Vegetable induced gene expression changes in anticarcinogenic pathways in human and mouse tissues

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**Vegetable induced gene expression changes
in anticarcinogenic pathways
in human and mouse tissues**

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Vegetable induced gene expression changes in anticarcinogenic pathways in human and mouse tissues

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Chapter 1

General introduction

1 Vegetables and Cancer Prevention

1.1 Vegetables

Historically, consumption of particular vegetables was thought to prevent or cure ailments ranging from headaches to heart disease. In fact, early medicine revolved largely around the prescription of specific plant food concoctions for certain disorders. The diverse medicinal properties of extracts, juices, or poultices obtained from plants have led to the cultivation of these in many parts of the world.¹⁻³ Botanically, a vegetable is any part of a plant not involved in the sexual reproduction of the plant. Vegetables are typically the cultivated or gathered leaves, roots, stalks, bulbs and flowers of plants. Some foods that are culinary vegetables are botanically classified as fruits; these include avocados, cucumbers, aubergines, peppers, tomatoes, pumpkins and courgettes. Vegetables include artichokes, asparagus, beets, broccoli, Brussels sprouts, cabbage, carrots, cauliflower, chard, endive, fennel, garlic, kohlrabi, leeks, lettuce, mushrooms, okra, onions, parsley, parsnips, radishes, rhubarb, spinach, turnips, legumes and various green leafy vegetables. Vegetables may be grouped into categories that are either botanically or culinary meaningful, for example: 'green leafy' (spinach, lettuce), 'cruciferous' (broccoli, cauliflower, kohlrabi, Brussels sprouts, cabbage), 'allium' (onions, garlic, scallions, chives and leeks), 'raw' (uncooked vegetables), 'orange' (carrots, pumpkin, winter squash), and legumes (peas, beans).^{1,4,5}

1.2 Epidemiological Evidence of Cancer Prevention by Vegetables

As early as 1933, a case-control study by Stocks and Karn in the UK suggested an inverse association between intakes of certain vegetables against all cancer sites.⁶ Since that time, over 250 epidemiological studies (case-control, cohort or ecological correlations) have been conducted worldwide to investigate the relationship between vegetable and fruit consumption and cancer risk. In most of these studies, fruit and vegetables are considered collectively, although different types of vegetables and fruits have been investigated separately. This literature on vegetables and fruit consumption and the prevention of cancer has been reviewed in several scientific papers.^{1,3,7,8} Prevention of stomach cancer has been the most studied, followed by cancers of the colon, esophagus, lung, oral cavity and pharynx, rectum and breast; more than ten studies have focused on each of these sites. About 80% of these studies found a significant protective effect of overall consumption of vegetables and/or fruit, or at least of some types of vegetables and fruits for cancers of the following targets: stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas, colon, and skin. Prostate cancer is the only cancer for which the majority of

studies have not reported at least one statistically significant inverse association. On the whole, evidence that vegetables are protective is stronger than for fruits, but this may simply reflect the generally higher consumption of vegetables worldwide.^{1, 3, 7, 8}

In 1997, the World Cancer Research Fund and the American Institute for Cancer Research (WCRF/AICR) jointly published an extensive, global review of the role of food and nutrition in the prevention of cancer.⁹ Vegetables and fruits received considerable attention in this review. Based on statistical and other considerations, the WCRF/AICR panel ranked the evidence that a high intake of vegetables and fruits decreased the risk of cancer as shown in Table 1. Consumption of higher levels of vegetables and fruits is associated consistently with a reduced risk of cancer at most sites. The association is most marked for epithelial cancers-particularly those of the alimentary and respiratory tracts-and, currently, is weak to nonexistent for hormone-related cancers.⁴ Diets containing substantial amounts (> 400 g/day) of a variety of vegetables and fruits may reduce the overall incidence of cancer by over 20 percent.¹⁰

Table 1. Ranking of cancer risk reduction at various sites from vegetables and fruits.⁹

	Convincing	Probable	Possible
Vegetables and fruits combined	Oral cavity	Larynx	Ovary
	Pharynx	Pancreas	Cervix
	Esophagus	Breast	Endometrium
	Stomach	Bladder	thyroid
	Lung		
Vegetables only	Colon		Liver
	Rectum		Prostate
			Kidney

Next to this assessment of cancer risk reduction, an evaluation can be made of which specific vegetables and fruits are responsible for the beneficial effects. Consumption of vegetables in general and raw vegetables in particular has most often been found to be inversely related with the risk of any type of cancer. More than 85 percent of studies which investigated raw vegetables reported a protective association. Data for allium vegetables, carrots, green vegetables, cruciferous vegetables, and tomatoes are also fairly consistent: for each of these types of vegetables, at least 70 percent of studies showed a protective association. Table 2 summarizes the evidence about the most protective vegetable types.^{1, 4}

Studies published since the WCRF/AICR review confirm and extend the recommendations for vegetables and fruit.^{11, 12} Most recently, The World Health Organization and the Food and Agriculture Organization of the United Nations have released a report of their Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases which suggested that a high intake of vegetables and fruit probably reduces the risk of cancers of the oral cavity, esophagus, stomach and colorectum.¹³

Table 2. Evidence relating particular types of vegetables and fruits to cancer risk reduction.^a

	Convincing	Probable	Possible
Vegetables in general	Stomach Colon Esophagus Lung Oral cavity and pharynx Rectum	Larynx Pancreas Breast Bladder	Ovary Endometrium Cervix Liver Prostate Kidney Thyroid
Allium vegetables	Stomach	Colon Rectum	
Tomatoes	Stomach Esophagus Lung	Colon Oral cavity and pharynx Rectum	
Green (leafy) vegetables	Stomach Colon Esophagus Lung Oral cavity and pharynx	Breast Bladder	
Legumes	Stomach		
Raw vegetables	Stomach Esophagus	Colon Oral cavity and pharynx Pancreas	
Carrots	Stomach Lung Oral cavity and pharynx Rectum	Breast Bladder Colon	
Cruciferous	Rectum Colon	Thyroid	Oral cavity and pharynx
Fruits in general	Stomach Lung Oral cavity and pharynx Breast Pancreas	Colon Esophagus Rectum	Bladder
Citrus fruits	Stomach Esophagus Oral cavity and pharynx	Rectum	Breast Pancreas

However, it is stated that the support for a broad and strong protective effect of higher vegetable and fruit intake has been weakened by the results from recent prospective studies¹⁴⁻¹⁹, which have not supported important protective effects for cancers of the lung and breast, and have suggested that the reduction in colorectal cancer risk may be modest. These discordant results, which add to concerns about the potential for bias in case-control studies, also suggests the need for some caution regarding conclusions about intake of fruits and vegetables and the risk of oral, esophageal and stomach cancers, which have not been adequately examined in large prospective studies. Although support for a broad and strong protective effect of higher fruit and vegetable intake against cancer incidence has been weakened, modest benefits of increasing fruit and vegetable intake have not been excluded and probably do exist. Further research is needed without epidemiological bias using sensitive biomarkers to strengthen the findings. Investigations at the molecular and submolecular levels may identify the genetic and biochemical mechanisms by which vegetables affect cancer risk.

1.3 Experimental Evidence of Cancer Prevention by Vegetables: Animal Studies

Laboratory animal models are used to test the effects of vegetables on cancer. The two species most often used to investigate the effects of vegetables and vegetable components on different stages of the carcinogenic process are the mouse and rat. In particular, the C57BL6 mouse is used, because of the possibility of using transgenic and knockout variants.²⁰⁻²³ Most of the studies are conducted in which cancer has been experimentally induced (usually via a chemical carcinogen or irradiation) in animals fed specified amounts of certain vegetables or vegetable components.²³⁻³⁵ In most studies, it was found that animals fed vegetables or vegetable constituents experienced fewer tumours^{23, 31, 36}, fewer metastases³⁶, less DNA damage³⁷, higher levels of enzymes involved in the detoxification of carcinogens^{28, 32, 37-39}, or other indicatives of a lower cancer risk.^{24, 27, 40, 41} However, to obtain statistically significant results, carcinogens and nutrients are both commonly administered to animals in doses far higher than in any conceivable real-life equivalent. The extrapolation of results from animal studies to humans is further hampered by species differences⁴², the use of carcinogens, and the use of genetically susceptible animals. Main advantages of animal studies are that laboratory conditions can be accurately controlled in ways impossible in any human environment, and that genetic variability between individuals can largely be avoided. Many insights into anticarcinogenic mechanism have been gained by these means. However, the genetic pathways through which vegetables exert their effect are still mostly unknown, because few studies have investigated the effect of vegetables and vegetable constituents on gene expression changes in target organs. In

section 2.2.4 and 2.3.4, this topic will be described in more detail for two target organs, i.e. the colon and rectum and the lung.

1.4 Mechanisms of Anticarcinogenesis

Many potentially mechanisms have been proposed to account for protection against cancer by vegetables. Numerous constituents found in vegetables, including micronutrients (nutritive compounds, e.g. carotenoids, vitamin C and E, folic acid, selenium), dietary fiber and phytochemicals (non-nutritive bioactive compounds with no known nutritional value, e.g. flavonoids, indoles, isothiocyanates, glucosinulates), and interactions among these constituents might contribute to the ability of these foods to reduce cancer risk. Evidence for the cancer-preventing actions of these agents mainly comes from animal studies and *in vitro* studies, which have been shown that they are capable of preventing cancer either directly or via complex interactions with the body's metabolic and molecular processes.^{4, 43-}

⁴⁵ It is thought that the potent anticarcinogenic agents act in concert to prevent cancer ('teamwork'), rather than that a single substance is responsible for the protective effect ('magic bullet').⁴⁶

Because cancer is a multistep process occurring over an extended time frame, there is a number of possible stages at which the process could be halted, inhibited, delayed or even reversed.⁴⁷ The multiple phases involved in the pathogenesis of cancer offer the basis of most classifications of anticarcinogenic mechanisms of agents present in vegetables.⁴⁸⁻⁵¹ Some mechanisms are reiterated several times in different phases of the process, while others are strictly interconnected or partially overlapping. Furthermore, the agents, which are possibly responsible, have both complementary and overlapping mechanisms of action. Table 3 gives an overview of a selection of proposed anticarcinogenic mechanisms, together with examples of protective agents, their specific mode of action and their vegetable source. Here, the mechanisms are divided into two categories; i.e., I) blocking mechanisms (inhibition of mutation and cancer initiation), and II) suppressing mechanisms (suppression of promotion, progression, invasion, and metastasis) (classification taken from Wattenberg (1992)⁵², supplemented with information from De Flora (1998)⁴⁹, Kelloff et al (1999)⁵⁰). In most of the presented mechanisms, modulation of gene expression plays a role, however, the molecular targets at the genome level and the involved genetic pathways are mostly unknown.

Table 3. Anticarcinogenic mechanisms of agents present in vegetables.

Process of anticarcinogenesis	Protective agent	Mode of action	Vegetable source	Ref.
I) Blocking mechanisms				
Prevention of carcinogen uptake	Dietary fiber	Adsorption of carcinogens	All vegetables	53
Inhibition of non-enzymatic endogenous carcinogen formation	Vitamin C	Scavenging of nitrite to prevent formation of nitrosamines	All vegetables	54
	Flavonoids	Inhibition of the conversion of nitrate to nitrite	Allium vegetables	54
Inhibition of enzymatic carcinogen formation/activation: modulation of biotransformation enzymes	Isothiocyanates, indoles	Inhibition of activation by phase-I enzymes; Induction of phase-I detoxification and phase-II conjugation pathways	Cruciferous vegetables	34, 55, 56
	Flavonoids	Inhibition of activation by phase-I enzymes	Allium vegetables	57, 58
	Organosulfur compounds	Inhibition of phase-I and induction of phase-II enzymes	Allium vegetables	59
Blocking, quenching or scavenging of reactive metabolites	Flavonoids	Direct scavenging of reactive oxygen species and free radicals; inhibition of xanthine oxidase activity	Allium vegetables	57, 58
	Isothiocyanates	Scavenging of free radicals	Cruciferous vegetables	60, 61
	Vitamin C	Scavenging of free radicals; Quenching of singlet oxygen	All vegetables	54, 62
	Carotenoids	Quenching of singlet oxygen; Protection of DNA nucleophilic sites	Orange and Green leafy vegetables	63-65
	Vitamin E	Intracellular antioxidant: protection of cell membranes from lipid peroxidation by trapping peroxy radicals	Green vegetables	66
Induction of DNA repair and -synthesis	Protease inhibitors	Inducing DNA repair by enhancing poly(ADP-ribosyl)transferase	Legumes	67
	Flavonoids	Inhibition of DNA topoisomerase II	Allium vegetables	58
II) Suppressing mechanisms				
Inhibition of cell proliferation and replication (cell cycle arrest)	Quercetin	Blocking of G1/S transition of the cell cycle	Allium vegetables	35, 68, 69
	Protease inhibitors	Repression of the NF- κ B signaling pathway	Legumes	70
	β -carotene	Inhibition of polyamine metabolism by downregulating ornithine decarboxylase	Orange and Green leafy vegetables	71-73
	Dietary fiber	Inhibition of secondary bile acids	All vegetables	53
	Indoles	Inhibition of cyclin-dependent kinase 6	Cruciferous vegetables	74
	Flavone	Downregulation of Cyclin B and E, induction of p21	Allium vegetables	75

Table 3. (Continued) Anticarcinogenic mechanisms of agents present in vegetables.

Process of anticarcinogenesis	Protective agent	Mode of action	Vegetable source	Ref.
Induction of cell apoptosis	Isothiocyanates,	Induction of caspases	Cruciferous vegetables	88, 76
	Indoles			
	Flavonoids	Induction of p53, Bax, and reduction of Bcl-2	Allium vegetables	75
	Organosulfur compounds	Inhibition of NF- κ B and bcl-X _L and induction of pro-apoptotic Bax	Allium vegetables	89
Induction of cell differentiation	Beta-carotene,	Upregulation of retinoid receptor expression;	Orange and Green leafy vegetables	77
	Vitamin A	induction of transforming growth factor- β		
	Flavonoids	Inhibition of abl oncogene tyrosine kinase	Allium vegetables	78, 79
Protection of intercellular communication between normal cells	Carotenoids	Upregulation of connexin-43 gene expression	Orange and Green leafy vegetables	80, 81
	Protease inhibitors	Protection of extracellular matrix	Legumes	82
Modulation of immune system:	Flavonoids	Inhibition of cyclooxygenase and lipoxygenase activities	Allium vegetables	57
	• Inhibition of impairment of the immune system		Green vegetables	83
	Vitamin E			
	• Stimulation of efficient and effective immune system	Induction of antibody production, natural killer cell activity and macrophage phagocytosis	All vegetables	84
	Vitamin C	Production of interferon	Orange and Green leafy vegetables	85
	Vitamin A	Induction of antibody response, proliferation of T-lymphocytes, production of interferon	Orange and Green leafy vegetables	86
Beneficial effect on hormone status	Carotenoids	Inhibition of nuclear factor kappa B activity		
	Apigenin,	Inhibition of estrone formation	Allium vegetables	58
	kaempferol			
	Indoles	Increasing estrogen conjugation	Cruciferous vegetables	87
	Isoflavones	Binding to estrogen receptors	Legumes	88
Inhibition of tumor induced neo-vascularization (angiogenesis)	Flavonoids	Inhibition of tyrosine kinases, inhibition of vascular endothelial growth factor and transforming growth factor- β expression	Allium vegetables	57, 89
Inhibition of metastasis: activation of antimetastasis genes	Indoles	Induction of E-cadherin, α -, β - and γ -catenin, and BRCA1	Cruciferous vegetables	90

2 Target Organs

2.1 Introduction

The epidemiological proof for cancer prevention by vegetables is most evident for cancers of the alimentary and respiratory tract. The colon and rectum, and the lungs are of particular interest in these studies. The high incidence of both types of cancer (see sections 2.2 and 2.3), together with the epidemiological rationale of prevention of these cancers by vegetables, makes these two types of cancer highly convenient for elucidating the mechanisms of cancer prevention by vegetables. Furthermore, these two organ systems differ in respect to the part/components of the vegetables that reaches the tissues. In contrary of the lungs, the colon and rectum are both sides of contact, i.e., as the vegetables pass through the colon and rectum, they come into close contact with the intestinal lining. The substances that reach the lung (and also the colon and rectum), have been modulated by the first-pass metabolism: substances that are absorbed via intestinal blood capillaries will first have to pass the liver before they reach the systemic circulation. Here, many substances are extensively metabolized.

In this paragraph, the tissue characteristics (histology), the epidemiology and etiology, the genes and involved pathways in cancer development, and a summary of the knowledge of modulation of cancer risk by vegetables for the colon and rectum, and lungs, are described.

2.2 The Colon and Rectum

2.2.1 *Histology of the Human Colon and Rectum*

The large intestine is part of the alimentary tract, and is subdivided into the cecum, colon (ascending, transverse, descending and sigmoid), rectum, and anus. It has three functions: 1) concentration of fecal effluent through water and electrolyte absorption; 2) storage and controlled elimination of feces; and 3) digestion and absorption of undigested food (complex sugars). The colon accounts for almost the entire length of the large intestine (125 cm). Its wall is composed of four broad layers: the mucosa, submucosa, muscularis externa, and serosa (Figure 1). The lumen is lined by an epithelium, deep to which is a loose connective tissue known as the lamina propria. Surrounding this connective tissue coat is the muscularis mucosae, composed of an inner circular layer and an outer longitudinal layer of smooth muscle. The epithelium, lamina propria, and muscularis mucosae are collectively called the mucosa. The epithelium is richly endowed with crypts of Lieberkühn which consists of surface absorptive cells, goblet cells, regenerative cells and

cells of the diffuse neuroendocrine system (DNES). The number of goblet cells increases from the cecum to the sigmoid colon, but the surface absorptive cells are the most numerous cell type. Rapid mitotic activity of the regenerative cells replaces the epithelial lining of the crypts and of the mucosal surface every 6 to 7 days. The mucosa is surrounded by a dense, irregular fibroelastic connective tissue layer, the submucosa. The muscularis externa consists of an outer longitudinal layer, which is not continuous along the surface but is gathered into three narrow ribbons of muscle fascicles, known as taeniae coli. The serosa envelops the muscularis externa and displays numerous fat-filled pouches, called appendices epiploicae. Histologically, the rectum resembles the colon, but the crypts of Lieberkühn are deeper and number fewer per unit area.⁹¹

Figure 1. Schematic diagram of the colon, crypts of Lieberkühn, and associated cells.⁹¹

2.2.2 Epidemiology and Etiology of Colorectal Cancer

Worldwide, colorectal cancer (CRC) is the third most common incidence cancer and cause of death from cancer. Approximately 943,000 new cases were diagnosed and 491,000 people died of CRC in the year 2000. The peak incidence of the disease is in the seventh

decade and it is fairly equally distributed between men and women. Incidence rates vary around the world; the developed world (Europe, Japan, Australia, New Zealand and North America) accounts for almost 65 percent of the total global incidence. Central and South America, Asia and Africa are areas of low risk, however, the incidence is now increasing in these regions.⁸ Migrant and temporal trend studies show that rates in migrants from low- to high-risk countries tend to increase to the rates of the host countries within one or two generations, or sometimes even as early as within the migrating generation itself. This indicates that environmental factors play an important role in the etiology of CRC.^{92, 93}

Increased CRC risk is associated with dietary (intake of excess fat, sugar and alcohol, low amount of vegetables and fiber) and lifestyle factors (low physical activity, high body mass, smoking), typical for Western societies.^{8, 94-96} Based on epidemiological studies, Doll and Peto (1981) assessed that 90 percent of CRC mortality was attributed to dietary factors.⁹⁷ This estimation was adjusted to 70 percent (range 50-80) by Willet in 1995.⁹⁸ CRC can largely be prevented by dietary changes. Especially diets containing considerable amounts of a variety of vegetables (> 400 g/day) may reduce the relative risk of CRC by 40 percent (range 30-50).⁹⁹

Besides exogenous factors, CRC may be the result of hereditary factors or a combination of genetic and environmental factors. Patients with a familial risk - those who have two or more first- or second-degree relatives (or both) with CRC - make up approximately 20 percent of all patients with CRC, whereas about 5 percent of the total annual burden of CRC is caused by an autosomal dominant manner. This last category can be divided into two major forms of hereditary CRC, i.e., familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC). FAP (incidence <0.5 percent) is an autosomal dominant syndrome associated with mutations of the adenomatous polyposis coli (APC) gene, which results in the development of hundreds of polyps by the third decade of life. CRC inevitably occurs unless the colon is prophylactically removed. HNPCC is more common than FAP (incidence 4-6 percent) and is a much more heterogeneous syndrome with lifetime risk of 70-90 percent of developing CRC. It is caused by a germline mutation in any of the mismatch repair (MMR) genes hMLH1 (human mutL homolog 1), hMSH2, hMSH3 and hMSH6 (human mutS homolog 2, 3 and 6), hPMS1 or hPMS2 (human postmeiotic segregation 1 and 2).¹⁰⁰⁻¹⁰² The experience derived from these two hereditary syndromes has been helpful in advancing our understanding of the molecular basis of CRC.

2.2.3 *Colorectal Cancer: Genetic Pathways involved in the Vogelstein Model*

For about a decade, the model proposed by Fearon and Vogelstein has been the paradigm of the genetic alterations involved in the development of CRC.^{103, 104} In this model, CRC develops as the result of the progressive accumulation of genetic and epigenetic changes that lead to the transformation of normal colonic epithelium to colon adenocarcinoma,

with the adenomatous polyp as an intermediate step in this sequence. The loss of genomic stability is an important step in this process by contributing to the occurrence of mutations.

Sporadic CRC arises mainly through two distinct pathways. In the first, chromosome instability, the initial inactivation of the APC tumor suppressor gene (chromosome 5q) is followed by the accumulation of alterations in additional oncogenes (K-RAS on chromosome 12p) and tumor suppressor genes (chromosome 18q: DCC, Deleted in Colorectal Cancer; chromosome 17p: TP53, p53 gene). The second, associated with microsatellite instability (MSI), occurs in 15-20% of sporadic CRC. Alterations have been found to cluster in genes encoding enzymes involved in the repair of DNA mismatches (in particular hMLH1 and hMSH2). These alterations provide a growth advantage and lead to clonal expansion of the distorted cells.¹⁰³⁻¹⁰⁷

Since the development of the Vogelstein model, a lot of information has become available on the function of the key genes in this model, as well as on their interactions. Additional genetic events have been identified and the specific molecular pathways which are perturbed by each of the mutations (Figure 2). Additionally, the importance of epigenetic mechanisms, like DNA methylation, that regulate gene expression is becoming increasingly clear.¹⁰⁶⁻¹⁰⁹ The genetic pathways, which are mostly investigated, are summarized below.

Wingless/Wnt signaling pathway: One of the central tumor-promoting effects of APC mutations is to lead to overactivation of the Wingless/Wnt signaling pathway, with the subsequent expression of genes that favor cell growth. APC mutations disrupt the association of APC with β -catenin, resulting in excessive amounts of β -catenin and overactivation of the Wnt signaling pathway. Consequently, genes that promote tumor formation are transcribed. Translocation of cytoplasmic β -catenin to the nucleus results in its interaction with other transcription factors like T-cell factor/lymphoid enhancing factor (TCF/Lef). TCF-4 is the predominant TCF family member expressed in colonic epithelium. Relevant targets up-regulated by TCF-4 identified to date include CYCLIN D1, C-MYC, MATRILYSIN, C-JUN, FRA-1, urokinase-type plasminogen activator receptor, and the peroxisome proliferator activator receptor delta (PPAR δ). Other genes that are regulated by the Wnt signaling pathway and are upregulated in colon cancer include the WISP genes, WISP-1 and WISP-3. Consistent with the concept that overexpression of β -catenin is a central tumor-promoting effect of APC mutation, oncogenic mutations in the β -catenin gene (CTNNB1) have been observed in some CRC.¹⁰⁶⁻¹⁰⁹

K-RAS pathway: The K-RAS mutations appear to occur after APC mutations are formed and are associated with advanced adenomatous lesions. K-RAS pathways transduce signals from extracellular growth factors to regulate progression through the cell cycle and proliferation. K-RAS mutations are associated with upregulation of DNA methyltransferase, CYCLIN-D1, and GASTRIN.¹⁰⁶⁻¹⁰⁹

TGF- β /SMAD pathway: Transforming growth factor beta (TGF- β) is a multifunctional cytokine that can induce growth inhibition, apoptosis, and differentiation in intestinal epithelial cells. The downstream transcriptional targets of the TGF- β signaling pathways are the so-called SMAD proteins, which have been shown to regulate genes encoding plasminogen activator inhibitor 1, the cyclin dependent kinase inhibitors p15, p21 and p27, cyclin D1, c-myc and TGF- β itself. Mutational inactivation of SMAD2 and SMAD4 has been observed in a high percentage of pancreatic cancer and in 5-10% of colon cancers. Next to DCC, these two tumor suppressor genes are the targets of 18q loss of heterozygosity (LOH).¹⁰⁶⁻¹⁰⁹

TP53 pathway: TP53 mutations appear to be late events in the colon adenoma-carcinoma sequence that may mediate the transition from adenoma to carcinoma. It is expressed at very low levels in cells until it is activated via incompletely understood mechanisms by genotoxic stress. Its activation results in the transcription of genes that directly regulate cell cycle progression and apoptosis. These genes include p21^{WAF1/CIP1}, GADD45, MDM2, 14-3-3- σ , BAX, TSP1, KILLER/DR5, FAS/APO1, CYCLIN G, and others. Expression of many of these genes effectively halts DNA replication, induces DNA repair and inhibits angiogenesis.^{106, 107}

DNA mismatch repair pathway: Somatic inactivation of the mismatch repair system additionally gives rise to approximately 15% of sporadic colon cancers. In either instance the resulting colon cancers display the phenotype of MSI. The predominant mechanism for inactivating MMR was found to be the epigenetic silencing of the hMLH1 promoter owing to aberrant promoter methylation. The most frequently targeted gene for mutation in this pathway is the TGF- β receptor type II tumor suppressor gene (TGFBR2). Other less frequently targeted genes include the IGF2 receptor, BAX, and CASPASE 5, genes encoding enzymes that regulate apoptosis; E2F4 and TCF-4, transcription factors; MSH3 and MSH6, DNA mismatch repair genes; RIZ, the retinoblastoma protein-interacting zinc finger gene; and CDX2, an intestinal homeobox factor. Importantly, MSI and the subsequent target gene mutations appear to occur throughout the adenoma-to-carcinoma progression.^{100, 105-107}

Figure 2. Overall scheme of key genetic events (genetic alterations and involved pathways) in colorectal tumorigenesis. The key genetic events can be grouped into two broad categories: A) Eighty to eighty-five percent of tumors exhibit chromosomal instability; B) The remaining fifteen to twenty percent of tumors are characterized by microsatellite instability.

2.2.4 Modulation of Colorectal Cancer (Risk) by Vegetables at the Genome Level

Numerous studies have been conducted to get more insight into the mechanisms underlying prevention of CRC risk by vegetables or vegetable components. This paragraph will give an overview of a selection of *in vitro*-, animal-, and human studies in which the effect of vegetables or vegetable components on CRC risk is investigated, especially focusing on gene or protein expression changes; these are summarized in Table 4.

In vitro studies: Most of the *in vitro* studies involved in investigating the effect of vegetables on CRC risk use colon carcinoma cell lines as model system and one or more specific vegetable constituents as test compounds. Furthermore, they investigate the effect of these test compounds on cell proliferation, cell cycle arrest, apoptosis and the expression of genes or proteins involved in these processes. In these studies it was found that specific vegetable components such as flavonoids, indoles, isothiocyanates and β -carotene are able to induce cell cycle arrest and apoptosis and to reduce cell proliferation and that this coincides with specific modulation of genes and/or proteins, such as cyclins and caspases.^{68, 72, 75, 76, 110}

Animal studies: In animal studies, a limited number of studies has investigated the effect of vegetable or vegetable components on gene and/or protein expression changes in the colon.^{28, 31, 39, 111, 112} In these studies, modulation of (mostly biotransformation) genes and enzymes in the colon was observed. In most of the animal studies, the effect of vegetables or vegetable components on protein and gene expression is investigated in the liver^{26, 32, 33, 37, 40, 113} and small intestine^{26, 113} or on other outcomes than protein and gene expression in the colon, such as development of intestinal neoplasia^{23, 41, 114, 115} and aberrant crypt foci.²⁴

Human studies: In only two human studies the effect is investigated of vegetables or vegetable components on modulation of the expression of genes or protein in the colon.^{111, 116} Also, in other tissues such as lymphocytes^{116, 117} and urine¹¹⁸⁻¹²⁰, the investigations are limited. More research has been done in the field of human dietary intervention trials in which the effect of nutritional interventions on the recurrence of precancerous polyps has been investigated. The results of these trials are, however, disappointing.^{14, 121, 122} In most of the studies, no reduction of the number of adenomas was found after an intervention with vegetables or -components.

In conclusion, the number of *in vitro*, animal and human studies in which the effect of vegetables on gene expression and/or protein expression changes at target level is examined is limited. Most studies focus on surrogate tissues and only a few genes and/or proteins are investigated. Studies in which the effect of vegetables on the expression of multiple genes involved in various cancer preventive pathways in colon tissue are needed to elucidate the effects of vegetables.

Table 4. Selection of *in vitro*, animal and human colon studies involved in modulation of gene or protein expression by vegetables or vegetable components.

Model system	Vegetable or -component	Methods	Main results	Ref.
I) <i>In vitro</i> studies				
HT-29 cells	Flavone (150 μ M)	Cell proliferation-, cell differentiation-, apoptosis assays RT-PCR analysis	Reduced cell proliferation, induction of cell differentiation and apoptosis Inhibition of COX-2, NF- κ B, cyclin E and B, anti-apoptotic bcl-X _L ; Induction of p21, pro-apoptotic bax; no change p53	75
HT-29 cells	Phenethyl isothiocyanate (5-50 μ M)	Apoptosis assays Western blot analysis	Induction of apoptosis; activation of caspase-3, -9; release of cytochrome c Induction of JNK, c-Jun and N-terminal kinase, extracellular signal regulated protein kinase, p38 kinase	110
COLO 320 cells HSR cells LS-174 cells HT-29 cells WiDr cells	β -carotene (0-50 μ M)	Cell growth inhibition Cell cycle analysis Apoptosis assay Immunohistochemistry	Inhibition of cell growth Induction of cell cycle arrest Induction of apoptosis Inhibition of cyclin A, Bcl-2 and bcl-X _L ; no changes in p21 and p27, and Bax	72
LS-174 cells Caco-2 cells	Four different indoles Three different isothiocyanates (IC ₅₀ conc.)	Apoptosis assay Western blot analysis Northern blot analysis RT-PCR analysis Genotoxicity assay	Induction of apoptosis Induction of cytochrome P4501A1 by the indoles Induction of AKR1C1, NQO1, and GCS _B by the isothiocyanates Less DNA damage after pre-incubation	76
HT-29 cells	Sulforaphane (15 μ M)	Apoptosis assay Cell cycle analysis Western blot analysis	Induction of apoptosis Induction of cell cycle arrest No change in p53, induction of pro-apoptotic bax, induction of cyclin A and B1	68

Table 4. (Continued) Selection of *in vitro*, animal and human colon studies involved in modulation of gene or protein expression by vegetables or vegetable components.

Model system	Vegetable or -component	Methods	Main results	Ref.
II) Animal studies				
C57BL/6 mice	20% soybean diet for 14 weeks; or 250 µg genistein orally (single dose), sacrifice 24 h later	Western blot analysis RT-PCR analysis	Induction of CYP27B1 and inhibition of CYP24 mRNA and protein expression after both the soybean diet and the genistein gavage	112
Wister rats	Organosulfur compounds: diallyl sulfide, diallyl disulfide, dipropyl sulfide, or dipropyl disulfide (1 mmol/kg/day) by gavage (4days)	Protein assays Immunoblot assay	Induction of UDP-glucuronyl transferase and microsomal epoxide hydrolase by diallyl disulfide Induction of quinone reductase and GST activity by diallyl disulfide and diallyl sulfide	28
C57BL/6j- Min/+ mice	Diets for 9 weeks: Caffeic acid phenethyl ester (CAPE) (0.03 or 0.15%); or Curcumin (0.1%); or Quercetin (2%); or Rutin (2%)	Apoptosis analysis Enterocyte proliferation assay Immunohistochemistry	In CAPE and curcumin fed mice: Decreased tumor formation Increased enterocyte apoptosis Proliferation of enterocyte back to normal levels Inhibition of β -catenin protein expression in the enterocytes	31
Wister rats	10% broccoli diet for 1 week	Nylon blot analysis Western blot analysis	Induction of CYP1A1 mRNA and protein Reduction of CYP1B mRNA, induction of CYP1B protein	39
ICR(Ha) mice	Lyophilized broccoli (1 g/kg) orally, sacrifice at 1 and 2 days post-treatment	Protein activity assay Western blot analysis	Induction of glutathione-S-transferase μ and π activity and protein	111
III) Human studies				
Human subjects at increased risk	Broccoli supplemented diet: tablets (6 g/day); or Cruciferous free diet for 14 days	Protein activity assay	No difference in GST- μ and π activity	111
Healthy human subjects	300 g Brussels sprouts/day	Western blot analysis	Induction of GST- μ and π levels in the rectum	116

2.3 The Lungs

2.3.1 Histology of the Human Lungs

The lungs are part of the respiratory system and comprise the intrapulmonary bronchi (secondary and tertiary bronchi) primary bronchioles, terminal bronchioles, respiratory bronchioles and alveoli (Figure 3).

Figure 3. Schematic diagram of the respiratory system.¹²⁵

The secondary bronchi lead to the lobes of the lung; the left lung has two lobes and thus has two secondary bronchi; the right lung has three lobes and thus has three secondary bronchi. As secondary bronchi enter the lobes of the lung, they subdivide into smaller branches, tertiary bronchi. Each tertiary bronchus splits and leads to a discrete section of the lung tissue known as bronchopulmonary segment. As they decrease in diameter, they eventually lead to bronchioles.¹²³

The intrapulmonary bronchus consists of three layers: mucosa, submucosa, and adventitia. The mucosal lining is composed of the respiratory epithelium, the subepithelial connective tissue (lamina propria), and a bundle of elastic fibers separating the mucosa from the submucosa. The respiratory epithelium consists of six cell types: goblet cells, ciliated columnar cells, basal cells, brush cells, serous cells, and DNES cells. Two distinct smooth muscle layers spiraling in opposite direction are located at the interface of the lamina propria and submucosa. The submucosa is composed of a dense irregular fibroelastic connective tissue housing numerous mucous and seromucous glands. The adventitia is also composed of fibroelastic connective tissue. The epithelial lining of bronchioles ranges from ciliated simple columnar with occasional goblet cells in larger bronchioles, to simple cuboidal with occasional Clara cells and no goblet cells in smaller bronchioles. The lamina propria of bronchioles has no glands, only smooth muscle cells which are surrounded by elastic fibers. Respiratory bronchioles are similar in structure to terminal bronchioles, but their wall is interrupted by the presence of alveoli, where gaseous exchange can occur. Each alveolus is a small outpouching of respiratory bronchioles, alveolar ducts, and alveolar sacs. Walls of alveoli are composed of squamous epithelium consisting of two types of cells: type-I pneumocytes and type-II pneumocytes.¹²³

2.3.2 *Epidemiology and Etiology of Lung Cancer*

Lung cancer is the most common malignant disease worldwide, and is the major cause of death from cancer. It was a rare disease until the beginning of the 20th century. Since then, the occurrence of lung cancer has increased rapidly and it now accounts for approximately 902,000 new cases each year among men and 337,000 among women. An estimated 1,100,000 people died of lung cancer in the year 2000.⁸ In both men and women, the incidence is low before age 40, and increases up to at least 70. The difference in incidence between men and women correlates with the difference in overall pattern of smoking tobacco, which is the predominant risk factor. However, for the same lifetime exposure to tobacco, risks are almost similar in men and women. In men, more than 80 percent of lung cancer cases are caused by smoking; in women the attributable risk is less: about 70 percent in Northern Europe, 45 percent worldwide.^{8, 124} The geographical and temporal patterns of lung cancer incidence are overwhelmingly determined by the use of tobacco. The highest incidence rates are recorded in North America, Europe, Japan, Australia, New Zealand and China and lowest in South America, Southern Asia and Africa.¹²⁵

Next to smoking, the other causes of lung cancer are known: environmental tobacco smoke (passive smoking); occupational exposures to asbestos, arsenic, chloromethyl ethers, chromium-VI, and nickel; residential and occupational exposures to radon; and exposures to carcinogenic air pollutants.¹²⁴⁻¹²⁶ Furthermore, risk factors include dietary factors^{124, 127} and genetic factors.¹²⁸⁻¹³⁰

In 1981, Doll and Peto assessed that 20 percent of lung cancer mortality was attributed to dietary factors.⁹⁷ Willet (1995) came to the same conclusion after revising the epidemiological studies and taking into account the studies published since.⁹⁸ Lung cancer can be prevented by dietary changes. Diets high in vegetables and fruits convincingly decrease risk; diets high in selenium, vitamin C and E possibly and diets high in carotenoids probably decrease risk; whereas diets high in total fat and alcohol possibly increase risk^{124, 127} (Table 1). Especially diets containing considerable amounts of a variety of vegetables (> 400 g/day) may reduce the relative risk of lung cancer by 45 percent.⁹⁹

Evidence from studies of familial aggregation of lung cancer suggests that genetic factors are involved in human lung tumor development. A positive familial history of lung cancer has been identified as a risk factor.¹³¹ More specifically, segregation analysis of lung cancer pro-band families indicates that a Mendelian codominant inheritance of a rare major autosomal gene is involved.¹³² In addition to the gene at this locus, several other genetic factors have been linked to lung cancer susceptibility. Genetic variants or polymorphisms have been identified that are hypothesized to affect lung cancer risk. In particular, genetic differences in the genes (CYP2D6, CYP1A1 and GSTM1) responsible for metabolism of tobacco carcinogens have been implicated in susceptibility of lung cancer.^{133, 134} Finally, lung cancer also appears at an increased rate in several genetic syndromes including the Li-Fraumeni syndrome, hereditary retinoblastoma, familial breast cancer, and the Bloom syndrome.¹²⁹

2.3.3 Lung Cancer: Genetic Alterations and Involved Pathways

During the past two decades, considerable efforts have been made to the identification of the genes that drive lung cancer development. Histologically apparent lung cancer is due to the sequential accumulation of specific genetic and morphologic changes to the normal epithelial cells of the lung. The major histological types of lung cancer are 1) non-small cell lung cancers (NSCLC), which represent about 80 percent of lung cancers and are divided into squamous cell carcinoma, adenocarcinoma, large cell carcinoma and mixed types, and 2) small cell lung cancers (SCLC), which represent about 20 percent of lung cancers. Some of the genetic alterations are specific to one of the two major lung cancer types and there are those that are common in both.^{128, 135-137} Until now, no single or combination of genetic events has been identified in 100 percent of lung cancer tissues.

Table 5 gives an overview of the main genetic alterations in lung cancer and the relative abundance in NSCLC and SCLC, respectively.¹²⁸ Particularly, these comprise

mutations or deletions of tumor suppressor genes (resulting in inactivation) and proto-oncogenes (resulting in activation), and epigenetic inactivation of these genes via DNA methylation (hypermethylation of gene promoter regions). The role of these genes and their involved pathways are shortly described.

Changes in several tumor suppressor genes are related to the development of lung cancer, including the APC and TP53 (See paragraph 2.2.3 for involved genetic pathways). Distinct from CRC, APC is not mutated in lung cancer, but it is inactivated through hypermethylation of the promoter region.^{128, 136, 137}

The p16-cyclin D1-cyclin-dependent kinase 4 (CDK4)-retinoblastoma (RB) pathway is disturbed in both SCLC and NSCLC. This pathway is central to controlling the G1 to S phase transition of the cell cycle. One of the four gene products that comprise this pathway is mutated or functionally altered in many human cancers; the two tumor suppressor gene products affected in lung cancer are p16 in NSCLC and RB in SCLC. Cyclin D1 overexpression can coexist with these changes, RB binds to the E2F transcription factor and therefore E2F cannot activate genes needed to initiate the S phase. Moreover, this complex also represses the transcription of other target genes. Phosphorylation of RB by cyclin D1/CDK4-6 releases E2F, which initiates the S phase. This pathway can be turned on by mutations inactivating RB, inactivating p16, overexpression of cyclin D1, or overexpression of CDK4. Other tumor suppressor genes affected in lung cancer are, for example, the fragile histidine triad gene (FHIT), RASSF1A, and CDH13. These genes undergo promoter hypermethylation, causing loss of expression. Other tumor suppressor genes present on chromosome 3p are FUS1, SEMA3B, 101F6, and NPRL2 which are affected by LOH.^{128, 134-137}

Next to changes in tumor suppressor genes, proto-oncogenes are affected in such a way that cell growth is stimulated. Furthermore, autocrine and paracrine growth factor stimulatory loops exist in lung cancer. Several, but not all components of these stimulatory pathways are proto-oncogene products. An important positive signaling pathway is gastrin-releasing peptide (GRP) and its receptor family. This growth stimulatory loop has a role in lung development and repair, but it also becomes 'reactivated' in lung cancer, particularly SCLC. Another important autocrine loop is the stem cell factor/tyrosine kinase receptor-oncogene KIT (SCF/KIT), also active in SCLC. An autocrine loop specific for NSCLC is the hypocyte growth factor/metastasis proto-oncogene (HGF/MET). The HGF stimulates mitogenesis or motogenesis of human bronchial epithelial, alveolar type II. Furthermore, NSCLC demonstrate abnormalities of the neuregulin receptors, ERBB1 and ERBB2, which are part of a family of transmembrane receptor tyrosine kinases. On ligand binding, ERBB receptors homo- or heterodimerize, thereby inducing intrinsic kinase activities that initiate intracellular signal transduction cascades, which regulates epithelial proliferation and differentiation. Overexpression of ERBB correlates with increased tumorigenicity of bronchial epithelial cells and metastatic potential of NSCLC.^{128, 136}

K-RAS is the most frequently activated RAS gene in lung cancer, resulting in inappropriate signaling for continued cell division (See involved pathway paragraph 2.2.3).^{128, 130, 136, 137} RAS signaling ultimately activates nuclear proto-oncogenes such as MYC, which, on heterodimerization, transcriptionally activates downstream genes that drive cells to grow. Activation occurs by gene amplification or by transcriptional dysregulation, both leading to protein overexpression.^{128, 130, 136, 137}

Other changes relevant in lung carcinogenesis are increased BCL-2 expression, telomerase activity, microsatellite instability and promoter hypermethylation. The product of the breakdown cluster lymphoma (BCL2) anti-apoptotic proto-oncogene is the key player in apoptosis, highly expressed in SCLC, making it less sensitive for apoptosis.^{128, 136}

Table 5. Major genetic alterations in lung cancer.¹²⁸

	SCLC (%)	NSCLC (%)
Tumor suppressor genes		
P53 abnormalities		
Mutation with 17p13 LOH	75-100	~50
Abnormal p53 expression	40-70	40-60
P16-cyclin D1-CDK4-RB pathway lesions		
P16 mutation or DNA methylation with 9p21 LOH	<0.1	10-40
Absent p16 expression	0-10	30-70
Absent RB expression with 13q14 LOH	~90	15-30
APC (5q21 LOH) DNA methylation	26	46
Chromosome 3p LOH (several sites)	100	90
RAR- β 3p24 (DNA methylation)	76	40
RASSF1A 3p21.3 (DNA methylation)	90	30-40
FHIT 3p14.2 (DNA methylation and deletion)	64	~50
CDH13 (DNA methylation)	20	45
Proto-oncogenes and growth stimulation		
Putative autocrine loops	GRP/GRP receptor SCF/KIT	HGF/MET NDF/ERBB
RAS mutation	<1	15-20
MYC amplification	15-30	5-10
Other changes		
BCL2 expression	75-95	10-35
Telomerase activity	~100	80-85
Microsatellite instability	~35	~22
Promoter hypermethylation	marker development	marker development

Abbreviations: LOH: loss of heterozygosity; CDK4: cyclin-dependent kinase 4; RB: retinoblastoma; APC: adenomatous polyposis coli; RAR- β : retinoic acid receptor β ; RASSF1A: tumor suppressor RASSF1 isoform A; FHIT: fragile histidine triad; CDH13: cadherin 13 (H-cadherin); GRP: gastrin releasing receptor; SCF: stem cell factor; KIT: tyrosine kinase receptor-oncogene KIT; HGF: hepatocyte growth factor; MET: metastasis oncogene; NDF: new differentiation factor; ERBB: ERBB1: epidermal growth factor receptor; ERBB2: Her2/neu.

