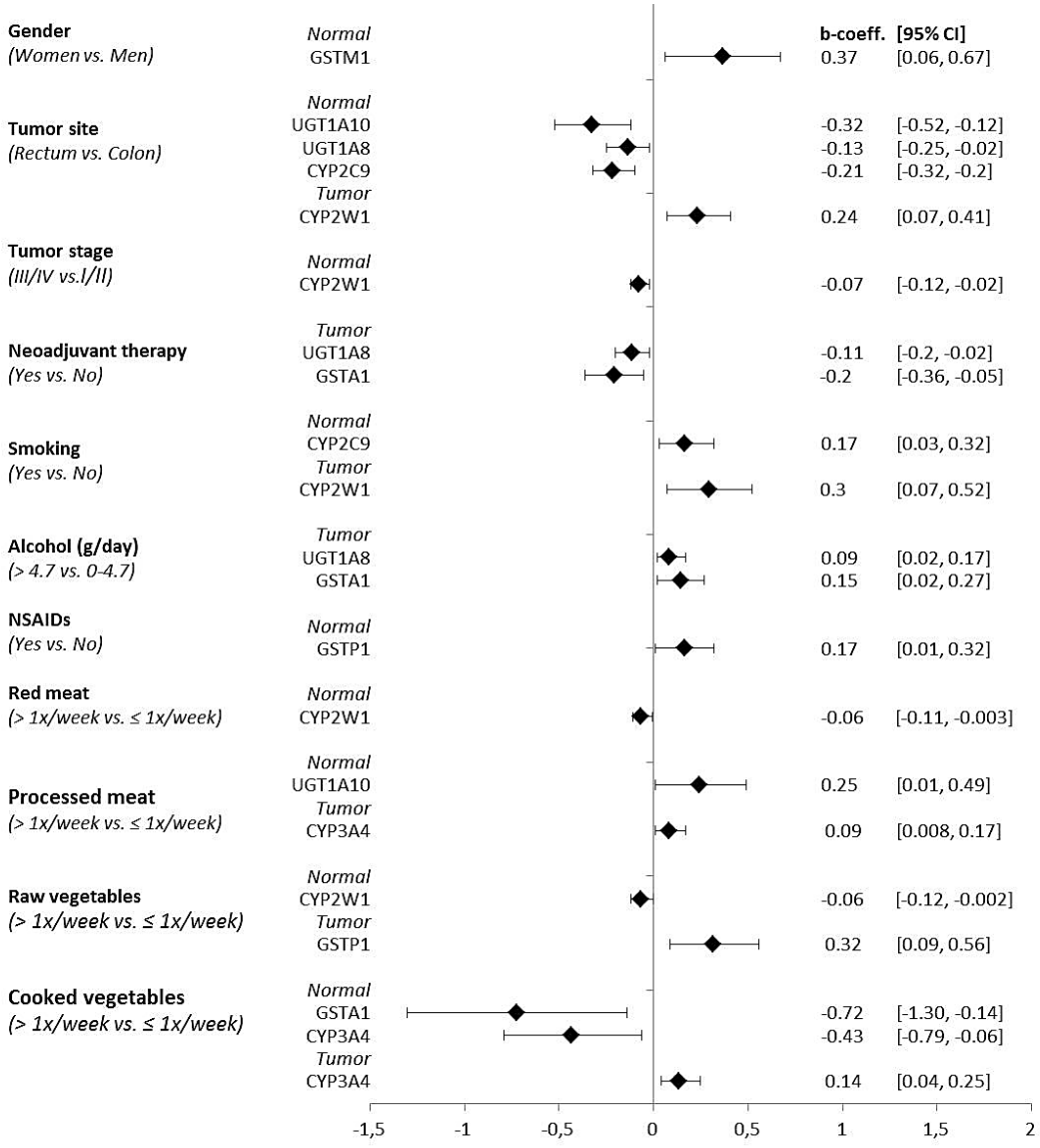

Figure 12: Associations of sociodemographic, lifestyle and dietary factors with the expression of XMEs

Multiple linear regression models were used to calculate the β-coefficients of the estimates. The bars represent the 95% confidence intervals (95% CI). All multivariable models were adjusted for age, gender, smoking status, neoadjuvant therapy, and tumor site. Further potential confounding variables were selected separately for each gene by backward elimination using an alpha-value of 0.1.

Note: All data shown are statistically significant with nominal p_n-values < 0.05. See Supplementary Table 5 for numeric data.

7 Discussion Aim 2

The aim of this study was 1) to compare the mRNA expression of eight different XMEs in colorectal carcinomas and adjacent normal mucosa in 71 CRC patients and 2) to investigate associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolism-related genes. There were two principal findings. First, significant differences in tumor vs. adjacent normal tissue in the expression of many genes involved in drug metabolism were observed. *GSTP1* and *CYP2W1* displayed higher expression in tumor samples, while *GSTM1, GSTA1, UGT1A8, UGT1A10, CYP3A4*, and *CYP2C9* displayed lower expression than normal mucosa. Secondly, several genes were differentially expressed comparing high and low categories of lifestyle and dietary variables, both in tumor and adjacent normal tissue. However, only one of the findings remained statistically significant after correction for multiple testing and is discussed after the next paragraph.

Given the fact that carcinogens and their metabolites are generally detoxificated by phase II enzymes and, pro-carcinogens are activated to ultimate carcinogens by phase I enzymes, the hypothesis was that the expression of the CYPs would be higher in the tumor tissue compared to the normal adjacent tissue and the expression of the phase II enzymes (GSTs and UGTs) would be lower in tumor tissue. The data is consistent with the hypothesis, that *GSTM1, GSTA1, UGT1A8, UGT1A10* were down-regulated in tumor tissue as compared to normal tissue, while *CYP2W1* was up-regulated in tumor tissue. However, not in accordance with the hypothesis, the results revealed that the expression of the phase I enzyme *CYP3A4* was slightly decreased in the tumor tissue, compared to expression in normal adjacent tissue and that *GSTP1* gene expression was increased in tumor tissue. The latter finding is supported by five previous studies detecting similarly significant increases in *GSTP1* mRNA and/or protein in human colorectal carcinomas [136, 233-236]. Miyanishi *et al*. demonstrated a close association of *KRAS* mutation with high levels of *GSTP1* mRNA [236]. However, the exact mechanism of the interaction between *KRAS* mutation and *GSTP1* overexpression remains still unclear.

The finding that mRNA expression of *CYP2W1* was up-regulated in tumor tissue compared to normal tissue is supported by three previous studies detecting high amounts of *CYP2W1* mRNA and protein in colonic and rectal tumors and very low expression levels in adjacent normal tissue [237-239]. Clinical analyses revealed CYP2W1 as a promising prognostic marker for CRC because *CYP2W1* gene expression was also shown to correlate with the degree of tumor malignancy and increased CYP2W1 protein levels predicts decreased 10-year survival in colon cancer patients [237, 240].

The second aim of this study was to investigate associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolism-related genes. These candidate genes were chosen prior to analysis based on literature reports and their biological roles in drug metabolism [203, 241-243] (Supplementary Table 3).