2.3.4 Modulation of Lung Cancer (Risk) by Vegetables at the Genome Level

Although the evidence from epidemiological studies is fairly convincing that vegetables and/or vegetable components decrease the risk of lung cancer, a small amount of experimental studies have been conducted in lung tissues or lung cells exposed to vegetables or vegetable components. This paragraph will give an overview of a selection of *in vitro*-, animal-, and human studies in which the effect of vegetables or vegetable components on lung cancer risk is investigated. Studies focusing on gene or protein expression changes are of particular interest; these are summarized in Table 6.

In vitro studies: A few studies have examined the effect of vegetable components on gene and/or protein expression in lung cells^{138, 139}, thereby solely focusing on effects on cell cycle regulation. Also, other indicatives of lung cancer risk have not been examined in large amounts in *in vitro* lung systems.

Animal studies: In animals, a limited number of studies has investigated the effect of vegetable or vegetable components on gene and/or protein expression changes in the lung.^{28, 140, 141} However, in these studies, modulation of (mostly biotransformation) genes and enzymes in the lung was detected. As already stated in paragraph 2.2.4, in animals, the effect of vegetables or vegetable components on protein and gene expression is investigated in the liver^{26, 32, 33, 37, 40, 113} and small intestine^{26, 113} or on other outcomes than protein and gene expression in the colon, such as development of lung tumors.¹⁴²⁻¹⁴⁴

Human studies: According to literature review, no human studies have investigated the effect of vegetables or vegetable components on modulation of the expression of genes or protein in the lung (medline, accessed July 3rd 2004). As explained in paragraph 2.2.4, the investigations in other tissues such as lymphocytes^{116, 117} and urine¹¹⁸⁻¹²⁰, are limited. More research has been done in the field of human dietary intervention trials in which the effect of nutritional interventions on the occurrence of lung cancer has been investigated. The results of these trials are, as in colon cancer prevention trials, however, unacceptable.^{126, 127, 145-147}

In summary, the number of *in vitro* and animal studies in which the effect of vegetables on gene expression and/or protein expression changes at target level is examined is limited. No human studies have been reported in which the effect of vegetables or vegetable components on intermediate biomarkers for lung cancer is investigated. In addition, results of human intervention trials measuring inhibiting effects of vegetables on lung cancer occurrence, are disappointing. Again, more research is desired in which the effect of vegetables on the expression of multiple genes involved in various cancer preventive pathways is investigated, to give more insights into the genetic mechanisms underlying lung cancer prevention.

Table 6. Selection of *in vitro*, animal and human lung studies involved in modulation of gene or protein expression by vegetables or vegetable components.

Model system	Vegetable or -component	Methods	Main results	Ref.
I) <i>In vitro</i> studies				
C10, E9, and 82-132 murine lung epithelial cells C3H10T1/2 murine fibroblasts	β -carotene (1-10 μ M, 1-5 days treatment)	Growth inhibition assays Gap junctional intercellular communication (GJIC) assay Protein assay	No effect on growth, GJIC and connexin 43 protein expression in C10, E9 and 82-132 cells Growth reduction, enhancement of GJIC and connexin 43 protein expression in C3H10T1/2 murine fibroblasts	139
A549 human lung adenocarcinoma cells	Flavone (30, 60 and 100 μ M, for 24, 48 or 72 h)	Cell growth assay Cell cycle analysis Western blot analysis Northern blot analysis	Inhibition of cell growth Cell cycle arrest Induction of p21 mRNA and protein Inhibition of phosphorylation of Retinoblastoma protein	138
II) Animal studies				
Wister rats	Organosulfur compounds: diallyl sulfide, diallyl disulfide, dipropyl sulfide, or dipropyl disulfide (1 mmol/kg) by gavage daily (4 days)	Protein assays Immunoblot assay	Induction of UDP-glucuronyl transferase, microsomal epoxide hydrolase, GST activity and quinone reductase by diallyl disulfide	28
Sprague-Dawley rats	Glucoraphanin (120 or 240 mg/kg) by gavage in a single dose or daily during 4 days	Protein assays ESR technique	Induction of UDP-glucuronyltransferase and GST Induction of CYP enzymes Induction of reactive oxygen species	140
A/J mice	Non-oxidative vitamin E (100 mg/kg) single dose	Cell proliferation assay Northern blot analysis Protein assay	Reduced cell proliferation Inhibition of ornithine decarboxylase activity and reduction of mRNA	141
III) Until today, no human studies are reported (Medline, accessed July 3rd, 2004)				

It is clear from paragraph 2.2 and 2.3 that in colorectal as well as in lung cancer development a lot of gene modulations are involved. However, the involved genetic pathways by which vegetables exert their beneficial effect, are grossly unknown. Therefore, more research is needed, in which the effects of vegetables on multiple genes representing different genetic pathways, are investigated. This can be achieved by using microarray technology, which enables rapid examination of the expression of hundreds to thousands of genes in a single experiment. This technology, together with its implication in nutritional research, is explained in paragraph 3.

3 DNA Microarray Technology in Food and Nutrition Sciences

3.1 Gene Expression Profiling

The profile of genes expressed by different cells determines their phenotype, and thus provides insights into the molecular basis for health and disease. Changes in the multi-gene pattern of expression can provide clues about regulatory mechanisms and broader cellular functions and biochemical pathways. Various methods are available for detecting and quantitating gene expression levels, including northern blots¹⁴⁸, differential display¹⁴⁹, reverse transcription polymerase chain reaction (RT-PCR)^{150, 151} and serial analysis of gene expression (SAGE).¹⁵² The advent of the DNA microarray technology as the newest technology on gene expression measurements has enabled rapid and simultaneous comparison of mRNA levels for multiple genes in a single experiment. The use of DNA microarrays to study gene expression profiles of biological samples began in 1995.¹⁵³ Since that time, the technology required for making and using arrays, research fields applying the technology and statistical tools for data analyses have been extensively reviewed.¹⁵⁴⁻¹⁶¹

3.2 Nutrigenomics

The DNA microarray technique has been increasingly utilized in various fields of life sciences. In nutrition research, microarrays have already been used to study retardation of aging by caloric restriction¹⁶², effects of high-fat diet¹⁶³, effects of isoflavones¹⁶⁴, and obesity and diabetes.¹⁶⁵ This new research area is termed nutritional genomics or nutrigenomics. The fundamental concepts of the field are that the progression from a healthy phenotype to a chronic disease phenotype must occur by changes in gene expression or by differences in activities of proteins and enzymes, and that dietary chemicals directly or indirectly regulate the expression of genomic information.¹⁶⁶ Applied

wisely, it will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phase of a diet-related disease and to what extent individual sensitizing genotypes contribute to such diseases. The ultimate goal of nutrigenomics is to allow effective dietary-intervention strategies to recover normal homeostasis and to prevent diet-related diseases.¹⁶⁷

3.3 Proteomics

A complementary technology to DNA microarrays for monitoring gene expression is provided by proteomics, a term generally used to encapsulate the quantitation of all proteins, the determination of their post-translational modifications and how these are dependent on cell state and environmental influences. Proteins are frequently functional molecules and could therefore provide a better view to understanding gene function and reflecting the post-transcriptional regulation level. Some messengers are transcribed but not translated, and the number of mRNA copies does not necessarily reflect the number of functional protein molecules.¹⁶⁸ However, for most genes, changes in mRNA abundance are related to changes in protein abundance.^{169, 170} Furthermore, protein-based approaches are generally more difficult, less sensitive and have a lower throughput than RNA-based ones. Many methods have been developed for monitoring protein levels either directly or indirectly. Currently, protein screening and detection is being performed by two-dimensional polyacrylamide gel electrophoresis (2-DE) coupled with annotation of the detected proteins by mass spectroscopy, usually matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). Automated methods have been devised for running several gels in parallel but the major drawback to this method is the time needed for annotating the proteins by mass spectroscopy and comparing sequences back to expressed sequence tag (EST) databases. To solve this problem, newer methods have been developed using protein microarrays, although these are relatively expensive and the number of proteins present on these arrays is still limited.¹⁶⁹ The importance of the protein-based methods is that they measure the final expression product rather than an intermediate. In addition, some of them enable the detection of post-translational protein modifications and protein complexes, and in some cases, yield information about protein localization, none of which are obtained directly by measurements of mRNA.¹⁷¹

3.4 Microarray Platforms

The basic concept behind all DNA microarrays is the precise positioning of DNA fragments (probes) at high density on a solid support so that they can act as molecular

detectors. In practice, DNA microarrays vary according to the solid support used (such as glass or filters), the surface modifications with various substrates, the type of DNA fragments on the array (such as cDNA, short (~25 base) or long (50-80 base) oligonucleotides or genomic fragments), whether the gene fragments are presynthesized and deposited or synthesized in situ, and the machinery used to place the fragments on the array (such as ink-jet printing, spotting, mask or micromirror-based in situ synthesis). Currently, combinations of these variables are used to generate different types of DNA microarrays: filter arrays, spotted glass slide arrays, and in situ synthesized oligonucleotide arrays.¹⁷²⁻¹⁷⁴ A key consideration is whether to adopt a do-it-yourself approach and spot arrays in the laboratory or to purchase arrays from a commercial supplier.

3.5 Microarrays used in the presented Studies of this Thesis

For the human study (Chapter 2), gene expression analysis was carried out using the commercially available PHASE-1 Microarray Human-600 (PHASE-1 Molecular Toxicology, USA), which contain 597 sequence verified human genes, representing a number of biologically relevant and control genes. The genes were selected based on relevance for responses of cells to xenobiotic compounds and knowledge of the involved processes. The presented pathways relate to apoptosis, cell cycle, cell proliferation, DNA damage and repair, inflammation, metabolism, oxidative stress, peroxisome proliferators, transport, cell environment, etc. These pathways and involved genes play an important role in cancer development. Target genes are single stranded, approximately 500 nucleotides in length and spotted in quadruplicate on glass microscope slides (Table 7).

For the mouse studies (Chapter 3-6), a mouse cDNA microarray was constructed based on the genes present on the PHASE-1 Microarray Human-600.

Table 7. Gene Categories present on the PHASE-1 Human 600 microarray.

Gene Categories	Types of Genes in Category
Apoptosis	Caspases, BAK, Bax, Fas, Cyclins, TNFs
Cell Cycle	Cyclins, DNA Binding Protein, Waf 1
Cell proliferation	Kinases, Transcription Factors, Growth Factors and Receptors, Connexins
DNA Damage/ Repair	DNA Repair Genes, ERCC's, GADDs, Helicases, Topoisomerases
Inflammation	Serum Amyloids, Interleukins, Adhesion Molecules, Chemokines
Metabolism	P450s, Glucuronidation and Glutathione Enzymes, Methyltransferases
Oxidative stress	O ₂ Response Genes, Superoxide Dimutase, Redox Enzymes
Peroxisome Proliferators	Peroxisomal Enzymes
Transport	Multi-drug Resistance Proteins, Organic Anion and Cation Transporters
Cell-environment	Connexins, Integrins, Selectins, Cadherins

4 Aim and Outline of the Thesis

It is clear from both epidemiological and experimental studies, that regular consumption of vegetables decreases the risk of cancers at many target sites. Convincing evidence exists for the gastrointestinal tract (oral cavity, pharynx, esophagus, stomach, colon and rectum) and respiratory tract (lung). The epidemiological evidence, however, has recently weakened and there are many gaps in our knowledge. Numerous potentially anticarcinogenic agents are present in vegetables, which have various potential mechanisms of action. In many of these postulated mechanisms, modulation of gene expression plays a role. However, this has only been investigated for a limited number of genes and mostly applied to pseudo-target cells like lymphocytes instead of the ultimate target organ. The molecular targets at the genome level are mostly unknown. Technologies are being developed that allow the simultaneous determination of the expression of many thousands of genes at the mRNA (transcriptomics) and protein (proteomics) levels. These tools make it possible to assess the effect of a specific diet on the expression of multiple genes and proteins simultaneously. It can help to identify and characterize the basic molecular pathways of gene regulation by vegetables. The hypothesis investigated in the present thesis is that an important contribution of the anticarcinogenic effects of vegetables in the colorectum and lung is through modulating the expression of genes involved in biological and genetic pathways that are relevant for carcinogenesis. Therefore, the aim is to provide more insight into the molecular mechanisms by which vegetables exert their anticarcinogenic effects, by focusing on the effects at the genome level in the relevant target cells.

Therefore, the research topics of the present thesis are:

- To identify genes and involved pathways which are modulated by vegetables *in vivo* in colorectal mucosa from healthy female volunteers and sporadic adenoma patients; and to study if sporadic adenoma patients, who are at higher risk for developing colorectal cancer, respond differently compared to healthy controls;
- To investigate the dose-dependent effect of a mixture of vegetables on gene expression in the colon mucosa of C57BL6 female mice, to mimic the human dietary intervention study in which two different doses of a vegetable mixture were used;
- To investigate the dose-dependent effect of vegetables on protein expression in the colon mucosa of C57BL6 female mice;
- To study the contribution of four different vegetables on gene expression in colon mucosa of C57BL6 female mice;

- To investigate the systemic effect of different doses of a vegetable mixture and the role of the four specific vegetables in this mixture on gene expression in lung tissue of C57BL/6 female mice.

The outline of the thesis is as follows:

Chapter 2: Adenoma patients are at higher risk for developing colorectal cancer compared to healthy subjects. This chapter describes the results of a human dietary intervention study in which the effect of a decreased or increased intake of a mixture of vegetables (cauliflower, carrots, peas and onions) during two weeks on gene expression was investigated in biopsies from normal colorectal mucosa of female healthy volunteers and sporadic adenoma patients by applying cDNA microarray technology. Similarities and differences are described.

Chapter 3: The C57BL/6 mouse model is frequently used to study the effect of diet and/or gene expression. To investigate whether the effect of vegetables on gene expression changes in the colon is dose dependent, C57BL/6 female mice were fed one of four different diets containing different doses of a vegetable mixture consisting of the same combination and type of vegetables used in the human dietary intervention study.

Chapter 4: A proteomics approach was followed to analyze the proteome changes induced in the colon mucosa of the female C57BL/6 mice the vegetable mix study described in Chapter 3.

Chapter 5: Individual vegetables can have a particular effect on gene expression. Therefore, the role of the four specific vegetables used in the mix study described in Chapter 4 on gene expression in the colon is investigated in female C57BL/6 mice.

Chapter 6: From epidemiological evidence it is known that lung cancer can be protected by vegetables. The effects of vegetables on gene expression in this organ are studied in the same female C57BL/6 mice described in Chapters 3 and 5. The dose dependent effect of the mixture and the contribution of the specific vegetables are discussed.

Chapter 7: The main findings of the studies described in this thesis are discussed and summarized.

References

1. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* 1996;96:1027-39.
2. Fenwick GR, Heaney RK, Mullin WJ. Glucosinolates and their breakdown products in food and food plants. *Crit Rev Food Sci Nutr* 1983;18:123-201.
3. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* 1991;2:325-57.
4. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Chapter 6.3: Vegetables and Fruits. World Cancer Research Fund & American Institute for Cancer Research. Washington: BANTA Book group, 1997:436-446.
5. Bennion M. Chapter 14: Vegetables. In: *The science of food*. New York: John Wiley & Sons, 1980:217-244.
6. Stocks P, Karn MN. A co-operative study of the habits, home life, dietary and family histories of 450 cancer patients and of an equal number of control patients. *Ann Eugen Human Genet* 1933;5:30-33.
7. Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer* 1992;18:1-29.
8. World Health Organization & International Agency for Research on Cancer. *World Cancer Report*. IARC Press, 2003.
9. Food, Nutrition and the Prevention of Cancer: a global perspective. Washington: World Cancer Research Fund & American Institute for Cancer Research, 1997.
10. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Chapter 9.1: Prevention and its benefits. World Cancer Research Fund & American Institute for Cancer Research. Washington: BANTA Book group, 1997:538-548.
11. Brennan P, Fortes C, Butler J, Agudo A, Benhamou S, Darby S, Gerken M, Jokel KH, Kreuzer M, Mallone S, Nyberg F, Pohlabein H, Ferro G, Boffetta P. A multicenter case-control study of diet and lung cancer among non-smokers. *Cancer Causes Control* 2000;11:49-58.
12. Voorrips LE, Goldbohm RA, Verhoeven DT, van Poppel GA, Sturmans F, Hermus RJ, van den Brandt PA. Vegetable and fruit consumption and lung cancer risk in the Netherlands Cohort Study on diet and cancer. *Cancer Causes Control* 2000;11:101-15.
13. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser 2003;916:i-viii, 1-149.
14. Schatzkin A, Lanza E, Corle D, Lance P, Iber F, Caan B, Shike M, Weissfeld J, Burt R, Cooper MR, Kikendall JW, Cahill J. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group [see comments]. *N Engl J Med* 2000;342:1149-55.
15. Michels KB, Edward G, Joshipura KJ, Rosner BA, Stampfer MJ, Fuchs CS, Colditz GA, Speizer FE, Willett WC. Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers [In Process Citation]. *J Natl Cancer Inst* 2000;92:1740-52.
16. Voorrips LE, Goldbohm RA, van Poppel G, Sturmans F, Hermus RJ, van den Brandt PA. Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer.[In Process Citation]. *Am J Epidemiol* 2000;152:1081-92.
17. Key TJ, Allen NE, Spencer EA, Travis RC. Nutrition and breast cancer. *Breast* 2003;12:412-6.

18. Feskanih D, Ziegler RG, Michaud DS, Giovannucci EL, Speizer FE, Willett WC, Colditz GA. Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. *J Natl Cancer Inst* 2000;92:1812-23.
19. Smith-Warner SA, Spiegelman D, Yaun SS, Adami HO, Beeson WL, van den Brandt PA, Folsom AR, Fraser GE, Freudenheim JL, Goldbohm RA, Graham S, Miller AB, Potter JD, Rohan TE, Speizer FE, Toniolo P, Willett WC, Wolk A, Zeleniuch-Jacquotte A, Hunter DJ. Intake of fruits and vegetables and risk of breast cancer: a pooled analysis of cohort studies. *Jama* 2001;285:769-76.
20. Orner G, Dashwood W, Blum C, Di, az G, Li Q, Al-Fageeh M, Tebbutt N, Heath J, Ernst M, Dashwood R. Response of Apc(min) and A33(DeltaNbeta-cat) mutant mice to treatment with tea, sulindac, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Mutat Res* 2002;506:507-121.
21. Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of beta-catenin signaling by a combination of tea plus sulindac. *Carcinogenesis* 2003;24:263-7.
22. Shattuck-Brandt RL, Varilek GW, Radhika A, Yang F, Washington MK, DuBois RN. Cyclooxygenase 2 expression is increased in the stroma of colon carcinomas from IL-10(-/-) mice. *Gastroenterology* 2000;118:337-45.
23. van Kranen HJ, van Iersel PW, Rijnkels JM, Beems DB, Alink GM, van Kreijl CF. Effects of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the ApcMin mouse. *Carcinogenesis* 1998;19:1597-601.
24. Rijken PJ, Timmer WG, van de Kooij AJ, van Benschop IM, Wiseman SA, Meijers M, Tijburg LB. Effect of vegetable and carotenoid consumption on aberrant crypt multiplicity, a surrogate end-point marker for colorectal cancer in azoxymethane-induced rats. *Carcinogenesis* 1999;20:2267-72.
25. Rijnkels JM, Alink GM. Effects of a vegetables-fruit mixture on liver and colonic 1,2-dimethylhydrazine-metabolizing enzyme activities in rats fed low- or high-fat diets. *Cancer Lett* 1998;128:171-5.
26. Wortelboer HM, de Kruif CA, van Iersel AA, Noordhoek J, Blaauw BJ, van Bladeren PJ, Falke HE. Effects of cooked brussels sprouts on cytochrome P-450 profile and phase II enzymes in liver and small intestinal mucosa of the rat. *Food Chem Toxicol* 1992;30:17-27.
27. Edenharder R, Frangart J, Hager M, Hofmann P, Rauscher R. Protective effects of fruits and vegetables against in vivo clastogenicity of cyclophosphamide or benzo[a]pyrene in mice. *Food Chem Toxicol* 1998;36:637-45.
28. Guyonnet D, Siess MH, Le Bon AM, Suschetet M. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicol Appl Pharmacol* 1999;154:50-8.
29. Larsen-Su S, Williams DE. Dietary indole-3-carbinol inhibits FMO activity and the expression of flavin-containing monooxygenase form 1 in rat liver and intestine. *Drug Metab Dispos* 1996;24:927-31.
30. Talalay P, Fahey JW. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J Nutr* 2001;131:3027S-33S.
31. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000;21:921-7.
32. Bonnesen C, Stephensen PU, Andersen O, Sorensen H, Vang O. Modulation of cytochrome P-450 and glutathione S-transferase isoform expression in vivo by intact and degraded indolyl glucosinolates. *Nutr Cancer* 1999;33:178-87.

33. Katchamart S, Williams DE. Indole-3-carbinol modulation of hepatic monooxygenases CYP1A1, CYP1A2 and FMO1 in guinea pig, mouse and rabbit. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;129:377-84.
34. Smith TJ, Yang CS. Effect of organosulfur compounds from garlic and cruciferous vegetables on drug metabolism enzymes. *Drug Metabol Drug Interact* 2000;17:23-49.
35. Deschner EE, Ruperto J, Wong G, Newmark HL. Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis* 1991;12:1193-6.
36. Belman S. Onion and garlic oils inhibit tumor promotion. *Carcinogenesis* 1983;4:1063-5.
37. Sorensen M, Jensen BR, Poulsen HE, Deng X, Tygstrup N, Dalhoff K, Loft S. Effects of a Brussels sprouts extract on oxidative DNA damage and metabolising enzymes in rat liver. *Food Chem Toxicol* 2001;39:533-40.
38. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. *J Nutr* 2002;132:3490S-3493S.
39. Vang O, Jensen H, Autrup H. Induction of cytochrome P-450IA1, IA2, IIB1, IIB2 and IIE1 by broccoli in rat liver and colon. *Chem Biol Interact* 1991;78:85-96.
40. Zeng H, Davis CD, Finley JW. Effect of selenium-enriched broccoli diet on differential gene expression in min mouse liver(1,2). *J Nutr Biochem* 2003;14:227-231.
41. Kim DJ, Shin DH, Ahn B, Kang JS, Nam KT, Park CB, Kim CK, Hong JT, Kim YB, Yun YW, Jang DD, Yang KH. Chemoprevention of colon cancer by Korean food plant components. *Mutat Res* 2003;523-524:99-107.
42. Kararli TT. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm Drug Dispos* 1995;16:351-80.
43. Potter JD. Cancer prevention: epidemiology and experiment. *Cancer Lett* 1997;114:7-9.
44. Greenwald P, Clifford CK, Milner JA. Diet and cancer prevention. *Eur J Cancer* 2001;37:948-65.
45. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991;2:427-42.
46. Temple NJ, Kaiser Gladwin K. Fruit, vegetables, and the prevention of cancer. Research challenges. *Nutrition* 2003;19:467-70.
47. Manson MM, Gescher A, Hudson EA, Plummer SM, Squires MS, Prigent SA. Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicol Lett* 2000;112-113:499-505.
48. Steele VE, Boone CW, Lubet RA, Crowell JA, Holmes CA, Sigman CC, Kelloff GJ. Preclinical drug development paradigms for chemopreventives. *Hematol Oncol Clin North Am* 1998;12:943-61, v-vi.
49. De Flora S. Mechanisms of inhibitors of mutagenesis and carcinogenesis. *Mutat Res* 1998;402:151-8.
50. Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Boone CW, Malone WA, Hawk ET, Lieberman R, Lawrence JA, Kopelovich L, Ali I, Viner JL, Sigman CC. Progress in cancer chemoprevention. *Ann N Y Acad Sci* 1999;889:1-13.
51. Dragsted LO, Strube M, Larsen JC. Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacol Toxicol* 1993;72:116-35.
52. Wattenberg LW. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res* 1992;52:2085s-2091s.
53. Jacobs LR. Fiber and colon cancer. *Gastroenterol Clin North Am* 1988;17:747-60.
54. Bartsch H, Ohshima H, Pignatelli B. Inhibitors of endogenous nitrosation. Mechanisms and implications in human cancer prevention. *Mutat Res* 1988;202:307-24.

55. Lampe JW, Peterson S. Brassica, biotransformation and cancer risk: genetic polymorphisms alter the preventive effects of cruciferous vegetables. *J Nutr* 2002;132:2991-4.
56. van Poppel G, Verhoeven DT, Verhagen H, Goldbohm RA. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv Exp Med Biol* 1999;472:159-68.
57. Nijveldt RJ, van Nood E, van Hooft DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 2001;74:418-25.
58. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 2002;22:19-34.
59. Bianchini F, Vainio H. Allium vegetables and organosulfur compounds: do they help prevent cancer? *Environ Health Perspect* 2001;109:893-902.
60. Verhoeven DT, Verhagen H, Goldbohm RA, van den Brandt PA, van Poppel G. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chem Biol Interact* 1997;103:79-129.
61. Murillo G, Mehta RG. Cruciferous vegetables and cancer prevention. *Nutr Cancer* 2001;41:17-28.
62. Awasthi YC, Singhal SS, Awasthi S. Mechanisms of anticarcinogenic effects of antioxidant nutrients. In: Watson RR, Mufti SI, eds. *Nutrition and Cancer Prevention*. Boca Raton, Florida: CRC Press, Inc., 1996:139-172.
63. Collins AR, Olmedilla B, Southon S, Granado F, Duthie SJ. Serum carotenoids and oxidative DNA damage in human lymphocytes. *Carcinogenesis* 1998;19:2159-62.
64. van Poppel G, Goldbohm RA. *Food and cancer prevention: the role of beta-carotene and cancer prevention*. *Am J Clin Nutr* 1995;62:1393S-1402S.
65. De Flora S, Zancchi P, Izotti H, Hayatsu H. Mechanisms of food-borne inhibitors of genotoxicity relevant to cancer prevention. In: Hayatsu H, ed. *Mutagens in Food. Detection and Prevention*. Boca Raton: CRC Press, 1991:157-180.
66. Dutta A, Dutta SK. Vitamin E and its role in the prevention of atherosclerosis and carcinogenesis: a review. *J Am Coll Nutr* 2003;22:258-68.
67. Troll W, Wiesner R, Frenkel K. Anticarcinogenic action of protease inhibitors. *Adv Cancer Res* 1987;49:265-83.
68. Gamet-Payrastré L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J, Terce F. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res* 2000;60:1426-33.
69. Johnson IT. Anticarcinogenic effects of diet-related apoptosis in the colorectal mucosa. *Food Chem Toxicol* 2002;40:1171-8.
70. Adams J. Proteasome inhibition: a novel approach to cancer therapy. *Trends Mol Med* 2002;8:S49-54.
71. Frommel TO, Mobarhan S, Doria M, Halline AG, Luk GD, Bowen PE, Candel A, Liao Y. Effect of beta-carotene supplementation on indices of colonic cell proliferation. *J Natl Cancer Inst* 1995;87:1781-7.
72. Palozza P, Serini S, Maggiano N, Angelini M, Boninsegna A, Di Nicuolo F, Ranelletti FO, Calviello G. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by beta-carotene through down-regulation of cyclin A and Bcl-2 family proteins. *Carcinogenesis* 2002;23:11-8.

73. Phillips RW, Kikendall JW, Luk GD, Willis SM, Murphy JR, Maydonovitch C, Bowen PE, Stacewicz-Sapuntzakis M, Wong RK. beta-Carotene inhibits rectal mucosal ornithine decarboxylase activity in colon cancer patients. *Cancer Res* 1993;53:3723-5.
74. Cover CM, Hsieh SJ, Tran SH, Hallden G, Kim GS, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J Biol Chem* 1998;273:3838-47.
75. Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* 2000;60:3823-31.
76. Bonnesen C, Eggleston IM, Hayes JD. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res* 2001;61:6120-30.
77. McBurney MW, Costa S, Pratt MA. Retinoids and cancer: a basis for differentiation therapy. *Cancer Invest* 1993;11:590-8.
78. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. *Med Res Rev* 2003;23:519-34.
79. Honma Y, Okabe-Kado J, Kasukabe T, Hozumi M, Umezawa K. Inhibition of abl oncogene tyrosine kinase induces erythroid differentiation of human myelogenous leukemia K562 cells. *Jpn J Cancer Res* 1990;81:1132-6.
80. Bertram JS. Carotenoids and gene regulation. *Nutr Rev* 1999;57:182-91.
81. Bertram JS, Bortkiewicz H. Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. *Am J Clin Nutr* 1995;62:1327S-1336S.
82. Schelp FP, Pongpaew P. Protection against cancer through nutritionally-induced increase of endogenous proteinase inhibitors—a hypothesis. *Int J Epidemiol* 1988;17:287-92.
83. Meydani SN, Meydani M, Blumberg JB, Leka LS, Siber G, Loszewski R, Thompson C, Pedrosa MC, Diamond RD, Stollar BD. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *Jama* 1997;277:1380-6.
84. Siegel BV. Enhancement of interferon production by poly(rI)-poly(rC) in mouse cell cultures by ascorbic acid. *Nature* 1975;254:531-2.
85. Reifen R. Vitamin A as an anti-inflammatory agent. *Proc Nutr Soc* 2002;61:397-400.
86. Hughes DA. Effects of carotenoids on human immune function. *Proc Nutr Soc* 1999;58:713-8.
87. Michnovicz JJ, Bradlow HL. Altered estrogen metabolism and excretion in humans following consumption of indole-3-carbinol. *Nutr Cancer* 1991;16:59-66.
88. Messina M, Barnes S. The role of soy products in reducing risk of cancer. *J Natl Cancer Inst* 1991;83:541-6.
89. Tosetti F, Ferrari N, De Flora S, Albini A. Angioprevention¹: angiogenesis is a common and key target for cancer chemopreventive agents. *Faseb J* 2002;16:2-14.
90. Meng Q, Qi M, Chen DZ, Yuan R, Goldberg ID, Rosen EM, Auburn K, Fan S. Suppression of breast cancer invasion and migration by indole-3-carbinol: associated with up-regulation of BRCA1 and E-cadherin/catenin complexes. *J Mol Med* 2000;78:155-65.
91. Gartner LP, Hiatt JL. Chapter 17: Digestive System: Alimentary Canal. *Color textbook of histology*, second edition. Philadelphia: W.B. Saunders Company, 2001:379-409.
92. Troisi RJ, Freedman AN, Devesa SS. Incidence of colorectal carcinoma in the U.S.: an update of trends by gender, race, age, subsite, and stage, 1975-1994. *Cancer* 1999;85:1670-6.
93. Parkin DM. Epidemiology of cancer: global patterns and trends. *Toxicol Lett* 1998;102-103:227-34.
94. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999;91:916-32.

95. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Chapter 4.10: Colon, rectum. World Cancer Research Fund & American Institute for Cancer Research. Washington: BANTA Book group, 1997:216-251.
96. Bruce WR, Giacca A, Medline A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev* 2000;9:1271-9.
97. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 1981;66:1191-308.
98. Willett WC. Diet, nutrition, and avoidable cancer. *Environ Health Perspect* 1995;103 Suppl 8:165-70.
99. van't Veer P, Jansen MC, Klerk M, Kok FJ. Fruits and vegetables in the prevention of cancer and cardiovascular disease. *Public Health Nutr* 2000;3:103-7.
100. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med* 2003;348:919-32.
101. Syngal S, Clarke G, Bandipalliam P. Potential roles of genetic biomarkers in colorectal cancer chemoprevention. *J Cell Biochem Suppl* 2000;34:28-34.
102. Levin B. Genetic syndromes as potential targets for chemoprevention of colorectal neoplasia. *J Cell Biochem Suppl* 2000;34:19-22.
103. Fearon ER, Jones PA. Progressing toward a molecular description of colorectal cancer development. *Faseb J* 1992;6:2783-90.
104. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
105. Fujiwara T, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B, Hamilton SR. Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. *Am J Pathol* 1998;153:1063-78.
106. Grady WM, Markowitz SD. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet* 2002;3:101-28.
107. Ilyas M, Straub J, Tomlinson IP, Bodmer WF. Genetic pathways in colorectal and other cancers. *Eur J Cancer* 1999;35:335-51.
108. Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000;119:854-65.
109. Arends JW. Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 2000;190:412-6.
110. Hu R, Kim BR, Chen C, Hebbar V, Kong AN. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis* 2003;24:1361-7.
111. Clapper ML, Szarka CE, Pfeiffer GR, Graham TA, Balshem AM, Litwin S, Goosenberg EB, Frucht H, Engstrom PF. Preclinical and clinical evaluation of broccoli supplements as inducers of glutathione S-transferase activity. *Clin Cancer Res* 1997;3:25-30.
112. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens Regulate Vitamin D Metabolism in the Mouse Colon: Relevance for Colon Tumor Prevention and Therapy. *J Nutr* 2002;132:3490S-3493S.
113. Spornins VL, Venegas PL, Wattenberg LW. Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J Natl Cancer Inst* 1982;68:493-6.
114. Mori H, Tanaka T, Sugie S, Yoshimi N, Kawamori T, Hirose Y, Ohnishi M. Chemoprevention by naturally occurring and synthetic agents in oral, liver, and large bowel carcinogenesis. *J Cell Biochem Suppl* 1997;27:35-41.

115. Kato T, Hancock R, Mohammadpour H, McGregor B, Manalo P, Khaiboullina S, Hall M, Pardini L, Pardini R. Influence of omega-3 fatty acids on the growth of human colon carcinoma in nude mice. *Cancer Lett* 2002;187:169.
116. Nijhoff WA, Grubben MJ, Nagengast FM, Jansen JB, Verhagen H, van Poppel G, Peters WH. Effects of consumption of Brussels sprouts on intestinal and lymphocytic glutathione S-transferases in humans. *Carcinogenesis* 1995;16:2125-8.
117. Persson I, He L, Fang C, Normen L, Rylander R. Influence of vegetables on the expression of GSTP1 in humans—a pilot intervention study (Sweden). *Cancer Causes Control* 2000;11:359-61.
118. Lampe JW, King IB, Li S, Grate MT, Barale KV, Chen C, Feng Z, Potter JD. Brassica vegetables increase and apiaceous vegetables decrease cytochrome P450 1A2 activity in humans: changes in caffeine metabolite ratios in response to controlled vegetable diets. *Carcinogenesis* 2000;21:1157-62.
119. Kall MA, Vang O, Clausen J. Effects of dietary broccoli on human in vivo drug metabolizing enzymes: evaluation of caffeine, oestrone and chlorzoxazone metabolism [see comments]. *Carcinogenesis* 1996;17:793-9.
120. Pantuck EJ, Pantuck CB, Anderson KE, Wattenberg LW, Conney AH, Kappas A. Effect of brussels sprouts and cabbage on drug conjugation. *Clin Pharmacol Ther* 1984;35:161-9.
121. Faivre J, Couillaud C, Belghiti C. Intervention trials on colorectal cancer prevention. *IARC Sci Publ* 1996;115-24.
122. MacLennan R, Macrae F, Bain C, Battistutta D, Chapuis P, Gratten H, Lambert J, Newland RC, Ngu M, Russell A, et al. Randomized trial of intake of fat, fiber, and beta carotene to prevent colorectal adenomas. *The Australian Polyp Prevention Project [see comments]. J Natl Cancer Inst* 1995;87:1760-6.
123. Gartner LP, Hiatt JL. Chapter 15: Respiratory System. *Color textbook of histology*, second edition. Philadelphia: W.B. Saunders Company, 2001:343-364.
124. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Chapter 4.5: Lung. World Cancer Research Fund & American Institute for Cancer Research. Washington: BANTA Book group, 1997:130-147.
125. Alberg AJ, Samet JM. Epidemiology of lung cancer. *Chest* 2003;123:21S-49S.
126. Omenn GS. Chemoprevention of lung cancer: the rise and demise of beta-carotene. *Annu Rev Public Health* 1998;19:73-99.
127. Ziegler RG, Mayne ST, Swanson CA. Nutrition and lung cancer. *Cancer Causes Control* 1996;7:157-77.
128. Fong KM, Minna JD. Molecular biology of lung cancer: clinical implications. *Clin Chest Med* 2002;23:83-101.
129. You M, Bergman G. Preclinical and clinical models of lung cancer chemoprevention. *Hematol Oncol Clin North Am* 1998;12:1037-53.
130. Szabo E, Mulshine J. Epidemiology, prognostic factors, and prevention of lung cancer. *Curr Opin Oncol* 1993;5:302-9.
131. Ooi WL, Elston RC, Chen VW, Bailey-Wilson JE, Rothschild H. Increased familial risk for lung cancer. *J Natl Cancer Inst* 1986;76:217-22.
132. Sellers TA, Bailey-Wilson JE, Elston RC, Wilson AF, Elston GZ, Ooi WL, Rothschild H. Evidence for mendelian inheritance in the pathogenesis of lung cancer. *J Natl Cancer Inst* 1990;82:1272-9.
133. Vineis P, Malats N, Lang A, d'Errico A, Caporaso N, Cuzick J, Boffetta P. Metabolic polymorphisms and susceptibility to cancer. *IARC Scientific Publications No.148*, 1999.

134. Spivack SD, Fasco MJ, Walker VE, Kaminsky LS. The molecular epidemiology of lung cancer. *Crit Rev Toxicol* 1997;27:319-65.
135. Zochbauer-Muller S, Gazdar AF, Minna JD. Molecular pathogenesis of lung cancer. *Annu Rev Physiol* 2002;64:681-708.
136. Sekido Y, Fong KM, Minna JD. Molecular genetics of lung cancer. *Annu Rev Med* 2003;54:73-87.
137. Sanchez-Cespedes M. Dissecting the genetic alterations involved in lung carcinogenesis. *Lung Cancer* 2003;40:111-21.
138. Bai F, Matsui T, Ohtani-Fujita N, Matsukawa Y, Ding Y, Sakai T. Promoter activation and following induction of the p21/WAF1 gene by flavone is involved in G1 phase arrest in A549 lung adenocarcinoma cells. *FEBS Lett* 1998;437:61-4.
139. Banoub RW, Fernstrom M, Ruch RJ. Lack of growth inhibition or enhancement of gap junctional intercellular communication and connexin43 expression by beta-carotene in murine lung epithelial cells in vitro. *Cancer Lett* 1996;108:35-40.
140. Paolini M, Perocco P, Canistro D, Valgimigli L, Pedulli GF, Iori R, Croce CD, Cantelli-Forti G, Legator MS, Abdel-Rahman SZ. Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. *Carcinogenesis* 2004;25:61-7.
141. Yano T, Yano Y, Yajima S, Kumadaki I, Ichikawa T, Otani S, Hagiwara K. The suppression of ornithine decarboxylase expression and cell proliferation at the promotion stage of lung tumorigenesis in mice by alpha-tocopheryloxybutyric acid. *Biochem Pharmacol* 2001;61:1177-81.
142. Khanduja KL, Gandhi RK, Pathania V, Syal N. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. *Food Chem Toxicol* 1999;37:313-8.
143. Chung FL, Morse MA, Eklind KI, Xu Y. Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis by compounds derived from cruciferous vegetables and green tea. *Ann N Y Acad Sci* 1993;686:186-201; discussion 201-2.
144. Chung FL, Kelloff G, Steele V, Pittman B, Zang E, Jiao D, Rigotty J, Choi CI, Rivenson A. Chemopreventive efficacy of arylalkyl isothiocyanates and N-acetylcysteine for lung tumorigenesis in Fischer rats. *Cancer Res* 1996;56:772-8.
145. Omenn GS, Goodman G, Thornquist M, Grizzle J, Rosenstock I, Barnhart S, Balmes J, Cherniack MG, Cullen MR, Glass A, et al. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers. *Cancer Res* 1994;54:2038s-2043s.
146. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med* 1994;330:1029-35.
147. Kelley MJ, McCrory DC. Prevention of lung cancer: summary of published evidence. *Chest* 2003;123:50S-59S.
148. Thomas PS. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci U S A* 1980;77:5201-5.
149. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992;257:967-71.
150. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-93.
151. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002;29:23-39.

152. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. *Science* 1995;270:484-7.
153. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray [see comments]. *Science* 1995;270:467-70.
154. Ball CA, Sherlock G, Parkinson H, Rocca-Sera P, Brooksbank C, Causton HC, Cavalieri D, Gaasterland T, Hingamp P, Holstege F, Ringwald M, Spellman P, Stoeckert CJ, Jr., Stewart JE, Taylor R, Brazma A, Quackenbush J. Standards for microarray data. *Science* 2002;298:539.
155. Butte A. The use and analysis of microarray data. *Nat Rev Drug Discov* 2002;1:951-60.
156. Cheung VG, Morley M, Aguilar F, Massimi A, Kucherlapati R, Childs G. Making and reading microarrays. *Nat Genet* 1999;21:15-9.
157. Debouck C, Goodfellow PN. DNA microarrays in drug discovery and development. *Nat Genet* 1999;21:48-50.
158. Habeck M. New approach to gene expression analysis. *Drug Discov Today* 2003;8:427-8.
159. Holloway AJ, Van Laar RK, Tothill RW, Bowtell DD. Options available-from start to finish-for obtaining data from DNA microarrays II. *Nat Genet* 2002;32 Suppl 2:481-9.
160. Kerr MK, Churchill GA. Statistical design and the analysis of gene expression microarray data. *Genet Res* 2001;77:123-8.
161. Lemieux B, Aharoni A, Schena M. Overview of DNA chip technology. *Molecular Breeding* 1998;4:277-89.
162. Lee CK, Klopp RG, Weindrich R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction [see comments]. *Science* 1999;285:1390-3.
163. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R, Nair KS. Impact of high-fat diet and antioxidant supplement on mitochondrial functions and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab* 2002;282:E1055-61.
164. Chen CC, Shieh B, Jin YT, Liao YE, Huang CH, Liou JT, Wu LW, Huang W, Young KC, Lai MD, Liu HS, Li C. Microarray profiling of gene expression patterns in bladder tumor cells treated with genistein. *J Biomed Sci* 2001;8:214-22.
165. Nadler ST, Attie AD. Please pass the chips: genomic insights into obesity and diabetes. *J Nutr* 2001;131:2078-81.
166. Kaput J, Rodriguez RL. Nutritional genomics: the next frontier in the postgenomic era. *Physiol Genomics* 2004;16:166-77.
167. Muller M, Kersten S. Opinion: Nutrigenomics: goals and strategies. *Nat Rev Genet* 2003;4:315-22.
168. Russo G, Zegar C, Giordano A. Advantages and limitations of microarray technology in human cancer. *Oncogene* 2003;22:6497-507.
169. Cunningham MJ. Genomics and proteomics: the new millennium of drug discovery and development. *J Pharmacol Toxicol Methods* 2000;44:291-300.
170. Sydor JR, Nock S. Protein expression profiling arrays: tools for the multiplexed high-throughput analysis of proteins. *Proteome Sci* 2003;1:3.
171. Lockhart DJ, Winzler EA. Genomics, gene expression and DNA arrays. *Nature* 2000;405:827-36.
172. Murphy D. Gene expression studies using microarrays: principles, problems, and prospects. *Adv Physiol Educ* 2002;26:256-70.
173. Barrett JC, Kawasaki ES. Microarrays: the use of oligonucleotides and cDNA for the analysis of gene expression. *Drug Discov Today* 2003;8:134-41.
174. Goldsmith ZG, Dhanasekaran N. The microrevolution: applications and impacts of microarray technology on molecular biology and medicine (review). *Int J Mol Med* 2004;13:483-95.

Chapter 2

Altered vegetable intake affects pivotal carcinogenesis pathways in colon mucosa from adenoma patients and controls

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Abstract

The evidence from epidemiological and experimental studies that vegetables reduce the risk of colorectal cancer is convincing. However, the involved genes and genetic pathways are not clear. The aim of this study was to identify genes that are modulated *in vivo* in colorectal mucosa by vegetables, and to investigate whether colon adenoma patients respond differently compared to healthy controls. Twenty female adenoma patients and eight healthy controls were randomly split into two groups of ten and four persons respectively, receiving either a 50% decreased (= 75 g/day) or doubled (= 300 g/day) intake of vegetables for two weeks. In order to assess effects on gene expression at target level, colorectal biopsies were collected before and after the intervention. Total RNA was isolated from the biopsies to measure gene expression of 597 genes relevant for responses to xenobiotics by microarray technology, followed by confidence analyses to identify differentially expressed genes. Mainly genes related to cell cycle control and genes for oxidoreductase activities were over-represented in the list of modulated genes. Twenty genes were modulated which are known to be related to (colon)carcinogenesis. Seven genes were similarly modulated in patients and controls, for example *fos* proto-oncogene and ornithine decarboxylase. Thirteen genes were modulated differently in patients compared to controls, including cyclooxygenase-2 and Human mdm2-A in patients and cytochrome P450-27B1, -2C19, -2D6, -2C9, and -3A4 in controls. Almost all the effects on modulating the expression of genes by altering vegetable intake can be mechanistically linked to cellular processes that explain either prevention of colorectal cancer risk by high vegetable intake or increased colorectal cancer risk by low vegetable intake. Furthermore, it seems that vegetables in patients affect genes involved in late stage of colorectal cancer, whereas in controls genes involved in the initiation phase are modulated.

Introduction

Worldwide, colorectal cancer (CRC) is one of the most common cancers. Approximately 943,000 new cases were diagnosed and 491,000 people died of CRC in the year 2000.¹ Although early detection of CRC is important, prevention has more potential for control of cancer.²

The predominantly modifiable determinant of CRC is the diet. The most consistent finding is the association of vegetables with reduced risk of CRC.¹⁻⁴ Numerous potentially anticarcinogenic agents are present in vegetables which have various potential mechanisms of action in the initiation and later stages of carcinogenesis. Compounds classified as blocking agents can prevent, or greatly reduce, initiation of carcinogenesis by altering the profile of both phase I and II drug metabolizing enzymes, by scavenging reactive oxygen and other free radical species and by altering rates of DNA repair. Suppressing agents affect later stages of carcinogenesis by reducing cell proliferation. This involves modulation of signal transduction pathways, leading to altered gene expression, cellcycle arrest or apoptosis and thereby preventing the accumulation of damaged cells.^{2, 5-7}

A genetic model for CRC has been presented by Fearon and Vogelstein⁸ which has been subsequently extended with additional genetic events and specific molecular pathways⁹ presenting a network of molecular genetic pathways.⁸⁻¹⁰ These genetic pathways and the involved genes are obvious molecular targets for the protection against CRC by vegetables. Nowadays available microarray technology makes it feasible to assess the effect of a specific diet on the expression of multiple genes simultaneously.¹¹

The aim of this study was to identify genes that are modulated *in vivo* in colorectal mucosa by vegetables, and to investigate whether colon adenoma patients respond differently in comparison to healthy controls. Therefore, a human dietary intervention study was carried out in which the effect of an increased or decreased intake of vegetables on gene expression was investigated in biopsies from normal colorectal mucosa of female sporadic adenoma patients and healthy controls by applying microarray technology. A descriptive evaluation was made of the gene expression results with respect to their possible role in mechanisms underlying CRC risk.

Materials and Methods

Subjects and Study Design

The study population was recruited at the Maasland Hospital Sittard, the Netherlands. Twenty female patients (mean age 61.5 ± 7.7 years, range 53-78 years) and eight healthy female controls (mean age 48.1 ± 10.2 years, range 33-65 years) were selected from the

colonoscopy control schedule. Eligible patients had a history of colorectal adenomas but no familial CRC syndrome or inflammatory bowel disease; eligible controls had no history of colorectal adenomas or other colorectal disorders. No colorectal mucosal diseases were permitted in patients as well as in controls. Existing adenomas were endoscopically removed during the examination. These two groups were chosen to investigate whether the effect of the intervention is different for patients, compared to controls. It is plausible that vegetables exert a different effect in persons who are at higher risk for the development of CRC.

Both patients and controls were randomly split into two groups of ten and four persons respectively, receiving either a low (75 g/day) or high (300 g/day) vegetable diet for a period of two weeks. The length of this intervention period was chosen based on results of other studies, in which the effect of a vegetable intervention on gene expression changes was examined in various tissues including lymphocytes and colorectal mucosa. In these studies, the length of intervention was equal or even shorter than two weeks and still a modulation of gene expression (mostly biotransformation genes) was reported.¹²⁻¹⁶ Next, taking into account that the mean intake of vegetables of adult females in the Netherlands is approximately 150 g/day¹⁷, this implies that, on average, subjects in the low vegetable group decreased their vegetable intake by 50% and subjects in the high vegetable group doubled their vegetable intake. A frozen vegetable mix was provided, consisting of 30% wt/wt cauliflower, 30% wt/wt peas, 30% wt/wt carrots and 10% wt/wt onions (Findus, Sweden) together with instructions for preparation. During the intervention period, subjects followed their normal dietary habits with, however, adapting their vegetable consumption in that sense that they were allowed to consume only the provided vegetables.

Four rectal biopsies and 20 ml peripheral blood were sampled at the first endoscopic examination and at the day following the last day of the intervention period. Biopsies were taken from healthy mucosal tissue of the rectum 10 cm above the level of the anus, immediately frozen in liquid nitrogen and stored at -80 °C until use. The rectum and colon are regarded as one anatomical and functional entity and therefore rectal biopsies represent colonic environment.¹⁸ Aliquots of blood plasma were stored at -20 °C until analyses. In order to assess the compliance with the intervention, levels of α - and β -carotene, retinol and α -tocopherol in plasma were determined by the method of Hess et al¹⁹ by HPLC-UV. By means of questionnaires, the dietary and life-style habits on two consecutive days before and during the intervention period were recorded. Energy and nutrient intake were calculated using the computerized Dutch Nutrient Data bank.²⁰

This study was approved by the Medical Ethical Committee of the Maasland Hospital in Sittard and written informed consent was obtained from the participants prior to the start of the study.

Total RNA Isolation and cDNA Probe Synthesis

Four biopsies (ca. 20 mg in total) from each subject were pooled and ground to a powder in a stainless steel mortar under liquid nitrogen and homogenized in 800 μ l TRIZOL Reagent (Gibco/BRL). Total RNA was extracted according to the manufacturer's instructions. The RNeasy® Mini Kit (Qiagen) was used to purify total RNA from salts and residual DNA. Quantity of each RNA sample was measured by a spectrophotometer and varied from 25 to 50 μ g. Integrity was determined by a Bioanalyzer (Agilent Technologies Netherlands B.V., The Netherlands). All samples contained intact total RNA with an rRNA ratio (28S/18S) > 1.5.

Cyanine 3- (Cy3) and Cyanine 5- (Cy5) labeled cDNA probes were prepared using 10 μ g total RNA from each subject before and after the intervention period, respectively, by the method of Hasseman et al.²¹ Each subject acted as its own control, meaning that the fluorescent-labeled cDNAs of each subject before and after the intervention were mixed. A second microarray experiment was carried out for each subject whereby the dyes were switched (flip-dye experiment).

Microarray Experiments

Gene expression analysis was on the PHASE-1 Microarray Human-600 (PHASE-1 Molecular Toxicology, Santa fee, USA), which contain 597 sequence verified human genes, representing biologically relevant and control genes. Each array batch passed the slide quality index in which several parameters such as signal to noise ratios, COVs, laser intensities, and others were taken into account. PHASE-1 has routinely confirmed their microarrays by using RT-PCR, histological analysis and other accurate measures of expression. The genes were selected based on relevance for responses of cells to xenobiotic compounds and knowledge of the involved processes. The presented pathways relate to inflammation, DNA damage and repair, oxidative stress, cell signaling, cell proliferation, detoxification, metabolism, transcription and apoptosis, which all relate to the genetic model of CRC.⁸⁻¹⁰ Target genes are single stranded, approximately 500 nucleotides in length and spotted in quadruplicate on glass microscope slides.

Labeled cDNA probe of each subject was hybridized to the PHASE-1 microarray according to the manufacturer's instructions. Slides were scanned on a GMS 418 Array Scanner (Affymetrix, High Wycombe, UK). Both Cy3 (532 nm) and Cy5 (635 nm) channels were scanned at a photomultiplier setting of 65%. Laser power was adjusted until there were no saturated spots. The images obtained (resolution 10 micron; 16 bit tiff image) were processed with ImaGene 5.0 software (Biodiscovery, Inc., Los Angeles, USA) to measure mean signal intensities for spots and local background.

Microarray Data Analyses

Data were transferred to GeneSight 4.0 (Biodiscovery) for analysis. Flagged spots were excluded. For each spot, local background intensity was subtracted. The background corrected mean intensities were log transformed (base 2). Next, the expression difference for each spot was calculated by subtracting the log transformed mean intensity from the sample before the intervention from the log transformed mean intensity from the sample after the intervention. Expression differences were normalized using Lowess and the four replicates on each array of each gene were combined to a mean expression difference with exclusion of outliers (> 2 standard deviations).

To identify up- and down-regulated genes within a vegetable group 99.9% confidence intervals were calculated (method based on Kerr et al²²). This very stringent interval was chosen to reduce the chance on false positive genes to 0.1%. Cut-off levels were set at $+ 0.25$ and $- 0.25$ respectively, because most of the genes were located within these boundaries.

The list of up- and down-regulated genes was further analyzed by means of the online software application Expression Analysis Systematic Explorer (EASE; <http://david.niaid.nih.gov/david/ease.htm>).²³ EASE automates the process of biological theme determination and performs a statistical analysis of gene categories in the provided gene list to find those gene categories that are the most over-presented in the list of differentially expressed genes by a Fisher exact test.

Additional Statistical Analyses

Statistical analysis of the questionnaires- and plasma-data was carried out using SPSS version 6.1.1 for Macintosh. The Shapiro-Wilks test was used to test normality of the datasets. Potential differences between the groups were assessed using the non-parametric Mann-Whitney U test or unpaired t-test. The non-parametric Wilcoxon Matched-Pairs Signed Ranks Test or the paired t-test was used to assess the differences within a vegetable group. All the tests were two-sided, and a p-value < 0.05 was considered to indicate statistical significance.

Results*Dietary Questionnaires and Plasma Values*

The results of the dietary questionnaires and plasma values are presented in Table 1. Mean age of the patients was higher compared to controls ($p < 0.01$). No other significant differences between the groups before the intervention were observed. Mean vegetable intake was indeed approximately 150 g/day (in agreement with¹⁷), meaning that the subjects increased or decreased their intake during the intervention by a factor 2.

Table 1. Reported daily dietary intake before and during the intervention period and plasma levels of α - and β -carotene, retinol and α -tocopherol before and after the intervention period.¹

Dietary intake	Patients (N = 20)				Controls (N = 8)			
	Low (75 g/day) vegetable group (N = 10)		High (300 g/day) vegetable group (N = 10)		Low (75 g/day) vegetable group (N = 4)		High (300 g/day) vegetable group (N = 4)	
	Before the intervention	During the intervention	Before the intervention	During the intervention	Before the intervention	During the intervention	Before the intervention	During the intervention
Energy (kcal/day)	1618±110	1487±110	2134±236	2113±151	1919±152	1955±109	2026±209	1707±125
Total Fat (g/day)	66.5±6.8	55.4±7.8	97.2±15.5	90.6±10.9	96.1±15.8	88.8±15.1	94.3±17.2	68.5±9.1
Saturated (g/day)	23.7±2.3	19.8±2.9	35.3±5.9	32.0±4.7	35.1±5.8	34.6±6.3	33.8±3.9	24.2±2.8
Monounsaturated (g/day)	25.0±2.9	19.2±2.9	38.6±6.2	33.3±4.2	36.1±6.1	31.1±5.3	36.6±6.5	26.7±3.1
Polysaturated (g/day)	14.7±1.7	12.7±1.8	17.2±3.0	19.8±2.8	20.0±3.6	18.0±2.6	18.6±5.7	13.8±3
Linoleic Acid (g/day)	10.6±1.4	9.7±1.6	12.8±2.2	14.6±2.6	12.4±2.3	11.8±1.5	13.7±4.6	13.3±3.1
Cholesterol (mg/day)	142±22	131±18	245±37	227±28	144±15	162±18	185±40	186±39
Total Protein (g/day)	78.0±5.6	68.9±4.5	101±8.2	95.8±3.0 ³	103±11	69.3±6.8	75.1±5.8	79.1±3.2
Vegetable protein (g/day)	24.1±2.3	23.1±1.9	31.4±3.6	34.4±2.2 ³	31.1±5.9	25.4±4.6	25.5±2.6	33.3±2.6
Total Carbohydrates (g/day)	171±16	169±13	211±21	225±15	175±32	168±12	188±18	199±6
Monosaccharides + disaccharides (g/day)	80.9±8.4	82.7±11.6	100±10	107±10	93.8±26.2	77.9±5.4	81.1±18.4	74.3±9.4
Polysaccharides (g/day)	88.2±10.0	83.5±6.0	109±12	116±6 ³	79.0±15.3	89.3±10.1	106±3	124±8
Total Fiber (g/day)	15.5±1.6	14.0±1.1	18.0±1.7	22.1±1.5 ^{3,4}	18.3±2.7	14.0±2.2	12.0±0.8	17.8±0.7
Alcohol (g/day)	3.7±2.3	6.0±4.1	2.4±2.0	2.5±2.0	2.5±2.5	2.7±1.9	5.6±5.6	5.0±5.0
Retinol (mg/day)	0.5±0.1	0.6±0.1	0.9±0.2	1.0±0.1	0.8±0.1	0.7±0.2	0.6±0.2	0.8±0.1
Vitamin C (mg/day)	83.5±9.2	95.0±13.3	106±19	165±25	86.0±23.1	100±27	47.5±18.7	92.7±18
Calcium (mg/day)	794±138	803±117	1119±81	1088±105	923±142	807±161	723±44.4	983±90
B-carotene (mg/day)	1.1±0.2	1.0±0.1	1.4±0.3	3.2±0.1 ^{3,4}	0.9±0.4	1.0±0.1	0.7±0.2	3.1±0.1
Total vegetables (g/day) ²	134±20	75±0	152±14	300±0 ⁵	151±18.6	75±0	134±21.2	300±0
Total fruit (servings/day)	1.9±0.2	2.1±0.3	2.3±0.5	2.0±0.5	1.8±0.6	1.9±0.6	1.4±0.1	1.3±0.3

Table 1. (Continued) Reported daily dietary intake before and during the intervention period and plasma levels of α - and β -carotene, retinol and α -tocopherol before and after the intervention period.¹

Plasma levels	Patients (N = 20)				Controls (N = 8)			
	Low (75 g/day) vegetable group (N = 10)		High (300 g/day) vegetable group (N = 10)		Low (75 g/day) vegetable group (N = 4)		High (300 g/day) vegetable group (N = 4)	
	Before the intervention	After the intervention	Before the intervention	After the intervention	Before the intervention	After the intervention	Before the intervention	After the intervention
α -Carotene ($\mu\text{g/ml}$)	0.04 $\pm$ 0.02	0.05 $\pm$ 0.01	0.04 $\pm$ 0.01	0.12 $\pm$ 0.02 ^{2,7}	0.07 $\pm$ 0.05	0.07 $\pm$ 0.04	0.07 $\pm$ 0.02	0.13 $\pm$ 0.04
β -Carotene ($\mu\text{g/ml}$)	0.23 $\pm$ 0.04	0.20 $\pm$ 0.03	0.22 $\pm$ 0.04	0.36 $\pm$ 0.06 ^{2,7}	0.29 $\pm$ 0.15	0.27 $\pm$ 0.12	0.25 $\pm$ 0.11	0.51 $\pm$ 0.20
Retinol ($\mu\text{g/ml}$)	0.96 $\pm$ 0.11	0.96 $\pm$ 0.08	0.82 $\pm$ 0.06	0.89 $\pm$ 0.07	0.83 $\pm$ 0.02	0.88 $\pm$ 0.05	0.94 $\pm$ 0.10	0.95 $\pm$ 0.12
α -Tocopherol ($\mu\text{g/ml}$)	15.3 $\pm$ 1.6	15.4 $\pm$ 1.7	13.9 $\pm$ 1.6	13.2 $\pm$ 1.4	10.70 $\pm$ 1.43	9.88 $\pm$ 1.23	12.96 $\pm$ 1.10	12.62 $\pm$ 1.56

¹Plus-minus values are means $\pm$ SEM.

²Standard vegetable amount intake during the intervention period.

³P<0.001; significantly higher than mean intake during the intervention in the 75 g vegetable group.

⁴P<0.001; significantly higher than mean intake before the intervention in the 300 g vegetable group.

⁵P<0.0001; significantly higher than mean intake during the intervention in the 75 g vegetable group and before the intervention in the 300 g vegetable group.

⁶P<0.05; significantly higher than mean plasma level in the 75 g vegetable group after the intervention.

⁷P<0.01; significantly higher than mean plasma intake in the 300 g vegetable group before the intervention.

The intake of total fiber, β -carotene ($P<0.001$) and total vegetables ($P<0.0001$) was significantly higher during the intervention as compared to the intake before the intervention in the high vegetable group. No significant changes in intake of other dietary compounds or life style habits were recorded. After the intervention, α - and β -carotene levels in plasma were significantly elevated in the high vegetable group compared to the levels before the intervention in the high vegetable group ($P<0.01$). For controls, similar findings were observed; however, because of the smaller number of subjects, differences were not significant.

Gene Expression

Table 2 shows a list of up- and down-regulated genes for each group. The expression differences of all genes are plotted against the mean expression in the pre-intervention samples in Figures 1A-1D. These scatter plots reveal that most genes are located within a range of -0.25 - $+0.25$, only a few are located outside these boundaries and were identified as significantly up- or down-regulated by the confidence analysis.

Table 2. Genes significantly up- and down-regulated in the vegetable groups (confidence interval 99.9%; up- and down-regulation level: >0.25 , and <-0.25 , respectively).

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Controls		Patients	
		Expression level ³	Difference ⁴	Expression level ³	Difference ⁴
Up-regulation low (75 g/day) vegetable group					
NM_000477	Albumin (ALB)			2.98±0.36	0.44±0.56
X03350	Alcohol dehydrogenase beta-1-subunit (ADH1-2)			8.66±0.22	0.49±0.15
M24317	Alcohol dehydrogenase beta-1-subunit (ADH2)			9.97±0.19	0.45±0.13
XM_003498	Alpha-fetoprotein (AFP)			3.25±0.34	0.38±0.36
XM_005207	Carbonic anhydrase III, muscle specific (CA3)			2.85±0.39	0.72±0.46
XM_001734	Prostaglandin-endoperoxide synthase 2 (PTGS2)			6.80±0.13	0.54±0.12
L01131	Decorin gene, exon 8 (DCN)			8.68±0.21	0.54±0.10
M98478	Human transglutaminase (TGM2)			7.80±0.13	0.58±0.13
XM_009010	Complement component C3 (C3)	6.20±0.44	0.68±0.19	6.84±0.37	0.47±0.40
XM_012575	Metallothionein 1G (MT1G)	9.88±0.37	0.93±0.45	9.69±0.26	0.93±0.28
X97260	Metallothionein isoform 2 (MT2A)	10.21±0.31	0.86±0.38	10.14±0.26	0.84±0.28
XM_003872	Methylacyl-CoA racemase alpha (AMACR)	9.74±0.15	0.99±0.18	9.90±0.16	0.61±0.24
NM_002738	Protein kinase C beta1 (PKCB1)	4.31±0.28	0.81±0.16	4.93±0.16	0.43±0.16

Table 2. (Continued)

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Controls		Patients	
		Expression level ³	Difference ⁴	Expression level ³	Difference ⁴
Up-regulation low (75 g/day) vegetable group					
K01396	Serine (or cysteine) proteinase inhibitor, clade A, member 1 (SERPINA1)	8.92±0.36	0.68±0.23		
M13699	Ceruloplasmin (ferroxidase) (CP)	2.20±0.52	0.88±0.21		
M61857	Cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9)	2.34±0.26	1.80±0.70		
AF016641	Homo sapiens MHC class I antigen HLA-B (HLA-B*1537)	13.69±0.18	0.65±0.26		
NM_005957	5,10-methylenetetrahydrofolate reductase (MTHFR)	5.22±0.33	0.58±0.10		
XM_045678	Mitochondrial ribosomal protein S11 (MRPS11)	9.66±0.25	0.63±0.24		
S69738	Monocyte chemotactic protein-1 (MCP-1)	5.57±0.09	0.97±0.48		
X14474	Microtubule-associated tau protein (MAPT)	2.36±0.35	1.39±0.61		
X71354	Vesicular monoamine transporter (VMAT)	5.34±0.53	0.34±0.05		
Up-regulation high (300 g/day) vegetable group					
X03350	Alcohol dehydrogenase beta-1-subunit (ADH1-2)			8.87±0.20	0.45±0.10
U33199	Human mdm2-A (MDM2)			2.73±0.27	0.41±0.18
XM_009010	Complement component C3 (C3)			6.19±0.42	0.51±0.25
D78341	Cyclin G1 (CCNG1)	8.27±0.21	0.31±0.14	8.68±0.23	0.42±0.09
L01131	Decorin gene, exon 8 (DCN)	8.36±0.51	0.56±0.20	8.29±0.29	0.59±0.13
XM_012575	Metallothionein 1G (MT1G)	9.69±0.43	0.74±0.41	9.83±0.16	0.53±0.27
X97260	Metallothionein isoform 2 (MTA2)	10.08±0.4	0.71±0.37	10.23±0.13	0.47±0.27
AB032261	Stearyl-CoA desaturase, Liver (SCD)	3.73±0.46	0.59±0.21		
AB005038	Cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1)	6.62±0.60	0.51±0.10		
L19871	Activating transcription factor 3 (ATF3)	6.60±0.57	0.64±0.32		
M29882	Apolipoprotein AII (APOA2)	4.92±0.54	0.61±0.24		
XM_001859	C-reactive protein (CRP)	3.31±0.57	0.44±0.23		
XM_001578	E-selectin (SELE)	2.89±0.52	0.34±0.14		

Table 2. (Continued)

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Controls		Patients	
		Expression level ³	Difference ⁴	Expression level ³	Difference ⁴
Down-regulation low (75 g/day) vegetable group					
NM_002417	Ki67 antigen (MKI67)			4.84±0.16	-0.74±0.14
M10617	Liver fatty acid binding protein (L-FABP)			12.90±0.17	-0.53±0.14
U33199	Human mdm2-A (MDM2)			3.46±0.31	-0.31±0.17
NM_020299	Aldo-keto reductase family 1, member B10 (AKR1B10)	4.78±0.57	-1.05±0.36	4.75±0.22	-0.57±0.34
AF016582	Checkpoint kinase-1 (CHK-1)	6.06±0.31	-0.71±0.14	6.47±0.19	-0.70±0.24
X51688	Cyclin A2 (CCNA2)	7.01±0.33	-0.86±0.35	7.20±0.26	-0.52±0.17
J05016	Disulfide isomerase related protein (EiRp72)	10.19±0.25	-0.68±0.18	10.22±0.18	-0.49±0.09
AJ271729	Glucose-regulated protein (HSPA5 gene)	11.31±0.43	-0.70±0.17	11.21±0.19	-0.56±0.18
NM_000558	Hemoglobin alpha 1 (HBA1)	7.58±0.38	-0.67±0.11	7.53±0.21	-0.53±0.12
AB005038	Cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1)	7.34±0.40	-1.06±0.06		
XM_001393	Peroxisome oxidoreductase 1 (PRDX1)	7.55±0.28	-1.14±0.60		
XM_004453	Homo sapiens H2A histone family, member I (H2AFI)	12.02±0.24	-0.70±0.18		
XM_004419	Homo sapiens H3 histone family, member B (H3FB)	13.31±0.27	-0.64±0.11		
AJ001417	Solute carrier family 22 (extraneuronal monoamine transporter), member 3 (SLC22A3)	7.47±0.11	-1.47±0.11		
X02308	Thymidylate synthase (TYMS)	8.15±0.19	-0.65±0.07		
Down-regulation high (300 g/day) vegetable group					
M16650	Ornithine decarboxylase (ODC1)	9.11±0.35	-1.41±0.44	8.65±0.27	-0.78±0.17
K00650	Fos proto-oncogene (C-FOS)	7.46±0.54	-2.07±0.54	6.80±0.48	-0.87±0.19
M10617	Liver fatty acid binding protein (L-FABP)	12.82±0.10	-0.75±0.37		
AF057039	Homo sapiens putative renal organic anion transporter 1 (hROAT1)	5.42±0.46	-0.67±0.22		
J04718	Proliferating cell nuclear antigen gene (PCNA)	9.10±0.16	-1.15±0.22		
M61854	Cytochrome P450, family 2, subfamily C, polypeptide 19 (CYP2C19)	3.85±0.28	-1.52±0.50		

Table 2. (Continued)

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Controls		Patients	
		Expression level ³	Difference ⁴	Expression level ³	Difference ⁴
Down-regulation high (300 g/day) vegetable group					
X08006	Cytochrome P450, family 2, subfamily D, polypeptide 6 (CYP2D6)	4.46±0.32	-2.18±0.84		
M18907	Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)	4.76±0.63	-1.80±0.76		

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.

²NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

³Expression level is mean Δ log intensity from the samples before the intervention. Plus-minus values are means $\pm$ SEM.

⁴Up- and down-regulation level: ≥ 0.25 and ≤ -0.25 , respectively. Difference is the mean Δ log difference (the Δ log transformed mean intensity from the samples before the intervention subtracted from the Δ log transformed mean intensity from the samples after the intervention) within a vegetable group.

The complete differential gene list was analyzed using EASE. Taken all differentially expressed genes into account (52), regardless of subject group and diet, 13 gene categories were significantly over-presented: four out of the five significantly over-presented biological processes are related to cell cycle control and seven of the eight molecular functions comprise oxidoreductase activities. Splitting the genes by means of subject group, the cell cycle control effect remains significant. The significant effect of the oxidoreductase activity category remains only in the 300 g vegetable control group. According to literature review of all genes, a lot of the genes which are related to (colon)carcinogenesis are present in the significant gene categories. In addition, genes which were not present in the significant gene categories, but are thought to be relevant to (colon) carcinogenesis according to the literature review are also taken into account.

Genes with different functions were modulated, only the 20 genes which are related to (colon)carcinogenesis are explained in more detail in Table 3. Seven of these genes were similarly modulated in both patients and controls. Methyl-CoA racemase alpha (AMACR) and protein kinase C beta 1 (PKCB1) were up-regulated in the low vegetable group; cyclin A2 (CCNA2) and checkpoint kinase-1 (CHK1) were down-regulated. In the high vegetable group, cyclin G1 (CCNG1) was up-regulated, and fos proto-oncogene (C-FOS) and ornithine decarboxylase 1 (ODC1) were down-regulated. Other genes which were modulated in either patients or controls include alpha-fetoprotein (AFP), prostaglandin-endoperoxide synthase 2 (PTGS2) or cyclooxygenase-2 (COX-2), cytochrome P450 2C9 (CYP2C9), Ki67 antigen, 5,10-methylenetetrahydrofolate reductase (MTHFR), activating transcription factor 3 (ATF3), Human mdm2-A (MDM2), cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), peroxiredoxin 1

(PRDX1), proliferating cell nuclear antigen gene (PCNA), cytochrome P450 2C19 (CYP2C19), -2D6 (CYP2D6) and -3A4 (CYP3A4).

A: Patients 75g/day

B: Patients 300g/day

Figure 1. Scatter plot views of gene expression: Mean $^2\log$ expression intensities before the intervention versus mean $^2\log$ difference. Black squares and triangles indicate statistically significant genes with a down-regulation level of -0.25 and up-regulation level of 0.25 , respectively as determined by means of 99.9% confidence intervals. Transparent squares indicate genes that are not significantly up- or down-regulated. (A) Scatter plot view of genes in the low vegetable patient group: 13 genes are up-regulated; 10 genes are down-regulated. (B) Scatter plot view of genes in the high vegetable patient group: 7 genes are up-regulated; 2 genes are down-regulated. (C) Scatter plot view of genes in the low vegetable control group: 15 genes are up-regulated; 12 genes are down-regulated. (D) Scatter plot view of genes in the high vegetable control group: 10 genes are up-regulated; 8 genes are down-regulated.

C: Controls 75g/day

D: Controls 300g/day

Figure 1. (Continued)

Table 3. Function of differentially expressed genes known to be involved in CRC.

Abbreviation ¹	Function in relation to carcinogenesis ²
AFP	Tumor associated fetal protein that primarily promotes growth in <i>in vitro</i> and <i>in vivo</i> models. AFP can serve as a dual regulator of cell proliferation (up- and down-regulation of growth)
PTGS2	Converts arachidonic acid into bioactive prostanoids that are believed to play a role in carcinogenesis by promoting cell proliferation and neoangiogenesis, inhibiting apoptosis
AMACR	Plays an important role in bile acid biosynthesis and β -oxidation of branched fatty acids. Overexpression of AMACR occurs in tumors that have been linked to high fat diet such as prostate and colon cancer
PKCB1	Isoform of serine/threonine protein kinases, which is involved in signal transduction pathways and regulates growth and differentiation in eukaryotic cells. PKC isoforms are often overexpressed in cancer
CCNG1	Cyclins bind to cyclin dependent protein kinases (CDK), which control the cell cycle. There is no proven CDK partner for cyclin G1. Conflicting roles have been assigned to CCNG1 in growth control
CCNA2	CCNA2 activates two different CDKs and is associated with cellular proliferation. CCNA2 is overexpressed in many types of cancers and is associated with poor prognosis.
MDM2	Nuclear phosphoprotein which forms a complex with p53. It regulates p53 by targeting its destruction
CHK-1	CHK-1 is an essential component of the DNA damage and -replication checkpoint. It directly phosphorylates Cdc25A protein phosphatase during an unperturbed cell cycle that is required for cells to delay cell cycle progression in response to double strand breaks
KI67	Marker for tumor proliferation rate. It provides estimates of the growth fraction of many normal and malignant tissues, including the colon
ODC1	First enzyme in polyamine synthesis. Polyamine metabolism is an integral component of carcinogenesis in epithelial tissues. There is a strong association between high levels of polyamines and rapid proliferation. Increases in ODC are often associated with initiation of normal cell growth and with sustained neoplastic cell growth
C-FOS	This proto-oncogene has been implicated in cell growth, differentiation and development. It is classified as an early growth-regulated gene
CYP2C9	Phase-1 biotransformation enzyme, capable of metabolizing a number of clinically drugs and of metabolically activating polycyclic aromatic hydrocarbons and heterocyclic aromatic amines
MTHFR	Regulates metabolism of folate. Low activity of this enzyme is associated with reduced colon cancer risk.
CYP27B1	This gene encodes for an enzyme which is responsible for the conversion of vitamin D to the active hormonal metabolite 1,25-dihydroxyvitamin D ₃ (1,25-D ₃) which may act as antimitotic and prodifferentiating agent
ATF3	ATF3 is an immediate early response gene that is induced in cells exposed to a variety of stress stimuli. It is a target gene of p53. The role of ATF3 in carcinogenesis is not clear
PRDX1	Antioxidant enzyme, which reduces hydrogen peroxide and alkyl hydroperoxides and thereby functions as a tumor suppressor

Table 3. (Continued)

Abbreviation ¹	Function in relation to carcinogenesis ²
PCNA	Marker for tumor proliferation rate. It provides estimates of the growth fraction of many normal and malignant tissues, including the colon
CYP2C19	Phase-1 biotransformation enzyme, metabolizes a number of prescribed drugs
CYP2D6	Phase-1 biotransformation enzyme, metabolizes a number of clinically important drugs
CYP3A4	Phase-1 biotransformation enzyme, metabolizes clinically used drugs and carcinogens such as aflatoxin B1, 6-aminochrysene, benzo[a]pyrene, ³⁸ and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)

¹NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

²NCBI (National Center for Biotechnology Information): Locuslink and OMIM: <http://www.ncbi.nlm.nih.gov/>.

Discussion

The current study describes the transcriptome changes induced in normal colorectal mucosa of female adenoma patients and healthy controls who received a decreased or increased amount of vegetables for a period of two weeks.

The results of the dietary questionnaires and plasma values support that the effects on gene expression found in this study truly are the result of changing the intake of vegetables.^{17, 24}

The differences in expression level before and after the two-week intervention period were relatively small, but distinct as based on confidence analyses. Only the genes which are known to be related to (colon)carcinogenesis are discussed here. First, genes which were significantly modulated in both patients and controls are reviewed. Next, the most interesting genes which were significantly modulated in either patients or controls are discussed. Table 4 summarizes the effects on expression of these genes according to subject group and in relation to possible effects on carcinogenesis.

Seven genes were similarly modulated in both study groups, the patients and healthy controls, which substantiate the importance of dietary control on these genes. In the low vegetable group, the induction of AMACR and PKCB1 and reduction of CHK1 and CCNA2 can be explained by a decreased intake of inhibitors or stimulators, respectively. Overexpression of AMACR is apparent in tumors that have been linked to high-fat diet such as CRC. As AMACR is involved in lipid metabolism it is speculated that this could lead to alterations in oxidant balance of a cell leading to DNA damage, malignant transformation and other parameters of cell disturbance.²⁵ PKC isoforms are often overexpressed in disease states such as cancer.²⁶ PKC activation has been implicated in tumor promotion of colonic epithelial cells by endogenous and dietary factors such as bile acids and free fatty acids.²⁷ CHK1 is an essential component of the DNA damage and

–replication checkpoint. It is required for cells to delay cell cycle progression in response to double strand breaks.²⁸ CCNA2 can activate two different cyclin dependent protein kinases (CDKs) and is associated with cellular proliferation. It is overexpressed in many types of cancers with high proliferative activity and is associated with a poor prognosis.²⁹ Thus the effects of reduced vegetable intake on AMACR, PKCB1 and CHK1 are expected to enhance cancer risk, whereas for CCNA2 to induce cancer risk suppression.

In the high vegetable group, CCNG1 was up-regulated, and C-FOS and ODC1 were down-regulated for both patients and controls. CCNG1 is a transcriptional target of p53 and its up-regulation coincides with activation of p53. Activated p53 suppresses growth by activating genes that trigger growth arrest or apoptosis. It is speculated that p53 activation is achieved by CCNG1 by stoichiometrically limiting the binding between p53 and MDM2, a down-regulator of p53, or by altering the phosphorylation status of MDM2 via phosphatase A2.³⁰ Noteworthy, also MDM2 expression is regulated by p53.³⁰⁻³² For patients, down-regulation of MDM2 occurred in the low vegetable group, up-regulation of MDM2 and CCNG1 was observed in the high vegetable group. C-FOS protein interacts with the protein of another proto-oncogene, C-JUN to form a heterodimer. This complex is able to recognize and bind specific sequence of nucleotides referred to as the AP-1 binding site located in the promoter region of genes which are expressed in transformed rapidly growing cells.³³ C-FOS is overexpressed in the preneoplastic colonic lesions aberrant crypt foci.³³ ODC1 is a transcriptional target of C-MYC and a modifier of adenomatous polyposis coli (APC)-dependent tumorigenesis. Most sporadic colon adenomas acquire mutations in the APC gene and show defects in APC-dependent signaling. Wild-type APC suppresses C-MYC, activates the C-MYC antagonist MAD1, and reduces the expression of ODC1 resulting in a decrease in polyamine synthesis and reduced tumorigenesis. β -Carotene inhibits ODC1 expression.³⁴ Intake and plasma levels of β -carotene were significantly increased in the high vegetable group and could thus explain the ODC1 inhibition. For all these genes the modulatory effects of increased vegetable intake on their expression might result in a decreased cell growth and as a consequence in lower colon cancer risk.

Patients already have a genetic predisposition to develop adenomas as compared to controls and that could explain why we found 13 genes modulated differently in patients compared to controls. The most interesting ones are discussed here. Already mentioned is the effect of vegetables on MDM2 expression in patients. Furthermore, COX-2, up-regulated in patients on low vegetable intake, is an established target for preventive interventions, as non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to suppress colon carcinogenesis through the inhibition of COX-2.³⁵ Inhibition of COX-2 expression by vegetable components has previously been reported.^{36, 37} In a study of Plummer et al (1999), COX-2 induction by the colon tumor promoters tumor necrosis factor α or fecapentaene-12 in human colon cells was diminished by the chemopreventive

agent curcumin.³⁶ Wenzel et al (2000) found that that dietary flavone was able to diminish COX-2 mRNA in the human carcinoma cell line HT-29 after a 48h incubation.³⁷ In the present study, the observed up-regulation of COX-2 could be the result of a decreased intake of possible COX-2 inhibitors and agrees with the observed cancer risk suppression by dietary vegetables.

Table 4. Presentation of differentially expressed genes known to be involved in CRC, the direction of the effect, and the theoretical effect of this on cancer risk.

Gene Name by NCBI (Abbreviation) ¹	Reduced vegetable intake		Increased vegetable intake	
	Effect on expression	Theoretical effect on cancer risk	Effect on expression	Theoretical effect on cancer risk
In patients and controls				
AMACR	+	+		
PKCB1	+	+		
CHK1	-	+		
CCNA2	-	-		
CCNG1			+	-
C-FOS			-	-
ODC1			-	-
In patients only				
COX-2	+	+		
MDM2			+	?
In controls only				
MDM2	-	?		
CYP2C9	+	+		
CYP2C19			-	?
CYP2D6			-	?
CYP3A4			-	-
CYP27B1	-	+	+	-

¹NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov>.

In controls, but not in patients, several phase-I biotransformation genes were modulated. In the low vegetable group CYP2C9 was up-regulated and in the high vegetable group CYP2C19, -2D6 and 3A4 were down-regulated. The CYP genes code for enzymes that function in a wide variety of metabolic pathways involving both endogenous and exogenous compounds. In endogenous processes, CYP enzymes are involved in the biosynthesis and catabolism of signaling molecules such as steroid hormones, retinoic acid, and vitamin D.³⁸ CYP27B1 codes for the enzyme which converses vitamin D to the active hormonal metabolite 1,25-dihydroxyvitamin D₃, which may act as an antimitotic and pro-differentiating agent in colon cancer cells.³⁹ The expression of CYP27B1 was down-regulated in the low and up-regulated in the high vegetable group. In addition to their role in endogenous processes, CYP enzymes catalyze the biotransformation of a wide variety of

xenobiotic compounds which require metabolic activation to form ultimate carcinogens or toxicants. By blocking the formation of ultimate carcinogens, initiation of carcinogenesis can be prevented.³⁸ CYP2C9^{38, 40} and CYP3A4 are responsible for the metabolism of a number of drugs, and the (colon)carcinogens polycyclic aromatic hydrocarbons and heterocyclic aromatic amines.⁴¹ Also CYP2C19 and CYP2D6 metabolize a number of drugs, but have not been thoroughly studied in relation to cancer susceptibility.³⁸ Once again, all these dietary effects on gene expression of CYP450 enzymes by altering vegetable intake agree with the presumed cancer-modulating properties of vegetables.

In conclusion, almost all the effects on the expression of genes by altering vegetable intake can be mechanistically linked to cellular processes that explain prevention of colon-cancer risk by high vegetable intake or higher colon-cancer risk by low vegetable intake (see Table 4). The only exception to this is CCNA2, which maybe is involved in currently unknown but relevant processes. Furthermore, it seems that for patients, genes are modulated which are involved in the late stages of CRC like MDM2 and COX-2, whereas for controls genes are modulated involved in initiating events, i.e. the CYP450 genes. This human study is the first in which the vegetable intake was investigated to modulate the expression of genes involved in carcinogenesis in colorectal mucosa. An increased intake of vegetables resulted in down-regulation of genes promoting cell proliferation and bioactivation of procarcinogens, and in up-regulation of genes involved in cell growth arrest; in contrast, a decreased intake of vegetables results in down-regulation of genes inhibiting cell growth and up-regulation of genes promoting cellular differentiation and bioactivation of procarcinogens. Some of the modulated genes are already targets in colon-chemoprevention trials, like COX-2. Possibly, the others are new targets for chemoprevention. Further research is needed to investigate what would be the relative impact of preventive modulation of these particular genetic pathways in reducing CRC risk.

References

1. World Health Organization & International Agency for Research on Cancer. *World Cancer Report*. Lyon. IARC Press, 2003.
2. Dashwood RH. Early detection and prevention of colorectal cancer (review). *Oncol Rep* 1999;6:277-81.
3. World Cancer Research Fund. *Food, Nutrition and the Prevention of Cancer: a Global Perspective*. Chapter 4.10: Colon, rectum. Washington: BANTA Book group, 1997:216-251.
4. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. *Epidemiol Rev* 1993;15:499-545.
5. Wattenberg LW. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res* 1992;52:2085s-2091s.
6. Manson MM, Gescher A, Hudson EA, Plummer SM, Squires MS, Prigent SA. Blocking and suppressing mechanisms of chemoprevention by dietary constituents. *Toxicol Lett* 2000;112-113:499-505.
7. Reddy BS. The Fourth DeWitt S. Goodman lecture. Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention. *Cancer Epidemiol Biomarkers Prev* 2000;9:239-47.
8. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
9. Arends JW. Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 2000;190:412-6.
10. Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000;119:854-65.
11. Muller M, Kersten S. Opinion: Nutrigenomics: goals and strategies. *Nat Rev Genet* 2003;4:315-22.
12. Kall MA, Vang O, Clausen J. Effects of dietary broccoli on human in vivo drug metabolizing enzymes: evaluation of caffeine, oestrone and chlorzoxazone metabolism [see comments]. *Carcinogenesis* 1996;17:793-9.
13. Lampe JW, Chen C, Li S, Prunty J, Grate MT, Meehan DE, Barale KV, Dightman DA, Feng Z, Potter JD. Modulation of human glutathione S-transferases by botanically defined vegetable diets [In Process Citation]. *Cancer Epidemiol Biomarkers Prev* 2000;9:787-93.
14. Lampe JW, King IB, Li S, Grate MT, Barale KV, Chen C, Feng Z, Potter JD. Brassica vegetables increase and apiaceous vegetables decrease cytochrome P450 1A2 activity in humans: changes in caffeine metabolite ratios in response to controlled vegetable diets. *Carcinogenesis* 2000;21:1157-62.
15. Nijhoff WA, Grubben MJ, Nagengast FM, Jansen JB, Verhagen H, van Poppel G, Peters WH. Effects of consumption of Brussels sprouts on intestinal and lymphocytic glutathione S-transferases in humans. *Carcinogenesis* 1995;16:2125-8.
16. Pool-Zobel BL, Bub A, Liegibel UM, Treptow-van Lishaut S, Rechkemmer G. Mechanisms by which vegetable consumption reduces genetic damage in humans. *Cancer Epidemiol Biomarkers Prev* 1998;7:891-9.
17. Anonymous. *Zo eet Nederland. Resultaten van de voedselconsumptiepeiling 1997-1998*. Voedingscentrum Den Haag, 1998.
18. Gartner LP, Hiatt JL. Chapter 17: Digestive System: Alimentary Canal. *Color textbook of histology*, second edition. Philadelphia: W.B. Saunders Company, 2001:379-409.