Although the overall statistical analysis did not reveal significant differences after correction for multiple testing, a trend toward significance for lower *CYP2C9* expression in rectal cancer patients compared to colon cancer patients in normal tissue was detected. This result is consistent with two other studies, detecting lower *CYP2C9* expression in healthy rectal biopsy samples than in colon biopsy samples [215, 244]. CYP2C9 is the most abundant CYP isoform of its sub-family and, the second most expressed CYP in human liver and intestine. It is involved in the metabolism of numerous substrates including the highly carcinogenic HA 2-amino-3,4-dimethylimidazo(4,5-f)quinolone or the PAH dibenzo(a,h)anthracene. The enzyme also plays a key role in the metabolism of NSAIDs and, individuals with several variant alleles demonstrated decreased metabolic clearance compared with individuals with the wild-type enzyme [245]. Due to the limited sample size (and the resultant loss of sufficient power), interactions of *CYP2C9* expression with NSAID use, PAHs or HAs were not detected but this could be the subject of larger studies in the future.

Furthermore I comment briefly on the nominally significant results on the associations of lifestyle and risk factors with the expression of xenobiotic metabolism-related genes in both, normal and tumor colorectal tissue. Several genes were differentially expressed between high and low categories of sociodemographic, lifestyle and dietary variables, both in tumor and adjacent normal tissue. One of the most interesting result was that regular consumption of cooked vegetables (>1x/week) was strongly associated with decreased expression of *CYP3A4* in the normal mucosa of CRC patients.

Consumption of vegetables, especially of the family Cruciferae has been associated with a decreased risk of colorectal adenomas and cancer [246]. Cruciferous vegetables contain high amounts of glucosinolates which are hydrolyzed to the biologically active isothiocyanates [247]. Isothiocyanates are known for their antitumorigenic features and are also known to down-regulate *CYP3A4* mRNA expression in human intestinal cells and human hepatocytes [214]. In a human dietary intervention study where *CYP3A4* expression was investigated in biopsies from normal colorectal mucosa, *CYP3A4* was downregulated in healthy controls as a consequence of high vegetable diet, but not in patients with sporadic adenoma [202]. Another interesting result of this study was that smoking was associated with higher *CYP2C9* expression in normal tissue of smokers compared to non-smokers. Tobacco smoke contains a variety of carcinogenic compounds such as PAHs, HAs and nitrosamines which require metabolic activation by different enzymatic pathways which is often initiated by CYP enzymes and primarily by CYP2C9 [248]. In addition PAHs are also potent inducers of CYP expression [249]. Therefore it can be hypothesized that a higher *CYP* expression may lead to increased carcinogen activation and thus increased disease risk among smokers. *CYP2C9* expression and its association with smoking status in the human colon and rectum have rarely been studied. Among these prior studies, one reported that carriers of *CYP2C9* variants, with lower enzyme activity, have a reduced adenoma risk than smokers with wild-type *CYP2C9* [203]. Furthermore, it has been shown that *CYP2C9* was significantly induced in smoker compared to non-smoker in lung and larynx tissue [250, 251].

This illustrates that some of the observed associations are consistent with the literature and biologically meaningful. However, there are probably some false positives among these associations which need to be confirmed in further studies.

Associations of environmental factors with XMEs are of particular interest to fully understand the complex mechanism underlying CRC etiology. However, one of the biggest issues to overcome is to obtain a greater sample size needed to investigate these interactions with sufficient power to adjust for multiple testing. Therefore, the current study should be regarded as a pilot study. However, it is unique in that alterations in gene expression patterns of CYPs, GSTs and UGTs could be monitored in colorectal tumors and compared, in parallel, to normal adjacent tissues. A further limitation of this study is that no genotype information of the eight genes studied was available.

Besides single nucleotide polymorphisms and copy number variants, there are two null polymorphisms (GSTM1*0 and GSTT1*0) that are of particular interest which result in complete absence of the respective GSTM enzyme activity [130]. About 50% of the Caucasian population carry a deletion for *GSTM1* gene and it has been postulated that variant-carriers have increased susceptibility to carcinogens and are more likely to develop cancer, including CRC [167, 252].

Strengths of the study include a detailed assessment of sociodemographic-, lifestyle-factors and dietary data, and the availability of both tumor and adjacent normal tissue from the same patient.

In conclusion, this study shows that colorectal tumor tissue and histologically normal tissue adjacent to tumors showed significant differences in the expression of eight genes involved in drug metabolism.

8 Conclusions and future perspectives

CRC is the most common type of gastrointestinal cancer and a major cause of morbidity and mortality throughout the world. Today we know that exposure to exogenous chemicals (xenobiotics) combined with a modified ability to detoxify carcinogens such as PAHs, which are ubiquitous environmental, dietary, and tobacco carcinogens, increases the risk of developing cancer. The phase I XMEs like CYPs and epoxide hydroxylase usually activate the pro-carcinogens through oxidation and dehydrogenation, thereby converting them into reactive metabolites. These metabolites react irreversibly with macromolecules such as proteins and nucleic acids, leading to mutations and finally to carcinogenesis. Phase II metabolic enzymes such as GSTs and *N*-acetyltransferases generally result in inactivation or detoxification of these reactive metabolites. These pathways are, however, also known to activate other toxic and carcinogenic chemicals, such as amines, to electrophilic forms. Equilibrium between expression and activity levels of these XMEs of both phase I and II therefore determines the relative level of detoxification of carcinogens.

It is therefore important to evaluate the different layers of XME abundance (mRNA, protein, and enzyme activity) in the target tissue of one and the same patient, to avoid inter-individual variability, and to identify organ-specific patterns. The unique study design and biospecimen availability within the ColoCare cohort (sampling of both normal and tumor tissue) and a comprehensive collection of data on relevant lifestyle factors, diet and clinical parameters, is well suited to this type of analyses. In this thesis, two different aims dealing with similar issues were addressed: Aim 1 included the analysis of expression and activity levels of phase I and phase II XMEs in the normal tissue of CRC patients, their correlations, and associations with lifestyle, dietary and clinical parameters, while Aim 2 addressed mRNA analysis of these XMEs and associations with the same factors, albeit in tumor and normal tissue. In both fields of investigations, interesting associations were observed.

One of the most intriguing findings was that CYP and GST mRNA level did not correlate with their protein expression or enzymatic activity, even though these parameters were determined in the same patient. For the UGTs, a weak, but statistically significant positive association was observed between UGT1A8 protein and mRNA abundance and UGT1A10 mRNA levels and UGT activity.

Between UGT1A10 protein level and UGT activity, a statistically significant negative correlation was found. It could also be demonstrated that total GST activity was found to be similar in GSTM1 negative and positive groups, though GSTM1 isoenzyme activity should be undetectable in the GSTM1-null individuals, illustrating that mRNA level are not necessarily correlated with enzymatic activities.

Another interesting result of this work was that investigation of associations between regular consumption of cooked vegetables and *CYP3A4* expression in the normal mucosa of CRC patients, showed two contrasting results: For patients participating in the ColoCare study sub-population 1 (Aim 1), regular consumption of cooked vegetables (>1x/week) was associated with increased CYP3A4 protein level, while patients participating in the ColoCare study sub-population 2 (Aim 2) showed decreased *CYP3A4* mRNA level after consumption of cooked vegetables. As it was indicated in the previous results of this work (Results Aim 1; pages 73-75), mRNA expression does not necessarily reflect expression at the protein level and inter-individual variabilities in translation may play a major role. Because this study was limited in size, chance findings cannot be ruled out and, after FDR-adjustment for multiple testing, these associations did not remain significant. Future, independent validations in a second prospective CRC cohort of larger sample size are needed.

Furthermore results of this thesis showed considerable differences between colon and rectum in the expression and activity of several XMEs in the normal mucosa of CRC patients.