19. Hess D, Keller HE, Oberlin B, Bonfanti R, Schuep W. Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *Int J Vitam Nutr Res* 1991;61:232-8.
20. NEVO Table: Dutch Food Composition Table. The Hague: Bureau for Nutritional Education, 1984.
21. Hasseman J, Chen E, Yang I, Quackenbush J. Aminoallyl labeling of RNA for microarrays. <http://www.tigr.org/tdb/micoarray/protocolsTIGR.shtml> (Accessed June 21, 2001), 2001.
22. Kerr MK, Martin M, Churchill GA. Analysis of variance for gene expression microarray data. *Journal of Computational Biology* 2000;7:819-837.
23. Hosack DA, Dennis G, Jr., Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome Biol* 2003;4:R70.
24. Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL, Potter JD. Plasma carotenoids as biomarkers of vegetable and fruit intake. *Cancer Epidemiol Biomarkers Prev* 1994;3:493-500.
25. Kuefer R, Varambally S, Zhou M, Lucas PC, Loeffler M, Wolter H, Mattfeldt T, Hautmann RE, Gschwend JE, Barrette TR, Dunn RL, Chinnaiyan AM, Rubin MA. alpha-Methylacyl-CoA racemase: expression levels of this novel cancer biomarker depend on tumor differentiation. *Am J Pathol* 2002;161:841-8.
26. Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. *Curr Drug Targets* 2000;1:163-83.
27. Kahl-Rainer P, Sedivy R, Marian B. Protein kinase C tissue localization in human colonic tumors suggests a role for adenoma growth control. *Gastroenterology* 1996;110:1753-9.
28. Zhao H, Watkins JL, Pownica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc Natl Acad Sci U S A* 2002;99:14795-800.
29. Yasmeen A, Berdel WE, Serve H, Muller-Tidow C. E- and A-type cyclins as markers for cancer diagnosis and prognosis. *Expert Rev Mol Diagn* 2003;3:617-33.
30. Zhao L, Samuels T, Winckler S, Korgaonkar C, Tompkins V, Horne MC, Quelle DE. Cyclin G1 has growth inhibitory activity linked to the ARF-Mdm2-p53 and pRb tumor suppressor pathways. *Mol Cancer Res* 2003;1:195-206.
31. Momand J, Wu HH, Dasgupta G. MDM2--master regulator of the p53 tumor suppressor protein. *Gene* 2000;242:15-29.
32. Zheleva DI, Lane DP, Fischer PM. The p53-Mdm2 pathway: targets for the development of new anticancer therapeutics. *Mini Rev Med Chem* 2003;3:257-70.
33. Stopera SA, Davie JR, Bird RP. Colonic aberrant crypt foci are associated with increased expression of c-fos: the possible role of modified c-fos expression in preneoplastic lesions in colon cancer. *Carcinogenesis* 1992;13:573-8.
34. Phillips RW, Kikendall JW, Luk GD, Willis SM, Murphy JR, Maydonovitch C, Bowen PE, Stacewicz-Sapuntzakis M, Wong RK. beta-Carotene inhibits rectal mucosal ornithine decarboxylase activity in colon cancer patients. *Cancer Res* 1993;53:3723-5.
35. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002;8:289-93.
36. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* 1999;18:6013-20.

37. Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* 2000;60:3823-31.
38. Vincis P, Malats N, Lang A, d'Errico A, Caporaso N, Cuzick J, Boffetta P. Metabolic polymorphisms and susceptibility to cancer. IARC Scientific Publications No.148, 1999.
39. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. *J Nutr* 2002;132:3490S-3493S.
40. Martinez C, Garcia-Martin E, Ladero JM, Sastre J, Garcia-Gamito F, Diaz-Rubio M, Agundez JA. Association of CYP2C9 genotypes leading to high enzyme activity and colorectal cancer risk. *Carcinogenesis* 2001;22:1323-6.
41. Snyderwine EG, Turesky RJ, Turteltaub KW, Davis CD, Sadrieh N, Schut HA, Nagao M, Sugimura T, Thorgeirsson UP, Adamson RH, Thorgeirsson SS. Metabolism of food-derived heterocyclic amines in nonhuman primates. *Mutat Res* 1997;376:203-10.

Chapter 3

Dose dependent effect of a vegetable mixture on gene expression in colon mucosa of C57BL6 female mice

Submitted

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Abstract

There is abundant epidemiological evidence that colorectal cancer (CRC) risk is decreased by consumption of vegetables. However, the molecular targets at the genome level are mostly unknown. The aim of the present study was to identify genes which are dose dependently modulated by vegetables, in order to provide more insight into the genetic mechanisms through which vegetables exert their beneficial effects. Female C57BL6 mice were randomly assigned to one of four dietary groups. Each group was fed one of four different diets, containing a 0%, 10%, 20% and 40% wt/wt vegetables mixture respectively, for a period of two weeks. The vegetables mixture consisted of cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt). In order to assess gene expression changes at target level, colonic mucosal cells were collected after sacrifice. Total RNA was isolated and microarray technology was used to measure the expression levels of 602 genes simultaneously. For 39 genes a significant dose-dependent effect was detected, although in general the relationship was not linear, 17 of these genes are likely to be involved in CRC protective mechanisms. For almost all of these genes, the altered expression can be explained by reduced cancer risk. Most statistical differences were observed between the highest vegetable dose group (40%) and one of the other diet groups. A diet high in vegetables positively modulates genes hypothetically involved in protective mechanisms at various stages of CRC development. Next to genes which are already known to be induced by vegetables (components), new genes were identified which may play a role in CRC prevention by vegetables in humans.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, with about 943,000 new cases per year.¹ Mortality has declined steadily, indicating the beneficial effects of improved therapy and early detection. However, incidence of this type of cancer is generally increasing, pointing out the need for additional preventive measures.² It has been suggested that as much as 90% of CRC cases might be prevented by changes in diet.³ There is abundant epidemiological evidence that CRC development is strongly influenced by food and nutrition. Particularly, the consumption of diets high in vegetables, decreases the risk of CRC.^{4, 5} The evidence from animal studies is less clear, but in the majority of these studies, it was found that vegetables or vegetable components reduce CRC risk.^{5, 9}

A large number of potentially anticarcinogenic substances is present in vegetables, which can influence the process of carcinogenesis via various mechanisms. For protection against CRC by fiber, which is present in all vegetables and is particularly high in pulses like beans and peas, proposed mechanisms comprise diluting and binding of carcinogens in the digestive tract, decreasing transit time of faecal bulk, inhibiting cell proliferation by diluting bile acids^{10, 11} and influencing indirectly the transcription rates of genes.^{10, 12} Allium vegetables, which comprise onions and garlic, contain organosulfur compounds like diallyl sulfide which can modify carcinogen activation by inhibition of phase I biotransformation enzymes¹³ and induction of phase II detoxification enzymes.^{11, 14} Other phytochemicals known to have an effect on the metabolic activation of procarcinogens, are isothiocyanates and indoles, breakdown products of glucosinolates present in cruciferous vegetables, which include cauliflower, broccoli, cabbage and Brussels sprouts: induction as well as inhibition of detoxification enzymes by these compounds has been described.^{15, 19}

Another important mechanism by which vegetables can protect against DNA damage, is scavenging of reactive oxygen species and other free radicals. Antioxidants like β - and α -carotene present in orange vegetables like carrots and pumpkin, are capable of protecting the DNA from free radical damage^{11, 20}. Furthermore, these agents are able to suppress cell proliferation by upregulation of connexin 43, a gene responsible for maintaining intercellular gap junctional communication, which is associated with decreased proliferation.^{20, 21}

In many of these postulated mechanisms, modulation of gene expression plays a role. Regulation of gene expression occurs by complex arrays of response elements that influence the rate of transcription and thereby regulate the abundance of proteins. Vegetable components can influence gene expression by direct interaction with regulatory elements in the genome, leading to a change in the transcription rate of a given gene.¹²

Gene expression modulation by vegetables and vegetable components has only been investigated in laboratory animals for a limited number of genes and often in relation

to exposure of carcinogens. Many of the molecular targets at the genome level are unknown. In this study, the effect of different doses of a mixture of vegetables on gene expression in colon mucosa of female C57BL/6 mice was investigated. This mouse model is frequently used in studies investigating dietary modulation at the molecular level, including gene expression, and it provides the basis of multiple transgenic mouse models, which can be used in future studies to investigate the specific role of a particular gene.^{6, 7, 22-26} Microarray technology was used to measure the expression levels of 602 genes simultaneously. Identification of genes dose-dependently modulated by vegetables can give more insight into the genetic mechanisms through which vegetables may exert their beneficial effects.

Materials and Methods

Animals and Diet

Eight-week-old female C57BL/6 mice (Charles River Laboratories, Lyon, France) were randomly assigned to one of four dietary groups, each consisting of seven animals. During 7 days of acclimatization, all the animals received the standard control diet. Next, each group was fed one of four different diets, containing a 0% (referred to as control diet), 10%, 20% and 40% wt/wt vegetables mixture respectively, for a period of two weeks. The vegetables mixture used consisted of cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt). Up to 20% vegetables were also used in previous mice vegetables studies, though no multiple vegetable dose diets were tested.^{9, 26, 27} Diets were refreshed every two days and provided as powdered feed. The 20% casein reference diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets. The vegetables were purchased as a single batch at the supermarket and separately cooked under household conditions. After freezing (-20 °C), the vegetables were lyophilized, ground and combined. Before the vegetables mixture was mixed with the basal diet, it was analyzed for macronutrient content (Hope Farms). The vegetables mixture added to the basal diet was at the expense of carbohydrates. Diets were adjusted for the amount of carbohydrates with dextrose/cellose and cellulose (dicacel) (Hope Farms), resulting in similar energy densities. No antioxidants or preservatives were added. After preparation, the diets were stored at -20 °C in air-closed plastic bags until use. The composition of the different diets is presented in Table 1.

The animals were maintained in the laboratory animal care facilities at Maastricht University under controlled environmental conditions (temperature 21 ± 1 °C, relative humidity 50% $\pm$ 10, 12-hr light/dark cycle). Body weights of the mice were recorded weekly. The study was approved by the Institutional Committee of Animal Experimentation Maastricht University.

Table 1. Composition of the four diets in g/kg.

Ingredients	Control diet ¹	10% Vegetable diet ²	20% Vegetable diet ²	40% Vegetable diet ²
Cerelose/Dextrose	515	422	329	143
DL-Methionine	2	2	2	2
NaCl	3	3	3	3
Choline Cl. 50%	4	4	4	4
Soya oil	50	50	50	50
KCl	7	7	7	7
KH ₂ PO ₄	7	7	7	7
MgO	2	2	2	2
CaHPO ₄ ·2H ₂ O	13	13	13	13
CaCO ₃	10	10	10	10
Corn Starch	100	100	100	100
Dicacel/Cellulose	77	70	63	49
Casein	200	200	200	200
MgSO ₄ ·7H ₂ O	4	4	4	4
Standard vitamin premix	3	3	3	3
Standard micronutrient premix	3	3	3	3
Vegetables mixture ³	0	100	200	400

¹Control diet is the basal 20% casein reference diet.

²In the vegetable diets, the basal diet was adjusted for cerelose/dextrose and dicacel/cellulose resulting in similar energy densities for all diets.

³Composition (% wet weight): cauliflower (30%), carrots (30%), peas (30%) and onions (10%).

Tissue Sampling

Mice were sacrificed by bleeding the *vena cava inferior* under Nembutal (Sanofi Sante, Maasluis, the Netherlands) anesthesia. Nembutal was administered subcutaneously in the neck at a dose of 60 mg/kg body weight. The large intestine was removed and placed on a specially made plastic box, which was kept at 4 °C. After removing the rectum, the colon was opened longitudinally with fine scissors, and mucus and faeces were removed. Colonic mucosal cells were incubated in Trizol™ (GIBCO Life Technologies, Breda, The Netherlands) for 3 minutes and scraped off the muscle layer with the edge of a sterile glass slide. Cells were transferred into 800 µl Trizol™, homogenized by resuspension and stored at -80 °C until total RNA isolation.

Total RNA Isolation and cDNA Probe Synthesis

Total RNA was extracted according to the manufacturer's instructions. The RNeasy® Mini Kit (Qiagen, Westburg, Leusden, The Netherlands) together with a DNase treatment was used to purify total RNA from salts and residual DNA. Quantity of each RNA sample was

measured by a spectrophotometer and varied from 30 to 100 µg per mouse. Integrity was determined by a Bioanalyzer (Agilent Technologies Netherlands B.V., Amsterdam, The Netherlands). All samples contained intact total RNA with an rRNA ratio (28S/18S) > 1.5.

The 0% vegetable diet group was considered as the control group; the vegetable diet groups were referred to as the dose groups. Three total RNA pools per diet group were prepared by pooling equal amounts of total RNA from two or three mice. Cyanine 3- (Cy3) and Cyanine 5- (Cy5) labeled cDNA probes were prepared using 10 µg total RNA from each pool, by the method of Hasseman et al.²⁸

cDNA Microarray Preparation

This study is part of a project that investigates the effects of vegetables on gene expression in colon mucosa of humans and mice. For the human study, the expression levels of genes in colon mucosa were measured using the PHASE-1 Microarray Human-600 (PHASE-1 Molecular Toxicology, Santa fee, USA). A mouse cDNA microarray was constructed based on the genes present on the PHASE-1 Microarray Human-600. These genes represent a dedicated selection of biologically relevant gene sequences involved in inflammation, DNA damage and repair, oxidative stress, cell signaling, cell proliferation, metabolism, transcription and apoptosis.

In total, 602 mouse cDNA clones from the I.M.A.G.E. consortium were selected using Homologene of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/HomoloGene/>). The largest group of cDNAs consisted of 358 cDNA clones, originating from the National Institute on Aging (NIA, Bethesda, USA), and was provided by the Genome Centre Maastricht, The Netherlands. The remaining 244 cDNA clones were obtained from the Resource Centre/Primary Database (RZPD, Berlin, Germany). All cDNA clones were provided as bacterial glycerol stocks and amplification of the cDNA inserts was performed by PCR in 96-well format in a Tgradient Thermocycler (Whatman Biometra, Westburg, Leusden, The Netherlands).

Amplification of the NIA cDNA inserts was carried out using 1.25 µl bacterial glycerol stock as template in a 125 µl reaction mixture consisting of 1x PCR buffer (Amersham Biosciences, Roosendaal, the Netherlands), 0.2 mM dNTPs (Invitrogen Life Technologies, Breda, The Netherlands), victorase (Genome Centre, Maastricht University, Maastricht, The Netherlands) and 0.25 pmol/µl vector primers (15K-forward primer 5'-CCAGTCACGACGTTGTAAAACGAC-3'; and 15K-reverse primer 5'-GTGTGGAATTGTGAGCGGATAACAA-3'; Eurogentec, Seraing, Belgium). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 2 minutes.

To amplify cDNA inserts from the RZPD clones, two different primer sets were used. For amplification of the pT7T3D-pac1, pSPORT1, pCMV-SPORT2, Bluescribe and pBluescript SK vectors, M13-primers were used (M13-forward primer 5'-

CCCAGTCACGACGTTCTAAAACG-3'; and M13-reverse primer 5'-AGCGGATAACAATTTTCACACAGG-3', Qiagen). PCR was performed in a 125 µl reaction mixture containing 0.125 µl bacterial glycerol stock, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM M13 primers and 0.5 U Taq DNA polymerase (Amersham Biosciences). Thermal cycling conditions consisted of an initial denaturation at 94 °C for 10 minutes, followed by 30 cycles of 94 °C for 15 seconds, 56 °C for 15 seconds and 72 °C for 45 seconds, and a final extension at 72 °C for 10 minutes. Sugano primers (Sugano-forward 5'-CTGCTCCTCAGTGGATGTTGCCTTTAC-3'; Sugano-reverse 5'-CAGGTTTCAGGGGGAGGTGTGG-3'; Qiagen) were used to amplify the pME18S-FL3 vector. PCR was carried out in a 125 µl reaction mixture containing 0.625 µl bacterial glycerol stock, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 4 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM M13 primers and 2.5 U Taq DNA polymerase (Amersham Biosciences). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 2 minutes, and a final extension at 72 °C for 5 minutes.

PCR products were verified by gel electrophoresis and purified using the QIAquick® 96-well purification kit (Qiagen). Purified PCR products were lyophilized and dissolved in 50% DMSO in 384 well plates (Genetix, Hampshire, UK). Spotting of the PCR products onto UltraGAPSTTM slides (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) was performed at the Genome Centre Maastricht using a pin-tool based spotting robot (Microgrid II, BioRobotics, Huntingdon, UK). DNA spots were arrayed in triplicate in order to reduce intra-assay variability in a 6x8 configuration. Printed slides were baked at 80 °C for 3 hours and stored in a desiccator at room temperature until use.

cDNA Microarray Hybridizations

For the hybridizations, within each diet group three pools were formed, each consisting of equal amounts of total RNA of two or three mice. By creating three pools per diet group instead of one, biological variability can be taken into account. This allows for a broader generalizability of the results. For each of the three sets of pools from different diet groups, a loop design was constructed with four microarrays, as follows: D0j→D1j→D2j→D3j→D0j (the pools for control, 10%, 20% and 40% diet denoted, respectively, by D0j, D1j, D2j and D3j with j= 1, 2 or 3; arrows join the samples put on the same array and indicate the sample labeled with Cy5). The loop is repeated three times, and then analyzed together, to reflect the possible variability in the experimental data. The statistical power of this design for estimating the dose-response profile is higher compared to the classical reference design in which each diet group is compared with the control group. Furthermore, fewer arrays are needed by using this design.²⁹⁻³¹ In total 12 arrays were used. Cy3 and Cy5 labeled cDNA probes of two groups were mixed according to this design and hybridized to the cDNA microarray by the method of Hasseman et al.²⁸ Slides

were scanned on a GMS 418 Array Scanner (Affymetrix, High Wycombe, UK). The images obtained (resolution 10 micron; 16 bit tiff image) were processed with ImaGene 5.0 (Biodiscovery, LA, USA) to measure mean signal intensities for spots and local background.

cDNA Microarray Data Processing and Statistical Analysis

Dose-dependency was analyzed using ANOVA models.³² Following the suggestions of Kerr et al. (2000), no background-signal correction was applied, as it appeared to add extra unnecessary variability to the data. In the analysis, the base-2 logarithmically transformed data were used. Due to computational limitations, the models were fit in two stages. The ANOVA model included a normalization step, taking into account both the global (across-genes) and local (gene-specific) normalization.³³

To investigate the differences in the gene expression levels, all pairwise differences between the treatment groups were examined. For each gene, the Tukey procedure was used to correct for multiple comparisons.³⁴ To control the overall (across genes) probability of false positive findings at around 5%, the procedure was applied using 0.0001 significance level per gene. Moreover, since the analysis of the fit of the model revealed possible problems with the assumption on the normality of the error terms, the critical values for the Tukey procedure were selected using an empirical distribution obtained by bootstrap rather than the theoretical distribution.³⁵

Real-time RT-PCR

To verify the cDNA microarray results, 21 gene expression differences, representing six genes which were responsive to vegetables, were analyzed by real-time RT-PCR. First strand cDNA was generated according to the Superscript™ II RNase H⁻ Reverse Transcriptase protocol (Invitrogen, Life Technology) using 1 µg total RNA. Forward and reverse primers for real-time PCR amplification were designed with Primer Express software version 1.5 (Applied Biosystem, Foster City, USA) using default settings. To avoid amplifying of contaminating genomic DNA, primers were chosen, either residing on different exons, thereby spanning at least 1 intron in the genomic sequence or on spanning the splicing junction site, therefore containing sequences from two adjacent exons. A list of validated genes and the normalization gene and their chosen primers is given in Table 2. To normalize cDNA loading and PCR variations, the signals of the selected genes were corrected with the signals from 18S mRNA as internal reference.^{36, 37} Therefore, the amount of target gene is divided by the amount of 18S of the sample to obtain a unit-less normalized target value.

PCR reactions consisted of 12.5 µl 2x SYBR Green I qPCR Mastermix Plus, 0.75 µl SYBR Green I (Eurogentec), 2.5 µl 3 µM forward and reverse primer (Qiagen), 1.75 µl milliQ water and 5 µl cDNA template (total 10 ng template for validated genes; total 16 pg template for the normalization gene 18S).

Table 2. Primer sequences of the validated genes and the 18S normalization gene.

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Forward Primer	Reverse Primer
A1193731	Tumor necrosis factor receptor superfamily, member 6 (TNFRSF6)	5'-GCTGCGCCTCGTGTGAA-3'	5'-GCGATTTCTGGGACTTTGTTTC-3'
A1152753	Caspase 4, apoptosis-related cysteine protease (CASP4)	5'-CGGGCAACCTTGACGAGAT-3'	5'-TTCAAGCTGAAGAAATGAGATTCAGTT-3'
BG063729, BG063771	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	5'-CCACCCACCCCAGCAA-3'	5'-GAAATTGTGAGGGAGATGCTCAGT-3'
AA107664	Signal transducer and activator of transcription 1 (STAT1)	5'-GACCACGCCCTTTGGGAAGTA-3'	5'-TCGCTTAGGGTCGTCAAGCT-3'
A1226017	Sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1)	5'-CCCTCAGAGTCTGCTGGATCA-3'	5'-GGAGACAACCACATCCTTTTGC-3'
BG066160, BG074606	Ornithine aminotransferase (OAT)	5'-GTGAGAGGGGAAAGGGTTGCTAA-3'	5'-CAGGCACACCTTCCAAGCAT-3'
	18S	5'-AGTCCCTGCCCTTTGTACACA-3'	5'-GATCCGAGGGCCTCACTAAAC-3'

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.²NCBI (National Center for Biotechnology Information); <http://www.ncbi.nlm.nih.gov/>.

All PCR reactions were performed in triplicate in 96-well optical plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) in the ABI Prism® 7700 Sequence Detection System. Data were analyzed using the ABI PRISM 7700 sequence detection system software version 1.9.1 by means of the Relative Standard Curve Method.

Additional Statistical Analysis

Statistical analysis of bodyweight of the mice was carried out using SPSS version 6.1.1 for Macintosh. Data were analyzed by means of analysis of variance followed by Student's *t*-test. A two-sided *p*-value < 0.05 was considered to indicate statistical significance.

Results

This study investigates the transcriptome differences induced in the colon of female C57BL/6 mice fed one of four different diets, containing a 0%, 10%, 20% and 40% wt/wt vegetables mixture respectively, for a period of two weeks. Mean body weights per week per group are presented in Table 3. During the week prior to the start of the intervention (week 0), all animals received the control diet. At the end of this week, there were no statistical significant differences in mean body weights between the groups. After the intervention, mean body weights were significantly increased within each group ($P < 0.05$), but no significant differences between the groups were observed. Because weight gain was similar between the groups, these results indicate that there was no difference in food intake. Furthermore, these results show that the diets did not affect normal growth.

Table 3. Mean body weights (g) per week per group.¹

	Week 0 ²	Week 1 ³	Week 2 ³
Control group	18.6 ± 0.6	19.6 ± 0.7	20.3 ± 0.7 ⁴
10% group	18.6 ± 1.3	19.7 ± 0.9	20.2 ± 0.8 ⁴
20% group	19.1 ± 0.6	20.0 ± 0.4	20.8 ± 0.3 ⁴
40% group	18.6 ± 0.4	19.9 ± 0.7	20.6 ± 1.0 ⁴

¹Body weights are expressed as mean ± SE.

²Acclimatization week, animals received control diet.

³Intervention period.

⁴Statistics: analysis of variance followed by Student's *t*-test. $P < 0.05$: body weight significantly higher compared to week 0.

The expressions of 602 genes were measured simultaneously by means of cDNA microarrays. Microarray data analysis compared the levels of expression between the diet groups and tested whether these differences were significant. In total, a significant difference in gene expression was found for 96 diet group comparisons, representing 39

genes. These genes, their matching comparisons and the differences are listed in Table 4. Remarkably, almost all the statistical differences are between the highest vegetable dose group (40%) and one of the other diet groups. Biological functions and involved processes for the differentially expressed genes are diverse.

Table 4. Differentially expressed genes.

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Comparison	Difference cDNA microarray ³	Difference RT-PCR ⁴
BG073773	Translation elongation factor EF-1 alpha-1 chain (EFHU1)	40-0	-0.28 ± 0.08	
AI193731	Tumor necrosis factor receptor superfamily, member 6 (TNFRSF6)	40-10	0.86 ± 0.12	1.57 ± 0.28
		40-20	0.89 ± 0.10	1.12 ± 0.07
W61433	SHB (Src homology 2 domain containing) adaptor protein B (SHB)	40-0	0.64 ± 0.11	
		40-10	0.64 ± 0.12	
		40-20	0.74 ± 0.11	
BG064736	Ribonucleotide reductase M1 polypeptide (RRM1)	40-0	0.59 ± 0.10	
		40-10	0.60 ± 0.12	
		40-20	0.95 ± 0.10	
BG063508	MYB binding protein (P160) 1a (MYBBP1a)	40-0	-0.54 ± 0.07	
		40-10	-0.53 ± 0.09	
BG067241	ATP-binding cassette, sub-family B (MDR/TAP), member 1B (ABCB1b)	10-0	1.17 ± 0.11	
		20-10	-0.59 ± 0.10	
		40-10	-1.10 ± 0.11	
BG072904	RAD51 homolog (<i>S.cerevisiae</i>) (RAD51)	40-0	0.61 ± 0.13	
		40-10	0.58 ± 0.15	
		40-20	0.86 ± 0.13	
		20-10	-0.29 ± 0.13	
BG064900	Stearoyl-Coenzyme A desaturase 2 (SCD2)	40-0	0.54 ± 0.10	
		40-20	0.61 ± 0.10	
		20-10	-0.43 ± 0.10	
BG064604	Aldolase B, fructose-biphosphate (ALDOB)	40-10	-0.53 ± 0.13	
		40-20	-0.46 ± 0.11	
BG063870	Beta actin, cytoplasmic (ACTB); clone H3011G02	40-0	0.65 ± 0.15	
		40-10	0.75 ± 0.17	
		40-20	0.80 ± 0.15	
BG064339	Beta actin, cytoplasmic (ACTB); clone H3018D10	40-0	0.65 ± 0.15	
		40-20	0.90 ± 0.09	
AI152753	Caspase 4, apoptosis-related cysteine protease (CASP4)	40-10	1.87 ± 0.14	2.78 ± 0.13
		40-20	1.28 ± 0.12	3.18 ± 0.13
		20-0	-0.78 ± 0.14	-0.45 ± 0.23
		20-10	0.59 ± 0.12	0.40 ± 0.13
		10-0	-1.38 ± 0.13	-0.05 ± 0.23

Table 4. (Continued)

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Comparison	Difference cDNA microarray ³	Difference RT-PCR ⁴
NM_0080841	Integrin beta 1 (fibronectin receptor beta) (ITGB1)	40-0	0.72 ± 0.09	
		40-10	0.69 ± 0.11	
		40-20	0.77 ± 0.09	
AA796818	Interferon, alpha-inducible protein (G1P2)	40-0	3.25 ± 0.13	
		40-10	2.01 ± 0.16	
		40-20	4.24 ± 0.13	
		20-10	-2.23 ± 0.13	
		20-0	-0.99 ± 0.16	
		10-0	1.24 ± 0.14	
BG063771	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); clone H303E11	40-0	0.71 ± 0.09	2.55 ± 0.22
		40-10	0.55 ± 0.11	2.59 ± 0.23
		40-20	0.65 ± 0.09	1.42 ± 0.31
BG063729	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); clone H3012A11	40-0	0.89 ± 0.08	2.55 ± 0.22
		40-10	0.74 ± 0.10	2.59 ± 0.23
		40-20	0.75 ± 0.08	1.42 ± 0.31
BG066160	Ornithine aminotransferase (OAT); clone H3039F05	40-0	-1.03 ± 0.12	-0.07 ± 0.56
		40-10	-0.79 ± 0.14	-0.78 ± 0.10
		40-20	-0.85 ± 0.12	-0.55 ± 0.09
BG074606	Ornithine aminotransferase (OAT); clone H3137B06	40-0	-0.75 ± 0.18	-0.07 ± 0.56
		40-20	-0.77 ± 0.18	-0.55 ± 0.09
AI226017	Sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1)	40-0	-1.28 ± 0.11	-1.29 ± 0.16
		40-10	-1.62 ± 0.12	3.07 ± 0.18
		40-20	-1.57 ± 0.11	3.69 ± 0.16
BG072707	Spermidine/spermine N1-acetyl transferase 1 (SAT)	40-20	0.63 ± 0.12	
BG063081	Thymosin, beta 10 (TMSB10)	40-0	0.54 ± 0.12	
		40-10	0.63 ± 0.14	
		40-20	0.80 ± 0.12	
AA107664	Signal transducer and activator of transcription 1 (STAT1)	40-0	1.45 ± 0.13	3.91 ± 0.25
		40-10	1.39 ± 0.15	2.81 ± 0.34
		40-20	2.48 ± 0.13	4.17 ± 0.11
		20-0	-1.04 ± 0.15	-0.26 ± 0.25
		20-10	-1.10 ± 0.13	-4.17 ± 0.34
AA798640	Tryptophan hydroxylase 1 (TPH)	40-20	-1.18 ± 0.12	
		20-0	0.86 ± 0.13	
		20-10	0.69 ± 0.11	
BG065392	Cathepsin B (CTSB)	40-20	0.46 ± 0.07	
AI119367	Caspase 7 (CASP7)	40-10	0.73 ± 0.11	
		40-20	0.73 ± 0.09	

Table 4. (Continued)

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Comparison	Difference cDNA microarray ³	Difference RT-PCR ⁴
BG079564	Leukemia inhibitory factor (LIF)	40-20	-0.49 ± 0.07	
BG075853	Selenoprotein P, plasma 1 (SEPP1)	40-0	-1.10 ± 0.14	
		40-10	-1.18 ± 0.16	
		40-20	-1.07 ± 0.14	
AA500809	Peripheral myelin protein (PMP22)	40-0	0.80 ± 0.12	
BG066650	Proteasome (prosome, macropain) 28 subunit, alpha (PSME1)	40-20	0.92 ± 0.14	
BG065380	Pyruvate kinase, muscle (PKM2)	40-0	0.62 ± 0.07	
		40-20	0.59 ± 0.07	
BG073104	Ribosomal protein S17 (RPS17)	40-0	-0.50 ± 0.09	
AI256565	Hydroxyprostaglandin dehydrogenase 15 (NAD) (HPGD)	40-10	-0.91 ± 0.16	
		40-20	-0.66 ± 0.14	
BG071325	Calbindin 3 (CALB3)	40-20	-2.68 ± 0.23	
AI157359	P311 protein (P311)	40-20	-0.93 ± 0.14	
BG064747	Glutamic-pyruvate transaminase (GPT)	10-0	0.93 ± 0.14	
BG086656	Aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1)	40-0	0.96 ± 0.12	
		10-0	1.39 ± 0.13	
AA759852	Solute carrier family 26, member 3 (SLC26A3)	40-0	1.31 ± 0.18	
BG070529	Caspase 3, apoptosis related cysteine protease (CASP3)	40-0	0.76 ± 0.12	
		40-20	0.85 ± 0.12	
AA510124	Chemokine (C-X-C motif) ligand 10 (CXCL10)	40-20	1.28 ± 0.18	
W29265	Glutathione S-transferase, alpha 2 (Yc2) (GSTA2)	40-20	1.38 ± 0.12	
BG064142	Hypoxia inducible factor 1, alpha subunit (HIF1A)	40-0	1.33 ± 0.15	
		40-20	1.11 ± 0.15	
BG064535	Squalene epoxidase (SQLE)	40-0	0.83 ± 0.10	
		40-20	0.90 ± 0.10	

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.²NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.³Estimated difference in mean ²log-transformed intensity ± SE.⁴Normalized difference in mean ²log-transformed intensity ± SE. Twenty-one gene differences were validated with RT-PCR. Empty cells indicate gene differences which were not taken into account.

At least 17 genes are, according to literature review, likely to play a role at different stages during CRC development and are discussed in more detail. They are involved in biotransformation [Sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1), Glutathione S-transferase, alpha 2 (Yc2) (GSTA2), Hydroxyprostaglandin dehydrogenase 15 (NAD) (HPGD), Aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1)], apoptosis [Tumor necrosis factor receptor superfamily, member 6 (TNFRSF6), Caspase 4, apoptosis-related cysteine protease (CASP4), Caspase 7 (CASP7), Caspase 3, apoptosis

related cysteine protease (CASP3), cathepsin B (CTSB), Thymosin beta 10 (TMSB10), Signal transducer and activator of transcription 1 (STAT1), DNA repair [RAD51 homolog (*S.cerevisiae*) (RAD51)], polyamine metabolism [Ornithine aminotransferase (OAT), Spermidine/spermine N1-acetyl transferase 1 (SAT)], and tumor suppression [ribonucleotide reductase M1 polypeptide (RRM1), Solute carrier family 26, member 3 (SLC26A3), MYB binding protein (P160) 1a (MYBBP1a)].

In Figure 1, the estimated differences per vegetable diet group compared to the 0% vegetable diet group (set to 0) of these genes are depicted. This figure shows that most effects of the vegetables on gene expression occur in the highest vegetable dose group, and that in general dose-response relationship was no clear linear. For the expression of the genes SULF1A1, RAD51, TMSB10, STAT1, OAT and RRM1 the effect of vegetables in the highest dose group is significantly different from the expression in the other vegetable groups. This seems also true for GSTA2, HPGD, TNFRSF6, CASP3, CASP4, CASP7, CTSB, SAT, and MYB, although not all differences between the highest vegetable dose group and the other dose groups are statistically significant. These results indicate that there is a relatively high dose of vegetables necessary to exert a measurable effect on colonic gene expression. The only exceptions to this are the ALDH1A1 and SLC26A3 genes. For these genes the effect on expression is similar in all three vegetable groups.

Figure 1. Estimated difference in mean $\log$ -transformed intensity $\pm$ SE per diet group (x-axis) for the genes that are likely to play a role at different stages during CRC development. The 0% diet intensity (set to 0) serves as the reference. Asterisk indicates significant differences between diet group and control (0% diet group). SULF1A1: Sulfotransferase family 1A, phenol-preferring, member 1; GSTA2: Glutathione S-transferase, alpha 2 (Yc2); HPGD: Hydroxyprostaglandin dehydrogenase 15 (NAD); ALDH1A1: Aldehyde dehydrogenase family 1, subfamily A1; RAD51: RAD51 homolog (*S.cerevisiae*); TNFRSF6: Tumor necrosis factor receptor superfamily, member 6; CASP3: Caspase 3, apoptosis related cysteine protease; CASP7: Caspase 7; CASP4: Caspase 4, apoptosis-related cysteine protease; CTSB: Cathepsin B; TMSB10: Thymosin beta 10; STAT1: Signal transducer and activator of transcription 1; SAT: Spermidine/spermine N1-acetyl transferase 1; OAT: Ornithine aminotransferase; RRM1: Ribonucleotide reductase M1 polypeptide; SLC26A3: Solute carrier family 26, member 3; C-MYB, C-myb proto-oncogene. See Table 4 for other statistical details.

HPGD

ALDH1A1

RAD51

TNFRSF6

CASP3

CASP7

CASP4

CTSB

Figure 1. (Continued)

Figure 1. (Continued)

Real-time RT-PCR was used to validate 21 gene expression differences, representing six genes responsive to vegetables, as identified by cDNA microarray analyses. The results are shown in Table 4. These data confirm the results obtained by microarray analyses and verify the reliability of this cDNA microarray approach to identify genes that are modulated by vegetables.

Discussion

In the present study, a cDNA microarray approach was used to analyze the dose-dependent changes in gene expression in response to diets supplied with different doses of a mixture of vegetables in colon mucosa of mice, in order to provide insight into the mechanisms underlying CRC prevention by vegetables.

CRC develops as the result of the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium into colon adenocarcinoma, as presented in the genetic model by Vogelstein.³⁸ At each of the presented stages, various genetic mechanisms are possible by which vegetables and/or vegetable components can inhibit progression to the next stage.

The results of this study show a dose-dependent effect of vegetables on gene expression, although in general the relationship was not linear. At least 17 genes which may play a role at different stages during CRC development were significantly modulated at this dose. Table 5 summarizes the effects on gene expression in relation to possible effects on carcinogenesis.

The first line of defense against initiation of CRC is the ability of the colon tissue to intercept and detoxify potentially DNA damaging xenobiotic or endogenous substances. In this study, three genes involved in xenobiotic metabolism were affected, i.e. SULT1A1, GSTA2 and ALDH1A1. SULT1A1 and GSTA2 encode both for phase-II biotransformation enzymes, but these enzymes differ in their mode of action. Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs and xenobiotic compounds and play an important role in detoxification of these compounds. However, they also play an important role in the metabolism and bioactivation of many dietary and environmental mutagens, including heterocyclic aromatic amines (HCA) implicated in the pathogenesis of colorectal and other cancers.^{39, 40} Several food components have already been shown to be potent inhibitors of SULT1A1 like quercetin^{41, 42}, grape juice⁴² and curcumin⁴³. In contrast to SULT1A1, the GSTA2 enzyme is able to detoxify HCA, next to a number of other electrophilic carcinogenic metabolites like benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide and aflatoxin B1-8,9-oxide⁴⁴, by coupling to glutathione.⁴⁵ In addition to this major activity, GSTs from

the alpha class, like GSTA2, are known to catalyze glutathione peroxidase reactions and are therefore an essential component of the cellular antioxidant defense mechanism.⁴⁶ The GST enzymes are inducible by drugs, carcinogens, antioxidants and other dietary components.^{44, 47} In this study, the expression of SULT1A1 was inhibited in the highest dose group, compared to the other diet groups, whereas the expression of GSTA2 was increased in the highest dose group. Next to these effects on phase-II biotransformation genes, vegetables induced another detoxifying gene, i.e. ALDH1A1. This gene encodes for an enzyme which belongs to the family of several isoenzymes involved in the metabolism of exogenous and endogenous aldehydes. This particular isoenzyme is the next enzyme after alcohol dehydrogenase in the major pathway of alcohol metabolism. It oxidizes acetaldehyde to acetic acid and thereby protects cells from the adverse effects of acetaldehyde. There is increasing evidence that acetaldehyde is highly toxic, mutagenic and carcinogenic.⁴⁸ In this study, the expression of ALDH1A1 was significantly higher in the 10% and 40% vegetable groups compared to the control group. The effects of vegetables on the expression of SULT1A1, GSTA2 and ALDH1A1 support the model that high vegetable consumption results in decreased colon cancer risk, by inhibition of formation and detoxification of colon carcinogens.

Table 5. Presentation of differentially expressed genes known to be involved in (colon)carcinogenesis, the direction of the effect in the 40% vegetable group compared to control, the theoretical effect of this on cancer risk, and the involved pathways.

Gene Name by NCBI (Abbreviation) ¹	Effect on expression in the highest (40%) vegetable group compared to control	Theoretical effect on cancer risk	Involved pathway
SULT1A1	–	–	Biotransformation
GSTA2	+	–	
HPGD	–	–	
ALDH1A1	+	–	
RAD51	+	–	DNA repair
TNFRSF6	+	–	Apoptosis
CASP4	+	–	
CASP7	+	–	
CASP3	+	–	
CTSB	+	–	
TMSB10	+	–	
STAT1	+	–	
OAT	–	+	Polyamine metabolism
SAT	+	–	
RRM1	+	–	Tumor invasion
SLC26A3	+	–	
MYBBP1a	–	+	

¹NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

A gene involved in generation of DNA reactive compounds is the HPGD gene, which was down-regulated in the highest vegetable group. It encodes for an enzyme which metabolizes a number of prostaglandins and non-prostanoid compounds. The products of the non-prostanoids compounds are generally highly reactive α,β -unsaturated aldehydes and ketones, which may cause carcinogenesis. HPGD is a target for many non-steroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase-2 inhibitors, which have been shown to decrease the risk of colon cancer in both epidemiological and experimental studies. Also phytophenolic compounds such as curcumin, flavonoids and epigallocatechin gallate were found to inhibit HPGD enzyme activity.^{49, 50} The mechanism of chemoprevention by these compounds could be by inhibition of HPGD mRNA resulting in a diminished HPGD enzyme activity, leading to a decreased production of the highly reactive α,β -unsaturated aldehydes and ketones in the colon tissues. Down-regulation of HPGD in the highest vegetable group indicates the presence of vegetable (components) acting as HPGD inhibitors.

If a genotoxic reaction with DNA has occurred, cancer initiation can still be prevented by repairing this lesion. One DNA repair gene was up-regulated, namely RAD51. This gene encodes for the key enzyme for homologous recombination which is an evolutionarily conserved mechanism for the repair of double strand breaks and for generation of genetic diversity. Disruption of the RAD51 gene leads to an increased number of chromosome aberrations, including chromosome breaks and finally in cellular lethality, suggesting that this gene is important for maintenance genome stability.^{51, 52} Vegetables as inducers of this DNA repair gene have not previously been described, but in this study up-regulation of this gene was observed in the highest vegetable group compared to the other diet groups and suggests that vegetables could protect cells from DNA damage by increasing the DNA repair capacity.

Apoptosis (programmed cell death) is a common process in the intestinal crypt, by removing differentiated cells which form the surface epithelium. In the stem cell and proliferation zone of the colorectal crypt, apoptosis occurs to a lesser extent. Here, genetically damaged stem cells are removed before they can undergo clonal expansion. Stimulation of apoptosis by vegetables provides a protective mechanism against CRC, by eliminating genetically damaged cells before they can transform to tumor cells. Seven genes known to be involved in apoptosis were affected by vegetables in this study. Apoptosis is mediated via cell surface receptors (APO1/CD95 and the tumor necrosis factor (TNF) receptor family) and the mitochondria. Activation of the cell surface receptor by an extracellular ligand causes an intracellular region, the death effector domain, to form a cytosolic complex with pro-caspase-8, followed by a release of caspase-8, which initiates downstream activation of effector caspases 3⁵³, 4⁵⁴ 6 and 7.⁵³ The activation of these caspases results in programmed cell death.⁵³ TNFRSF6, CASP4, CASP7, and CASP3 were up-regulated in the highest vegetable dose group. CTSB can trigger the mitochondrial

pathway of apoptosis. This protease is released from lysosomes into the cytosol during intracellular cytotoxic-signaling cascades and thereby increasing the cytochrome c release from mitochondria.⁵⁵ Cytochrome c forms a protein complex with the apoptosis activating factor-1 that recruits procaspase 9, resulting in its activation. Caspase 9 activates caspase 3, initiating a caspase cascade that leads to cell death.^{53, 56} CTSB can also trigger apoptosis in a caspase-independent pathway, whereby CTSB functions as the dominant execution protease.⁵⁷ This mechanism could represent a backup program in tumor cells, because here the standard apoptosis pathway is frequently impaired and the expression of CTSB is increased. However, CTSB has another but opposing role in malignancy, namely enhancing the invasiveness of the tumor cells.⁵⁸ TMSB10, a small actin-binding protein, has also dual functions: programmed cell death⁵⁹ and tumor invasion.⁶⁰ The seventh gene affected by vegetables which is involved in apoptosis is STAT1. The mechanism by which STAT1 induces apoptosis is not clear, but may involve indirect up-regulation of Fas and caspase activation.⁶¹ In the colon mucosa of mice receiving the highest vegetable dose diet the TNFRSF6, CASP4, CASP7, CASP3, CTSB, TMSB10 and STAT1 genes were up-regulated and could thereby provide a protective mechanism against neoplasia by removing through apoptosis genetically damaged stem cells before they can proliferate.⁵³

Next to genes involved in apoptosis, genes concerned with cell proliferation and invasion were modulated by vegetables. Two genes involved in polyamine metabolism were modulated. Polyamines are ubiquitous cellular polycations essential for optimal rates of cell growth and differentiation.⁶² Increased polyamine content has been shown to correlate with higher CRC risk. Mutations in the adenomatous polyposis coli gene results in higher polyamine content in the intestinal mucosa.⁶³⁻⁶⁵ SAT, up-regulated in the highest vegetable group, is the key enzyme in polyamine catabolism⁶⁴, causing polyamine contents to drop. NSAIDs are able to activate SATs and are already used as chemopreventive agents for colon carcinogenesis.⁶⁴ OAT, down-regulated in the highest vegetable group, is also involved in polyamine metabolism. The protein-product of this gene converses ornithine to glutamate semialdehyde, thereby reducing intracellular ornithine contents. Ornithine is the substrate of ornithine decarboxylase (ODC), which is the rate-limiting enzyme in polyamine synthesis.⁶⁶ A decrease in OAT activity could contribute to an increase in ornithine available for ODC, leading to increased polyamine synthesis. The result of the effect of vegetables on polyamine metabolism and subsequent cell growth is not clear and has to be further investigated.

Several genes involved in tumor suppression were modulated by vegetables. Until now, no relation between vegetables and expression has previously been described for one of these genes, i.e the RRM1 gene, which was up-regulated in the highest vegetable group. This gene encodes for the large subunit (M1) of ribonucleotide reductase, the heterodimeric enzymes that catalyzes the rate-limiting step in deoxyribonucleotide synthesis, providing a balanced supply of precursors for DNA synthesis and repair.⁶⁷ Aside

from this role, RRM1 functions as a metastasis suppressor gene by suppressing invasion, migration and *in vivo* metastasis formation through upregulation of the phosphatase and tensin homolog (PTEN) tumor-suppressor gene, when overexpressed in human and mouse cancer cell lines.⁶⁸ SLC26A3 or DRA (down-regulated in adenoma) is an important tumor-suppressor gene associated with CRC risk and was up-regulated in the highest vegetable group compared to the control group. This gene is expressed exclusively in normal colon tissue⁶⁹, and it encodes a membrane specific anion transporter⁷⁰ for transporting sulfate and chloride into the cell.^{71, 72} Next to this, it induces growth-suppression in colon cells, what correlates with inhibition of colon tumor progression.⁷³ Up-regulation of this colon tumor suppressor gene by vegetables provides another mechanism by which vegetables can protect against colon cancer risk by inducing growth inhibition. However, next to this beneficial effect on the DRA tumor suppressor gene, the MYBBP1a gene was down-regulated in the highest vegetable group, which inhibits the c-myc proto-oncogene (C-MYB) protein. C-MYB is predominantly expressed in immature hemopoietic cells where it plays an important role in hemopoietic cell proliferation and differentiation.⁷⁴ C-MYB expression is restricted to just a few tissues and the role in these tissues is less clear. MYBBP1a is ubiquitously expressed in human and mouse cell lines and tissues and therefore it is thought that this gene has a broader cellular function than only being a myb-interacting protein.⁷⁵ The result of this modulation in relation to colon cancer development remains unclear and needs to be further investigated.

Although there is a gap between gene expression and CRC risk, it is found that vegetables exert an effect on intermediate biomarkers for CRC risk such as aberrant crypt foci and intestinal polyps. The aberrant crypt foci are considered as precursor lesions that would progress to overt CRC. In a study of Rijken et al (1999), rats fed different diets supplemented with peas, spinach, sprouts or a mix of different vegetables experienced a reduction in aberrant crypt foci (ACF) multiplicity.⁹ Furthermore, Kim et al (2003) investigated the effect of indole-3-carbinol (constituent of cruciferous vegetables) supplemented diets on ACF in C57BL/6j mice. Total number of ACF was significantly decreased in these animals compared to animals which received the standard diet. Next to an effect on ACF, a reduction of intestinal polyp development was observed in C57BL/6j-*apc*^{Min/+} mice fed the indole-3-carbinol supplemented diets.⁶ Mahmoud et al (2000) also reported a decrease in intestinal polyp development in C57BL/6j-*apc*^{Min/+} mice fed diets supplemented with the plant phenolics caffeic acid phenethyl ester or curcumin.⁷

In this study, the dose of vegetables necessary to exert a measurable effect on mouse colonic gene expression is relatively high, i.e. 40% of the diet. In the human population (females, age range 22-65 years), approximately 20% of the total daily food intake comprise vegetables and fruits, accounting for 270 g (~150 g vegetables and ~120 g fruits).⁷⁶ In this respect, it can be stated that on average, humans probably consume not enough vegetables and fruit to activate cancer preventive pathways. This is in line with the

latest recommendation made by The World Health Organization, which advises an intake of at least 400 g of vegetables and fruits (accounting for approximately 30% of the total daily food intake) to reduce the risk of cancer of the gastrointestinal tract.⁷⁷

In summary, the results of this study show a dose-dependent effect of vegetables on gene expression changes in the colon mucosa of female mice. Genes were modulated which are involved in protective mechanisms at the various stages of CRC development. A diet with a high content of vegetables modulates genes involved in inhibition of carcinogen formation, increasing DNA repair capacity, induction of apoptosis, and in reducing cell growth and -tumor invasion. Next to genes which are already known to be induced by vegetables (components), new genes were identified, i.e. the detoxification gene *ALDH1A1*, the DNA repair gene *RAD51* and the apoptosis genes *TNFRSF6*, *CASP4*, *CASP7*, *CASP3*, *CTSB*, *TMSB10* and *STAT1*, the metastases suppressor gene *RRM1* and the tumor suppressor gene *SLC26A3*. These genes may play an important role in CRC prevention by vegetables and may provide new molecular targets for CRC prevention. The possible importance of these genes and their modulation by vegetables has to be further investigated.

References

1. World Health Organization & International Agency for Research on Cancer. World Cancer Report. Lyon. IARC Press, 2003.
2. Greenwald P. Colon cancer overview. *Cancer* 1992;70:1206-15.
3. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 1981;66:1191-308.
4. World Cancer Research Fund. Food, Nutrition and the Prevention of Cancer: a Global Perspective. Chapter 4.10: Colon, rectum. Washington: BANTA Book group, 1997:216-251.
5. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* 1996;96:1027-39.
6. Kim DJ, Shin DH, Ahn B, Kang JS, Nam KT, Park CB, Kim CK, Hong JT, Kim YB, Yun YW, Jang DD, Yang KH. Chemoprevention of colon cancer by Korean food plant components. *Mutat Res* 2003;523-524:99-107.
7. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnoli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000;21:921-7.
8. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens Regulate Vitamin D Metabolism in the Mouse Colon: Relevance for Colon Tumor Prevention and Therapy. *J Nutr* 2002;132:3490S-3493S.
9. Rijken PJ, Timmer WG, van de Kooij AJ, van Benschop IM, Wiseman SA, Meijers M, Tijburg LB. Effect of vegetable and carotenoid consumption on aberrant crypt multiplicity, a surrogate end-point marker for colorectal cancer in azoxymethane-induced rats. *Carcinogenesis* 1999;20:2267-72.
10. Lupton JR, Turner ND. Potential protective mechanisms of wheat bran fiber. *Am J Med* 1999;106:24S-27S.
11. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991;2:427-42.
12. Cousins RJ. Nutritional regulation of gene expression. *Am J Med* 1999;106:20S-23S; discussion 50S-51S.
13. Wargovich MJ. Experimental evidence for cancer preventive elements in foods. *Cancer Lett* 1997;114:11-7.
14. Guyonnet D, Siess MH, Le Bon AM, Suscheter M. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicol Appl Pharmacol* 1999;154:50-8.
15. Wortelboer HM, de Kruif CA, van Iersel AA, Noordhoek J, Blaauboer BJ, van Bladeren PJ, Falke HE. Effects of cooked brussels sprouts on cytochrome P-450 profile and phase II enzymes in liver and small intestinal mucosa of the rat. *Food Chem Toxicol* 1992;30:17-27.
16. Larsen-Su S, Williams DE. Dietary indole-3-carbinol inhibits FMO activity and the expression of flavin-containing monooxygenase form 1 in rat liver and intestine. *Drug Metab Dispos* 1996;24:927-31.
17. Katchamart S, Williams DE. Indole-3-carbinol modulation of hepatic monooxygenases CYP1A1, CYP1A2 and FMO1 in guinea pig, mouse and rabbit. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;129:377-84.
18. Sorensen M, Jensen BR, Poulsen HE, Deng X, Tygstrup N, Dalhoff K, Loft S. Effects of a Brussels sprouts extract on oxidative DNA damage and metabolising enzymes in rat liver. *Food Chem Toxicol* 2001;39:533-40.

19. Clapper ML, Szarka CE, Pfeiffer GR, Graham TA, Balshem AM, Litwin S, Goosenberg EB, Frucht H, Engstrom PF. Preclinical and clinical evaluation of broccoli supplements as inducers of glutathione S-transferase activity. *Clin Cancer Res* 1997;3:25-30.
20. Bertram JS, Bortkiewicz H. Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. *Am J Clin Nutr* 1995;62:1327S-1336S.
21. Bertram JS. Carotenoids and gene regulation. *Nutr Rev* 1999;57:182-91.
22. Bates MD, Erwin CR, Sanford LP, Wiginton D, Bezerra JA, Schatzman LC, Jegga AG, Ley-Ebert C, Williams SS, Steinbrecher KA, Warner BW, Cohen MB, Aronow BJ. Novel genes and functional relationships in the adult mouse gastrointestinal tract identified by microarray analysis. *Gastroenterology* 2002;122:1467-82.
23. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy. *J Nutr* 2002;132:3490S-3493S.
24. Kawai J, Shinagawa A, Shibata K, Yoshino M, Itoh M, Ishii Y, Arakawa T, Hara A, Fukunishi Y, Konno H, Adachi J, Fukuda S, Aizawa K, Izawa M, Nishi K, Kiyosawa H, Kondo S, Yamanaka I, Saito T, Okazaki Y, Gojobori T, Bono H, Kasukawa T, Saito R, Kadota K, Matsuda H, Ashburner M, Batalov S, Casavant T, Fleischmann W, Gaasterland T, Gissi C, King B, Kochiwa H, Kuehl P, Lewis S, Matsuo Y, Nikaido I, Pesole G, Quackenbush J, Schriml LM, Staubli F, Suzuki R, Tomita M, Wagner L, Washio T, Sakai K, Okido T, Furuno M, Aono H, Baldarelli R, Barsh G, Blake J, Boffelli D, Bojunga N, Carninci P, de Bonaldo MF, Brownstein MJ, Bult C, Fletcher C, Fujita M, Gariboldi M, Gustincich S, Hill D, Hofmann M, Hume DA, Kamiya M, Lee NH, Lyons P, Marchionni L, Mashima J, Mazzarelli J, Mombaerts P, Nordone P, Ring B, Ringwald M, Rodriguez I, Sakamoto N, Sasaki H, Sato K, Schonbach C, Seya T, Shibata Y, Storch KF, Suzuki H, Toyo-oka K, Wang KH, Weitz C, Whittaker C, Wilming L, Wynshaw-Boris A, Yoshida K, Hasegawa Y, Kawaji H, Kohtsuki S, Hayashizaki Y. Functional annotation of a full-length mouse cDNA collection. *Nature* 2001;409:685-90.
25. Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of beta-catenin signaling by a combination of tea plus sulindac. *Carcinogenesis* 2003;24:263-7.
26. van Kranen HJ, van Iersel PW, Rijnkels JM, Beems DB, Alink GM, van Kreijl CF. Effects of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the ApcMin mouse. *Carcinogenesis* 1998;19:1597-601.
27. Rijnkels JM, Alink GM. Effects of a vegetables-fruit mixture on liver and colonic 1,2-dimethylhydrazine-metabolizing enzyme activities in rats fed low- or high-fat diets. *Cancer Lett* 1998;128:171-5.
28. Hasseman J, Chen E, Yang I, Quackenbush J. Aminoallyl labeling of RNA for microarrays. <http://www.tigr.org/tdb/micoarray/protocolsTIGR.shtml> (Accessed June 21, 2001), 2001.
29. Kerr MK. Design considerations for efficient and effective microarray studies. *Biometrics* 2003;59:822-828.
30. Dobbin K, Shih JH, Simon R. Questions and answers on design of dual-label microarrays for identifying differentially expressed genes. *J Natl Cancer Inst* 2003;95:1362-9.
31. Churchill GA. Fundamentals of experimental design for cDNA microarrays. *Nat Genet* 2002;32 Suppl 2:490-5.
32. Kerr MK, Martin M, Churchill GA. Analysis of variance for gene expression microarray data. *Journal of Computational Biology* 2000;7:819-837.

33. Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS. Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol* 2001;8:625-37.
34. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. *Applied Linear Statistical Models* (Fourth Edition). Mass, 1996.
35. Kerr MK, Afshari CA, Bennett L, Bushel P, Martinez J, N.J. W, Churchill GA. Statistical analysis of a gene expression microarray experiment with replication. *Statistica Sinica* 2002;12:203-217.
36. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-93.
37. Aerts JL, Gonzales MI, Topalian SL. Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. *Biotechniques* 2004;36:84-6, 88, 90-1.
38. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
39. Gamage NU, Duggleby RG, Barnett AC, Tresillian M, Latham CF, Liyou NE, McManus ME, Martin JL. Structure of a human carcinogen-converting enzyme, SULT1A1. Structural and kinetic implications of substrate inhibition. *J Biol Chem* 2003;278:7655-62.
40. Wong CF, Liyou N, Leggett B, Young J, Johnson A, McManus ME. Association of the SULT1A1 R213H polymorphism with colorectal cancer. *Clin Exp Pharmacol Physiol* 2002;29:754-8.
41. De Santi C, Pietrabissa A, Mosca F, Rane A, Pacifici GM. Inhibition of phenol sulfotransferase (SULT1A1) by quercetin in human adult and foetal livers. *Xenobiotica* 2002;32:363-8.
42. Tamura H, Matsui M. Inhibitory effects of green tea and grape juice on the phenol sulfotransferase activity of mouse intestines and human colon carcinoma cell line, Caco-2. *Biol Pharm Bull* 2000;23:695-9.
43. Vietri M, Pietrabissa A, Mosca F, Spisni R, Pacifici GM. Curcumin is a potent inhibitor of phenol sulfotransferase (SULT1A1) in human liver and extrahepatic tissues. *Xenobiotica* 2003;33:357-63.
44. Morel F, Schulz WA, Sies H. Gene structure and regulation of expression of human glutathione S-transferases alpha. *Biol Chem Hoppe Seyler* 1994;375:641-9.
45. Coles B, Nowell SA, MacLeod SL, Sweeney C, Lang NP, Kadlubar FF. The role of human glutathione S-transferases (hGSTs) in the detoxification of the food-derived carcinogen metabolite N-acetoxy-PhIP, and the effect of a polymorphism in hGSTA1 on colorectal cancer risk. *Mutat Res* 2001;482:3-10.
46. Yang Y, Sharma R, Zimniak P, Awasthi YC. Role of alpha class glutathione S-transferases as antioxidant enzymes in rodent tissues. *Toxicol Appl Pharmacol* 2002;182:105-15.
47. t Hoen PA, Rooseboom M, Bijsterbosch MK, van Berkel TJ, Vermeulen NP, Commandeur JN. Induction of glutathione-S-transferase mRNA levels by chemopreventive selenocysteine Se-conjugates. *Biochem Pharmacol* 2002;63:1843-9.
48. Seitz HK, Matsuzaki S, Yokoyama A, Homann N, Vakevainen S, Wang XD. Alcohol and cancer. *Alcohol Clin Exp Res* 2001;25:137S-143S.
49. Cho H, Tai HH. Inhibition of NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) by cyclooxygenase inhibitors and chemopreventive agents. *Prostaglandins Leukot Essent Fatty Acids* 2002;67:461-5.
50. Cho H, Tai HH. Thiazolidinediones as a novel class of NAD(+) -dependent 15-hydroxyprostaglandin dehydrogenase inhibitors. *Arch Biochem Biophys* 2002;405:247-51.
51. Stark JM, Hu P, Pierce AJ, Moynahan ME, Ellis N, Jasin M. ATP hydrolysis by mammalian RAD51 has a key role during homology-directed DNA repair. *J Biol Chem* 2002;277:20185-94.

52. Kanamoto T, Hellman U, Heldin CH, Souchelnytskyi S. Functional proteomics of transforming growth factor-beta1-stimulated Mv1Lu epithelial cells: Rad51 as a target of TGFbeta1-dependent regulation of DNA repair. *Embo J* 2002;21:1219-30.
53. Johnson IT. Anticarcinogenic effects of diet-related apoptosis in the colorectal mucosa. *Food Chem Toxicol* 2002;40:1171-8.
54. Kamada S, Washida M, Hasegawa J, Kusano H, Funahashi Y, Tsujimoto Y. Involvement of caspase-4(-like) protease in Fas-mediated apoptotic pathway. *Oncogene* 1997;15:285-90.
55. Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest* 2000;106:1127-37.
56. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA, Lemasters JJ. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. *Mol Cell Biol* 1998;18:6353-64.
57. Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M, Jaattela M. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol* 2001;153:999-1010.
58. Emmert-Buck MR, Roth MJ, Zhuang Z, Campo E, Rozhin J, Sloane BF, Liotta LA, Stetler-Stevenson WG. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am J Pathol* 1994;145:1285-90.
59. Hall AK. Thymosin beta-10 accelerates apoptosis. *Cell Mol Biol Res* 1995;41:167-80.
60. Santelli G, Califano D, Chiappetta G, Vento MT, Bartoli PC, Zullo F, Trapasso F, Viglietto G, Fusco A. Thymosin beta-10 gene overexpression is a general event in human carcinogenesis. *Am J Pathol* 1999;155:799-804.
61. Xu X, Fu XY, Plate J, Chong AS. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 1998;58:2832-7.
62. Pegg AE. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res* 1988;48:759-74.
63. Martinez ME, O'Brien TG, Fultz KE, Babbar N, Yerushalmi H, Qu N, Guo Y, Boorman D, Einspahr J, Alberts DS, Gerner EW. Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc Natl Acad Sci U S A* 2003;100:7859-64.
64. Babbar N, Gerner EW. Polyamines as modifiers of genetic risk factors in human intestinal cancers. *Biochem Soc Trans* 2003;31:388-92.
65. Wang W, Liu LQ, Higuchi CM. Mucosal polyamine measurements and colorectal cancer risk. *J Cell Biochem* 1996;63:252-7.
66. Han X, Kazarinoff MN, Seiler N, Stanley BA. Rat colon ornithine and arginine metabolism: coordinated effects after proliferative stimuli. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G389-99.
67. Wright JA, Chan AK, Choy BK, Hurta RA, McClarty GA, Taggar AY. Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase, and the significance to DNA synthesis. *Biochem Cell Biol* 1990;68:1364-71.
68. Gautam A, Li ZR, Bepko G. RRM1-induced metastasis suppression through PTEN-regulated pathways. *Oncogene* 2003;22:2135-42.

69. Schweinfest CW, Henderson KW, Suster S, Kondoh N, Papas TS. Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. *Proc Natl Acad Sci U S A* 1993;90:4166-70.
70. Byeon MK, Westerman MA, Maroulakou IG, Henderson KW, Suster S, Zhang XK, Papas TS, Vesely J, Willingham MC, Green JE, Schweinfest CW. The down-regulated in adenoma (DRA) gene encodes an intestine-specific membrane glycoprotein. *Oncogene* 1996;12:387-96.
71. Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, Shull GE. Renal and intestinal absorptive defects in mice lacking the NHE3 Na⁺/H⁺ exchanger. *Nat Genet* 1998;19:282-5.
72. Rajendran VM, Binder HJ. Cl-HCO₃ and Cl-OH exchanges mediate Cl uptake in apical membrane vesicles of rat distal colon. *Am J Physiol* 1993;264:G874-9.
73. Antalis TM, Reeder JA, Gotley DC, Byeon MK, Walsh MD, Henderson KW, Papas TS, Schweinfest CW. Down-regulation of the down-regulated in adenoma (DRA) gene correlates with colon tumor progression. *Clin Cancer Res* 1998;4:1857-63.
74. Mucenski ML, McLain K, Kier AB, Swerdlow SH, Schreiner CM, Miller TA, Pietryga DW, Scott WJ, Jr., Potter SS. A functional c-myc gene is required for normal murine fetal hepatic hematopoiesis. *Cell* 1991;65:677-89.
75. Keough R, Woollatt E, Crawford J, Sutherland GR, Plummer S, Casey G, Gonda TJ. Molecular cloning and chromosomal mapping of the human homologue of MYB binding protein (P160) 1A (MYBBP1A) to 17p13.3. *Genomics* 1999;62:483-9.
76. Anonymous. Zo eet Nederland. Resultaten van de voedselconsumptiepeiling 1997-1998. Voedingscentrum Den Haag, 1998.
77. Diet, nutrition and the prevention of chronic diseases. World Health Organ Tech Rep Ser 2003;916:i-viii, 1-149, backcover.

Chapter 4

Identification of proteins differentially expressed in colon mucosa of C57BL6 female mice by altered vegetable intake

In Preparation

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Abstract

Colorectal cancer is one of the most common types of cancer in the world. Over 70% of the cases might be prevented by changes in diet and life style. Vegetable consumption is thought to have a protective effect against colorectal cancer and a positive relationship has already been found in both mouse and human studies. Until now, not much is known about the targets of preventive actions by vegetables at the genome and proteome level. In the current study, the effect of increased vegetable intake on the protein expression in the colonic mucosa of healthy mice was studied in order to identify the proteins of which the levels are affected by increased vegetable consumption. Mice were fed four different diets, containing respectively 0%, 10%, 20% or 40% of vegetables, and total cellular protein from colonic mucosal cells was analyzed by means of 2D-gelelectrophoresis. We found 30 proteins that were differentially expressed in one or more vegetable groups as compared to the control group. Six of these proteins could be identified by MALDI-TOF mass spectrometry; namely MLRN, CAH-1, HMG-1, PAP3, GAPDH and OSCP. Except for OSCP, alterations in the levels of these proteins coincide with a pivotal role in the protection against colon cancer. We therefore conclude that the observed altered protein levels following increased vegetable intake, provide functional support for the protective effects of vegetables against colorectal cancer.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer in the Western world. Although mortality has declined, the incidence is generally increasing and preventive measures are therefore needed.¹ The etiology of CRC is complex and involves both genetic and environmental factors. Known risk factors include a positive family history, age, meat and alcohol consumption and fat intake. It is thought that over 70% of CRC cases might be preventable by modification of the diet and life style risk factors² and inverse associations are reported with physical activity, vegetable intake, and, although less consistent, with fruit consumption.³⁻⁵

Vegetables contain a large number of potentially anticarcinogenic compounds that can affect the process of carcinogenesis via various mechanisms. Using mice and rats, many studies have been performed investigating the role of individual vegetable derived compounds in the protection against CRC. The majority shows a protective effect of vegetable components against CRC.⁶⁻⁸

Vegetables can exert their protective effect against CRC via several mechanisms like, for example, free radical scavenging, modulation of enzyme levels and transcriptional regulation. So far, the effect of vegetable consumption on gene and protein levels has not yet been extensively studied and the targets at the genome and proteome level are largely unknown. Some studies have already been performed describing the effect of individual vegetable components on gene expression in mouse models and human cell lines.^{9, 10} However, often these effects were studied in relation to, for example, exposure to carcinogens.^{9, 11} These studies present evidence for a protective effect of single vegetable components. In order for genes to exert their effect, changes on the transcriptomic level will have to be translated to the proteome and eventually to the metabolome. Several studies have already been performed exploring the effect of consumption of single food components on protein expression in the colon and colonic cell lines.¹²⁻¹⁴ These studies focused, for example, on the regulation of specific enzymes involved in (the protection against) CRC or expression of proteins important for cell cycle regulation and apoptosis. However, not much is yet known about the effect of vegetable consumption on the total protein expression pattern.

In a previous study of our group, the effect of vegetable consumption on gene expression changes in colon mucosa from female mice was described (**Chapter 3**). In the current study, the effect of increased vegetable levels in the diet on the protein expression in the colon mucosa of these female mice was studied, in order to identify the proteins for which the expression levels are affected by increased vegetable consumption. Mice were fed four different diets, containing respectively 0%, 10%, 20% or 40% vegetables (mixture of cauliflower, carrots, peas and onions). Each vegetable represents a subclass of

vegetables that are known to interact via different mechanism with the process of carcinogenesis. Cauliflower belongs to the family of cruciferous vegetables. They contain isothiocyanates and indoles, breakdown products of glucosinolates that are present in these vegetables. These compounds affect the levels of detoxification enzymes and modulate the levels of phase-I and -II metabolizing enzymes.¹⁵⁻¹⁷ Carrots contain antioxidants that protect the cell from free radical damage and suppress cell proliferation.^{10, 18-20} Dietary fibers dilute and bind carcinogens in the digestive tract, decrease transit time of faecal bulk and inhibit cell proliferation. Peas are known to contain large amounts of fiber and are therefore added to the vegetable diets. Onion is a diallyl vegetable, containing organosulfur compounds that can modify carcinogen activation by changing biotransforming and detoxification enzyme levels.^{18, 19, 21, 22}

In the current study, total protein was isolated from colonic mucosal cells and the proteins were separated by 2D-gelelectrophoresis. Proteins that showed differential expression in one or more of the vegetable groups compared to control were selected for identification by MALDI-TOF mass spectrometry. Five out of six identified proteins might play a role in the protection against CRC. This may provide new insights in the biological mechanisms that possibly are related to or even directly involved in the CRC risk reduction properties of vegetables.

Materials and Methods

Animals and Diets

The number and type of animals, the development of the vegetable diets and treatment of the animals in the present study was previously described in **Chapter 3** (Section Materials and Methods; Animals and diet). In short, eight-week-old female C57BL6 mice (Charles River Laboratories, France) were randomly assigned to one of four different diets, containing a 0% (referred to as control diet), 10%, 20% and 40% wt/wt vegetables mixture respectively, for a period of two weeks (7 mice per group). The vegetables mixture used in the present mice study consisted of cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt). The composition of the different diets is given in Table 1.

The animals were maintained under controlled environmental conditions (temperature 21 ± 1 °C, relative humidity $50\% \pm 10$, 12-hr light/dark cycle). Body weights of the mice were recorded weekly. The study was approved by the Institutional Committee of Animal Experimentation of the University of Maastricht.

Protein Isolation

Protein from mouse colon mucosal cells was isolated from the residuals after the isolation of RNA using Trizol™ (GIBCO Life Technologies, Breda, The Netherlands) according to the manufacturers' instructions. Total RNA was used for gene expression studies (**Chapter 3**). After precipitation of the RNA, the supernatant was collected and stored at -80°C for isolation of total protein according to the manual, except that two additional wash steps of the protein pellet were performed in 0.3 M guanidine hydrochloride in 95% ethanol and one additional wash in 95% ethanol to reduce contamination. Total protein from two to three mice was pooled obtaining in total 3 pools per diet group similar to the pools in the gene expression studies (**Chapter 3**). Protein concentrations were determined using the DC Protein Assay (Bio-Rad, Veenendaal, The Netherlands).

Table 1. Composition of the four diets in g/kg.

Ingredients	Control diet ¹	10% Vegetable diet ²	20% Vegetable diet ²	40% Vegetable diet ²
Cerelose/Dextrose	515	422	329	143
DL-Methionine	2	2	2	2
NaCl	3	3	3	3
Choline CL 50%	4	4	4	4
Soya oil	50	50	50	50
KCl	7	7	7	7
KH ₂ PO ₄	7	7	7	7
MgO	2	2	2	2
CaHPO ₄ ·2H ₂ O	13	13	13	13
CaCO ₃	10	10	10	10
Corn Starch	100	100	100	100
Dicacel/Cellulose	77	70	63	49
Casein	200	200	200	200
MgSO ₄ ·7H ₂ O	4	4	4	4
Standard vitamin premix	3	3	3	3
Standard micronutrient premix	3	3	3	3
Vegetables mixture ³	0	100	200	400

¹Control diet is the basal 20% casein reference diet.

²In the vegetable diets, the basal diet was adjusted for cerelose/dextrose and dicacel/cellulose resulting in similar energy densities for all diets.

³Composition (% wet weight): cauliflower (30%), carrots (30%), peas (30%) and onions (10%).

2D-Gelelectrophoresis

100 µg of total protein of each pool was loaded on Immobiline Dry Strips (pH 3-10, 24 cm long) for the first dimension. Isoelectric focusing was performed on an IPGphor

electrophoresis unit (Amersham Biosciences, Buckinghamshire, England) at 20°C. The strips were rehydrated overnight in 350 µl of 8 M urea, 2% w/v CHAPS, 65 mM DTT, 0.5% v/v IPG buffer 3-10 at 30 V. Isoelectric focusing was performed using the following program: 500 V for 1 h, 1000 V for 1 h, 1000-8000 V for 2 h and a final step of 8000 V to reach a total of 56250 Vh. After focusing, IPG strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT and for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% w/v iodoacetamide, and were placed onto a slab gel and sealed with a 0.5% w/v agarose solution in Laemmli buffer with a trace of bromophenol blue. The second-dimension run was carried out on 12.5% SDS-PAGE gels. Electrophoresis was conducted at a constant voltage of 200 V for 5 h in a 24 cm Dodeca Cell (Bio-Rad) (Rabilloud et al., 1997; Gorg et al., 2000). In-gel proteins were silver-stained according to Humphery-Smith et al. (2000). Gel images were obtained with a GS-800 calibrated densitometer (Bio-Rad).

Spot Selection

Protein expression patterns were analyzed using the PDQuest 7.1.1 program (Bio-Rad). The average spot density from the three replicate gels of each diet group was calculated and the protein spots were analyzed for differential protein expression upon consumption of vegetables by comparison of these average values to those of the control diet. Proteins that showed at least two times enhanced or decreased expression were selected for identification.

In-gel Protein Digestion

Protein spots were excised from the gel and processed on a MassPREP digestion robot (Waters, Manchester, UK) according to the following protocol: for silver stained spots 0.5% H₂O₂ was used for destaining. Cysteines were reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 30 minutes followed by alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 minutes at room temperature (RT). Spots were washed with 100 mM ammonium bicarbonate to remove excess reagents and dehydrated with 100% acetonitrile. Trypsin (6 ng/µl) in 50 mM ammonium bicarbonate was added to the gel plug and incubated at 37 °C for 5 hours. The peptides were extracted with 1% formic acid / 2% acetonitrile.

Mass Spectrometry

For MALDI-TOF mass spectrometry 1.5 µl of each peptide mixture and 0.5 µl matrix solution (10 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1%TFA) was spotted automatically onto a 96 well-format target plate. The spots were allowed to air dry for homogenous crystallization. Spectra were obtained using a M@lDi-LR mass spectrometer (Waters, Manchester, UK). The instrument was operated in positive

reflectron mode. Acquisition mass range was 900-3000 Da. The instrument was calibrated on 6-8 reference masses from a tryptic digest of alcohol dehydrogenase. Next, a nearpoint lockmass correction for each spot was performed using ACTH 1-39 (MH+ 2465.199) to achieve maximum mass accuracy. Typically 100 shots were combined and background subtracted. A peptide mass list was generated for subsequent database search.²³

Database Search of MALDI-TOF Peptide Spectra

The peptide mass list was searched with Mascot (<http://www.matrixscience.com>) against the Swiss-Prot database (<http://expasy.ch/sprot>) for protein identification. The search parameters were as follows: one mis-cleavage was tolerated; carbamidomethylation was set as a fixed modification; and oxidation of methionine as an optional modification. The peptide mass tolerance was set to 100 ppm. No restrictions were made on the protein molecular weight and the isoelectric point. A protein was regarded identified when the search was found to be statistically significant ($p < 0.05$) in Mascot.

Immunoblotting

Total cellular proteins were separated by SDS-PAGE (12% acrylamide) followed by immunoblotting. 10 µg of total protein was dissolved in 1% SDS and 30 mM DTT, 15 mM Tris-HCl buffer (pH 6.8), 10% glycerol and 0.006% bromophenol blue (final concentrations). SDS-polyacrylamide gelelectrophoresis was performed in 12% (w/v) slab gels using the (mini)ProteanII equipment (Bio-Rad). After separation, the proteins were electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) in blotting buffer (25 mM Tris, 200 mM glycine, 20% methanol) using the (mini)Trans-blot (Bio-Rad). Subsequently free protein binding sites on the nitrocellulose filter were blocked with 5% BSA (w/v) and 0.1% Tween-20 (v/v) in PBS for 1 hour at RT under gently agitation. The blots were incubated with the first antibody in PBS, 0.05% Tween-20 for 1 hour at RT or o/n at 4°C. Antibodies used were: anti-GAPDH 1:10.000 (Campro, Veenendaal, The Netherlands), anti-HMG1 1:5.000 (United States Biological, Swampscott, USA) and anti-CAH1 1:20.000 (United States Biological). After rinsing in excess PBS, 0.05% Tween-20, detection was accomplished using an appropriate chemoluminescent secondary antibody (Abcam, Cambridge, UK); rabbit-anti-mouse IgG peroxidase-conjugated 1:10.000 in PBS, 0.05% Tween-20 for GAPDH or 1:5.000 for HMG-1 and rabbit-anti-goat IgG peroxidase-conjugated 1:60.000 in PBS, 0.05% Tween-20 for CAH-1, for 1 hour at RT. After rinsing in excess PBS, 0.05% Tween-20 the blots were incubated for 5 min at RT with ECL plus Western blotting detection reagent (Amersham Biosciences) according to the manufacturer's instructions. The signal was recorded using a LAS 3000 camera in combination with the Las-3000 pro program (Fuji, Tilburg, The Netherlands). Molecular weight values were estimated using standard molecular weight

markers. Protein band densities were calculated and corrected for background using Aida version 3.5 (Raytest Benelux B.V. Tilburg, The Netherlands).

Statistical Analysis

Statistical analysis of protein expression was performed using the Student's t-test carried out with SPSS software version 6.1 for Macintosh. A p-value < 0.05 was considered as statistical significant.

Results

2D-Gelelectrophoresis and MALDI-TOF Mass Spectrometry

Mice received one of four different diets, containing a 0%, 10%, 20% or 40% wt/wt vegetables mixture respectively, for a period of two weeks. Differential protein expression in colonic mucosal cells was analysed by means of 2D-gelelectrophoresis. Single gels were prepared from pooled total cellular protein obtained from two to three animals. In total, three gels were run for each diet. Spot densities were calculated as a measure for protein expression and the expression in the diet groups was compared to that of mice receiving the control diet (0% vegetables).

Figure 1. Silver stained 2D-gel of proteins isolated from mouse colonic mucosal cells. The spots for which the protein identity was revealed are indicated on the gel (see Table 2 for protein function).

Spots that showed at least two times increased or decreased protein expression compared to control were selected for protein identification using MALDI-TOF mass spectrometry. From the 30 excised proteins, 6 could be identified. Figure 1 shows an example of a 2D gel together with the identified proteins. An example of a peptide spectrum of one of the identified proteins is depicted in Figure 2. These spectrometric data were used to search the Swiss-Prot protein database in order to reveal the identity of the proteins.

Figure 2. Trypic MALDI-TOF spectrum of CAH-1.

The expression patterns of the identified proteins are shown in Figure 3 and their properties have been summarized in Table 2. Spot 1222 was identified as the smooth muscle isoform of myosin regulatory light chain 2 (MLRN). MLRN expression was very similar to control in both the 10% and 20% vegetable groups, but showed an increased expression for the 40% vegetable group. Spot 5316 represents carbonic anhydrase I (CAH-1) and was upregulated in all vegetable groups, although this upregulation was statistically significant only for the 20% vegetable group. The peptide fragment pattern obtained from protein spot 5419 after trypsin digestion was found to match the high-mobility group protein 1 (HMG-1). The expression of this protein was decreased upon vegetable consumption, but this was not statistically significant. Spot 7076 was identified as pancreatitis-associated precursor protein 3 (PAP3). This protein was absent in colon mucosa from control mice, displayed only low expression in the 10% and 20% vegetable groups, but was significantly increased in the 40% vegetable group. Spot 8641 was found to

represent glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All vegetable groups showed reduced expression of GAPDH compared to control mice. The peptide fragment mass spectrum obtained from protein spot 9241 was found to match the mitochondrial ATP synthase oligomycin sensitivity conferral precursor protein (OSCP), and its protein expression was reduced in all vegetable groups compared to control.

Table 2. Protein spots isolated from 2D-gels identified by MALDI-TOF mass spectrometry in combination with searches in the Swiss-Prot protein database.¹

Spot #	AC # ¹	Swiss-prot abbreviation ¹	Abbreviation ¹	Protein name ¹
1222	Q9CQ19	MLRN_MOUSE	MLRN	myosin regulatory light chain 2, smooth muscle isoform
5316	P13634	CAH1_MOUSE	CAH-1	carbonic anhydrase I
5419	P07155	HMG1_MOUSE	HMG-1	high mobility group protein 1
7076	O09049	PAP3_MOUSE	PAP3	pancreatitis-associated protein 3 (precursor)
8641	P16858	G3P_MOUSE	GAPDH	glyceraldehyde 3-phosphate dehydrogenase
9241	Q9DB20	ATPO_MOUSE	OSCP	ATP synthase oligomycin sensitivity conferral protein, mitochondrial (precursor)

¹(<http://expasy.ch/sprot>)

Immunoblotting

Immunoblotting was performed with three out of six identified proteins, as for these antibodies were commercially available. Figure 4A shows the protein expression patterns of respectively HMG-1, GAPDH and CAH-1, and Figure 4B shows the relative protein expression of the identified proteins in the vegetable groups compared to control. The 25 kDa band for HMG-1 showed a dose dependent upregulation compared to control, which was found to be significant for the 10% and 40% vegetable groups, but not the 20% vegetable group. This is in contradiction to its 2D-expression pattern where HMG-1 showed no clear change in protein expression. GAPDH showed a band at 36 kDa. The 2D-protein expression pattern indicated decreased protein expression in all vegetable groups. The immunoblot results for GAPDH showed a highly variable expression pattern suggesting that there is no dose dependent effect of vegetable consumption on GAPDH expression. Possibly, the strong variation in the expression pattern masks a possible change in protein expression upon consumption of the vegetable-enriched diets. CAH-1 showed a single band at 28 kDa (Figure 4A) and was significantly upregulated in all vegetable groups compared to control (Figure 4B) as this was also observed in the 2D-gel electrophoresis expression pattern.

Figure 3. (A) Expression pattern of the identified spots from 2D-gels. (B) Relative protein expression of the identified spots compared to control. Average protein level for controls was set at 100%; * $p < 0.05$. MLRN: smooth muscle isoform of myosin regulatory light chain 2; CAH-1: carbonic anhydrase 1; HMG-1: high-mobility group protein 1; PAP3: pancreatitis-associated precursor protein 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; OSCP: mitochondrial ATP synthase oligomycin sensitivity conferral precursor protein. X-axis: C: control diet, 10%: 10% α -diet, 20%: 20% α -diet, 40%: 40% α -diet. The third spot of the control group for HMG-1 was regarded as missing value.

Figure 3. (Continued)

A

Figure 4. Immunoblot analysis of total colonic mucosal protein. (A) Blots were screened with antibodies: A1: HMG-1: high-mobility group protein 1, A2: GAPDH: glyceraldehyde-3-phosphate dehydrogenase; and A3: CAH-1: carbonic anhydrase I. The lanes were loaded with 10 μ g of total protein. (B) Protein expression relative to control of HMG-1, GAPDH and CAH-1. Average protein levels for control was set at 100%. X-axis: C: control diet, 10%: 10%-diet, 20%: 20%-diet, 40%: 40%-diet. * $p < 0.05$.

Figure 4. (Continued)

Discussion

Diet plays an important role in CRC. Meat consumption and high fat intake, for example, are known risk factors. On the other hand, vegetable consumption is thought to protect against CRC. So far, not much is known about the changes at the genome and proteome level that may be related to or involved in this protective mechanism. To study the effect of consumption of a vegetable rich diet on protein expression, we analyzed the expression levels of many proteins in colonic mucosal cells from mice following a two weeks diet with different vegetable content. Total cellular protein was isolated and the proteins were separated by means of 2D-gelelectrophoresis and identified by MALDI-TOF mass spectrometry. We were able to identify six proteins that showed a change in expression level, namely HMG-1, GAPDH, CAH-1, PAP3, OSCP and MLRN. HMG-1, GAPDH and CAH-1 were further analysed by immunoblotting. All six proteins will be discussed in detail below.

The HMG-1 protein is both a nuclear factor and a secreted protein. In the nucleus HMG-1 binds to DNA and interacts with several transcription factors to regulate transcription²⁴, outside the cell it is thought to be a mediator of differentiation.^{25, 26} Analysis of HMG-1 protein expression using 2D-gelelectrophoresis showed a decrease in protein expression for all vegetable groups compared to control, although this was not statistically significant. This is in contradiction with the expression pattern observed from the immunoblotting experiments, where we found that HMG-1 expression was significantly upregulated upon increased vegetable consumption, which was dose-dependent. This difference in protein expression pattern between 2D-gelelectrophoresis

and immunoblotting might be explained by posttranslational modification of the protein. HMG-1 is thought to be regulated by phosphorylation.²⁷ Hypophosphorylation enhances transport to the nucleus and subsequent binding to DNA.²⁷ The same protein but with different phosphorylation status will appear on different locations on the gel. For this moment it is unknown whether we detected the phosphorylated or hypophosphorylated isoform of HMG-1. Since immunoblotting reflects total protein expression, its expression pattern observed may differ from that observed upon 2D-gelelectrophoresis. The cells analyzed in this study are healthy colonic mucosal cells, containing dividing stem cells and many differentiating crypt and epithelial cells. Since phosphorylated HMG-1 is important in cellular differentiation, increased expression of this isoform might enhance cell differentiation, which suppresses aberrant cell growth of poorly differentiated cells like many (pre-)cancerous cells, thereby reducing the risk for CRC. However, for the moment we do not know which isoform is increased upon vegetable consumption.

GAPDH is a cytoplasmic protein that has long been considered as a classical glycolytic enzyme, playing an important role in energy production.²⁸ The active glycolytic enzyme consists of a tetramer of four identical subunits. However, mammalian GAPDH displays a wide functional diversity including membrane transport and fusion, microtubuli assembly, nuclear RNA export, protein phosphotransferase and kinase reactions, translational control of gene expression, DNA replication and repair and apoptosis.²⁹ Our 2D-gelelectrophoresis experiments indicated a substantially decreased protein expression in all vegetable groups compared to control, although this decrease was not statistically significant. The immunoblotting results on the other hand showed a large variation between the various sub-pool samples due to which no clear differences in protein expression between the four diets can be observed. So far, we can not explain this variation. For the six proteins that were identified by MALDI-TOF mass spectrometry, GAPDH was the only protein of which the gene was present in the pool of genes that were analysed in the microarray study (**Chapter 3**). In this study, an increased GAPDH gene expression in the 40% vegetable group was found, but the expression in the 10% and 20% vegetable groups did not change. The outcome of these results was confirmed by RT-PCR. This pattern differs from that which was found in the present study in the 2D-gelelectrophoresis. Although Piechaczyk et al. (1984) observed similar changes in expression for GAPDH protein and mRNA levels in several rat organs under normal conditions³⁰, no such relation was found in our experiments. The difference between the mRNA levels and protein levels may be due to different subforms of GAPDH. To exert the wide variety of GAPDH functions, the different subforms of the protein need to be distinguished by the cell. This might be regulated by alternate splicing or posttranslational modification and give protein products that differ in isoelectric point (pI) and/or molecular weight.^{29, 31} This might also explain the difference in protein expression as observed from the 2D-gelelectrophoresis and the immunoblotting experiments. Considering the role of GAPDH

in DNA replication, DNA repair and apoptosis²⁹, GAPDH might be an interesting candidate for protection against CRC. However, the evidence so far is too premature to draw definitive conclusions.