Generally, expression levels and activities were found to be lower in the colon, primarily regarding UGT1A8 and CYP2C9 mRNA expression and UGT activity.

In summary, it can be concluded that in normal tissue of CRC patients:

- Correlations between XMEs' mRNA, protein and enzyme activities are moderate to poor
- Colon and rectum showed considerable differences regarding expression and activities of several XMEs
- Regular consumption of cooked vegetables was associated with CYP3A4 gene expression and protein levels

Future perspectives

To better understand xenobiotic metabolic pathways in the human colon and rectum there is an emerging recognition of the need for multidisciplinary studies. In this work, I have attempted to analyze changes in the metabolism of xenobiotics at the level of mRNA, protein and enzyme in normal and tumor tissue of CRC patients. Unfortunately, it was not possible to analyze all these parameters in an integrated fashion. For example, ColoCare study sub-population 1 provided only normal tissue adjacent to the tumor to investigate correlations between mRNA, protein and enzyme. Although study- population 2 provided both, normal and tumor tissue, the amount of tissue from study sub-population 2 was too small to analyze protein levels and enzyme activities in addition to gene expression. These correlations are at least equally important in tumor tissue, but also the most difficult to obtain, since larger sample volumes are needed. To overcome this problem, there is a need for new technologies to quantify very small amounts of tissue. These methods, once developed and validated should, in the next step, enable to quantify large amounts of samples (high-throughput). Simultaneous analyses of large sample sizes are particular important in epidemiological studies, to achieve the power required to detect significant associations.

Further subjects of interest in the future are genotype-phenotype correlations. Traditional methods for phenotyping XMEs involve the measurement of enzymatic activities using diagnostic substrates. This has become less popular because it is more labor intensive than genotyping, where only blood is needed. To determine levels of expression and function (the phenotype) it is important to access the target-tissue where the phenotype is actually expressed. Until now, genotype-phenotype correlation studies in humans are scarce. As transcriptomic, proteomic and metabolomic information are better reflectors of phenotypes than genomic sequences alone, combining genomic information with longitudinal monitoring of these omics should enable researchers to obtain real-time information of a person’s physiological status. This information will lead to a better understanding of molecular mechanisms of human disease and individual response to drugs.

9 References

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List of communications

Parts of this thesis are either published or in preparation for publication in peer-reviewed journals.

Articles in peer reviewed journals

Beyerle J, Frei E, Stiborova M, Habermann N, Ulrich CM. Biotransformation of xenobiotics in the human colon and rectum and its association with colorectal cancer. *Drug metabolism reviews* 2015**:** 1-23.

Articles in preparation

Beyerle J, Salou M, Boehm J, Gigic B, Frei E, Schrotz-King P, Habermann N, Scherer D, Skender S, Stiborova M, Becker N, Herpel E, Schneider M, Ulrich A, Schirrmacher P, Chang-Claude J, Brenner H, Hoffmeister M, Owen RW, and Ulrich CM. Evaluation of differentially expressed xenobiotic metabolizing enzymes in normal and tumor colorectal tissue *In preparation* 2016

Conference abstracts

Beyerle J, Habermann N, Frei E, Staffa J, Schrotz-King P, Gigic B, Stiborova M, Hoffmeister M, Brenner H, Ulrich A, Herpel E, Schneider M, Ulrich CM. Expression and activity of glutathione S-transferases in the human colon and rectum – a pilot study. Poster presentation at the 4th edition of the Heidelberg Forum for Young Life Scientists

10 Appendix A: Supplementary Tables

Supplementary Table 1: Laboratory equipment

Equipment	Manufacturer
Tissue collection and isolation	
Mikro-Dismembrator S	Sartorius, Göttingen-Germany
Ultracentrifuge L8-70M	Beckman Coulter GmbH, Krefeld, Germany
Sorvall Super T21 centrifuge	Sorvall
Gene expression experiments	
Nanodrop spectrophotometer ND-1000	PeqLab Biotechnologie, Erlangen, Germany
7900HT Fast Real-Time PCR System	Applied Biosystems, Foster City, USA
Tprofessional Basic Thermocycler	Biometra, Göttingen, Germany
SpeedVac Concentrator	Eppendorf, Hamburg, Germany
Protein expression experiments	
Mini Trans-Blot®Electrophoretic Transfer Cell	Bio-Rad Laboratories, München-Germany
Mini-PROTEAN® Tetra Cell	Bio-Rad Laboratories, München-Germany
COMPACT™ X-ray film processor	PROTEC , Oberstenfeld-Germany
µQuant™ Microplate Spectrophotometer	BioTek, Bad Friedrichshall, Germany
ENDURO™ MiniMix™ Nutating Mixer	Labnet, Edison NJ, USA
Enzyme activities	
Agilent 1100 HPLC combined system consisting of a degaser, G1312A binary pump, G1330A autosampler, G1316A diode array and an MSD SL mass spectrometer	Agilent Technologies, Santa Clara, USA
Column: Gemini 250 mm x 10.0 mm, 5 µm	Phenomenex, Aschaffenburg, Germany
General laboratory equipment	
Thermomixer comfort	Eppendorf, Hamburg, Germany
Centrifuge Galaxy Ministar	VWR, Darmstadt, Germany
Heraeus Megafuge 40R	Thermo Scientific, Karlsruhe, Germany
Vortex Genie 2	Scientific Industries Inc., Bohemia, USA

Supplementary Table 2: Buffer and Reagents

Reagent	Company	Article number
Tissue collection and isolation		
Hank's Balanced Salts Solution (HBSS)	Biowest, Nuaillé - France	L0612-500
Formaldehyde solution 4%,buffered, pH 6.9	Merck, Darmstadt-Germany	1004965000
1/15 M Na/K phosphate buffer, pH 7.4	Merck, Darmstadt-Germany	1065801000
• 1/15 M Na_2HPO_4 x $2H_2O$	Merck, Darmstadt-Germany	1048731000
• 1/15 M KH_2PO_4	Merck, Darmstadt-Germany	1049360250
• 0.5% KCl		
RNA*later*	Sigma-Aldrich, Germany	R0901
Lowry protein assay		
1% BSA (0.01 g/10 ml)	Boehringer, Mannheim-Germany	775827
Folin-Ciocalteus (1:2.5)	Merck, Darmstadt-Germany	109001
2% Na_2CO_3 in (20g/l)	Merck, Darmstadt-Germany	106392
1N NaOH	Merck, Darmstadt-Germany	6498
1% $CuSO_4$ x $5H_2O$ (0.5 g/50 ml)	Merck, Darmstadt-Germany	102780
2% sodium potassium tartrate (1g/50 ml)	Merck, Darmstadt-Germany	108087
Protein expression experiments		
4x Laemmli Sample Buffer	Bio-Rad Laboratories, München-Germany	1610747
Precision Plus Protein WesternC Standard	Bio-Rad Laboratories, München-Germany	1610367
Precision Protein StrepTactin-HRP Conjugate	Bio-Rad Laboratories, München-Germany	1610381
Phosphate buffered saline (PBS) (10X) pH 7,4 (1 l)		
• 137 mM NaCl (80 g/l)	Sigma-Aldrich, Germany	31434
• 2.7 mM KCl (2 g/l)	Merck, Darmstadt-Germany	1049360500
• 8 mM Na_2HPO_4 x $2H_2O$ (14.2 g/l)	Merck, Darmstadt-Germany	1065801000
• 1.5 mM KH_2PO_4 (2 g/l)	Merck, Darmstadt-Germany	1048731000
• ddH_2O		
Running Buffer (5x) (1 l)		
• 25 mM Tris-Base (30.3 g/l)	Sigma-Aldrich, Germany	T1503
• 190 mM Glycine (144 g/l)	AppliChem, Darmstadt, Germany	A1067,1000
• 0.1% SDS (5 g/l)	AppliChem, Darmstadt, Germany	A1112,0100