CAH-1 is a member of a family of isoenzymes that catalyze the reversible hydration of carbon dioxide and participate in various biological processes like electrolyte transport and maintenance of pH. The isoenzymes differ in kinetic properties, tissue distribution and subcellular localization. CAH-1 is a cytoplasmic isoenzyme that is highly expressed in large intestinal mucosa³² and takes care of the maintenance of the intracolonic pH. In colorectal tumours CAH-1 is significantly less expressed than in normal mucosa.³³ Reduced expression of CAH-1 might lead to cellular acidic pH, thereby promoting cellular motility, and contribute to tumour growth and metastasis.³⁴ Our results from the 2D-experiments already indicated an increase in protein expression upon vegetable consumption, although this was only significant for the 20% vegetable group. The immunoblot experiments showed that CAH-1 protein expression is significantly upregulated in all vegetable groups compared to control. We therefore suggest that consumption of a vegetable rich diet contributes to maintenance of the intracolonic and cellular pH, which thereby might protect against CRC development.

PAP3 belongs to the regenerating gene (Reg) family that constitutes the calcium dependent lectin (C-type lectin) gene superfamily.³⁵ The Reg family represents a group of small secretory proteins that can function as acute phase reactants, lectins, anti-apoptotic factors and growth factors. The members of the Reg family can be grouped into three subclasses: type I, II and III.³⁵ There are at least three distinct type III Reg genes in the mouse genome: Reg III alpha (RegIII α), beta (RegIII β) and gamma (RegIII γ , i.e. PAP3).³⁶ The subclasses share considerable amino acid homologies including a conserved trypsin cleavage site. It is thought that the secreted forms represent the precursors and the cleaved forms the activated molecules.³⁷ Mouse type III Reg genes are expressed strongly in intestinal tracts and PAP3 in particular is expressed strongly in small intestine, moderately in colon and weakly in pancreas.^{35, 36} The type III genes are thought to be involved in the growth and regeneration of cells and tissues. In our 2D-electrophoresis experiments we identified the PAP3 precursor protein. It was upregulated in the 40% vegetable groups, while hardly any effect was observed in the 10% and 20% vegetable group. Our results suggest that only a high intake of vegetables (40%) stimulates the expression of PAP3 precursor protein. Considering the role of PAP3 in growth and regeneration of cells and tissues, we suggest that activation of the precursor might lead to increased amounts of active PAP3 protein, stimulating the proliferation of healthy cells and/or regeneration of damaged tissue and thus contribute to healthy colon tissue. However, to allow regeneration, it might be necessary to switch off apoptosis, thereby increasing the chance of survival of cells exhibiting genetic mutations in growth promoting or metastasis inducing genes.³⁸ Thus, upregulation of PAP3 precursor protein and thereby possibly of

PAP3, might contribute to proliferation and differentiation of healthy colon tissue although it also involves a risk for CRC. Upregulation of the precursor protein of PAP3, however, may not necessarily lead to a change in active protein levels. Additional experiments are therefore needed to unravel the regulation of PAP3 protein expression.

ATP production is one of the major chemical reactions in living organisms. ATP synthase is a key enzyme in cellular energy production. During ATP synthesis it uses a proton gradient and the associated membrane potential to synthesize ATP. The ATP synthases are rotary motor complexes that are composed of two discrete sectors (F_1 and F_0) that are considered to be two separate rotary motors working cooperatively. Oligomycin sensitivity-conferring protein (OSCP) is a component of the F_0 sector and is considered to serve as a link between the F_1 and F_0 sectors.³⁹ OSCP also has been implicated in the binding of F_1 to the membrane.^{40, 41} In our 2D-gel electrophoresis experiments we observed a downregulation of the OSCP precursor protein upon increased vegetable consumption that was only statistically significant for the 20% vegetable group. Considering the fact that OSCP precursor protein and the active OSCP protein are very likely to be located at different locations on the 2D-gel, reduced expression of OSCP precursor protein might indicate that the amount of precursor is decreased in favour of active, processed OSCP. The processed OSCP might then be used for assembly of ATP synthase and contribute to ATP production. At this moment there are no indications that OSCP might play a role in CRC.

Myosin is a major component of the contractile elements of smooth muscle and is composed of two identical heavy chains (200 kDa) and two sets of light chains of 20 and 17 kDa.⁴² The 20 kDa chain, called regulatory light chain (MLRN), plays a central role in the regulation of smooth muscle contraction.^{43, 44} In our 2D-gel electrophoresis experiments we found an increase, although not significant, of MLRN protein expression with increasing vegetable intake, most evident for the 40% vegetable group. This might indicate that vegetable consumption increases smooth muscle activity. Vegetables contain high amounts of dietary fibers and are known to stimulate bowel movements, decreasing the passage time through the gut.^{45, 46} Mutagenic agents might be present in faeces and decreased passage time reduces the risk of interaction with the colon tissue and protects against genetic damage. Increased MLRN protein expression might contribute to enhanced bowel movement and as a consequence reduce the passage time of mutagenic agents, lowering the risk for genetic damage and CRC.

In the current study, several proteins were identified that displayed differential expression levels in colon mucosa of mice following consumption of diets with different vegetable contents. These proteins play a role in processes like growth, differentiation and apoptosis. Disturbance of these processes plays an important role in carcinogenesis. We therefore conclude that altered protein levels following increased vegetable intake, support the protective effects of vegetables against CRC.

References

1. Greenwald P. Colon cancer overview. *Cancer* 1992;70:1206-15.
2. Platz EA, Willett WC, Colditz GA, Rimm EB, Spiegelman D, Giovannucci E. Proportion of colon cancer risk that might be preventable in a cohort of middle-aged US men. *Cancer Causes Control* 2000;11:579-88.
3. World Cancer Research Fund. Food, Nutrition and the Prevention of Cancer: a global perspective. Washington: BANTA Book group, 1997.
4. Slattery ML, Curtin KP, Edwards SL, Schaffer DM. Plant foods, fiber, and rectal cancer. *Am J Clin Nutr* 2004;79:274-81.
5. Riboli E, Norat T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr* 2003;78:559S-569S.
6. Finley JW. Reduction of cancer risk by consumption of selenium-enriched plants: enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli. *J Med Food* 2003;6:19-26.
7. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens Regulate Vitamin D Metabolism in the Mouse Colon: Relevance for Colon Tumor Prevention and Therapy. *J Nutr* 2002;132:3490S-3493S.
8. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000;21:921-7.
9. Symolon H, Schmelz EM, Dillehay DL, Merrill AH, Jr. Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1,2-dimethylhydrazine-treated CF1 mice and ApcMin/+ mice. *J Nutr* 2004;134:1157-61.
10. Bertram JS, Bortkiewicz H. Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. *Am J Clin Nutr* 1995;62:1327S-1336S.
11. Talalay P, Fahey JW. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J Nutr* 2001;131:3027S-33S.
12. Sengupta A, Ghosh S, Bhattacharjee S, Das S. Indian food ingredients and cancer prevention - an experimental evaluation of anticarcinogenic effects of garlic in rat colon. *Asian Pac J Cancer Prev* 2004;5:126-32.
13. Parnaud G, Li P, Cassar G, Rouimi P, Tulliez J, Combaret I, Gamet-Payrastra L. Mechanism of Sulforaphane-Induced Cell Cycle Arrest and Apoptosis in Human Colon Cancer Cells. *Nutr Cancer* 2004;48:198-206.
14. Chung JG, Lu HF, Yeh CC, Cheng KC, Lin SS, Lee JH. Inhibition of N-acetyltransferase activity and gene expression in human colon cancer cell lines by diallyl sulfide. *Food Chem Toxicol* 2004;42:195-202.
15. Katchamatt S, Williams DE. Indole-3-carbinol modulation of hepatic monooxygenases CYP1A1, CYP1A2 and FMO1 in guinea pig, mouse and rabbit. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;129:377-84.
16. Larsen-Su S, Williams DE. Dietary indole-3-carbinol inhibits FMO activity and the expression of flavin-containing monooxygenase form 1 in rat liver and intestine. *Drug Metab Dispos* 1996;24:927-31.
17. Smith TJ, Yang CS. Effect of organosulfur compounds from garlic and cruciferous vegetables on drug metabolism enzymes. *Drug Metabol Drug Interact* 2000;17:23-49.

18. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* 1996;96:1027-39.
19. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991;2:427-42.
20. Bertram JS. Carotenoids and gene regulation. *Nutr Rev* 1999;57:182-91.
21. Guyonnet D, Siess MH, Le Bon AM, Suschetet M. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicol Appl Pharmacol* 1999;154:50-8.
22. Wargovich MJ. Experimental evidence for cancer preventive elements in foods. *Cancer Lett* 1997;114:11-7.
23. Bouwman F, Renes J, Mariman E. A combination of protein profiling and isotopomer analysis using matrix-assisted laser desorption/ionization-time of flight mass spectrometry reveals an active metabolism of the extracellular matrix of 3T3-L1 adipocytes. *Proteomics*, in press.
24. Bianchi ME, Beltrame M. Upwardly mobile proteins. Workshop: the role of HMG proteins in chromatin structure, gene expression and neoplasia. *EMBO Rep* 2000;1:109-14.
25. Passalacqua M, Patrone M, Picotti GB, Del Rio M, Sparatore B, Melloni E, Pontremoli S. Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. *Neuroscience* 1998;82:1021-8.
26. Sparatore B, Passalacqua M, Patrone M, Melloni E, Pontremoli S. Extracellular high-mobility group 1 protein is essential for murine erythroleukaemia cell differentiation. *Biochem J* 1996;320 (Pt 1):253-6.
27. Poser I, Golob M, Buettner R, Bosserhoff AK. Upregulation of HMG1 leads to melanoma inhibitory activity expression in malignant melanoma cells and contributes to their malignancy phenotype. *Mol Cell Biol* 2003;23:2991-8.
28. Harris JI, Waters M. Glyceraldehyde-3-phosphate dehydrogenase. In: Boyer PD, ed. *The enzymes*. New York: Academic Press, 1976:1-49.
29. Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1999;1432:159-84.
30. Piechaczyk M, Blanchard JM, Marty L, Dani C, Panabieres F, El Saboury S, Fort P, Jeanteur P. Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucleic Acids Res* 1984;12:6951-63.
31. Mezquita J, Pau M, Mezquita C. Several novel transcripts of glyceraldehyde-3-phosphate dehydrogenase expressed in adult chicken testis. *J Cell Biochem* 1998;71:127-39.
32. Lonnerholm G, Selking O, Wistrand PJ. Amount and distribution of carbonic anhydrases CA I and CA II in the gastrointestinal tract. *Gastroenterology* 1985;88:1151-61.
33. Kivela AJ, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Pastorekova S, Pastorek J, Waheed A, Sly WS, Parkkila TS, Rajaniemi H. Differential expression of cytoplasmic carbonic anhydrases, CA I and II, and membrane-associated isozymes, CA IX and XII, in normal mucosa of large intestine and in colorectal tumors. *Dig Dis Sci* 2001;46:2179-86.
34. Chiang WL, Chu SC, Yang SS, Li MC, Lai JC, Yang SF, Chiou HL, Hsieh YS. The aberrant expression of cytosolic carbonic anhydrase and its clinical significance in human non-small cell lung cancer. *Cancer Lett* 2002;188:199-205.
35. Unno M, Yonekura H, Nakagawara K, Watanabe T, Miyashita H, Morizumi S, Okamoto H, Itoh T, Teraoka H. Structure, chromosomal localization, and expression of mouse reg genes, reg I and reg II. A novel type of reg gene, reg II, exists in the mouse genome. *J Biol Chem* 1993;268:15974-82.

36. Narushima Y, Unno M, Nakagawara K, Mori M, Miyashita H, Suzuki Y, Noguchi N, Takasawa S, Kumagai T, Yonekura H, Okamoto H. Structure, chromosomal localization and expression of mouse genes encoding type III Reg, RegIII alpha, RegIII beta, RegIII gamma. *Gene* 1997;185:159-68.
37. Graf R, Schiesser M, Scheele GA, Marquardt K, Frick TW, Ammann RW, Bimmler D. A family of 16-kDa pancreatic secretory stress proteins form highly organized fibrillar structures upon tryptic activation. *J Biol Chem* 2001;276:21028-38.
38. Macadam RC, Sarela AI, Farmery SM, Robinson PA, Markham AF, Guillou PJ. Death from early colorectal cancer is predicted by the presence of transcripts of the REG gene family. *Br J Cancer* 2000;83:188-95.
39. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* 1985;54:1015-69.
40. Sandri G, Wojtczak L, Ernster L. Cation-dependent reassembly of F0F1-ATPase in submitochondrial particles: evidence for a binding site for F1 on F0 in the absence of F6 and oligomycin sensitivity-conferring protein. *Arch Biochem Biophys* 1985;239:595-602.
41. Duszynski J, Dupuis A, Lux B, Vignais PV. Spectral properties of fluorescent derivatives of the oligomycin sensitivity conferring protein and analysis of their interaction with the F1 and F0 sectors of the mitochondrial ATPase complex. *Biochemistry* 1988;27:6288-96.
42. Harrington WF, Rodgers ME. Myosin. *Annu Rev Biochem* 1984;53:35-73.
43. Adelstein RS, Eisenberg E. Regulation and kinetics of the actin-myosin-ATP interaction. *Annu Rev Biochem* 1980;49:921-56.
44. Hartshorne DJ. Phosphorylation of myosin and the regulation of smooth muscle actomyosin. In: Dowben RM, Shay JW, eds. *Cell and Muscle motility II*. New York: Plenum Publishing Corp., 1982:188-220.
45. Cummings JH, Branch W, Jenkins DJ, Southgate DA, Houston H, James WP. Colonic response to dietary fibre from carrot, cabbage, apple, bran. *Lancet* 1978;1:5-9.
46. Wrick KL, Robertson JB, Van Soest PJ, Lewis BA, Rivers JM, Roe DA, Hackler LR. The influence of dietary fiber source on human intestinal transit and stool output. *J Nutr* 1983;113:1464-79.

Chapter 5

Gene expression modulation by specific vegetables in colon mucosa of C57BL6 female mice

Submitted

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Abstract

Consumption of cruciferous vegetables, carrots and onions has been shown to exhibit protective effects against colorectal cancer (CRC). The evidence for legumes is less clear, but promising. In a previous study, the combined effect of these four vegetables on gene expression changes in colon mucosa from female C57BL6 mice was examined. The aim of the present study was to investigate the contribution of these four individual vegetables on gene expression, to give more insight into their role in genetic mechanisms underlying CRC prevention. Female C57BL6 mice were randomly divided into five dietary groups (6 mice per group). Each group was fed a different diet, containing no vegetables, cauliflower, carrots, peas or onions, for two weeks. Colonic mucosal cells were collected after sacrifice. Total RNA was isolated and microarray technology was used to measure the expression levels of 602 genes simultaneously. Eighteen genes were significantly modulated by one or more of the specific vegetables. Ten of these are known to be involved in CRC and seven out of these ten were affected in such a way that CRC protective mechanisms may be stimulated. The four individual vegetables seem to have about the same potential of modulating genes in favor of CRC protection. Eleven similar genes were modulated in the present study as in the previous mixture study and for seven of them the modulation by the mixture could be explained by the effect of a particular vegetable. These similarly modulated genes are of particular interest because they are consistently affected and could play an important role in CRC prevention by vegetables.

Introduction

In Western countries, colorectal cancer (CRC) is one of the most common malignant diseases, accounting for 15.3 percent of all new cancer cases. Although treatment of CRC continues to improve, large-bowel cancer remains a major cause of illness and death.¹

Substantial evidence from epidemiological, clinical, and laboratory studies shows that risk of CRC cancer is closely related to diet and other lifestyle factors. Decreased intake of dietary fat, increased intake of vegetables and fruit and high physical activity is associated with decreased risk of developing CRC. The most consistent observation is that vegetable consumption is inversely associated with CRC risk.¹⁻⁷

Particularly, carrots, cruciferous and allium vegetables have been shown to exhibit protective effects. The evidence for legumes, like beans and peas, is promising, but inconclusive.^{3, 5, 7} These vegetables may be protective against CRC by providing the colon with a range of anticarcinogenic factors. These compounds act via various different proposed mechanisms, such as modulation of phase-I and -II metabolizing enzymes, induction of cell differentiation, inhibition of cell proliferation and scavenging of free radicals.^{3, 5, 7-11}

Although during the last decade an intensified investigation of the effects of diet on the colon has taken place¹²⁻¹⁸, the genetic pathways through which vegetable components exert their effects are mostly unknown. Furthermore, the number of experimental studies in which the effect of whole vegetables, instead of individual micronutrients or other bioactive compounds are examined, is limited. By investigating the effect of whole vegetables, the biological availability of the compounds from the food matrix and their possible interactions can be taken into account.

In a previous study, the effect of a diet providing different doses of a vegetable mixture consisting of cauliflower, carrots, peas and onions on gene expression changes in colon mucosa of female C57BL/6 mice was examined by means of cDNA microarray technology. (**Chapter 3**) The results of that study indicated that a diet with a high content of vegetables positively modulates genes involved in protective mechanisms at various stages of CRC development. In the present study, the contribution of the individual vegetables on gene expression changes in colon mucosa of female C57BL/6 mice is investigated. This will provide more information about the role of these specific vegetables in the genetic mechanisms underlying CRC prevention.

Materials and Methods

Animals and Diets

Eight-week-old female C57BL6 mice (Charles River Laboratories, Lyon, France) were randomly assigned to one of five dietary groups, each consisting of six animals. During 7 days of acclimatization, all the animals received the standard control diet. Next, each group was fed one of five different diets, containing no vegetables (referred to as control diet), cauliflower, carrots, peas or onions respectively, for a period of two weeks. This study succeeds a previously conducted mouse study in which the effect of a diet containing different doses of a vegetable mixture consisting of cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt) was investigated. (Chapter 3) The diet in the highest vegetable dose group contained 40% of this vegetable mixture. The amount of vegetables used in the present study is equal to the amount of these specific vegetables in the 40% vegetable dose group. In the vegetable mixture study, most of the statistical differences were observed between the highest vegetable dose group (40%) and one of the other diet groups. Diets were refreshed every two days and provided as powdered feed. The 20% casein reference diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the five different diets. The vegetables were from the same batch used in the previous study. This batch was purchased as a single batch at the supermarket and separately cooked under household conditions. After freezing (-20°C), the vegetables were lyophilized, ground and combined. Before the vegetables were mixed with the basal diet, they were analyzed for macronutrient content (Hope Farms). The vegetables added to the basal diet were at the expense of carbohydrates; therefore diets were supplied with dextrose/cerelose and cellulose (dicacel) (Hope Farms), resulting in similar energy densities. No antioxidants or preservatives were added. After preparation, the diets were stored at -20°C in air-closed plastic bags until use. The composition of the different diets is given in Table 1.

The animals were maintained in the laboratory animal care facilities at the University of Maastricht under controlled environmental conditions (temperature $21 \pm 1^{\circ}\text{C}$, relative humidity $50\% \pm 10$, 12-hr light/dark cycle). Body weights of the mice were recorded weekly. The study was approved by the Institutional Committee of Animal Experimentation of Maastricht University.

Tissue Sampling

Mice were sacrificed by bleeding the *vena cava inferior* under Nembutal (Sanofi Sante, Maasluis, the Netherlands) anesthesia. Nembutal was administered subcutaneously in the neck at a dose of 60 mg/kg body weight. The large intestine was removed and placed on a box, which was kept at 4°C . After removing the rectum, the colon was opened longitudinally with fine scissors and mucus and faeces were removed. Colonic mucosal

cells were incubated in Trizol™ (GIBCO Life Technologies) for 3 minutes and scraped off the muscle layer with the edge of a sterile glass slide. Cells were transferred into 800 µl Trizol™, homogenized by resuspension and stored at -80 °C until total RNA isolation.

Table 1. Composition of the five diets in g/kg.

Ingredients	Control diet ¹	Cauliflower diet ²	Carrots diet ²	Peas diet ²	Onions diet ²
Cerclose/Dextrose	515	451	452	303	488
DL-Methionine	2	2	2	2	2
NaCl	3	3	3	3	3
Choline CL 50%	4	4	4	4	4
Soya oil	50	50	50	50	50
KCl	7	7	7	7	7
KH ₂ PO ₄	7	7	7	7	7
MgO	2	2	2	2	2
CaHPO ₄ ·2H ₂ O	13	13	13	13	13
CaCO ₃	10	10	10	10	10
Corn Starch	100	100	100	100	100
Dicacel/Cellulose	77	71	67	63	73
Casein	200	200	200	200	200
MgSO ₄ ·7H ₂ O	4	4	4	4	4
Standard vitamin premix	3	3	3	3	3
Standard micro-nutrient premix	3	3	3	3	3
Vegetables ³	0	70	73	226	31

¹Control diet is the basal 20% casein reference diet.

²In the vegetable diets, the basal diet was adjusted for cerclose/dextrose and dicacel/cellulose resulting in similar energy densities for all diets.

³wet weight: cauliflower 1.8 kg; carrots 1.8 kg; peas 1.8 kg; onions 0.6 kg.

Total RNA Isolation and cDNA Probe Synthesis

Total RNA was extracted according to the manufacturer's instructions. The RNeasy® Mini Kit (Qiagen) together with a DNase treatment was used to purify total RNA from salts and residual DNA. Quantity of each RNA sample was measured by a spectrophotometer and varied from 20 to 90 µg per mouse. Integrity was determined by a Bioanalyzer (Agilent Technologies Netherlands B.V., The Netherlands). All samples contained intact total RNA with an rRNA ratio (28S/18S) > 1.5.

The 0% vegetable diet group was considered as the control group; the vegetable diet groups were referred to as the test groups. Three RNA pools per diet group were prepared by pooling equal amounts of total RNA from two mice. Cyanine 3- (Cy3) and

Cyanine 5- (Cy5) labeled cDNA probes were prepared using 10 µg total RNA from each pool, by the method of Hasseman et al.¹⁹

cDNA Microarray Preparation

This study is part of a project that investigates the effects of vegetables on gene expression in colon mucosa of humans and mice. For the human study, the expression levels of genes in colon mucosa were measured using the PHASE-1 Microarray Human-600 (PHASE-1 Molecular Toxicology, Santa fee, USA). A mouse cDNA microarray was constructed based on the genes present on the PHASE-1 Microarray Human-600. These genes represent a dedicated set of biologically relevant gene sequences involved in inflammation, DNA damage and repair, oxidative stress, cell signaling, cell proliferation, metabolism, transcription and apoptosis. Selection of mouse cDNA clones, amplification of cDNA inserts by PCR, verification and purification of PCR products and spotting of the microarrays was carried out as described in **Chapter 3** (Section Materials and Methods; cDNA Microarray Preparation).

cDNA Microarray Hybridizations

For the hybridizations, within each diet group three pools were formed, each consisting of equal amounts of total RNA of two or three mice. By creating three pools per diet group instead of one, biological variability can be taken into account, what allows for a broader generalizability of the results. For each of the three sets of pools from different diet groups, a reference hybridization design was constructed.²⁰ In this design, each test group is compared with the same pool from control group. Cy3 and Cy5 labeled cDNA probes of the test and control group were mixed and hybridized to the cDNA microarray by the method of Hasseman et al.¹⁹ The reference design was chosen because it allows for the same precision of all comparisons of test groups against the control. In order to remove potential bias to dye effects, a second microarray experiment was carried out for each couple, in which the dyes were switched (flip-dye experiment). In total, 24 cDNA microarray hybridizations were performed. Slides were scanned on a GMS 418 Array Scanner (Affymetrix, High Wycombe, UK). The images obtained (resolution 10 micron; 16 bit tiff image) were processed with ImaGene 5.0 software (Biodiscovery, Los Angeles, USA) to measure mean signal intensities for spots and local background.

cDNA Microarray Data Processing and Statistical Analysis

The data were analyzed using ANOVA models²¹, without background correction and using base-2 logarithmic transformation of the measured intensities. Due to computational limitations, the models were fit in two stages. The models included a normalization step, taking into account both the global (across-genes) and local (gene-specific) normalization.²² All pairwise differences between the five diet groups were examined. For each gene, the

Tukey procedure was used to correct for multiple comparisons.²³ To control the overall (across genes) probability of false positive findings, at around 5%, a p -value < 0.0001 was considered to indicate statistical significance of an individual pairwise comparison. The critical values for the Tukey procedure were selected using an empirical distribution obtained by bootstrap.²⁴

Real-time RT-PCR

To verify the cDNA microarray results, 14 gene expression differences, representing six genes which were responsive to vegetables, were analyzed by real-time RT-PCR, as described previously in **Chapter 3** (Section Materials and Methods; Real-time RT-PCR). A list of validated genes and the normalization gene and their primers is given in Table 2.

Additional Statistical Analysis

Statistical analysis of bodyweight of the mice was carried out using SPSS version 6.1.1 for Macintosh. Data were analyzed by means of analysis of variance followed by Student's t -test. A two-sided p -value < 0.05 was considered to indicate statistical significance.

Results

Mean body weights per week per group are presented in Table 3. Prior to the start of the intervention, all animals received the control diet (Week 0). At the end of this week, there were no statistically significant differences in mean body weights between the groups. After the intervention, mean body weights were significantly increased within each group ($P < 0.05$), but no significant differences between the groups were observed. Because the diets are isocaloric and weight gain was similar between the groups, these results indicate that there was no difference in food intake and that the diets did not affect normal growth.

Table 3. Mean body weights (g) per week per group.¹

	Week 0 ²	Week 1 ³	Week 2 ³
Control group (C)	17.6 $\pm$ 0.9	18.7 $\pm$ 1.0	19.4 $\pm$ 1.4 ⁴
Cauliflower group (T1)	18.3 $\pm$ 0.6	19.5 $\pm$ 0.6	20.7 $\pm$ 0.5 ⁴
Carrots group (T2)	17.9 $\pm$ 0.3	19.7 $\pm$ 0.7	20.2 $\pm$ 0.8 ⁴
Peas group (T3)	18.2 $\pm$ 0.6	19.3 $\pm$ 1.1	20.3 $\pm$ 1.1 ⁴
Onions (T4)	18.0 $\pm$ 0.8	18.9 $\pm$ 0.7	19.6 $\pm$ 0.7 ⁴

¹Body weights are expressed as mean $\pm$ SE.

²Acclimatization week, animals received the control diet.

³Intervention period.

⁴Statistics: analysis of variance followed by Student's t -test. $P < 0.05$; body weight significantly higher compared to week 0.

Table 2. Primer sequences of the validated genes and the 18S normalization gene.

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Forward Primer	Reverse Primer
BG073773	Translation elongation factor EF-1 alpha-1 chain (EFHU1)	5'-GGCAAGCCCATGTGTGTTG-3'	5'-CCTCATGTTCACGAACAGCAAA-3'
BG073067	Heat shock 60kDa protein 1 (chaperonin) (HSPD1)	5'-CCGAAGACGTTGACGGAGAA-3'	5'-TGCCACAACCTGAAGACCAA-3'
AI152753	Caspase 4, apoptosis-related cysteine protease (CASP4)	5'-CGGGCAACCTTGACGAGAT-3'	5'-TTCAAGCTGAAGAAATGAGATTCAGTT-3'
BG065221	Ornithine decarboxylase, structural (ODC)	5'-CAACATCATTGCCAAAAAAACC-3'	5'-TGTTCAATTTGACTCATCTTCATCGT-3'
W88005	Cyclin-dependent kinase inhibitor 1A (p21) (CDKN1A)	5'-GCAGATCCACAGCGATATCCA-3'	5'-GGTCGGACATCACCAGGATT-3'
BG074606	Ornithine aminotransferase (OAT)	5'-GTGAGAGGGAAAGGGTTGCTAA-3'	5'-CAGGCACACCTTCCAAGCAT-3'
	18 S	5'-AGTCCCTGCCCTTTGTACACA-3'	5'-GATCCGAGGGCCTCACTAAAC-3'

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.²NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

The expressions of 602 genes were measured simultaneously by means of cDNA microarrays. Microarray data analysis compared the levels of expression between the diet groups and tested whether these differences were significant. Thereby, a significant difference in gene expression was found for 34 diet group comparisons, representing 18 genes. These genes, their matching comparisons and the differences are listed in Table 4.

Table 4. Differentially expressed genes.

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Comparison	Difference cDNA microarray ³	Difference RT-PCR ⁴
BG073773	Translation elongation factor EF-1 alpha-1 chain (EFHU1)	T1-C	-0.23 ± 0.06	-1.46 ± 0.18
		T2-C	-0.19 ± 0.07	-0.82 ± 0.17
BG074767	Topoisomerase (DNA) II alpha 170 kDa (TOP2A)	T4-C	-1.05 ± 0.10	
BG076302	Interleukin 18 (interferon-gamma-inducing factor) (IL18)	T3-C	0.93 ± 0.11	
		T3-T2	1.11 ± 0.15	
BG073067	Heat shock 60kDa protein 1 (chaperonin) (HSPD1)	T1-C	-0.54 ± 0.09	-2.38 ± 0.15
		T2-C	-0.55 ± 0.09	-1.72 ± 0.21
		T3-C	-0.57 ± 0.19	-1.72 ± 0.15
		T4-C	-0.57 ± 0.08	-1.40 ± 0.15
AA396890	BCL2/adenovirus E1B 19kDa-interacting protein 1 (BNIP1)	T4-C	-0.24 ± 0.05	
		T2-C	-0.19 ± 0.05	
BG065603	Cytochrome c oxidase subunit IV isoform 1 (COX4I1)	T1-C	0.22 ± 0.06	
W88005	Cyclin-dependent kinase inhibitor 1A (p21) (CDKN1A)	T1-C	0.68 ± 0.09	0.44 ± 0.17
		T2-C	0.72 ± 0.09	1.00 ± 0.40
		T3-C	0.69 ± 0.09	0.57 ± 0.17
BG065221	Ornithine decarboxylase, structural (ODC)	T1-C	-0.52 ± 0.06	-1.73 ± 0.18
		T2-C	-0.51 ± 0.06	-1.26 ± 0.21
		T2-T3	-0.44 ± 0.08	
BG064900	Stearoyl-Coenzyme A desaturase 2 (SCD2)	T4-C	0.32 ± 0.07	
BG063870	Beta actin, cytoplasmic (ACTB); clone H3011G02	T1-C	0.74 ± 0.11	
AI152753	Caspase 4, apoptosis-related cysteine protease (CASP4)	T1-C	0.77 ± 0.12	1.31 ± 0.19
		T1-T4	1.14 ± 0.16	
		T2-T4	0.96 ± 0.16	
BG063771	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); clone H303E11	T1-T4	0.46 ± 0.10	
BG074606	Ornithine aminotransferase (OAT); clone H313B06	T1-C	-0.48 ± 0.09	-2.95 ± 0.10
		T2-C	-0.78 ± 0.09	-0.70 ± 0.22
		T2-T3	-0.59 ± 0.11	
		T2-T4	-0.58 ± 0.12	

Table 4. (Continued)

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Comparison	Difference cDNA microarray ³	Difference RT-PCR ⁴
BG063081	Thymosin, beta 10 (TMSB10)	T4-C	-0.79 ± 0.09	
BG075853	Selenoprotein P, plasma 1 (SEPP1)	T4-C	0.68 ± 0.10	
AA500809	Peripheral myelin protein (PMP22)	T1-C	0.69 ± 0.09	
		T2-C	0.70 ± 0.09	
BG086656	Aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1)	T1-C	-0.80 ± 0.11	
BG064142	Hypoxia inducible factor 1, alpha subunit (HIF1A)	T4-C	0.94 ± 0.09	

¹Ac#: GenBank accession numbers of the cDNA fragments present on the microarrays.

²NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

³Estimated difference in mean log₂-transformed intensity ± SE. (C: control group; T1: cauliflower group; T2: carrots group; T3: peas group; T4: onions group).

⁴Normalized difference in mean log₂-transformed intensity ± SE. Fourteen gene differences were validated with RT-PCR. Empty cells indicate gene differences which were not taken into account.

In Figure 1, the estimated differences of these genes per vegetable group as compared to the control group (set to 0) are depicted. Furthermore, a schematic overview of the genes with respect to the particular vegetables, by which they are differentially expressed, is visually described in a Venn diagram (Figure 2).

Heat shock 60kDa protein 1 (chaperonin) (HSPD1) is the only gene the expression of which was statistically significantly modulated in all vegetable groups compared to the control group. Next to HSPD1, six genes were affected by more than one, but not all vegetable groups, i.e. Translation elongation factor EF-1 alpha-1 chain (EFHU1), Ornithine decarboxylase, structural (ODC), Caspase 4, apoptosis-related cysteine protease (CASP4), Ornithine aminotransferase (OAT), Peripheral myelin protein (PMP22), all by cauliflower and carrots; Cyclin-dependent kinase inhibitor 1A (p21) (CDKN1A) in the cauliflower-, carrots- and peas group; and BCL2/adenovirus E1B 19kDa-interacting protein 1 (BNIP1) by carrots and onions. Ten genes were modulated in one specific vegetable group; onions had an effect on the expression of Topoisomerase (DNA) II alpha 170 kDa (TOP2A), Stearoyl-Coenzyme A desaturase 2 (SCD2), Thymosin, beta 10 (TMSB10), Selenoprotein P, plasma 1 (SEPP1) and Hypoxia inducible factor 1, alpha subunit (HIF1A); peas modulated Interleukin 18 (interferon-gamma-inducing factor) (IL18) gene expression; and cauliflower affected the expression of Cytochrome c oxidase subunit IV isoform 1 (COX4I1), Beta actin, cytoplasmic (ACTB) and Aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1). No genes were modulated solely by the carrots. The expression of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was statistically significantly different between the cauliflower group and the onions group, but

no difference was observed compared to the control group. Most genes (10) were modulated in the cauliflower group and least (3) in the peas group.

Figure 1. Estimated difference in mean $\log$ -transformed intensity $\pm$ SE per diet group (x-axis) (T1: cauliflower group; T2: carrots group; T3: peas group; T4: onions group) for all identified differentially expressed genes from Table 4. The control diet group intensity (set to 0) serves as the reference. EFHU1: Translation elongation factor EF-1 alpha-1 chain; TOP2A: Topoisomerase (DNA) II alpha 170 kDa; IL18: Interleukin 18 (interferon-gamma-inducing factor); HSPD1: Heat shock 60kDa protein 1 (chaperonin); BNIP1: BCL2/adenovirus E1B 19kDa-interacting protein 1; COX4I1: Cytochrome c oxidase subunit IV isoform 1; CDKN1A: Cyclin-dependent kinase inhibitor 1A (p21); ODC: Ornithine decarboxylase, structural; SCD2: Stearoyl-Coenzyme A desaturase 2; ACTB: Beta actin, cytoplasmic; CASP4: Caspase 4, apoptosis-related cysteine protease; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; OAT: Ornithine aminotransferase; TMSB10: Thymosin, beta 10; SEPP1: Selenoprotein P, plasma 1; PMP22: Peripheral myelin protein; ALDH1A1: Aldehyde dehydrogenase family 1, subfamily A1; HIF1A: Hypoxia inducible factor 1, alpha subunit. Asterisk indicates significant differences between diet group and control (0% vegetable diet group). See Table 4 for other statistical details.

Figure 1. (Continued)

Figure 1. (Continued)

Eleven genes were differentially expressed in the present study as well as in the vegetable mixture study. (**Chapter 3**; section Results) For seven of these eleven genes, the direction in which the genes were modulated ($\uparrow$ up- or $\downarrow$ down-) in the current test groups is similar as in the 40% group of the vegetable mixture study, i.e. EFHU1 ($\downarrow$), SCD2 ($\uparrow$), ACTB ($\uparrow$), CASP4 ($\uparrow$, although in the 40% group not significantly different compared to control group), OAT ($\downarrow$), PMP22 ($\uparrow$) and HIF1A ($\uparrow$). Opposite modulation was observed for the three genes TMSB10 ($\downarrow$ in current study, $\uparrow$ in previous study), ALDH1A1 ($\downarrow$ in current study, $\uparrow$ in previous study) and SEPP1 ($\uparrow$ in current study, $\downarrow$ in previous study). For GAPDH, a difference in expression was observed between the cauliflower group ($\uparrow$) and onions group, but not as compared to the control group, whereas in the vegetable mixture study an up-regulation was observed in the 40% vegetable dose group as compared to the control group. In Figure 3A-D, for each of the individual vegetable groups of the present study, the genes which are vegetable specific, vegetable mixture specific and overlapping between these two groups are visually described in a Venn diagram.

Figure 2. Venn diagram of the significantly differentially expressed genes, with respect to the vegetable group(s) in which they are differentially expressed.

Real-time RT-PCR was used to confirm 14 gene expression differences, representing six genes as responsive to vegetables, identified by cDNA microarray analyses. The results are presented in Table 4 and show in all cases the same direction of up- or down-effect. These data verify the results obtained by cDNA microarray analyses.

Figure 3. Venn diagram of the significantly differentially expressed genes for each of the individual vegetable groups of the present study and the 40% group of the previous vegetable mixture study (**Chapter 3**). The genes which are vegetable specific, or which are present in the vegetable specific and vegetable mixture groups, are shown. Genes in *Italic style* are modulated in opposite directions in the individual vegetable group as compared to the 40% mixture group. (A) Venn diagram of cauliflower group and 40% group of vegetable mixture. (B) Venn diagram of carrots group and 40% group of vegetable mixture. (C) Venn diagram of peas group and 40% group of vegetable mixture. (D) Venn diagram of onions group and 40% group of vegetable mixture.

Figure 3. (Continued)

Discussion

In the present study, the contribution of four individual vegetables on gene expression changes in colon mucosa of female C57BL/6 mice is investigated by means of cDNA microarray technology.

For 18 genes a significant difference in gene expression was found for 34 diet group comparisons. Table 5 summarizes the effects on gene expression in relation to possible effects on colon carcinogenesis risk. HSPD1 was the only gene which was significantly modulated (down-regulated) by all vegetables. HSPD1 encodes for a member of the family of chaperones, which can be defined as proteins which assist other proteins to reach their final active form.²⁵ Their level of expression is highly increased when a cell is subjected to stress conditions.²⁶ It could be that the vegetables provide the cell with compounds which maintain homeostasis, thereby reducing the call for stress-induced proteins resulting in their decreased transcription.

Most of the significantly modulated genes (↑ up- or ↓ down-regulated as compared to control) were in the cauliflower group, i.e. EFHU1 (↓), COX4I1 (↑), CDKN1A (↑), ODC (↓), ACTB (↑), CASP4 (↑), OAT (↓), PMP22 (↑), and ALDH1A1 (↓). EFHU1, ODC, OAT and PMP22 were also modulated in the carrots group and CDKN1A also in the carrots and peas group (see Figure 2). No roles for EFHU1, COX4I1, ACTB and PMP22 in possible protective mechanisms against CRC have previously been reported (see Table 5). Also, except for ACTB, no relation with vegetables or vegetable components has been described. ACTB, although widely used as internal standard for gene expression studies, has previously been shown to be regulated by nutritional interventions, including a vitamin A deficiency study.²⁷⁻²⁹

Table 5. Presentation of differentially expressed genes, the direction of the effect in the individual vegetable group compared to control, and the theoretical effect on cancer risk.

Gene Name by NCBI (Abbreviation) ¹	Cauliflower group	Carrots group	Peas group	Onions group	Theoretical effect on cancer risk
HSPD1	–	–	–	–	–
EFHU1	–	–			Unknown
COX4I1	+				Unknown
CDKN1A	+	+	+		–
ODC	–	–			–
ACTB	+				Unknown
CASP4	+				–
OAT	–	–			+
PMP22	+	+			Unknown
ALDH1A1	–				+
BNIP1		–		–	Unknown
TOP2A				–	–
SCD2				+	Unknown
TMSB10				–	+
SEPP1				+	Unknown
HIF1A				+	–
IL18			+		–

¹NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

CDKN1A, ODC and OAT are involved in cell growth, although via different mechanisms. CDKN1A encodes for a potent cyclin-dependent kinase (CDK) inhibitor, which binds to and inhibits the activity of cyclin-CDK2- or CDK4 complexes, thereby preventing G₁-S³⁰ and G₂-M³¹ transition, which results in cell cycle arrest. The expression of this gene is tightly regulated by the tumour suppressor protein p53.³⁰ Next to its ability to block cell cycle progression, CDKN1A mediates apoptosis.³² Several vegetable components have been shown to induce CDKN1A expression *in vitro*, like the flavonoids apigenin³³ and genistein³⁴, indole-3- carbinol³⁵ and isothiocyanates³⁶ present in Brassica vegetables like cauliflower, and beta-carotene³⁷ present in carrots. This is the first *in vivo* study, in which CDKN1A is reported to be significantly up-regulated by cauliflower, carrots and peas.

Two other genes implicated in cell growth are OAT and ODC. These two genes are involved in polyamine metabolism and were down-regulated in the cauliflower- and carrots group. The protein-product of OAT converses ornithine to glutamate semialdehyde, thereby reducing intracellular ornithine contents. Ornithine is the substrate of ODC, which is the first enzyme in polyamine synthesis. Increases in intracellular polyamine levels are associated with rapid growth, including tumorigenesis.^{38, 39} ODC is a transcriptional target of C-MYC and a modifier of adenomatous polyposis coli (APC)-

dependent tumorigenesis. Wild-type APC regulates the expression of C-MYC and its antagonist, MAD1, causing an inhibition of ODC expression, a decrease in polyamine metabolism, and reduced tumorigenesis.⁴⁰ Inhibition of ODC mRNA has been described for β -carotene⁴¹ and indole-3-carbonol⁴², present in carrots and cauliflower, respectively, which could explain the reduction in these two diet groups.

CASP4, up-regulated in the cauliflower group, is implicated in apoptosis (programmed cell death). Apoptosis is a general feature in the intestinal crypt, by eliminating differentiated epithelial cells which form the intestinal lining. Programmed cell death occurs also deep in the proliferative zone of the intestinal crypt. Here, genetically damaged stem cells are removed before they can undergo clonal expansion and thereby the occurrence of neoplasia can be prevented.⁴³ Sequential activation of caspases plays a central role in the execution phase of cell apoptosis.⁴⁴ Several studies have shown an induction of apoptosis by certain flavonoids^{33, 45}, glucosinolate breakdown products^{46, 47} and carotenoids⁴⁸⁻⁵⁰, however, no investigation of CASP4 expression was performed.

ALDH1A1 expression was significantly down-regulated in the cauliflower group. It encodes for an enzyme important in ethanol detoxification, metabolism of neurotransmitters, and synthesis of retinoic acid.^{51, 52} Ethanol and retinol share chemical similarities and can be oxidized to aldehydes (acetaldehyde and retinal, respectively), and these are oxidized to carboxylic acids (acetate acid and retinoic acid, respectively) by the same enzymes (alcohol dehydrogenase and aldehyde dehydrogenase).⁵² Epidemiological and experimental studies have revealed that acetaldehyde rather than ethanol itself is responsible for the adverse effects of alcohol consumption on CRC. Acetaldehyde is highly toxic, mutagenic and carcinogenic.⁵¹

Summarizing the effects in the cauliflower group, six out of ten modulated genes could be involved in CRC protective mechanisms; four (HSPD1, CDKN1A, ODC and CASP4) out of these six are affected in such a way that CRC protective mechanisms could be expected mostly related to cell growth regulation and apoptosis.

BNIP1 was down-regulated in the carrots and onions group. No modulation of BNIP1 mRNA or enzyme activity by vegetables or nutrition has been described yet. The protein product of the BNIP1 gene interacts with members of the BCL2 family, which consists of pro-apoptotic (such as BAX) and anti-apoptotic (such as BCL2) proteins. It is suggested that BNIP1 has pro-apoptotic properties, by interacting with BCL2 and BCL2L1. However, the precise function of BNIP1 is unclear.^{53, 54}

In total seven genes were affected by carrots. For five of these genes, CRC protective mechanisms are described. For three (HSPD1, CDKN1A and ODC) of these five genes, the direction of the regulation is in favour of CRC protection.