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Reagent	Company	Article number
Transfer Buffer (1 l)		
• 150 mM Glycine (11.25 g/l)	AppliChem, Darmstadt, Germany	A1067,1000
• 20 mM Tris-Base (2.43 g/l)	Sigma-Aldrich, Germany	T1503
• 20% Methanol (200 ml)	Sigma-Aldrich, Germany	322113
• 0.01% (w/v) SDS (0.1 g/l)	AppliChem, Darmstadt, Germany	A1112,0100
Wash Buffer		
• PBS		
• + 0.05% Tween-20	Alpha Diagnostics, San Antonio, USA	TW-100100
Blocking Buffer		
• PBS		
• + 5% Skim milk powder	Sigma-Aldrich, Germany	70166-500G
Ponceau S-solution (10 ml)		
• dH_2O (9 ml)		
• Ponceau S-solution (1 ml)	AppliChem, Darmstadt, Germany	A2935,0500
Nitrocellulose membrane Amersham™ Protran™ 0.45 µm NC	GE Healthcare, München, Germany	10600007
Acrylamide solution (40%) – Mix 19:1	AppliChem, Darmstadt, Germany	A3658
Cytochrome P450 activity		
67 mM potassium phosphate buffer, pH 7.4		
• 67 mM Na_2HPO_4 x $2H_2O$	Merck, Darmstadt-Germany	1065801000
• 67 mM KH_2PO_4	Merck, Darmstadt-Germany	1048731000
• 0.5% KCl	Merck, Darmstadt-Germany	1049360250
Cofactors:		
• 3 mM Glucose-6-phosphate (4.23 mg/ 5ml)	Sigma-Aldrich, Germany	G7879
• 1 mM NADP (3.94 mg/5ml)		
• 10 mM $MgCl_2$ (10.17 mg/5 ml)	Sigma-Aldrich, Germany	N7505
	Sigma-Aldrich, Germany	M8266
• 0.5 U Glucose-6-phosphate dehydrogenase (35 µl/5ml)	Sigma-Aldrich, Germany	G5760
+ 5 ml potassium phosphate buffer (67 mM)		
10 mM CEC in DMSO, diluted 1:200 in phosphate buffer to 50 µM, concentration in the incubation 5 µM	Sigma-Aldrich, Germany	UC455

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Reagent	Company	Article number
Glutathione *S*-transferase activity		
0.1 M Potassium phosphate buffer, pH 6.5		
• 0.1 M KH_2PO_4 (13.6 g/l)	Merck, Darmstadt-Germany	1065801000
• 0.1 M Na_2HPO_4 x $2H_2O$ (17.8 g/l)	Merck, Darmstadt-Germany	1048731000
20 mM CDNB (81 mg/20 ml)	Sigma-Aldrich, Germany	138630-5G
20 mM GSH (122.92 mg/20 ml)	Sigma-Aldrich, Germany	G4251-1G
GSH assay		
Glutathione (reduced form)	Sigma-Aldrich, Germany	G4251-1G
0.1 M postassium phosphate buffer with 1 mM EDTA, pH 7.0		
• 0.1 M KH_2PO_4 (6.8 g/500 ml)	Merck, Darmstadt-Germany	1048731000
• 0.1 M K_2HPO_4 (8.5 g/500 ml)	Merck, Darmstadt-Germany	1051011000
• 1 mM EDTA	Sigma-Aldrich, Germany	E6758-100G
DTNB	Sigma-Aldrich, Germany	D8130-1G
Glutathione reductase	Sigma-Aldrich, Germany	G3664-100UN
β-NADPH	Sigma-Aldrich, Germany	N7505-25MG
5% 5-Sulfosalicylic acid	Sigma-Aldrich, Germany	S2130-100G
UDP-glucuronosytransferase activity		
4-Nitrophenyl β-D-glucuronide	Sigma-Aldrich, Germany	73677
4-Nitrophenol (0.5 mM)	Sigma-Aldrich, Germany	1048
Triton™ X-100	Sigma-Aldrich, Germany	X100
UDPGA	Sigma-Aldrich, Germany	U6751
87 mM Tris-HCl, pH 7.4 (0.53 g/50 ml)	Sigma-Aldrich, Germany	T1503
15% Perchloric acid	Merck, Darmstadt-Germany	1005141000
1% BSA (0.01 g/10 ml)	Boehringer, Mannheim-Germany	775827

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Reagent	Company	Article number
Gene expression experiments		
High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor	Life Technologies, Darmstadt-Germany	4374966
TaqMan® Gene Expression Assays	Applied Biosystems, Foster City, USA	4331182
2X TaqMan® Gene expression Master Mix	Applied Biosystems, Foster City, USA	4369016

Abbreviations: BSA: bovine serum albumin; UDPGA: Uridine 5'-diphosphoglucuronic acid; EDTA: ethylenediaminetetraacetic acid; β-NADPH: β-Nicotinamide adenine dinucleotide 2‘-phosphate.

Supplementary Table 3: Candidate genes chosen for analysis

Assay ID	Gene	Gene expression log(2)
Hs02516990_s1	*UGT1A10*	5.48 ± 1.09
Hs01592482_m1	*UGT1A8*	4.63 ± 1.21
Hs00168310_m1	*GSTP1*	7.70 ± 0.60
Hs01683722_gH	*GSTM1*	4.08 ± 1.80
Hs00241417_m1	*CYP3A5*	4.20 ± 1.01
Hs01682803_mH	*CYP2C9*	4.46 ± 1.38

Note: All values are multiplied by factor 100 000 and log2 transformed. Mean ± SD.

Supplementary Table 4: Expression of genes linked to drug metabolism in normal colorectal mucosa samples

Gene	Location	Description
CYP2C9	10q24	Homo sapiens Cytochrome P450 family 2 subfamily C member 9,
CYP2W1	7p22.3	Homo sapiens Cytochrome P450 family 2 subfamily W member 1,
CYP3A4	7q21.1	Homo sapiens Cytochrome P450 family 3 subfamily A member 4,
GSTA1	6p12.1	Homo sapiens Glutathione S-transferase alpha 1, mRNA
GSTM1	1p13.3	Homo sapiens Glutathione S-transferase mu 1, mRNA
GSTP1	11q13	Homo sapiens Glutathione S-transferase pi 1, mRNA
UGT1A10	2q37	Homo sapiens UDP glucuronosyltransferase family 1 member A10,
UGT1A8	2q37	Homo sapiens UDP glucuronosyltransferase family 1 member A8,

Supplementary Table 5: Associations of sociodemographic, lifestyle and dietary factors with the expression of xenobiotic metabolizing enzymes

		UGT1A10 Tumor					UGT1A10 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=65)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.002 [-0.003, 0.007]	0.45	0.001 [-0.005, 0.007]	0.72	0.78	0.007 [-0.0005, 0.01]	0.07	0.009 [0.001, 0.017]	0.02	0.12
BMI		0.0008 [-0.01, 0.02]	0.91				-0.002 [-0.02, 0.02]	0.82			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	0.03 [-0.11, 0.16]	0.71	0.07 [-0.08, 0.22]	0.33	0.53	0.12 [-0.07, 0.32]	0.20	0.04 [-0.17, 0.25]	0.70	0.78
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.03 [-0.10, 0.16]	0.65	0.09 [-0.06, 0.23]	0.23	0.44	-0.25 [-0.42, -0.07]	0.006	-0.32 [-0.52, -0.12]	0.003	0.10
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.02 [-0.15, 0.11]	0.77				0.07 [-0.11, 0.26]	0.43	0.12 [-0.06, 0.31]	0.18	0.37
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.02 [-0.19, 0.16]	0.86	-0.06 [-0.25, 0.13]	0.55	0.71	0.12 [-0.13, 0.38]	0.34	0.20 [-0.05, 0.45]	0.11	0.30
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.06 [-0.09, 0.20]	0.43				-0.21 [-0.42. -0.01]	0.04			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.05 [-0.09, 0.20]	0.47				0.14 [-0.08, 0.37]	0.20			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.08 [-0.26, 0.10]	0.40	-0.10 [-0.30, 0.09]	0.30	0.52	-0.11 [-0.37, 0.16]	0.42	0.09 [-0.17, 0.34]	0.51	0.69
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.06 [-0.09, 0.21]	0.44				-0.21 [-0.43, 0.01]	0.07	-0.16 [-0.37, 0.05]	0.13	0.33
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.02 [-0.20, 0.16]	0.83				0.12 [-0.15, 0.37]	0.40	0.25 [0.01, 0.49]	0.04	0.18
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.004 [-0.17, 0.18]	0.96				-0.29 [-0.53, -0.05]	0.02	-0.16 [-0.46, 0.15]	0.30	0.53
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.17 [-0.04, 0.38]	0.11				-0.28 [-0.59, 0.03]	0.07			

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Supplementary Table 5 continued

		UGT1A8 Tumor					UGT1A8 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=63)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.002 [-0.0009, 0.004]	0.21	0.0005 [-0.002, 0.003]	0.72	0.78	0.003 [-0.001, 0.007]	0.18	0.004 [-0.001, 0.008]	0.12	0.31
BMI		-0.0005 [-0.007, 0.007]	0.89				0.001 [-0.01, 0.01]	0.83			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	-0.02 [-0.08, 0.04]	0.49	0.04 [-0.03, 0.12]	0.25	0.46	0.10 [-0.008, 0.20]	0.07	0.09 [-0.03, 0.20]	0.15	0.35
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.01 [-0.05, 0.07]	0.69	0.05 [-0.02, 0.12]	0.17	0.36	-0.11 [-0.21, -0.01]	0.03	-0.13 [-0.25, -0.02]	0.03	0.15
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.02 [-0.08, 0.04]	0.47				0.01 [-0.09, 0.11]	0.84			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.005 [-0.07, 0.08]	0.90	-0.02 [-0.11, 0.07]	0.62	0.75	0.02 [-0.12, 0.16]	0.77	0.04 [-0.11, 0.19]	0.60	0.74
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.06 [-0.003, 0.12]	0.06	0.09 [0.02, 0.17]	0.01	0.10	-0.13 [-0.24, -0.02]	0.02			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.05 [-0.02, 0.11]	0.15	0.07 [-0.0009, 0.14]	0.05	0.21	0.07 [-0.06, 0.19]	0.29			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.06 [-0.15, 0.02]	0.15	-0.11 [-0.20, -0.02]	0.02	0.12	-0.04 [-0.18, 0.11]	0.61	0.05 [-0.11, 0.20]	0.55	0.71
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.006 [-0.07, 0.06]	0.87				-0.11 [-0.23, 0.02]	0.09			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.02 [-0.10, 0.05]	0.57				0.06 [-0.08, 0.20]	0.41	0.12 [-0.03, 0.26]	0.11	0.30
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.03 [-0.04, 0.10]	0.42				-0.10 [-0.23, 0.04]	0.17			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.08 [-0.01, 0.17]	0.09				-0.09 [-0.26, 0.09]	0.33			

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Supplementary Table 5 continued

		GSTP1 Tumor					GSTP1 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=63)			Unadjusted Model (n=71)		Adjusted Model (n=61)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.005 [-0.002, 0.01]	0.15	0.005 [-0.004, 0.01]	0.26	0.46	0.001 [-0.004, 0.006]	0.66	-0.001 [-0.007, 0.005]	0.66	0.77
BMI		0.004 [-0.02, 0.03]	0.71				-0.003 [-0.02, 0.01]	0.69			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	0.08 [-0.11, 0.27]	0.39	-0.009 [-0.21, 0.20]	0.93	0.93	-0.04 [-0.17, 0.10]	0.57	0.03 [-0.12, 0.18]	0.67	0.77
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	-0.006 [-0.18, 0.17]	0.95	-0.05 [-0.25, 0.15]	0.63	0.75	0.08 [-0.04, 0.21]	0.19	0.10 [-0.05, 0.25]	0.17	0.36
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.15 [-0.32, 0.03]	0.10				0.09 [-0.03, 0.22]	0.15			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.09 [-0.16, 0.33]	0.47	0.16 [-0.01, 0.43]	0.22	0.43	-0.06 [-0.24, 0.11]	0.47	-0.11 [-0.31, 0.08]	0.24	0.45
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.002 [-0.20, 0.20]	0.99				0.04 [-0.10, 0.18]	0.59			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.03 [-0.18, 0.24]	0.80				0.11 [-0.03, 0.25]	0.12	0.17 [0.01, 0.32]	0.03	0.15
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.12 [-0.37, 0.14]	0.37	-0.10 [-0.36, 0.17]	0.48	0.69	0.12 [-0.06, 0.30]	0.19	0.05 [-0.14, 0.24]	0.60	0.74
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.06 [-0.28, 0.15]	0.57				-0.04 [-0.19, 0.12]	0.65			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.20 [-0.44, 0.04]	0.10				0.10 [-0.07, 0.28]	0.24			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.29 [0.06, 0.51]	0.01	0.32 [0.09, 0.56]	0.008	0.10	-0.10 [-0.27, 0.07]	0.26			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.18 [-0.11, 0.48]	0.22				-0.07 [-0.29, 0.14]	0.50			

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Supplementary Table 5 continued

		GSTA1 Tumor					GSTA1 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=61)			Unadjusted Model (n=71)		Adjusted Model (n=61)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.002 [-0.002, 0.006]	0.33	0.004 [-0.0006, 0.008]	0.09	0.27	0.005 [-0.008, 0.02]	0.48	0.007 [-0.008, 0.02]	0.34	0.54
BMI		0.002 [-0.01, 0.01]	0.74				-0.02 [-0.06, 0.02]	0.39			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	-0.03 [-0.14, 0.07]	0.53	0.07 [-0.06, 0.19]	0.31	0.52	0.38 [0.04, 0.71]	0.03	0.35 [-0.04, 0.74]	0.08	0.27
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.04 [-0.06, 0.14]	0.47	0.08 [-0.04, 0.19]	0.17	0.36	-0.25 [-0.57, 0.08]	0.13	-0.04 [-0.43, 0.35]	0.82	0.84
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.08 [-0.18, 0.02]	0.12				-0.05 [-0.38, 0.28]	0.76			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.09 [-0.23, 0.05]	0.19	-0.13 [-0.28, 0.01]	0.08	0.27	0.35 [-0.12, 0.81]	0.14	0.18 [-0.31, 0.67]	0.67	0.77
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.11 [0.0003, 0.23]	0.05	0.15 [0.02, 0.27]	0.02	0.12	-0.26 [-0.64, 0.12]	0.18			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.04 [-0.16, 0.08]	0.53				0.01 [-0.34, 0.36]	0.95			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.12 [-0.26, 0.02]	0.10	-0.20 [-0.36, -0.05]	0.01	0.10	-0.47 [-0.93, -0.02]	0.04	-0.31 [-0.81, 0.19]	0.22	0.43
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.11 [-0.01-0.23]	0.08	0.10 [-0.02, 0.22]	0.09	0.27	-0.16 [-0.58, 0.25]	0.43			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.002 [-0.14. 0.15]	0.98				-0.11 [-0.59, 0.37]	0.65			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.03 [-0.10, 0.17]	0.62				-0.15 [-0.61, 0.31]	0.52			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.13 [-0.05, 0.30]	0.15				-0.70 [-1.25, -0.15]	0.01	-0.72 [-1.30, -0.14]	0.02	0.12