Next to BNIP1 and HSPD1, onions affected the expressions of the genes TOP2A, SCD2, TMSB10, SEPP1 and HIF1A. No roles for SCD2, SEPP1 and HIF1A in possible protective mechanisms against CRC have been reported and no relation, except

for HIF1A, with vegetables or vegetable components has been described. HIF1A is a major regulator of the hypoxic response. During hypoxia, HIF1A translocates to the nucleus and facilitates transcription of genes involved in glycolysis and angiogenesis.^{55, 56} Until recently, hypoxia has been the only naturally occurring signal shown to activate this gene. However, it was shown that the dietary flavonoid quercetin was able to increase HIF1A protein under normal oxygen concentrations.⁵⁶ In the present study, HIF1A was up-regulated in the onions group, which can be caused by flavonoids present in the onions. The possible importance of these genes in CRC and their regulation by vegetables has to be further studied.

TOP2A catalyzes DNA topological reactions via a DNA breakage/reunion mechanism. These DNA topological reactions allow TOP2A to remove excess DNA supercoils.⁵⁷ The breakage/reunion reaction of TOP2A can be interrupted by many anticancer drugs, resulting in the accumulation of a topoisomerase II-DNA covalent intermediate: the cleavage complex. Accumulation of this complex causes tumour cell death.⁵⁸ The inhibition of TOP2A has been shown to be an important therapeutic mechanism in cancer chemotherapy. Nutritional inhibition of the TOP2A enzyme in colon cancer cells has been described for the isoflavone genistein, a major component of soy.⁵⁹

TMSB10 encodes for a G-actin binding protein which has dual functions: induction of programmed cell death and cell growth and -differentiation.⁶⁰⁻⁶² TMSB10 was significantly down-regulated in the onions group compared to the control group. Retinoic acid is known to increase TMSB10 mRNA and apoptosis in cancer cells.⁶³ However, in the present study, no difference in TMSB10 expression was found in the carrots group compared to the control group, suggesting that the amount of possible bioactive compounds like carotenoids and retinoids are too low to exert an effect on TMSB10 expression.

Reviewing the results on gene expression in the onions group, four out of seven genes were modulated which could be involved in CRC protective mechanisms. For three (HSPD1, TOP2A and TMSB10) of these four genes the expression was affected in such a way that CRC protective mechanisms could be stimulated by maintaining homeostasis and DNA stability, and by reducing cell growth and induction of cell differentiation.

IL18 was significantly up-regulated in the peas group compared to the control and carrots group. Expression in the cauliflower and onions group was lower, although not significantly. IL18 encodes for a type-1 T-helper cytokine which has antitumour activities by increasing apoptosis^{64, 65}, inhibiting angiogenesis⁶⁶ and maintaining homeostasis.⁶⁷ Little information is available about nutritional modulation of IL18. In two studies, a reduction in serum IL-18 was observed after a high fibre diet.^{68, 69}

Least modulations took place in the peas group, however, the modulation of these three genes (HSPD1, IL18 and CDKN1A) are expected to increase CRC protective mechanisms.

Next to this evaluation of the specific gene effects of the individual vegetables and their possible role in CRC prevention, it is interesting to compare the results of the current individual vegetable study with the previous performed vegetable mixture study described in **Chapter 3**. In the Venn diagrams (Figure 3A-D), the corresponding and differing genes between each vegetable group and the 40% vegetable mixture group are shown.

Eleven genes were significantly modulated in both studies. For seven of these eleven genes, the result in the vegetable mixture study can be expected from the results of the individual vegetables on the expression of these genes. The results of the present study reveal which vegetables were (mainly) accountable for these same effects. ACTB was modulated by the cauliflowers; OAT, EFHU1 and PMP22 were modulated by the cauliflowers and carrots; and, SCD2 and HIF1A were modulated by the onions; in the same direction as observed in the vegetable mixture study. CASP4 was significantly increased in the 40% vegetable mixture group as compared to the 10% and 20% vegetable mixtures group. In the present study, this gene was only significantly up-regulated in the cauliflower group. For three genes, i.e. ALDH1A1, TMSB10 and SEPP1, the effect of the vegetable mixture resulted in an opposite outcome as would be expected by the effects of the individual vegetables.

Seven genes which were modulated in the present study on individual vegetables were not modulated in the study on mixed vegetables. An explanation could be that the bioactive compounds present in the individual vegetables possibly neutralize each other in the mixture. Fourteen genes which were significantly affected in the 40% group of vegetable mixture study, were not significantly modulated in at least one of the individual vegetable groups of the present study. It is suggested that the individual vegetables contained not enough bioactive compounds to exert a measurable effect on gene expression. The combined effect could be additive or synergistic and therefore large enough to cause a gene expression effect.

Almost half of the affected genes which were modulated are currently not known to be involved in CRC protective mechanisms or known to be modulated by vegetables. Their possible role in CRC prevention and their modulation by vegetables are not clear and have to be further investigated. However, a number of genes was modulated in such a way CRC preventive mechanisms could be activated and this already occurred through diets relatively low in vegetable amount. The four individual vegetables seem to have about the same potential of modulating genes in favour of lower CRC risk, although via different mechanisms. Furthermore, a number of similar genes were modulated by the respective vegetables individually as well as mixed. These similar modulated genes are of particular interest because they are modulated in both studies and could play an important role in CRC prevention by vegetables.

References

1. World Health Organization & International Agency for Research on Cancer. World Cancer Report. Lyon: IARC Press, 2003.
2. Potter JD. Colorectal cancer: molecules and populations. *J Natl Cancer Inst* 1999;91:916-32.
3. World Cancer Research Fund. Food, Nutrition and the Prevention of Cancer: a global perspective. Washington: BANTA Book group, 1997.
4. World Health Organization. The World Health Report 1997: Conquering suffering Enriching humanity. Geneva: WHO, 1997.
5. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc* 1996;96:1027-39.
6. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* 1991;2:325-57.
7. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991;2:427-42.
8. Murillo G, Mehta RG. Cruciferous vegetables and cancer prevention. *Nutr Cancer* 2001;41:17-28.
9. van Poppel G, Verhoeven DT, Verhagen H, Goldbohm RA. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv Exp Med Biol* 1999;472:159-68.
10. Guyonnet D, Siess MH, Le Bon AM, Suschetet M. Modulation of phase II enzymes by organosulfur compounds from allium vegetables in rat tissues. *Toxicol Appl Pharmacol* 1999;154:50-8.
11. Bianchini F, Vainio H. Allium vegetables and organosulfur compounds: do they help prevent cancer? *Environ Health Perspect* 2001;109:893-902.
12. van Kranen HJ, van Iersel PW, Rijnkels JM, Beems DB, Alink GM, van Kreijl CF. Effects of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the ApcMin mouse. *Carcinogenesis* 1998;19:1597-601.
13. Rijnkels JM, Alink GM. Effects of a vegetables-fruit mixture on liver and colonic 1,2-dimethylhydrazine-metabolizing enzyme activities in rats fed low- or high-fat diets. *Cancer Lett* 1998;128:171-5.
14. Rijken PJ, Timmer WG, van de Kooij AJ, van Benschop IM, Wiseman SA, Meijers M, Tijburg LB. Effect of vegetable and carotenoid consumption on aberrant crypt multiplicity, a surrogate end-point marker for colorectal cancer in azoxymethane-induced rats. *Carcinogenesis* 1999;20:2267-72.
15. Schatzkin A, Lanza E, Corle D, Lance P, Iber F, Caan B, Shike M, Weissfeld J, Burt R, Cooper MR, Kikendall JW, Cahill J. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group [see comments]. *N Engl J Med* 2000;342:1149-55.
16. Gamet-Payraastre I, Li P, Lumeau S, Cassar G, Dupont MA, Chevrolleau S, Gasc N, Tulliez J, Terce F. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res* 2000;60:1426-33.
17. Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000;21:921-7.
18. Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res* 2000;60:3823-31.
19. Hasseman J, Chen E, Yang I, Quackenbush J. Aminoallyl labeling of RNA for microarrays. <http://www.tigr.org/tdb/micoarray/protocolsTIGR.shtml> (Accessed June 21, 2001), 2001.

20. Kerr MK. Design considerations for efficient and effective microarray studies. *Biometrics* 2003;59:822-828.
21. Kerr MK, Martin M, Churchill GA. Analysis of variance for gene expression microarray data. *Journal of Computational Biology* 2000;7:819-837.
22. Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS. Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol* 2001;8:625-37.
23. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. *Applied Linear Statistical Models* (Fourth Edition). Mass, 1996.
24. Kerr MK, Afshari CA, Bennett L, Bushel P, Martinez J, N.J. W, Churchill GA. Statistical analysis of a gene expression microarray experiment with replication. *Statistica Sinica* 2002;12:203-217.
25. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 2001;286:433-42.
26. Jindal S. Heat shock proteins: applications in health and disease. *Trends Biotechnol* 1996;14:17-20.
27. Yamada H, Chen D, Monstein HJ, Hakanson R. Effects of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. *Biochem Biophys Res Commun* 1997;231:835-8.
28. Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, Griffiths LR. Beta-actin--an unsuitable internal control for RT-PCR. *Mol Cell Probes* 2001;15:307-11.
29. Nur T, Peijnenburg AA, Noteborn HP, Baykus H, Reifen R. DNA microarray technology reveals similar gene expression patterns in rats with vitamin a deficiency and chemically induced colitis. *J Nutr* 2002;132:2131-6.
30. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817-25.
31. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998;282:1497-501.
32. Levkau B, Koyama H, Raines EW, Clurman BE, Herren B, Orth K, Roberts JM, Ross R. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. *Mol Cell* 1998;1:553-63.
33. Shukla S, Gupta S. Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells. *Mol Carcinog* 2004;39:114-26.
34. Kobayashi T, Nakata T, Kuzumaki T. Effect of flavonoids on cell cycle progression in prostate cancer cells. *Cancer Lett* 2002;176:17-23.
35. Firestone GL, Bjeldanes LF. Indole-3-carbinol and 3-3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. *J Nutr* 2003;133:2448S-2455S.
36. Chiao JW, Chung F, Krzeminski J, Amin S, Arshad R, Ahmed T, Conaway CC. Modulation of growth of human prostate cancer cells by the N-acetylcysteine conjugate of phenethyl isothiocyanate. *Int J Oncol* 2000;16:1215-9.
37. Palozza P, Serini S, Torsello A, Boninsegna A, Covacci V, Maggiano N, Ranelletti FO, Wolf FI, Calviello G. Regulation of cell cycle progression and apoptosis by beta-carotene in undifferentiated and differentiated HL-60 leukemia cells: possible involvement of a redox mechanism. *Int J Cancer* 2002;97:593-600.

38. Seiler N. Ornithine aminotransferase as a therapeutic target in hyperammonemias. *Adv Exp Med Biol* 1997;420:113-42.
39. Han X, Kazarinoff MN, Seiler N, Stanley BA. Rat colon ornithine and arginine metabolism: coordinated effects after proliferative stimuli. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G389-99.
40. Martinez ME, O'Brien TG, Fultz KE, Babbar N, Yerushalmi H, Qu N, Guo Y, Boorman D, Einspahr J, Alberts DS, Gerner EW. Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc Natl Acad Sci U S A* 2003;100:7859-64.
41. Phillips RW, Kikendall JW, Luk GD, Willis SM, Murphy JR, Maydonovitch C, Bowen PE, Stacewicz-Sapuntzakis M, Wong RK. beta-Carotene inhibits rectal mucosal ornithine decarboxylase activity in colon cancer patients. *Cancer Res* 1993;53:3723-5.
42. Shertzer HG, Senft AP. The micronutrient indole-3-carbinol: implications for disease and chemoprevention. *Drug Metabol Drug Interact* 2000;17:159-88.
43. Johnson IT. Anticarcinogenic effects of diet-related apoptosis in the colorectal mucosa. *Food Chem Toxicol* 2002;40:1171-8.
44. Kamada S, Washida M, Hasegawa J, Kusano H, Funahashi Y, Tsujimoto Y. Involvement of caspase-4(-like) protease in Fas-mediated apoptotic pathway. *Oncogene* 1997;15:285-90.
45. Mertens-Talcott SU, Talcott ST, Percival SS. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J Nutr* 2003;133:2669-74.
46. Gingras D, Gendron M, Boivin D, Moghrabi A, Theoret Y, Beliveau R. Induction of medulloblastoma cell apoptosis by sulforaphane, a dietary anticarcinogen from Brassica vegetables. *Cancer Lett* 2004;203:35-43.
47. Hu R, Kim BR, Chen C, Hebbar V, Kong AN. The roles of JNK and apoptotic signaling pathways in PEITC-mediated responses in human HT-29 colon adenocarcinoma cells. *Carcinogenesis* 2003;24:1361-7.
48. Palozza P, Calviello G, Serini S, Maggiano N, Lanza P, Ranelletti FO, Bartoli GM. beta-carotene at high concentrations induces apoptosis by enhancing oxy-radical production in human adenocarcinoma cells. *Free Radic Biol Med* 2001;30:1000-7.
49. Palozza P, Maggiano N, Calviello G, Lanza P, Piccioni E, Ranelletti FO, Bartoli GM. Canthaxanthin induces apoptosis in human cancer cell lines. *Carcinogenesis* 1998;19:373-6.
50. Palozza P, Serini S, Maggiano N, Angelini M, Boninsegna A, Di Nicuolo F, Ranelletti FO, Calviello G. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by beta-carotene through down-regulation of cyclin A and Bcl-2 family proteins. *Carcinogenesis* 2002;23:11-8.
51. Seitz HK, Matsuzaki S, Yokoyama A, Homann N, Vakevainen S, Wang XD. Alcohol and cancer. *Alcohol Clin Exp Res* 2001;25:137S-143S.
52. Crabb DW, Pinars J, Hasanadka R, Fang M, Leo MA, Lieber CS, Tsukamoto H, Motomura K, Miyahara T, Ohata M, Bosron W, Sanghani S, Kedishvili N, Shiraishi H, Yokoyama H, Miyagi M, Ishii H, Bergheim I, Menzl I, Parlesak A, Bode C. Alcohol and retinoids. *Alcohol Clin Exp Res* 2001;25:207S-217S.
53. Zhang H, Heim J, Meyhack B. Novel BNIP1 variants and their interaction with BCL2 family members. *FEBS Lett* 1999;448:23-7.

54. Yasuda M, Chinnadurai G. Functional identification of the apoptosis effector BH3 domain in cellular protein BNIP1. *Oncogene* 2000;19:2363-7.
55. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* 2003;23:359-69.
56. Wilson WJ, Poellinger L. The dietary flavonoid quercetin modulates HIF-1 alpha activity in endothelial cells. *Biochem Biophys Res Commun* 2002;293:446-50.
57. Watt PM, Hickson ID. Structure and function of type II DNA topoisomerases. *Biochem J* 1994;303 (Pt 3):681-95.
58. Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989;58:351-75.
59. Salti GI, Grewal S, Mehta RR, Das Gupta TK, Boddie AW, Jr., Constantinou AI. Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. *Eur J Cancer* 2000;36:796-802.
60. Lee SH, Zhang W, Choi JJ, Cho YS, Oh SH, Kim JW, Hu L, Xu J, Liu J, Lee JH. Overexpression of the thymosin beta-10 gene in human ovarian cancer cells disrupts F-actin stress fiber and leads to apoptosis. *Oncogene* 2001;20:6700-6.
61. Gutierrez-Pabello JA, McMurray DN, Adams LG. Upregulation of thymosin beta-10 by *Mycobacterium bovis* infection of bovine macrophages is associated with apoptosis. *Infect Immun* 2002;70:2121-7.
62. Santelli G, Califano D, Chiappetta G, Vento MT, Bartoli PC, Zullo F, Trapasso F, Viglietto G, Fusco A. Thymosin beta-10 gene overexpression is a general event in human carcinogenesis. *Am J Pathol* 1999;155:799-804.
63. Hall AK. Liarazole amplifies retinoid-induced apoptosis in human prostate cancer cells. *Anticancer Drugs* 1996;7:312-20.
64. Dinarello CA. Interleukin-18. *Methods* 1999;19:121-32.
65. Marino E, Cardier JE. Differential effect of IL-18 on endothelial cell apoptosis mediated by TNF-alpha and Fas (CD95). *Cytokine* 2003;22:142-8.
66. Coughlin CM, Salhany KE, Wysocka M, Aruga E, Kurzawa H, Chang AE, Hunter CA, Fox JC, Trinchieri G, Lee WM. Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. *J Clin Invest* 1998;101:1441-52.
67. Pages F, Berger A, Henglein B, Piqueras B, Danel C, Zinzindohoue F, Thiounn N, Cugnenc PH, Fridman WH. Modulation of interleukin-18 expression in human colon carcinoma: consequences for tumor immune surveillance. *Int J Cancer* 1999;84:326-30.
68. Esposito K, Pontillo A, Di Palo C, Giugliano G, Masella M, Marfella R, Giugliano D. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *Jama* 2003;289:1799-804.
69. Esposito K, Nappo F, Giugliano F, Di Palo C, Ciotola M, Barbieri M, Paolisso G, Giugliano D. Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. *Am J Clin Nutr* 2003;78:1135-40.

Chapter 6

Gene expression modulation in lung cancer preventive pathways by vegetables in female C57BL6 mice

In preparation

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Abstract

Worldwide, lung cancer is the most prevalent and lethal malignant disease. In addition to avoidance of the most predominant risk factor, i.e. tobacco use, consumption of high amounts of vegetable and fruits is the most effective means of preventing lung cancer. However, the molecular mechanisms underlying lung cancer risk prevention by vegetables are not clear. In the present study, the effect of vegetables on gene expression changes in the lungs of female C57BL/6 mice was investigated using cDNA microarray technology. Therefore, mice were fed one of eight different diets for a period of two weeks: 1) a diet containing no vegetables; a diet containing a vegetable mixture: 2) 10% wt/wt; 3) 20% wt/wt; or 4) 40% wt/wt; or a diet containing individual vegetables: 5) cauliflower; 6) carrots; 7) peas; or 8) onions. The vegetables mixture consisted of these four individual vegetables. After sacrifice, the lungs were removed and total RNA was isolated from the lungs for expression analysis of 602 genes. The results of this study suggest that individual vegetables have a higher potential of modulating genes (5 out of 8) in favor of lung cancer risk prevention, instead of the vegetable mixture (2 out of 7); the other gene modulations are expected to enhance lung cancer risk. The involved pathways are various and include cell growth, apoptosis, biotransformation and immune response. Furthermore, carrots were able to modulate most gene expressions, and most of these effects also favor lung cancer risk prevention. The current study provides more insight into the genetic mechanisms by which vegetables, in particular carrots, can prevent lung cancer risk.

Introduction

Lung cancer is the most prevalent and lethal malignant disease in the world, accounting for almost 16 percent of all new cancer cases. Approximately 90% of the people who develop lung cancer will die from it. No effective treatment is available; the five years survival rate for lung cancer patients is less than 15 percent. Thus the need for primary prevention of lung cancer is paramount.¹

The most effective means of preventing lung cancer is avoidance of tobacco use. Cigarette smoking accounts for about 90 percent of cases in men and about 80 percent of cases in women, with the rest due to occupational exposures including asbestos, arsenic, chloromethyl ethers, chromium-VI, and nickel; residential and occupational exposures to radon; and probably exposures to carcinogenic air pollutants in the general environment.² Next to avoiding or reducing these known causative exposures, consumption of diets high in vegetables and fruit is the most effective means for prevention of lung cancer.³ The most available and consistent evidence from prospective epidemiological studies is that particularly dark green and yellow-orange vegetables, rich in β -carotene and vitamin E, are the specific types of vegetables, which best protect against lung cancer. Limited evidence is available regarding the preventive potential of legumes like beans and peas against lung cancer.³

A lot of the research on vegetables and lung cancer has been motivated by the hypothesis that these vegetables contain relatively high amounts of specific micronutrients with specific anticarcinogenic potential. Observational epidemiologic studies have demonstrated a statistically significant decreased relative risk of lung cancer between the extreme quintiles or quartiles of vegetable intake and serum levels of these agents.^{4,5} These agents would have antioxidative capacities thereby protecting against oxidative DNA damage resulting in lower cancer risk.⁵⁻⁹ However, the results of the randomised controlled trials in which the effect of daily high doses of β -carotene, vitamin A and/or vitamin E administered orally during several years on lung cancer incidence was investigated, were disappointing.¹⁰⁻¹³ Instead of a protective effect, adverse effects on lung cancer development were reported. It has been suggested that higher concentrations of these antioxidants resulting from the supplementations have pro-oxidant effects, inducing DNA damage and membrane instability. Furthermore, the results of these trials also emphasize that it is not known whether the epidemiological associations are specific for the micronutrients or whether the micronutrient measurements are merely serving as a marker of the intake of other protective substances or even more healthful dietary habits in general. In addition, the complex mixture of numerous substances rather than a single constituent in vegetables could be responsible for the net effect.

The vegetable components that reach the lung may have been modulated by the first-pass metabolism: substances that are absorbed via intestinal blood capillaries will first have to pass the liver before they reach the systemic circulation. Here, many substances are extensively metabolized. Therefore, the lungs are regarded as a systemic organ to study the effect of vegetables, because they are subject to only systemic exposure of vegetable-derived components. The mechanisms by which vegetables protect against lung cancer could therefore be different compared to for instance the colon and rectum. The gastrointestinal tract, including the colon and rectum, is side of contact, i.e., as the vegetables pass through the tract, they come into close contact with the intestinal lining.

The specific mechanisms by which vegetables exert lung cancer risk prevention are unknown and need to be elucidated. The number of studies in which the effect of whole vegetables instead of a specific constituent has been examined is limited. Changes in the multi-gene expression patterns can provide clues about regulatory mechanisms and broader cellular functions and biochemical pathways. Current DNA microarray technology allows the simultaneous expression analysis of a large numbers of genes.¹⁴

Therefore, in the present study, microarray technology was used to gain more information about the effect of vegetables in the lungs at the level of the expression for multiple genes involved in various genetic pathways underlying cancer risk prevention. Two different approaches were taken: in the first approach, the dose-dependent effect of a mixture of four vegetables (cauliflower, carrots, peas and onions) on gene expression changes in the lung was examined; in the second approach, the contribution of the individual vegetables present in the vegetable mixture, was investigated.

Materials and Methods

Animals and Diet

The number and type of animals, the development of the vegetable diets and the treatment of the animals in the present study was previously described in **Chapter 3** and **5** (Section Materials and Methods; Animals and Diet). In short, eight-week-old female C57BL/6 mice (Charles River Laboratories, Lyon, France) were randomly assigned to one of eight different diets for a period of two weeks: 1) a diet containing no vegetables (control diet; two groups of six and seven animals received the control diet, referred to as control group I and II); a diet containing a vegetable mixture: 2) 10% wt/wt vegetables mixture; 3) 20% wt/wt vegetables mixture; or 4) 40% wt/wt vegetables mixture; or a diet containing individual vegetables: 5) cauliflower; 6) carrots; 7) peas; or 8) onions (six or seven animals per group). The vegetables mixture used consisted of the four individual vegetables used in diet 5-8, i.e. cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt).

Table 1. Composition of the eight diets in g/kg.

Ingredients	Control diet ¹	10% Vegetable diet ²	20% Vegetable diet ²	40% Vegetable diet ²	Cauliflower diet ²	Carrots diet ²	Peas diet ²	Onions diet ²
Cerelose/Dextrose	515	422	329	143	451	452	303	488
DL-Methionine	2	2	2	2	2	2	2	2
NaCl	3	3	3	3	3	3	3	3
Choline CL 50%	4	4	4	4	4	4	4	4
Soya oil	50	50	50	50	50	50	50	50
KCl	7	7	7	7	7	7	7	7
KH ₂ PO ₄	7	7	7	7	7	7	7	7
MgO	2	2	2	2	2	2	2	2
CaHPO ₄ ·2H ₂ O	13	13	13	13	13	13	13	13
CaCO ₃	10	10	10	10	10	10	10	10
Corn Starch	100	100	100	100	100	100	100	100
Dicacel/Cellulose	77	70	63	49	71	67	63	73
Casein	200	200	200	200	200	200	200	200
MgSO ₄ ·7H ₂ O	4	4	4	4	4	4	4	4
Standard vitamin premix	3	3	3	3	3	3	3	3
Standard micronutrient premix	3	3	3	3	3	3	3	3
Cauliflower ³	0	17.5	35	70	70			
Carrots ³	0	18.3	36.5	73		73		
Peas ³	0	56.5	113	226			226	
Onions ³	0	7.7	15.5	31				31

¹Control diet is the basal 20% casein reference diet.²In the vegetable diets, the basal diet was adjusted for cerelose/dextrose and dicacel/cellulose resulting in similar energy densities for all diets.³Wet weight: cauliflower 1.8 kg; carrots 1.8 kg; peas 1.8 kg; onions 0.6 kg.

The diet in the highest vegetable mixture group contained 40% of this vegetable mixture. The amount of vegetables used in the individual vegetable diets is equal to the amount of these specific vegetables in the 40% vegetable mixture group. The composition of the different diets is presented in Table 1.

The animals were maintained in the laboratory animal care facilities at Maastricht University under controlled environmental conditions (temperature 21 ± 1 °C, relative humidity $50\% \pm 10$, 12-hr light/dark cycle). Body weights of the mice were recorded weekly. The study was approved by the Institutional Committee of Animal Experimentation Maastricht University.

Tissue Sampling

Mice were sacrificed by bleeding the *vena cava inferior* under Nembutal (Sanofi Sante, Maasluis, the Netherlands) anesthesia. Nembutal was administered subcutaneously in the neck at a dose of 60 mg/kg body weight. The lungs were removed and quickly washed in ice-cold 1x PBS, immediately frozen in liquid nitrogen and stored at -80 °C until use. For total RNA isolation, frozen lung tissue was ground to a powder in a stainless steel mortar under liquid nitrogen and homogenized in 800 μ l TRIZOL Reagent (Gibco/BRL).

Total RNA Isolation and cDNA Probe Synthesis

Total RNA was extracted according to the manufacturer's instructions. The RNeasy® Mini Kit (Qiagen, Westburg, Leusden, The Netherlands) together with a DNase treatment was used to purify total RNA from salts and residual DNA. Quantity of each RNA sample was measured by a spectrophotometer and varied from 10 to 30 μ g per mouse. RNA integrity was determined by a Bioanalyzer (Agilent Technologies Netherlands B.V., Amsterdam, The Netherlands). All samples contained intact total RNA with an rRNA ratio (28S/18S) > 1.5 .

Three RNA pools per diet group were prepared by pooling equal amounts of total RNA from two or three mice. Cyanine 3- (Cy3) and Cyanine 5- (Cy5) labeled cDNA probes were prepared using 3 μ g total RNA from each pool, by the method of Hasseman et al.¹⁵

cDNA Microarray Preparation

This study is part of a project that investigates the effects of vegetables on gene expression in colon mucosa of humans and mice. For the human study, the expression levels of genes in colon mucosa were measured using the PHASE-1 Microarray Human-600 (PHASE-1 Molecular Toxicology, Santa fee, USA). A mouse cDNA microarray was constructed based on the genes present on the PHASE-1 Microarray Human-600. These genes represent a dedicated selection of biologically relevant gene sequences involved in inflammation, DNA

damage and repair, oxidative stress, cell signaling, cell proliferation, metabolism, transcription and apoptosis.

Selection of mouse cDNA clones, amplification of cDNA inserts by PCR, verification and purification of PCR products and spotting of the microarrays was carried out as described in **Chapter 3** (Section Materials and Methods; cDNA Microarray Preparation).

cDNA Microarray Hybridizations

For the hybridizations, within each diet group three pools were formed, each consisting of equal amounts of total RNA of two or three mice. By creating three pools per diet group instead of one, biological variability can be taken into account. This allows for a broader generalizability of the results.

For each of the three sets of pools from the different vegetable mixture groups, a loop design was constructed with four microarrays, as follows: $D0j \rightarrow D1j \rightarrow D2j \rightarrow D3j \rightarrow D0j$ (the pools for control I, 10%, 20% and 40% diet denoted, respectively, by $D0j$, $D1j$, $D2j$ and $D3j$ with $j = 1, 2$ or 3 ; arrows join the samples put on the same array and indicate the sample labeled with Cy5). The loop is repeated three times, and then analyzed together, to reflect the possible variability in the experimental data. The statistical power of this design for estimating the dose-response profile is higher compared to the classical reference design in which each diet group is compared with the control group. Furthermore, fewer arrays are needed by using this design.^{16, 17} In total 12 arrays were used. Cy3 and Cy5 labeled cDNA probes of two groups were mixed according to this design and hybridized to the cDNA microarray by the method of Hasseman et al.¹⁵

For each of the three sets of pools from the individual vegetable groups, a reference hybridization design was constructed.¹⁷ In this design, each vegetable group is compared with the same pool from control group II. Cy3 and Cy5 labeled cDNA probes of the vegetable and control group II were mixed and hybridized to the cDNA microarray by the method of Hasseman et al.¹⁵ The reference design was chosen because it allows for the same precision of all comparisons of vegetable groups against the control. In order to remove potential bias to dye effects, a second microarray experiment was carried out for each couple, in which the dyes were switched (flip-dye experiment). In total, 24 cDNA microarray hybridizations were performed.

Slides were scanned on a GMS 418 Array Scanner (Affymetrix, High Wycombe, UK). The images obtained (resolution 10 micron; 16 bit tiff image) were processed with ImaGene 5.0 software (Biodiscovery, Los Angeles, USA) to measure mean signal intensities for spots and local background.

cDNA Microarray Data Processing and Statistical Analysis

The data were analyzed using ANOVA models¹⁸, without background correction and using base-2 logarithmic transformation of the measured intensities. Following the suggestions of Kerr et al. (2000), no background-signal correction was applied, as it appeared to add extra unnecessary variability to the data. In the analysis, the base-2 logarithmically transformed data were used. Due to computational limitations, the models were fit in two stages. The ANOVA model included a normalization step, taking into account both the global (across-genes) and local (gene-specific) normalization.¹⁹

To investigate dose-dependency, all pairwise differences between the vegetable mixture groups were examined. To examine the contribution of the individual vegetable on gene expression, all pairwise differences between the individual vegetable groups were examined. For each gene, the Tukey procedure was used to correct for multiple comparisons.²⁰ To control the overall (across genes) probability of false positive findings, at around 5%, a p -value < 0.0001 was considered to indicate statistical significance of an individual pairwise comparison.

Real-time RT-PCR

To verify the cDNA microarray results, 10 gene expression differences, representing five genes which were responsive to vegetables, were analyzed by real-time RT-PCR, as described previously in **Chapter 3** (Section Materials and Methods; Real-time RT-PCR). A list of validated genes and the normalization gene and their chosen primers is given in Table 2.

Additional Statistical Analysis

Statistical analysis of bodyweight of the mice was carried out using SPSS version 6.1.1 for Macintosh. Data were analyzed by means of analysis of variance followed by Student's t -test. A two-sided p -value < 0.05 was considered to indicate statistical significance.

Results

Mean body weights per week per group are presented in Table 3. During the week prior to the start of the intervention (week 0), all animals received the control diet. At the end of this week, there were no statistical significant differences in mean body weights between the groups. After the intervention, mean body weights were significantly increased within each group ($P < 0.05$), but no significant differences between the groups were observed.

Table 2. Primer sequences of the validated genes and the 18S normalization gene.

AC# ¹	Gene Name by NCBI (Abbreviation) ²	Forward Primer	Reverse Primer
BG076621	Heat shock 70kD protein 5 (glucose-regulated protein) (HSPA5)	5'-GAAGGTTACCCATGCAGTTGTTACT-3'	5'-CAATAGTGCCAGCATCTTTGGTT-3'
BG088567	Insulin-like growth factor binding protein 3 (IGFBP3)	5'-GTCTAAGCGGGAGACAGAATACG-3'	5'-ACATTGAGGAACCTTCAGATGATTGAG-3'
BG063729	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	5'-CCACCCACCCAGCAA-3'	5'-GAAATTGTGAGGGAGATGCTCAGT-3'
A1256565	Hydroxyprostaglandin dehydrogenase 15 (NAD) (HPGD)	5'-GACCACGCCTTTGGGAAGTA-3'	5'-TCGCTTAGGGTCGTCAAGCT-3'
A1226017	Sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1)	5'-CCCTCAGAGTCTGCTGGATCA-3'	5'-GGAGACAACCACATCCTTTGTC-3'
18 S		5'-AGTCCCTGCCCTTTGTACACA-3'	5'-GATCCGAGGGCCTCACTAAAC-3'

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.²NCBI (National Center for Biotechnology Information); <http://www.ncbi.nlm.nih.gov/>.

Because weight gain was similar between the groups, these results indicate that there was no difference in food intake. Furthermore, these results show that the diets did not affect normal growth.

Table 3. Mean body weights (g) per week per group.¹

	Week 0 ²	Week 1 ³	Week 2 ³
Control group I	18.6 ± 0.6	19.6 ± 0.7	20.3 ± 0.7 ⁴
10% group	18.6 ± 1.3	19.7 ± 0.9	20.2 ± 0.8 ⁴
20% group	19.1 ± 0.6	20.0 ± 0.4	20.8 ± 0.3 ⁴
40% group	18.6 ± 0.4	19.9 ± 0.7	20.6 ± 1.0 ⁴
Control group II	17.6 ± 0.9	18.7 ± 1.0	19.4 ± 1.4 ⁴
Cauliflower group (T1)	18.3 ± 0.6	19.5 ± 0.6	20.7 ± 0.5 ⁴
Carrots group (T2)	17.9 ± 0.3	19.7 ± 0.7	20.2 ± 0.8 ⁴
Peas group (T3)	18.2 ± 0.6	19.3 ± 1.1	20.3 ± 1.1 ⁴
Onions (T4)	18.0 ± 0.8	18.9 ± 0.7	19.6 ± 0.7 ⁴

¹Body weights are expressed as mean ± SE.

²Acclimatization week, animals received control diet.

³Intervention period.

⁴Statistics: analysis of variance followed by Student's t-test. $P < 0.05$: body weight significantly higher compared to week 0.

By means of microarray technology, the expression levels of 602 genes were measured simultaneously. Data analysis revealed which genes were statistically significantly differentially expressed between the diet groups. In Table 4, a list of these genes is presented together with the gene expression differences between the particular diet groups.

In the vegetable mixture diet groups, a statistically significant difference in gene expression was found for 25 diet group comparisons, representing 18 genes. Remarkably, 23 of the 25 gene expression differences are between the highest vegetable mixture group (40%) and one of the other diet groups. According to literature review, only seven of the 18 differentially expressed genes are likely to play a role in lung cancer development. They are involved in various processes, including cell growth [insulin-like growth factor binding protein 3 (IGFBP3) (↓)], apoptosis [IGFBP3 (↓), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (↑), transglutaminase 2, C polypeptide (TGM2) (↓)], metabolism [topoisomerase (DNA) II alpha 170 kDa (TOP2A) (↑), glutamate-ammonia ligase (glutamine synthase) (GLUL) (↓)], and immune response [Fc receptor, IgE, high affinity I, gamma polypeptide (FCER1G) (↑), cathepsin S (CTSS) (↓)].

In the individual vegetable groups, a statistically significant difference in gene expression was found for 22 diet group comparisons, representing 11 genes. Three of these 11 genes were also statistically significantly different in the vegetable mixture groups, i.e. GLUL (↑), hemoglobin alpha, adult chain 1 (HBA-A1) (↓) and CTSS (↓).

Table 4. Differentially expressed genes.

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Vegetable mixture			Specific vegetables		
		Comparison ³	Difference cDNA microarray ⁴	Difference RT-PCR ⁵	Comparison ³	Difference cDNA microarray ⁴	Difference RT-PCR ⁵
BG079172	Topoisomerase (DNA) II alpha 170 kDa (TOP2A)	40-10	1.19 ± 0.21				
		40-20	0.82 ± 0.16				
C78483	Translationelongation factor EF-1alpha-1 chain (EFHU1)	40-20	0.22 ± 0.05				
BG085131	Ic receptor, IgE, high affinity I, gamma polypeptide (FCER1G)	40-20	0.46 ± 0.09				
BG063822	Pleckstrin homology domain containing, family C (with FERM domain) member 1 (PLEKHC1)	40-0	-0.52 ± 0.08				
		40-20	-0.39 ± 0.07				
BG088394	Selenoprotein P, plasma 1 (SEPP1)	40-20	-0.28 ± 0.05				
A1116399	Apolipoprotein A-II (APOA2)	40-20	-0.63 ± 0.10				
BG063515	Ferritin heavy chain (FTH)	40-20	-0.24 ± 0.05				
BG076621	Heat shock 70kD protein 5 (HSPA5)	40-20	-0.34 ± 0.07	-0.32 ± 0.15			
BG072171	Histidine decarboxylase (HDC)	20-10	0.48 ± 0.09				
BG088567	Insulin-like growth factor binding protein 3 (IGFBP3)	10-0	-0.71 ± 0.11	-0.80 ± 0.22			
		20-10	-0.82 ± 0.10	-0.86 ± 0.30			
		40-0	-0.45 ± 0.09	-0.37 ± 0.05			
		40-20	-0.56 ± 0.08	-0.86 ± 0.22			
BG073920	Lactate dehydrogenase 2, B (LDH2)	40-20	-0.28 ± 0.05				
BG063729	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); clone H3012A11	40-20	0.33 ± 0.06	0.63 ± 0.29			
BG078765	Glutamate oxaloacetate transaminase 2 (GOT2)	40-10	-0.45 ± 0.09				
W17665	Glutamate-ammonia ligase (glutamine synthase) (GLUL)	40-10	-0.73 ± 0.13		T2-C	0.62 ± 0.09	
					T2-T3	0.76 ± 0.11	
BG074617	Transglutaminase 2, C polypeptide (TGM2)	40-20	-0.27 ± 0.06				
W41608	Vesicle-associated membrane protein 1 (VAMP1)	40-20	-0.62 ± 0.11				
AA796818	Interferon, alpha-inducible protein (G1P2)				T1-C	-0.71 ± 0.14	

Table 4. (Continued)

Ac# ¹	Gene Name by NCBI (Abbreviation) ²	Vegetable mixture			Specific vegetables		
		Comparison ³	Difference cDNA microarray ⁴	Difference RT-PCR ⁵	Comparison ³	Difference cDNA microarray ⁴	Difference RT-PCR ⁵
AA109900	Hemoglobin alpha, adult chain 1 (HBA-A1)	40-10	-0.51 ± 0.09		T3-C	-0.57 ± 0.10	
		40-20	-0.35 ± 0.07		T3-T1	-0.74 ± 0.15	
W29976	Cathepsin S (CTSS)	40-0	-0.90 ± 0.16		T2-C	-0.71 ± 0.14	-0.05 ± 0.14
		40-20	-0.82 ± 0.14		T4-C	-0.82 ± 0.15	-0.96 ± 0.13
BX516671	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (SLC6A4)				T2-C	0.77 ± 0.09	
					T2-T3	0.91 ± 0.12	
AA396890	BCL2/adenovirus E1B 19kDa-interacting protein 1 (BNIP1)				T1-C	0.23 ± 0.10	
					T1-T2	0.29 ± 0.12	
					T1-T4	0.28 ± 0.12	
BG066439	Glutathione reductase 1 (GSR)				T3-C	0.23 ± 0.09	
					T3-T4	0.37 ± 0.12	
BG063992	Low density lipoprotein receptor (LDLR)				T3-C	0.20 ± 0.08	
					T3-T1	0.32 ± 0.13	
					T3-T4	0.35 ± 0.12	
AI256565	Hydroxyprostaglandin dehydrogenase 15 (NAD) (HPGD)				T2-C	0.55 ± 0.12	0.23 ± 0.19
					T2-T3	0.71 ± 0.14	
AI226890	Sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1)				T2-C	0.50 ± 0.10	0.78 ± 0.22
AI156918	Selenium binding protein 1 (SELENBP1)				T2-C	0.47 ± 0.08	
					T2-T3	0.60 ± 0.11	

¹AC#: GenBank accession numbers of the cDNA fragments present on the microarrays.

²NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

³C: control group; 10%: 10% vegetable mixture group; 20%: 20% vegetable mixture group; 40%: 40% vegetable mixture group; T1: cauliflower group; T2: carrots group; T3: peas group; T4: onions group.

⁴Estimated expression difference in mean ²log-transformed intensity ± SE.

⁵Normalized expression difference in mean ²log-transformed intensity ± SE. Ten gene differences were validated. Empty cells indicate genes which were not taken into account.

Most (10) of the 22 statistically significant gene expression differences occurred in the carrots group, representing six genes [GLUL ($\uparrow$), CTSS ($\downarrow$), solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (SLC6A4) ($\uparrow$), hydroxyprostaglandin dehydrogenase 15 (NAD) (HPGD) ($\uparrow$), sulfotransferase family 1A, phenol-preferring, member 1 (SULT1A1) ($\uparrow$) and selenium binding protein 1 (SELENBP1) ($\uparrow$)]. CTSS was the only gene, which was modulated by more than one individual vegetable. In addition to carrots, onions had an effect on the expression of this gene, which was also the single gene effect occurring in this group. Second to the carrots, peas caused seven gene expression differences, accounting for three genes, including HBA-A1 ($\downarrow$), glutathione reductase 1 (GSR) ($\uparrow$) and low density lipoprotein receptor (LDLR) ($\uparrow$). Finally, cauliflower modulated the expression of BCL2/adenovirus E1B 19kDa-interacting protein 1 (BNIP1) ($\uparrow$) and interferon, alpha-inducible protein (G1P2) ($\downarrow$), representing four gene expression differences. According to literature review, eight of these 11 genes might play a role in lung cancer development. In Figure 1, the estimated differences of the genes which are likely to play a role in prevention of lung cancer risk, according to literature review, per vegetable group as compared to the control group (set to 0) are illustrated.

Ten gene expression differences, representing five genes responsive to vegetables, were validated by real-time RT-PCR. The results are shown in Table 4. These data prove the results obtained by microarray analyses and verify the reliability of this cDNA microarray approach to identify genes that are modulated by vegetables.

Figure 1. Estimated difference in mean $\log_2$ -transformed intensity $\pm$ SE per diet group (x-axis) for all identified differentially expressed genes from Table 4 (10%: 10% vegetable mixture group; 20%: 20% vegetable mixture group; 40%: 40% vegetable mixture group; T1: cauliflower group; T2: carrots group; T3: peas group; T4: onions group). The control diet group intensity (set to 0) serves as the reference. IGFBP3: insulin-like growth factor binding protein 3; GAPDH: glyceraldehyde-3-phosphate d hydrogenase; TGM2: transglutaminase 2, C polypeptide; TOP2A: topoisomerase (DNA) II alpha 170 kDa; GLUL: glutamate-ammonia ligase (glutamine synthase); FCER1G: Fc receptor, IgE, high affinity I, gamma polypeptide; CTSS: cathepsin S; HPGD: hydroxyprostaglandin dehydrogenase 15 (NAD); SULT1A1: sulfotransferase family 1A, phenol-preferring, member 1; SELENBP1: selenium binding protein 1; GSR: glutathione reductase 1; LDLR: low density lipoprotein receptor; BNIP1: BCL2/adenovirus E1B 19kDa-interacting protein 1; G1P2: interferon, alpha-inducible protein. Asterisks without connection lines indicate statistically significant difference between vegetable group and control group. See Table 4 for other statistical details.

Figure 1. (Continued)

Figure 1. (Continued)

Discussion

In this study, the effect of different doses of a vegetable mixture and of the individual vegetables present in the mixture on gene expression changes in the lung of female C57BL/6 mice was investigated by means of microarray technology. First, the effects of the vegetable mixture will be discussed; next, the contribution of the four individual vegetables on gene expression changes in lung cancer risk preventive pathways.

At least 13 genes, which were modulated by one or more of the vegetable groups, could play a role in different mechanisms involved in preventing lung carcinogenesis. Table 5 summarizes the effects of the vegetable groups on the expression of these genes in relation to possible effects on prevention of lung cancer risk.