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Supplementary Table 5 continued

		GSTM1 Tumor					GSTM1 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=65)			Unadjusted Model (n=71)		Adjusted Model (n=65)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		-0.006 [-0.01, 0.003]	0.21	-0.004 [-0.01, 0.007]	0.47	0.68	-0.005 [-0.02, 0.005]	0.30	-0.002 [-0.01, 0.01]	0.74	0.79
BMI		0.03 [0.003, 0.06]	0.03				0.01 [-0.02, 0.04]	0.39			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	0.15 [-0.10, 0.39]	0.23	0.25 [-0.02, 0.53]	0.07	0.27	0.27 [0.003, 0.54]	0.05	0.37 [0.06, 0.67]	0.02	0.12
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.11 [-0.12, 0.34]	0.34	0.19 [-0.08, 0.46]	0.17	0.36	0.061 [-0.20, 0.32]	0.64	0.15 [-0.15, 0.44]	0.33	0.53
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.03 [-0.26, 0.20]	0.81				-0.15 [-0.41, 0.10]	0.23			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.09 [-0.42, 0.24]	0.58	-0.24 [-0.60, 0.11]	0.18	0.37	0.05 [-0.31, 0.42]	0.77	-0.12 [-0.51, 0.27]	0.54	0.71
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.12 [-0.37, 0.14]	0.36				-0.08 [-0.36, 0.20]	0.56			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.01 [-0.29, 0.27]	0.92				-0.04 [-0.34, 0.27]	0.81			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.05 [-0.28, 0.38]	0.78	-0.03 [-0.40, 0.33]	0.86	0.88	-0.03 [-0.40, 0.34]	0.87	-0.05 [-0.45, 0.35]	0.80	0.83
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.16 [-0.44, 0.13]	0.28				-0.13 [-0.45, 0.18]	0.40			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.10 [-0.43, 0.24]	0.56				-0.12 [-0.48, 0.24]	0.51			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.008 [-0.33, 0.31]	0.96				0.10 [-0.25, 0.45]	0.56			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.05 [-0.35, 0.45]	0.80				0.16 [-0.28, 0.60]	0.47			

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Supplementary Table 5 continued

		CYP3A4 Tumor					CYP3A4 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=63)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.0006 [-0.002, 0.003]	0.61	0.0006 [-0.002, 0.003]	0.69	0.77	0.001 [-0.007, 0.009]	0.74	0.006 [-0.003, 0.016]	0.20	0.40
BMI		0.003 [-0.004, 0.01]	0.38	0.007 [-0.001, 0.01]	0.09	0.27	-0.01 [-0.04, 0.01]	0.26			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	-0.04 [-0.11, 0.02]	0.15	-0.06 [-0.13, 0.005]	0.07	0.27	0.10 [-0.12, 0.31]	0.36	0.08 [-0.16, 0.33]	0.50	0.69
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	-0.01 [-0.07, 0.04]	0.64	-0.06 [-0.13, 0.005]	0.07	0.27	-0.15 [-0.34, 0.05]	0.14	-0.11 [-0.35, 0.13]	0.37	0.57
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	-0.005 [-0.06, 0.05]	0.85				-0.02 [-0.22, 0.18]	0.86			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.02 [-0.11, 0.06]	0.56	0.03 [-0.05, 0.12]	0.44	0.65	0.24 [-0.04, 0.53]	0.09	0.22 [-0.08, 0.53]	0.15	0.35
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.03 [-0.04, 0.09]	0.42				-0.07 [-0.30, 0.17]	0.58			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.02 [-0.04, 0.09]	0.48				-0.02 [-0.22, 0.18]	0.87			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.03 [-0.11, 0.05]	0.27	-0.02 [-0.11, 0.06]	0.56	0.71	-0.11 [-0.40, 0.18]	0.44	0.03 [-0.29, 0.34]	0.87	0.88
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.02 [-0.06, 0.09]	0.67				0.03 [-0.22, 0.29]	0.80			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.07 [-0.007, 0.16]	0.07	0.09 [0.008, 0.17]	0.03	0.15	-0.02 [-0.31, 0.27]	0.89			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.008 [-0.07, 0.09]	0.85				-0.04 [-0.32, 0.25]	0.80			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.10 [-0.003, 0.19]	0.06	0.14 [0.04, 0.25]	0.007	0.10	-0.45 [-0.78, -0.11]	0.009	-0.43 [-0.79, -0.06]	0.02	0.12

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Supplementary Table 5 continued

		CYP2C9 Tumor					CYP2C9 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=65)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		0.001 [-0.001, 0.003]	0.35	0.0009 [-0.002, 0.004]	0.54	0.71	0.003 [-0.0007, 0.007]	0.11	0.006 [0.001, 0.01]	0.01	0.10
BMI		0.0002 [-0.007, 0.007]	0.95				-0.007 [-0.02, 0.005]	0.27			
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	0.02 [-0.04, 0.08]	0.57	0.03 [-0.04, 0.10]	0.42	0.64	0.04 [-0.07, 0.15]	0.47	-0.02 [-0.14, 0.09]	0.68	0.77
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.02 [-0.04, 0.08]	0.47	0.04 [-0.03, 0.11]	0.26	0.46	-0.19 [-0.28, -0.09]	0.0002	-0.21 [-0.32, -0.10]	0.0005	0.05
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	0.005 [-0.05, 0.06]	0.85				-0.02 [-0.13, 0.08]	0.68			
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.0005 [-0.08, 0.08]	0.99	-0.01 [-0.11, 0.08]	0.75	0.80	0.11 [-0.42, 0.25]	0.16	0.17 [0.03, 0.32]	0.02	0.12
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	0.03 [-0.03, 010]	0.32				-0.06 [-0.19, 0.06]	0.29			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.008 [-0.08, 0.06]	0.82				0.11 [0.007, 0.22]	0.04			
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.02 [-0.10, 0.06]	0.63	-0.03 [-0.13, 0.06]	0.49	0.69	-0.07 [-0.22, 0.08]	0.34	0.07 [-0.07, 0.22]	0.31	0.52
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.005 [-0.08, 0.07]	0.88				0.06 [-0.08, 0.19]	0.39			
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.05 [-0.14, 0.03]	0.19				0.01 [-0.14, 0.16]	0.88			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.004 [-0.08, 0.08]	0.93				-0.04 [-0.19, 0.11]	0.58			
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.04 [-0.06, 0.14]	0.39				-0.22 [-0.40, -0.05]	0.01	-0.15 [-0.32, 0.02]	0.08	0.27

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Supplementary Table 5 continued

		CYP2W1 Tumor					CYP2W1 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=61)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Age		-0.002 [-0.008, 0.004]	0.54	-0.0009 [-0.008, 0.006]	0.79	0.83	0.0004 [-0.001, 0.002]	0.66	-0.001 [-0.003, 0.001]	0.35	0.55
BMI		0.01 [-0.008, 0.03]	0.27				-0.005 [-0.01, 0.0003]	0.06	-0.005 [-0.01, 0.001]	0.11	0.30
Gender	**Men**	Ref.		Ref.			Ref.		Ref.		
	Women	0.18 [0.02, 0.34]	0.03	0.07 [-0.13, 0.26]	0.50	0.69	0.008 [-0.04, 0.06]	0.73	-0.01 [-0.06, 0.04]	0.63	0.75
Site	**Colon**	Ref.		Ref.			Ref.		Ref.		
	Rectum	0.15 [-0.002, 0.30]	0.05	0.24 [0.07, 0.41]	0.007	0.10	0.04 [-0.003, 0.09]	0.07	0.04 [-0.01, 0.08]	0.14	0.34
Stage	**I/II**	Ref.		Ref.			Ref.		Ref.		
	III/IV	0.04 [-0.12, 0.19]	0.65				-0.005 [-0.09, -0.0008]	0.05	-0.07 [-0.12, -0.02]	0.005	0.10
Smoking	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.36 [0.16, 0.57]	0.0007	0.30 [0.07, 0.52]	0.01	0.10	0.02 [-0.04, 0.08]	0.60	-0.02 [-0.08, 0.05]	0.61	0.75
Alcohol	**0-4.7 g/day**	Ref.		Ref.			Ref.		Ref.		
	>4.7 g/day	-0.12 [-0.30, 0.05]	0.17	-0.17 [-0.36, 0.02]	0.08	0.27	0.004 [-0.05, 0.05]	0.87			
NSAIDs*	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	0.02 [-0.16, 0.21]	0.80				-0.02 [-0.07, 0.04]	0.49			

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Supplementary Table 5 continued

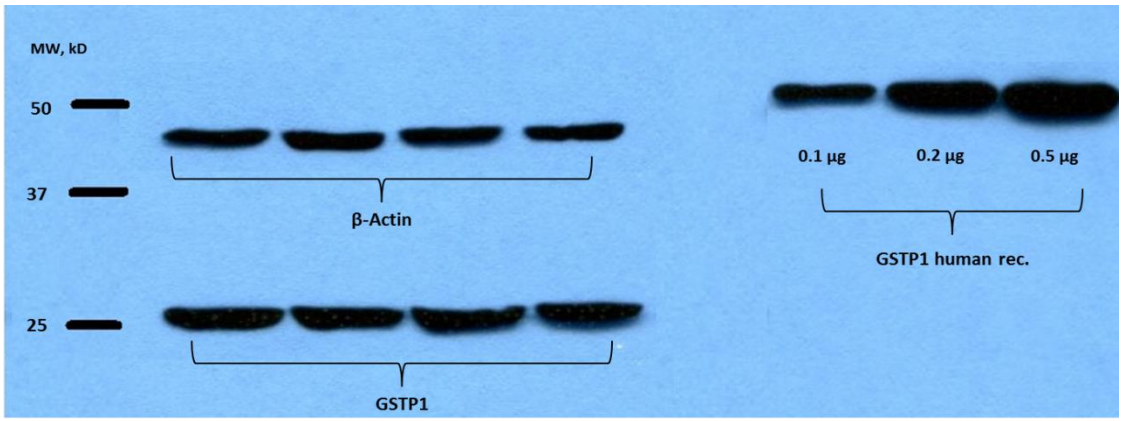
		CYP2W1 Tumor					CYP2W1 Normal				
		Unadjusted Model (n=71)		Adjusted Model (n=61)			Unadjusted Model (n=71)		Adjusted Model (n=63)		
		b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}	b-coeff. (95% CI)	*p*	b-coeff. (95% CI)	p_n	p_{FDR}
Neoadjuvant therapy	**No**	Ref.		Ref.			Ref.		Ref.		
	Yes	-0.14 [-0.36, 0.08]	0.21	-0.18 [-0.41, 0.05]	0.12	0.31	0.04 [-0.03, 0.10]	0.24	0.03 [-0.04, 0.09]	0.44	0.65
Red meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.06 [-0.14, 0.25]	0.56	0.15 [-0.03, 0.33]	0.10	0.29	-0.05 [-0.10, 0.005]	0.08	-0.06 [-0.11, -0.003]	0.04	0.18
Processed meat	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.11 [-0.12, 0.33]	0.35				0.03 [-0.03, 0.09]	0.28			
Raw vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	-0.01 [-0.23, 0.21]	0.92				-0.04 [-0.09, 0.23]	0.23	-0.06 [-0.12, -0.002]	0.04	0.18
Cooked Vegetables	**≤ 1x/week**	Ref.		Ref.			Ref.		Ref.		
	> 1x/week	0.11 [-0.16, 0.38]	0.41				-0.02 [-0.09, 0.05]	0.58			

* NSAID user took at least one pill per month in the past month before surgery (baseline).

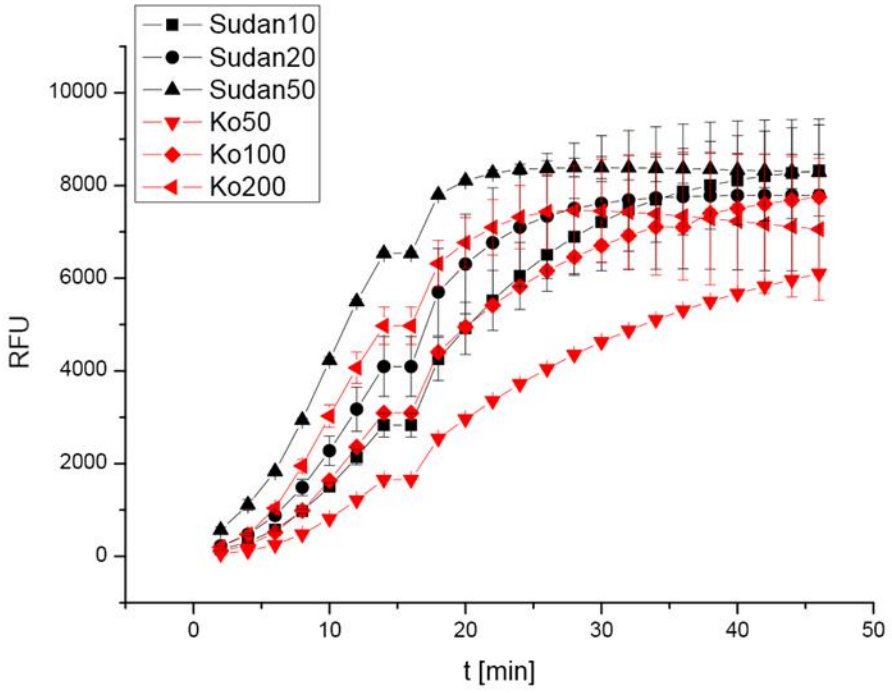
Note: Uni- and multivariable linear regression models were used for the estimation of the b-coefficients. All multivariable models adjusted for age (continuous), gender (men, women), current smoking status (yes, no), neoadjuvant therapy (yes, no), and tumor site (colon, rectum). Further potential confounding variables were selected separately for each gene, by backward elimination using an alpha-value of 0.1. The following covariates were included in the backward elimination: BMI (continuous), alcohol (g/day, 0-4.7, >4.7), stage (I/II, III/IV), regular NSAID use (yes, no), and consumption of red meat, processed meat-, cooked vegetables-, and raw vegetables (≤ 1x/week, > 1x week).

Abbreviations: p_n: nominal *p*-value; p_{FDR}: false-discovery-rate-adjusted *p*-value.

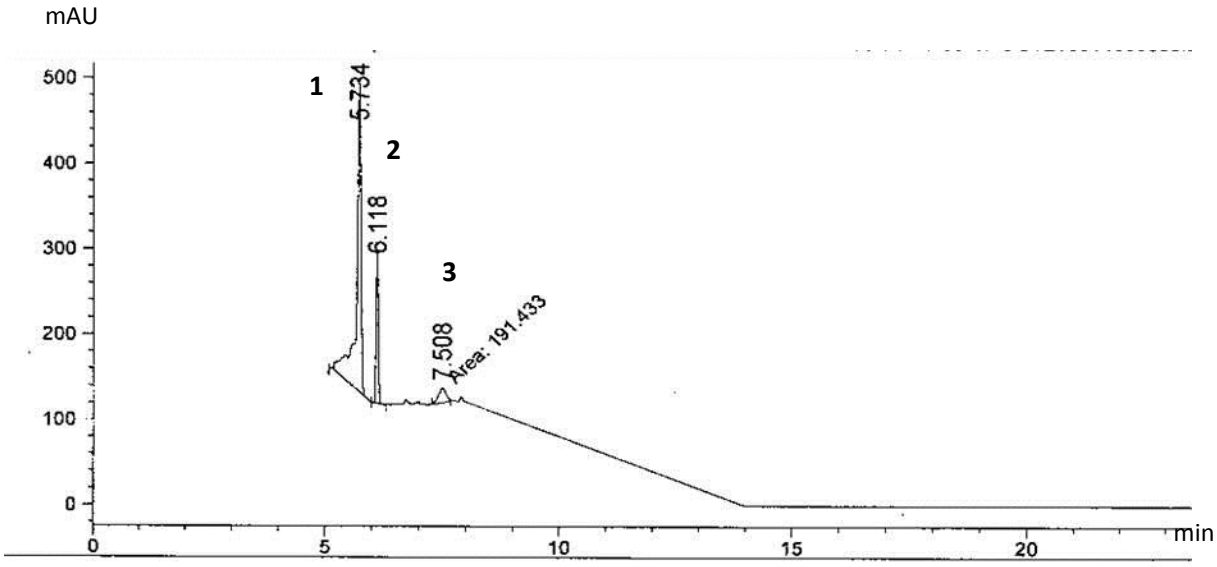
11 Appendix B: Supplementary Figures



Supplementary Figure 1: Western blot analyses of GSTP1 expression in normal mucosa of CRC patients
Known concentrations (0.1 µg, 0.2 µg, and 0.5 µg) of GSTP1 human recombinant proteins were used for quantification. β-Actin was used as loading control.MW: molecular weight; kD: kilodalton



Supplementary Figure 2: Fluorescence-based assay for the determination of CYP1A1/2 activity
In a 96-well plate the incubation mixture (200 µL) contained 67 mM potassium phosphate buffer (pH 7.4), 3 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, 10 mM MgCl2, 1 mM NADP, 5 µM CEC (dissolved in DMSO) and 50 µg, 20 µg and 10 µg of microsomal fractions from Sudan I induced microsomes and 200 µg, 100 µg and 50 µg of control microsomes.
Abbreviations: RFU: relative fluorescence units.



Supplementary Figure 3: HPLC-ESI-MS detection of UGT activity toward 4-NP in normal human colorectal microsomes.

Reactions were performed in the presence of 4-NP (0.5 mM) and colorectal microsomal proteins (250 µg) in a total volume of 400 µl for 60 min at 37°C. Peaks are: 1) UDPGA; 2) 4-NPG; 3) 4-NP.

Abbreviations: UDPGA: Uridine-diphosphate-glucuronic acid; 4-NPG: 4-nitrophenyl β-D-glucuronide; 4-NP: 4-nitrophenol; HPLC-ESI-MS: High-performance liquid chromatography-electrospray ionization mass spectrometry.

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List of Abbreviations

Abbreviation	Explanation
4-NP	4-nitrophenol
4-NPG	4-nitrophenyl β-D-glucuronide
ACF	Aberrant crypt foci
Ah receptor	Aryl hydrocarbon receptor
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
BMI	Body-Mass-Index
BSA	Bovine serum albumin
CD	Crohn's disease
CDNB	1-chloro-2,4-dinitrobenzene
CEC	3-cyano-7-ethoxycoumarin
CHC	3-cyano-7-hydroxycoumarin
CIN	Chromosomal instability
CRC	Colorectal cancer
CYP	Cytochrome-P-450 monooxygenase
DACHS	Darmkrebs: Chancen der Verhütung durch Screening
DCC	Deleted in colorectal carcinoma
DEET	N,N-diethyl-m-toluamide
DMEs	Drug-metabolizing enzymes
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithio-*bis*-2-nitrobenzoic acid
ECL	Enhanced chemiluminescence reagent
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmatic reticulum
FAP	Familial adenomatous polyposis
FC	Fold change
FDR	False-discovery-rate
FMO	Flavin monooxygenase
GI tract	Gastrointestinal tract

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Abbreviation	Explanation
GR	Glutathione reductase nicotinamide adenine dinucleotide phosphate
GSGG	Glutathione disulfide
GSH	Glutathione
GST	Glutathione *S*-transferase
HBSS	Hank`s Balanced Salt Solution
HCAs	Heterocyclic amines
HNPCC	Hereditary nonpolyposis colorectal cancer
HPLC	High-performance liquid chromatography
HPLC-ESI-MS	High-performance liquid chromatography-electrospray ionization mass spectrometry
HRP	Horseradish peroxidase
ICD-10	International Classification of Diseases (10th revision)
IgG	Immunoglobulin G
KRAS	Kirsten rat sarcoma
LC-MS	Liquid chromatography-mass spectrometry
MAO	Monoamine oxidase
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MLH1	*MutL homologue 1*
MMR	Mismatch repair system
MSH2	*MutS homologue 2*
MSI	Microsatellite instability
MT	Methyltransferase
n.a.	not available
NADPH	Nicotinamide adenine dinucleotide phosphate
NASH	Non-alcoholic steatohepatitis
NAT	N-acetyltransferase
NCT	National Center for Tumor Diseases
NSAIDs	Nonsteroidal anti-inflammatory drugs
p53	Tumor protein 53
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

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Abbreviation	Explanation
PM	Poor metabolizers
PTFE	Polytetrafluorethylene
qRT-PCR	Real-time quantitative PCR
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sig	significantly
SIM	selected ion monitoring mode
SSA	Sulfosalicylic acid
SSRI	Selective serotonin reuptake inhibitor
SULT	Sulfotransferase
TEMED	Tetramethylethylenediamine
TMB	3,3’,5,5’-Tetramethylbenzidine
TNB	5-thio-2-nitrobenzoic acid
UC	Ulcerative colitis
UDPGA	Uridine-diphosphate-glucuronic acid
UGT	UDP-glucuronosyltransferase
UM	Ultrarapid metabolizers
XMEs	Xenobiotic-metabolizing enzymes
β-ME	β-Mercaptoethanol

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