In the vegetable mixture groups, seven genes were modulated which could be involved in preventing lung cancer risk. One of these genes is concerned with cell growth, i.e. IGFBP3. The protein encoded by this gene belongs to one of the six members of the IGFBP family and regulates the activity of insulin-like growth factor 1 (IGF-1), thereby inhibiting the mitogenic and antiapoptotic action of IGF-1. IGFBP3 also inhibits cell growth and induces apoptosis independent of IGF-1 by inhibiting the p13K/Akt/PKB and MAPK signaling pathways.²¹ In mice, IGFBP-3 inhibited the growth of lung tumors

and in humans, a higher IGFBP-3 level in serum was associated with lower lung cancer risk. In *in vitro* and *in vivo*, the expression of IGFBP3 was induced by dietary factors like lycopene, retinoic acid, flavonoids and green tea.²² However, in the present study, IGFBP3 was downregulated in the 10% and 40% vegetable mixture groups. Possible other constituents than lycopene, retinoic acid and flavonoids are present in the vegetables which have a downregulating effect on the expression of this gene.

Next to IGFBP3, two other apoptosis genes were modulated in this study, i.e. GAPDH and TGM2. Besides playing an important role in glycolysis in the cytosol, GAPDH has diverse cellular functions, one of the most intriguing is likely to be the induction of apoptosis²³, although at present the mechanism of action is unknown.²⁴ TGM2 covalently cross-links cellular proteins and polyamines. Increased TGM2 expression is associated with apoptosis, probably by induction of massive Ca^{2+} -mediated intracellular cross-linking.²⁵ Furthermore, it has been found that TGM2 was upregulated in bronchial epithelial cells by retinoic acid, which has a role in cancer treatment and prevention by inducing growth suppression.²⁶ GAPDH was upregulated and TGM2 downregulated in the highest vegetable group in the current study. The results of the modulation by the vegetable mixture on IGFBP3, GAPDH and TGM2 are expected to be induction of cell growth and reduction of apoptosis, which are not in line with the proposed hypothesis of cancer risk prevention by vegetables.

Two genes, which were modulated by the vegetable mixture, are involved in metabolism, i.e. TOP2A and GLUL. TOP2A codes for an essential nuclear enzyme that carries out topological changes in DNA and plays important role in DNA replication, transcription and chromosome condensation.²⁷ Overexpression of TOP2A might contribute to accelerated cell proliferation, abortive G2 cell cycle checkpoints, tumor dedifferentiation and sensitivity to anticancer drugs. Although downregulation of TOP2A was reported by genistein²⁸, an isoflavone present in soybeans, in the present study, TOP2A was up-regulated in the highest vegetable dose group. The protein encoded by GLUL is responsible for catalyzing the formation of glutamine from glutamate using ammonium ions and ATP to maintain glutamine homeostasis. It plays an important role in controlling body pH and in removing ammonia from the body. It was shown that GLUL expression is increased in the lungs in response to trauma or infection. There is a paradox that GLUL expression is decreased in tumor cells and that large amounts of glutamine are required for tumor cell growth. It is hypothesized that reactivation of GLUL expression would have growth suppressive effects on tumor cells, however, this has not yet been resolved.²⁹ In the present study, GLUL expression was downregulated in the 40% vegetable mixture group, but upregulated in the carrots vegetable group. The modulation by the vegetable mixture on TOP2A and GLUL gene expression might result in increased cell proliferation and, again, these results are not in favor of lung cancer risk prevention.

Table 5. Presentation of differentially expressed genes likely to be involved in lung cancer preventive pathways, the direction of the effect in the vegetable groups, and the theoretical effect of this on cancer risk.

Abbreviation ¹	Vegetable mixture groups				Individual vegetable groups				
	10%	20%	40%	Effect on cancer risk	Cauliflower	Carrots	Peas	Onions	Effect on cancer risk
IGFBP3	-		-	+					
GAPDH			+	-					
TGM2			-	+					
TOP2A			+	+					
FCER1G			+	-					
GLUL			-	+		+			-
CTSS			-	+		-		-	+
HPGD						+			+
SULT1A1						+			-
SELENBP1						+			-
GSR							+		-
LDLR							+		+
BNIP1					+				-

¹NCBI (National Center for Biotechnology Information): <http://www.ncbi.nlm.nih.gov/>.

Two genes involved in immune response were modulated in the 40% vegetable mixture group, i.e. FCER1G and CTSS. FCER1G is one of the Fc receptors, which plays a role in effective immunity against malignant cells; probably by enhancement of Fc receptor mediated antibody-dependent cellular cytotoxicity resulting in tumor cytotoxicity.³⁰ CTSS has been characterized as a key enzyme in major histocompatibility complex class II mediated antigen presentation. Increased expression of CTSS in lung tumors was associated with better survival probability for lung cancer patients. The high expression could initiate modeling of antigen presentation and consequently the response to tumor antigens.³¹ However, increased CTSS has also been associated with autoimmune diseases. Inhibition of CTSS alters autoantigen presentation and inhibits development of organ-specific autoimmunity.³² FCER1G was upregulated, whereas CTSS was downregulated in the 40% vegetable mixtures group. CTSS was also downregulated in the individual carrots and onions vegetable group, which could be responsible for the observed effect in the 40% vegetable mixture group. The net effect of the modulation of FCER1G and CTSS by the vegetable mixture on immune response is not clear, because the effects on the immune system are opposite for these genes.

Summarizing, the effects on gene expression changes by the vegetable mixture, five out of the seven modulated genes involved in lung cancer preventive mechanisms are expected to result in increased lung cancer risk.

With respect to the individual vegetables, most of the statistically significant gene expression differences occurred in the carrots vegetable group. In addition to the already

discussed genes *GLUL* (↑) and *CTSS* (↓), also *SLC6A4* (↑), *HPGD* (↑), *SULT1A1* (↑) and *SELENBP1* (↑) gene expressions were modulated. No role for *SLC6A4* in lung cancer preventive mechanisms has been described. *HPGD* encodes for an enzyme, which metabolizes a number of prostanoids and non-prostanoid compounds. The products of the non-prostanoids compounds are generally highly reactive α,β -unsaturated aldehydes and ketones, which may cause carcinogenesis. *HPGD* is a target for many non-steroidal anti-inflammatory drugs, including cyclooxygenase-2 inhibitors. Also phytophenolic compounds such as curcumin, flavonoids and epigallocatechin gallate were found to inhibit colonic *HPGD* enzyme activity.³³ Modulation of lung *HPGD* by dietary compounds has not been reported yet. The protein product of *SULT1A1* belongs to a superfamily of enzymes, which catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs and xenobiotic compounds. They play an important role in chemical defense mechanisms against various xenobiotics but also bioactivates many dietary procarcinogens, including heterocyclic aromatic amines implicated in the pathogenesis of colorectal cancer.³⁴ In lung cancer, however, it was shown that a higher activity of this enzyme in the lung was associated with a decreased lung cancer risk.³⁵ *SELENBP1* encodes for a protein which belongs to the selenium containing proteins. Little information is available about the function of this protein in lung cancer or other cancers. Chen et al (2004) have found a that reduced selenium-binding protein 1 expression is associated with poor outcome in lung adenocarcinomas, possibly by increasing cell proliferation and decreasing differentiation.³⁶ As indicated by literature review, no information is available about gene expression modulation in the lung by consumption of carrots. However, few studies have investigated the effect of β -carotene and/or vitamin A on the expression of genes in the lungs of rats. It was found that pharmacological (i.e. high dose) β -carotene supplementation in rats resulted in increased expression of activator protein 1, proliferating cell nuclear antigen and cyclin D1, and reduced expression of retinoic acid receptor β in rat lung tissue. Modulations of these genes were associated with increased keratinized squamous metaplasia. No modulations were found after physiological (i.e. low dose) β -carotene supplementation.³⁷ Borràs et al (2003) have reported reduced expression of p53 and p21 and increased expression of c-jun and cyclin D1 in lung tissue of rats supplemented with vitamin A.³⁸ In the present study, the dose of β -carotene is expected to be comparable with physiological doses, because it is present in the biological source and not supplemented in high amounts. This could explain the relatively beneficial effects of carrots on gene expression changes in the mouse lung.

Summarizing the effect of carrots on gene expression changes, five out of six modulated genes could be involved in lung cancer protective mechanisms; three (*GLUL*, *SULT1A1* and *SELENBP1*) out of these five are affected in such a way that lung cancer protective mechanisms could be expected.

In addition to modulation of CTSS by carrots, onions were able to downregulate the expression of this gene. No other genes were affected by the onions. No information on lung cancer risk prevention for onions or other allium vegetables is present from epidemiological studies. *In vitro* and *in vivo*, a protective effect of allium compounds on lung cancer risk was reported.^{39, 40} With regard to modulation of gene expression, the allium compound flavone induced p21 gene expression in human lung adenocarcinoma cells.³⁹ The present study is the first in which onions modulated gene expression *in vivo*.

Three genes were modulated in the peas group, i.e. HBA-A1 (↓), GSR (↑) and LDLR (↑). No lung cancer preventive pathways in which HBA-A1 could play a role are currently known. GSR codes for an enzyme which reduces oxidized glutathione. Glutathione is involved in the antioxidative defense system against endogenous and exogenous pro-oxidants. Radicals and reactive oxygen species are inactivated by e.g. catalase, superoxide dismutase and glutathione peroxidases, which oxidize glutathione in the same step. Dietary broccoli has been shown to increase GSR expression in the liver, kidney and colon.⁴¹ This is the first study in which vegetables were demonstrated to induce GSR expression in the lungs and thereby to provide the lungs with more reduced oxidized glutathione which could result in an improved antioxidative defense in the lungs. The protein product of LDLR is a cell surface receptor that plays an important role in cholesterol homeostasis. Mammalian cells can obtain cholesterol by *de novo* synthesis (minor pathway) or by the uptake of lipoprotein derived cholesterol via the LDL receptor (major pathway). It has been shown that tumor cell lines have higher LDL receptor activity than the corresponding normal cells. Furthermore, LDL uptake has been shown to be higher in lung tumor tissue than in the corresponding tissue. The reasons for this are not clear, but it is hypothesized that cholesterol is needed for cell growth.⁴²

Taken all this together, two out of three genes modulated by the peas could be involved in lung cancer preventive pathways; only the effect on GSR could lead to lung cancer risk prevention by inducing antioxidative defense.

Finally, cauliflower was able to modulate the expression of the genes BNIP1 (↑) and GIP2 (↓). Only BNIP1 is likely to be involved in lung cancer preventive pathways. No modulation of BNIP1 mRNA or enzyme activity by vegetables or nutrition has been described yet. The protein product of the BNIP1 gene interacts with members of the BCL2 family, which consists of pro-apoptotic (such as BAX) and anti-apoptotic (such as BCL2) proteins. It is suggested that BNIP1 has pro-apoptotic properties, by interacting with BCL2 and BCL2L1.^{43, 44} It was shown that BNIP1 mRNA was downregulated in mouse lung tumor tissue, possibly leading to reduced apoptosis of tumor cells.⁴⁵ However, the precise function of BNIP1 is unclear.^{43, 44} Upregulation of BNIP1 by cauliflower could lead to increased apoptosis, which is generally regarded as a protective mechanism against cancer by removing genetically damaged cells before they can undergo clonal expansion.

Comparing the results from the vegetable mixtures groups with the individual vegetables on gene expression changes, it is clear that in general, other genes are modulated by the vegetables mixture as compared to the individual vegetables. Only three genes were modulated by both the vegetable mixture and one or more of the individual vegetable groups. The gene expression differences occurring in the vegetable mixture groups can not be explained (except for CTSS and HBA-A1) by the effects in the individual vegetables. It could be that the amount of bioactive compounds in individual vegetables is too small to modulate the expression of genes. Possible, the combined effect of micronutrients from different vegetables could be synergistic or additive and therefore large enough to exert a gene expression effect.

In the present study, the complete mouse lung was used for gene expression analysis. The lungs contain a heterogeneous population of cells, such as macrophages, various epithelial cells and smooth muscle cells. It can not be excluded that the effects on gene expression in specific lung cancer target cells by vegetables are diluted by the effects on gene expression in other cells. Isolation, however, of specific target cells has limitations as well. Protocols for the isolation of homogeneous populations of specific cells require a lot of time, thereby risking degradation of mRNA, and furthermore, these protocols comprise enzymatic digestion or agitation and centrifugation, which could also influence gene expression patterns. The relatively new technology of laser capture microdissection can overcome these drawbacks, however, this technology is still in its infancy.⁴⁶

In this study, 13 genes were modulated which could be involved in lung cancer risk preventive pathways. From Table 5, it becomes clear that for the vegetable mixture groups only 2 out of 7, and for the individual vegetables 5 out of 8 are modulated in such a way that lung cancer preventive pathways could be stimulated; the other gene modulations are expected to enhance lung cancer risk. These results do not provide a strong indication of favorable vegetable-induced gene expression modulations which could result in reduced lung cancer risk. But, it is very difficult to estimate the contribution of each gene expression modulation in carcinogenesis. The net effect of the gene expression modulations by the vegetables could still be in favor of lung cancer risk prevention. It seems that individual vegetables have a higher potential of modulating genes in favor of lower lung cancer risk, in stead of a combined effect. Furthermore, carrots modulated most gene expressions, and most of these effects were also in support of lung cancer risk prevention. These results are in line with the prospective epidemiological studies in which was shown that particularly dark green and yellow-orange vegetables, rich in β -carotene and vitamin E, are the specific types of vegetables, which have the most available and consistent evidence in reducing lung cancer risk. The current study provides more insight into the genetic mechanisms by which vegetables, in particular carrots, can prevent lung cancer risk.

References

1. World Health Organization & International Agency for Research on Cancer. World Cancer Report. Lyon: IARC Press, 2003.
2. Omenn GS. Chemoprevention of lung cancer: the rise and demise of beta-carotene. *Annu Rev Public Health* 1998;19:73-99.
3. World Cancer Research Fund. Food, Nutrition and the Prevention of Cancer: a global perspective. Chapter 4.5. Lung Washington: BANTA Book group, 1997:130-147.
4. Peto R, Doll R, Buckley JD, Sporn MB. Can dietary beta-carotene materially reduce human cancer rates? *Nature* 1981;290:201-8.
5. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* 1991;2:427-42.
6. Thompson HJ, Heimendinger J, Haegele A, Sedlacek SM, Gillette C, O'Neill C, Wolfe P, Conry C. Effect of increased vegetable and fruit consumption on markers of oxidative cellular damage. *Carcinogenesis* 1999;20:2261-6.
7. Zino S, Skeaff M, Williams S, Mann J. Randomised controlled trial of effect of fruit and vegetable consumption on plasma concentrations of lipids and antioxidants. *Bmj* 1997;314:1787-91.
8. Nishino H. Cancer prevention by natural carotenoids. *J Cell Biochem Suppl* 1997;27:86-91.
9. Dutta A, Dutta SK. Vitamin E and its role in the prevention of atherosclerosis and carcinogenesis: a review. *J Am Coll Nutr* 2003;22:258-68.
10. Omenn GS, Goodman G, Thornquist M, Grizzle J, Rosenstock L, Barnhart S, Balmes J, Cherniack MG, Cullen MR, Glass A, et al. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers. *Cancer Res* 1994;54:2038s-2043s.
11. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med* 1994;330:1029-35.
12. Kelley MJ, McCrory DC. Prevention of lung cancer: summary of published evidence. *Chest* 2003;123:50S-59S.
13. Ziegler RG, Mayne ST, Swanson CA. Nutrition and lung cancer. *Cancer Causes Control* 1996;7:157-77.
14. Barrett JC, Kawasaki ES. Microarrays: the use of oligonucleotides and cDNA for the analysis of gene expression. *Drug Discov Today* 2003;8:134-41.
15. Hasseman J, Chen E, Yang I, Quackenbush J. Aminoallyl labeling of RNA for microarrays. <http://www.tigr.org/tdb/micoarray/protocolsTIGR.shtml> (accessed June 21, 2001), 2001.
16. Churchill GA. Fundamentals of experimental design for cDNA microarrays. *Nat Genet* 2002;32 Suppl 2:490-5.
17. Kerr MK. Design considerations for efficient and effective microarray studies. *Biometrics* 2003;59:822-828.
18. Kerr MK, Martin M, Churchill GA. Analysis of variance for gene expression microarray data. *Journal of Computational Biology* 2000;7:819-837.
19. Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS. Assessing gene significance from cDNA microarray expression data via mixed models. *J Comput Biol* 2001;8:625-37.

20. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. *Applied Linear Statistical Models* (Fourth Edition). Mass, 1996.
21. Lee HY, Chun KH, Liu B, Wiehle SA, Cristiano RJ, Hong WK, Cohen P, Kurie JM. Insulin-like growth factor binding protein-3 inhibits the growth of non-small cell lung cancer. *Cancer Res* 2002;62:3530-7.
22. Ali O, Cohen P, Lee KW. Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. *Horm Metab Res* 2003;35:726-33.
23. Ishitani R, Tajima H, Takata H, Tsuchiya K, Kuwae T, Yamada M, Takahashi H, Tatton NA, Katsube N. Proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase: a possible site of action of antiapoptotic drugs. *Prog Neuropsychopharmacol Biol Psychiatry* 2003;27:291-301.
24. Berry MD, Boulton AA. Glyceraldehyde-3-phosphate dehydrogenase and apoptosis. *J Neurosci Res* 2000;60:150-4.
25. Griffin M, Casadio R, Bergamini CM. Transglutaminases: nature's biological glues. *Biochem J* 2002;368:377-96.
26. Ma Y, Koza-Taylor PH, DiMattia DA, Hames L, Fu H, Dragnev KH, Turi T, Beebe JS, Freemantle SJ, Dmitrovsky E. Microarray analysis uncovers retinoid targets in human bronchial epithelial cells. *Oncogene* 2003;22:4924-32.
27. Watt PM, Hickson ID. Structure and function of type II DNA topoisomerases. *Biochem J* 1994;303 (Pt 3):681-95.
28. Kurzer MS, Xu X. Dietary phytoestrogens. *Annu Rev Nutr* 1997;17:353-81.
29. Schraml P, Shipman R, Colombi M, Ludwig CU. Identification of genes differentially expressed in normal lung and non-small cell lung carcinoma tissue. *Cancer Res* 1994;54:5236-40.
30. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci U S A* 1998;95:652-6.
31. Kos J, Sekirnik A, Kopitar G, Cimerman N, Kayser K, Stremmer A, Fiehn W, Werle B. Cathepsin S in tumours, regional lymph nodes and sera of patients with lung cancer: relation to prognosis. *Br J Cancer* 2001;85:1193-200.
32. Saegusa K, Ishimaru N, Yanagi K, Arakaki R, Ogawa K, Saito I, Katunuma N, Hayashi Y. Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity. *J Clin Invest* 2002;110:361-9.
33. Cho H, Tai HH. Inhibition of NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) by cyclooxygenase inhibitors and chemopreventive agents. *Prostaglandins Leukot Essent Fatty Acids* 2002;67:461-5.
34. Bamber DE, Fryer AA, Strange RC, Elder JB, Deakin M, Rajagopal R, Fawole A, Gilissen RA, Campbell FC, Coughtrie MW. Phenol sulphotransferase SULT1A1*1 genotype is associated with reduced risk of colorectal cancer. *Pharmacogenetics* 2001;11:679-85.
35. Liang G, Miao X, Zhou Y, Tan W, Lin D. A functional polymorphism in the SULT1A1 gene (G638A) is associated with risk of lung cancer in relation to tobacco smoking. *Carcinogenesis* 2004;25:773-8.
36. Chen G, Wang H, Miller CT, Thomas DG, Gharib TG, Misek DE, Giordano TJ, Orringer MB, Hanash SM, Beer DG. Reduced selenium-binding protein 1 expression is associated with poor outcome in lung adenocarcinomas. *J Pathol* 2004;202:321-9.
37. Liu C, Wang XD, Bronson RT, Smith DE, Krinsky NI, Russell RM. Effects of physiological versus pharmacological beta-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets. *Carcinogenesis* 2000;21:2245-53.

38. Borras E, Zaragoza R, Morante M, Garcia C, Gimeno A, Lopez-Rodas G, Barber T, Miralles VJ, Vina JR, Torres L. In vivo studies of altered expression patterns of p53 and proliferative control genes in chronic vitamin A deficiency and hypervitaminosis. *Eur J Biochem* 2003;270:1493-501.
39. Bai F, Matsui T, Ohtani-Fujita N, Matsukawa Y, Ding Y, Sakai T. Promoter activation and following induction of the p21/WAF1 gene by flavone is involved in G1 phase arrest in A549 lung adenocarcinoma cells. *FEBS Lett* 1998;437:61-4.
40. Khanduja KL, Gandhi RK, Pathania V, Syal N. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. *Food Chem Toxicol* 1999;37:313-8.
41. Vang O, Rasmussen BF, Andersen O. Combined effects of complex mixtures of potentially anti-carcinogenic compounds on antioxidant enzymes and carcinogen metabolizing enzymes in the rat. *Cancer Lett* 1997;114:283-6.
42. Gueddari N, Favre G, Marmouget C, Soula G, Le Gaillard F. Involvement of tyrosine kinase activity in the low-density lipoprotein receptor expression in human lung adenocarcinoma cell line A549. *Biochimie* 1996;78:874-81.
43. Zhang H, Heim J, Meyhack B. Novel BNIP1 variants and their interaction with BCL2 family members. *FEBS Lett* 1999;448:23-7.
44. Yasuda M, Chinnadurai G. Functional identification of the apoptosis effector BH3 domain in cellular protein BNIP1. *Oncogene* 2000;19:2363-7.
45. Bonner AE, Lemon WJ, Devereux TR, Lubet RA, You M. Molecular profiling of mouse lung tumors: association with tumor progression, lung development, and human lung adenocarcinomas. *Oncogene* 2004;23:1166-76.
46. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. Laser capture microdissection. *Science* 1996;274:998-1001.

Chapter 7

Summary and general discussion

Summary and General Discussion

Food preparation and dietary habits are very relevant exogenous factors affecting cancer risk. The most consistent finding on diet as a determinant of cancer risk is the association between consumption of vegetables and fruit and reduced risk of several cancers. Convincing epidemiological evidence exists for cancer of the gastrointestinal tract (oral cavity, pharynx, esophagus, stomach, colon and rectum) and of the respiratory tract (lung).¹ In terms of incidence, lung cancer is the most common cancer worldwide, closely followed by cancer of the colorectum as the third most frequent.² Diets containing considerable amounts of a variety of vegetables (> 400 g/day) may reduce these types of cancer by 45 respectively 40 percent (range 30-50).³ The mechanisms by which vegetables exert their beneficial effects are various and studied in experimental systems. However, whether vegetables exert their effect by affecting genetic pathways is still mostly unknown, because only few studies have investigated the effect of vegetables and vegetable constituents on gene expression changes in target organs. Nowadays, microarray technology can be used to investigate the effect of a specific diet on the expression of multiple genes or proteins in a single experiment. It can help to identify the genetic mechanisms by which vegetables affect cancer risk at the molecular level.

The hypothesis investigated in the present thesis is that an important contribution of the anticarcinogenic effects of vegetables in the colorectum and lung is through modulating the expression of genes involved in biological and genetic pathways that are relevant for chemical carcinogenesis. Therefore, the aim is to provide more insight into the molecular mechanisms by which vegetables exert their anticarcinogenic effects, by focusing on the effects at the genome level in the relevant target cells.

The model of colorectal cancer (CRC) proposed by Fearon and Vogelstein consists of successive genetic changes, in which a number of genes are involved, including APC (adenopolyposis coli), K-RAS, DCC (deleted in colorectal cancer) and p53.⁴ During the last decade, additional genetic events and specific molecular pathways have been identified. It became clear that the intact or mutated key molecules of the Vogelstein model interact and form a network of molecular events affecting additional genes.^{5,6} These genetic pathways and the involved genes are obvious molecular targets for the protection against CRC by vegetables. To identify the genes which are modulated *in vivo* in colorectal mucosa from humans by vegetables, the study described in **Chapter 2** was carried out. In this human dietary intervention study the effect of a 50% decreased (= 75 g/day) or doubled intake (= 300 g/day) of a mixture of vegetables (cauliflower, carrots, peas and onions) during two weeks on gene expression changes in normal colorectal mucosa of female sporadic adenoma patients and healthy controls was investigated by means of

microarray analysis of 597 genes representing pathways relevant for carcinogenesis. The two study groups were chosen to investigate whether the effect of the intervention is different for patients compared to controls, as it is plausible that vegetables exert a different effect in persons who are at higher risk for the development of CRC. In total, comparison of pre- and post-intervention rectal biopsy samples revealed that 52 genes were differentially expressed, and according to literature review, 20 of these genes are likely to be related to (colon)carcinogenesis. In both patients and controls, seven genes were similarly modulated, for example *fos* proto-oncogene and ornithine decarboxylase. Thirteen genes were modulated differently in patients compared to controls, including cyclooxygenase-2 and *mdm2-A* in patients and cytochrome P450-27B1, -2C19, -2D6, -2C9, and -3A4 in controls. An increased intake of vegetables resulted in down-regulation of genes promoting cell proliferation and bioactivation of procarcinogens, and in up-regulation of genes involved in cell growth arrest; in contrast, a decreased intake of vegetables resulted in down-regulation of genes inhibiting cell growth and up-regulation of genes promoting cellular dedifferentiation and bioactivation of procarcinogens. Furthermore, it seems that vegetables in patients affect genes involved in late stage of CRC, whereas in controls genes involved in the initiation phase are modulated. This is the first human study in which the effect of a vegetable intervention on gene expression changes at target level has been investigated. The results show that almost all the effects on modulating the expression of genes by altering vegetable intake can be mechanistically linked to cellular processes that explain either prevention of colorectal cancer risk by high vegetable intake or increased colorectal cancer risk by low vegetable intake.

To confirm the human findings and further elucidate mechanisms, mouse studies were performed using a comparable approach and technique. Although mice are often used to investigate the effect of diet on carcinogenesis⁷⁻¹⁰, the number of studies in which the effect on gene expression is studied is limited, is mostly investigated in combination with carcinogen administration and is restricted to analyses of a few genes. Therefore, the mouse studies carried out in this thesis provide much information for the effect of specific vegetable diets on the expression of multiple biologically relevant genes in target tissues. To achieve this, a mouse cDNA microarray was constructed, which contained the same genes as present on the microarray used for the human study.

To mimic the human dietary intervention study, the mouse study illustrated in **Chapter 3** was performed in which the dose-dependent effect of a vegetable mixture was investigated on gene expression changes in colon mucosa. C57BL6 female mice were fed one of four different diets, containing no vegetables, 10%, 20% or 40% wt/wt vegetables mixture respectively, for a period of two weeks. The vegetables mixture consisted of the same combination and proportion of vegetables as used in the human intervention study, i.e. cauliflower (30% wet wt), carrots (30% wet wt), peas (30% wet wt), and onions (10% wet wt). The results of this study show a dose-dependent effect of vegetables on gene

expression, although in general this relationship is not linear. Almost all of the gene expression modulations occurred in the highest vegetable dose (40%) group. In total, the expressions of 39 genes were modulated, 17 of which are likely to play a role at different stages during CRC development, as indicated by literature review. Furthermore, for almost all these genes the altered expression can indeed explain reduced CRC risk. A diet high in vegetables positively modulated genes involved in different pathways, including inhibition of carcinogen formation, increasing DNA repair capacity, induction of apoptosis, and in reducing cell growth and tumor invasion. Many new genes were identified for which currently no modulation by vegetables has been reported, such as the detoxification gene *ALDH1A1*, several apoptosis genes (*TNFRSF6*, *CASP3*, -4 and -7, *CTSB*, *TMSB10* and *STAT1*) and the tumor suppressor genes *RRM1* and *SLC26A3*. Comparing the results of this mouse study with the results of the human study, only one gene is similarly affected by the vegetables in human and mouse colon, i.e. stearoyl-CoA desaturase (*SCD*). To date, two mouse (*SCD1* and *SCD2*) genes and a single human *SCD* gene have been cloned and characterized. *SCD* is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids. A proper ratio of saturated to unsaturated fatty acids contributes to membrane fluidity and cell-cell interaction. Abnormal alteration of this ratio has been shown to play a role in several physiological and disease states including diabetes, cardiovascular disease, obesity, hypertension, neurological disease, immune disorders, and cancer. However, causal relationships between *SCD* activity and these various disease states remain unclear. The activity of *SCD* is sensitive to for instance dietary changes, hormonal imbalance, temperature changes, alcohol and phenolic compounds.¹¹ *SCD* activity was decreased in rat liver during starvation and diabetes and was rapidly induced to high levels upon refeeding high carbohydrate diets or upon insulin administration.¹² The higher amount of carbohydrates present in the high vegetable diets could be responsible for the induction of *SCD* gene expression. The consequences of this on CRC risk are, however, indistinct. Although only one gene is similarly modulated in both studies, the results of both studies showed that a diet high in vegetables modulates genes in favor of CRC risk prevention via a number of similar pathways, i.e. inhibition of cell proliferation and detoxification of procarcinogens.

Despite the fact that gene expression studies are a fast and reproducible way to make an inventory of the effects of vegetables on gene expression changes, changes at the mRNA level are not necessarily proportional to the changes at the protein level because of differences in protein translation and degradation. Furthermore, proteins can undergo several functional posttranslational modifications like phosphorylation and glycosilation. Therefore, in addition to the gene expression analysis, a proteomic approach was undertaken to evaluate the effect of different doses of the vegetable mixtures on proteome changes in colon mucosa of female C57BL6 mice (**Chapter 4**). Differential protein expression was determined with 2D gel electrophoresis. Thirty proteins were found to be

differentially expressed, and six proteins could be identified by mass spectrometry, namely MLRN, CAH-1, HMG-1, PAP3, GAPDH and OSCP. Except for OSCP, alterations in the levels of these proteins coincide with a pivotal role in the protection against colon cancer. The proteins are involved in different processes like cell growth, cell differentiation and apoptosis.

Comparing the results from the proteomic study with the gene expression study, no correspondence was observed between the human gene colon study and the mouse proteomic colon study. Only GAPDH was found to be differentially expressed in both the mouse proteomic study as in the mouse gene expression study, although dissimilar. Protein analysis by 2D-gel electrophoresis indicated a decreased protein expression in all diets compared to control, although this was not statistically significant. The immunoblotting results on the other hand showed a large variation between the various sub-pool samples. The observed difference between the mRNA levels and protein levels may be due to different subforms of GAPDH. To exert the wide variety of GAPDH functions, the different subforms of the protein need to be distinguished by the cell. This might be regulated by alternate splicing or posttranslational modification and results in protein products that differ in isoelectric point (pI) and/or molecular weight. This might also explain the difference in protein expression as observed from the 2D-gel electrophoresis and the immunoblotting experiments.^{13, 14} Considering the role of GAPDH in DNA replication, DNA repair and apoptosis, GAPDH might be an interesting candidate for protection against colon cancer.¹⁴ However, this has to be further investigated. The cDNAs representing the mRNAs coding for the other proteins found in the proteomic study were not present on the microarray used in the gene expression studies. However, besides GAPDH, for none of the other genes present on the array, differentially expressed proteins were identified. It could be that the gene expression difference resulted in a protein difference too small to be identified in the proteomics analysis. Despite this, in addition to the gene expression results, the results of the proteomic study also indicate modulation of proteins in favor of prevention of CRC risk.

Next to the amount of vegetables, the type of vegetables can be of importance for inducing particular gene expression changes. Therefore, the study described in **Chapter 5** was carried out in which the effect of four individual vegetables, i.e. cauliflower, carrots, peas and onions, on the expression of genes in colon mucosa of female C57BL6 mice was examined. In total, 18 genes were differentially expressed by one or more of the specific vegetables: cauliflower was able to modulate most genes (10), closely followed by the onions (7) and carrots (7); least gene expression modulations occurred in the peas group (3). No genes were modulated solely by the carrots. According to literature review, ten of the 18 modulated genes are likely to be involved in CRC development, and seven out of these 10 are modulated in favor of CRC prevention. The four individual vegetables have about the same potential in modulating genes in support of lower CRC risk, although

mostly via different mechanisms. Genes involved in cell growth regulation and maintaining homeostasis were affected in all vegetable groups. Furthermore, cauliflower, carrots and peas affected genes involved in induction of apoptosis; onions modulated genes which could play a role in maintaining DNA stability and induction of cellular differentiation; and peas, in addition to increasing the expression of an apoptosis gene, induced an angiogenesis inhibiting gene. From epidemiological studies it is known that with respect to prevention of CRC especially cruciferous and green leafy vegetables play a role.¹ Indeed, cruciferous vegetables were able to induce most gene expression differences; however, most of these genes are currently not known to be involved in CRC protective mechanisms. The contribution of carrots, peas and onions in prevention of CRC risk could be of greater importance than until now considered.

In addition to this evaluation of vegetable specific gene modulations, a comparison can be made with the results of the studies described in **Chapters 2 and 3**. First, an evaluation will be made with the mouse vegetable mixture study described in **Chapter 3**. The amount of the individual vegetables is equal to the amount of these specific vegetables in the highest vegetable mixture group (40%) and therefore the contribution of the four individual vegetables to the combined effect of these vegetables on gene expression can be investigated. Eleven similar genes were modulated in both the vegetable mixture study and the individual vegetable study and for seven of them the modulation by the mixture could be explained by the effect of a particular vegetable. ACTB and CASP4 were modulated in the cauliflower group; OAT, EFHU1 and PMP22 were affected by cauliflower and carrots; and SCD2 and HIF1A were modulated by the onions. Although most of these genes (i.e. ACTB, EFHU1, PMP22 and SCD2) are at this time not known to be involved in CRC protective mechanisms, the modulation in both vegetables studies gives a strong indication of their importance. Their role, however, in CRC development has to be further investigated. Comparing the results of the individual vegetables study with the human study illustrated in **Chapter 2**, two genes were similarly affected, i.e. ODC and, again, SCD. The role of SCD has already been discussed. The fact that modulation of this gene was observed in three independent vegetable studies and that the modulation is found in mice as well as in humans, indicates a strong regulation by vegetables and makes this gene of particular interest for further research. In addition to SCD, ODC was similarly modulated in the human study compared to the mouse individual vegetable study. This gene was downregulated in the high vegetable diet groups in both patients and controls, and in the mouse cauliflower and carrots group. ODC catalyzes the first step in the polyamine biosynthetic pathway, a highly regulated pathway associated with rapid growth states, including tumorigenesis. The polyamines are ubiquitous cellular polycations essential for optimal rates of cell growth and -differentiation. Increases in intracellular polyamine levels are related to increased cell proliferation and higher CRC risk.^{15, 16} In normal colonic epithelium, polyamine levels are maintained at low levels, coordinated by the tumor

suppressor gene APC. APC mediates ODC expression via a C-MYC dependent mechanism affecting ODC transcription. Wild-type APC suppresses the level of β -catenin, thereby reducing the formation of a complex between β -catenin and the lymphoid-enhancing factor (LEF)/T-cell factor (Tcf) resulting in reduced expression of target genes like C-MYC and subsequent C-MYC target genes, like ODC.¹⁶ Inhibition of ODC by vegetable components has already been reported for β -carotene¹⁷, present in carrots, and indole-3-carbinol¹⁸, present in cauliflower. The results of the studies described in this thesis (**Chapter 2** and **4**) are in line with these previous investigations and provide more evidence of regulation of ODC expression by vegetables.

The polyamine biosynthetic pathway plays an important role in cell growth and differentiation in the intestine. Two other genes involved in this pathway were modulated in the vegetable mixtures study (**Chapter 3**), i.e. OAT and SAT; OAT was also affected in the cauliflower and carrots group (**Chapter 5**). OAT catalyzes the conversion of ornithine, which is the substrate for ODC, to glutamate semialdehyde, thereby reducing intracellular ornithine contents.¹⁹ Both in the highest vegetable mixture group (40%) as in the cauliflower and carrots group, the expression of OAT was downregulated, which could result in an increased ornithine available for ODC, leading to higher polyamine levels. However, SAT, a key gene involved in polyamine catabolism, was upregulated in the highest vegetable group (40%). This gene encodes for the key enzyme catalyzing the formation of shorter-chain amines from longer-chain ones, thereby reducing polyamine contents. The expression of the SAT gene is regulated by the K-RAS gene, which is an important proto-oncogene involved in development of CRC. Mutated K-RAS leads to a suppression of the nuclear hormone receptor peroxisomal proliferators-activated receptor γ (PPAR γ), which in turn leads to a decreased SAT expression.¹⁶ The modulation of ODC, OAT and SAT by the vegetables provides evidence for vegetables to exert part of their beneficial effects in CRC through affecting the polyamine biosynthetic pathway, leading to reduced cell proliferation.

In addition to prevention of CRC, epidemiological studies provide evidence for protection against lung cancer incidence by vegetables and fruits. Particularly dark green and yellow-orange vegetables, rich in β -carotene and vitamin E, are the specific types of vegetables, which best protect against lung cancer. In contrast to the colon and rectum, the lungs are not a site of direct contact, i.e. the vegetables pass the gastrointestinal tract but not the lungs as they are ingested. Furthermore, the dietary compounds that reach the lung may have been modulated by the first-pass metabolism in the liver. Therefore, the lungs are subject to only systemic exposure of vegetables-derived compounds and the mechanisms by which vegetables protect against lung cancer could therefore be different compared to that in the colon. To investigate the effect of vegetables on gene expression in the lung, the lungs of the mice used in the studies described in **Chapters 3** and **5**, were analyzed for gene expression changes (**Chapter 6**). In the vegetable mixture groups, 18

genes were differentially expressed. Similar as in the colon study (**Chapter 3**), most gene expression differences occurred between the highest vegetable dose group (40%) and one of the other diet groups. However, only seven of the 18 genes are, according to literature review, likely to be involved in mechanisms underlying lung cancer risk prevention. But, the hypothesized effects of gene expression modulation by the vegetable mixture are not always in favor of lung cancer risk prevention. The effects on IGFBP3 (↓), TGM2 (↓), TOP2A (↑), GLUL (↓) and CTSS (↓) are expected to enhance lung cancer risk, whereas the effects on GAPDH (↑) and FCER1G (↑) are expected to result in lung cancer risk prevention. In the individual vegetable groups, 11 genes were modulated, eight of which might be implicated in lung cancer preventive mechanisms, as indicated by literature review. Most gene modulations (6) took place in the carrots group, followed by peas (3) and cauliflower (2). Onions, together with carrots, were able to modulate the expression of only one gene, i.e. CTSS. The effect, however, on the expression of this gene is expected to increase lung cancer risk, by suppression of the immune system. Furthermore, also for the other modulated genes, the theoretical effects of the modulation by the individual vegetables on mechanisms underlying lung cancer prevention are not always beneficial. With respect to the effect of carrots on gene expression changes, five out of six modulated genes could be involved in lung cancer protective mechanisms; three (GLUL, SULT1A1 and SELENBP1) out of these five are affected in such a way that lung cancer protective mechanisms could be expected. For peas, two out of three modulated genes could be involved in lung cancer preventive pathways; only the effect on GSR could lead to lung cancer risk prevention by inducing the anti-oxidative defense. Finally, cauliflower was able to modulate the expression of two genes, i.e. BNIP1 (↑) and G1P2 (↓). Only BNIP1 is likely to be involved in lung cancer preventive pathways. Upregulation of BNIP1 by cauliflower could lead to increased apoptosis, which is generally regarded as a protective mechanism against cancer by removing genetically damaged cells before they can undergo clonal expansion. Taken all this together, vegetables are able to modulate genes involved in lung cancer preventive pathways, although, the modulation is not unambiguous in favor of reduced lung cancer risk. It is difficult, however, to predict the contribution of each gene modulation in the cancer process. The net effect of the gene expression modulation by the vegetables could still result in lung cancer risk prevention. Furthermore, genes which were not present on the microarray could play a role in lung carcinogenesis. More research is needed to investigate the effect of vegetables on gene expression changes in the lungs. Very interestingly, carrots were able to modulate most gene expression changes. Taken into account that it is known from epidemiological studies that in particular consumption of high amounts of carrots are able to protect against lung cancer, the results of the lung study provide rationale that indeed carrots are of importance in modulating this process.

Comparing the gene expression modulation occurring in colon with those in lung, corresponding and different genes were affected. Genes which are modulated in both

tissues provide evidence for similar mechanisms by which vegetables protect against both types of cancer. When comparing the mouse lung study with the mouse colon study (**Chapter 3, 4 and 5**), eight genes were modulated in both the mouse colon and lung and include EFHU1, G1P2, GAPDH, SULT1A1, SEPP1, HPGD, TOP2A and BNIP1, however, the modulation was opposite (except for GAPDH). As already mentioned, the dietary compounds that reach the lung may have been modulated by the first-pass metabolism in the liver and these metabolites could be responsible for the observed effects. No (other) explanation for this remarkable result can be given and this has to be further investigated. The role of EFHU1, G1P2, and SEPP1 in carcinogenesis is currently not known. GAPDH (also identified in the colon proteomic study as differentially expressed, **Chapter 4**) and BNIP1 are involved in apoptosis, TOP2A plays a role in DNA replication, and SULT1A1 and HPGD are involved in biotransformation. In addition to modulating of similar genes, genes were affected by vegetables in either colon or lung. The number of genes affected in lung tissue is smaller and no genes are overrepresented in mechanisms of cancer prevention. However, remarkably, carrots seem to have the greatest potential of modulating genes in the lung, whereas in colon the individual vegetables appear to have equal promise for modulating genes in favor of CRC prevention.

Overall, the studies presented in this thesis provide much information on vegetable induced gene expression changes in target tissue where cancer risk is reduced by increased vegetable intake. In particular, the studies regarding vegetable induced gene expression changes in the colon and rectum support the hypothesis investigated in this thesis. In colon, vegetables modulated a lot of genes involved in biological and genetic pathways in favor of prevention of CRC risk. Genes were modulated for which already a relationship with vegetables and/or cancer prevention has been reported. In this regard, the studies provide new evidence which strengthens this relationship. In addition to these 'known' genes, new genes were identified for which, until now, no modulation by vegetables has been described, and/or no link is known with cancer. Remarkably, the dose of vegetables necessary to exert a measurable effect on the expression of genes in favor of cancer preventive mechanisms is relatively high in mice. The evidence regarding lung cancer risk prevention by vegetable induced gene expression changes is less clear compared to the results of the colon studies. The role of carrots is of particular interest and most promising for modulating genes in support of reduced lung cancer risk. The results of the lung studies, however, provide too little information to either reject or confirm the hypothesis. Further research is needed in which the contribution of specific genes and pathways modulated by different vegetables types is investigated. Several genes are of particular interest for future studies. Especially SCD is of interest because the modulation by vegetables was observed in three independent vegetable studies in mice as well as in humans. In addition, the role of COX-2 mediated gene expression by vegetables is very

relevant, because of its established role in CRC risk. In addition to specific genes, several pathways are of particular interest. In both human and mouse studies, mostly genes involved in inhibition of cell growth are modulated by the vegetables. The affected pathways predominantly comprise the polyamine biosynthetic pathway and the apoptosis pathway. By using knockout and/or transgenic systems, the effect of vegetables on the contribution of the absence or presence of particular genes in the cancer process can be examined. Relatively new technologies like RNA interference and laser capture microdissection provide new possibilities in optimizing gene expression research. Furthermore, to investigate the effect of vegetables on gene expression changes in different stages of the carcinogenic process, studies in which vegetable are applied before and/or after administration of carcinogens can be carried out. In addition to dose-response studies, time-line studies could provide information about specific time-points and duration of vegetable induced gene expression effects. The results of these studies will provide a more complete and fundamental understanding of cancer aetiology, which is necessary for the development of improved prevention strategies based on dietary advice. Improved and more convincing evidence for the anticarcinogenic effects of vegetables may result in higher consumption of vegetables by the general public.

References

1. World Cancer Research Fund. Food, Nutrition and the Prevention of Cancer: a global perspective. Washington: BANTA Book group, 1997.
2. World Health Organization. World Cancer Report. Lyon: IARC Press, 2003.
3. Willett WC. Diet, nutrition, and avoidable cancer. *Environ Health Perspect* 1995;103 Suppl 8:165-70.
4. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.
5. Arends JW. Molecular interactions in the Vogelstein model of colorectal carcinoma. *J Pathol* 2000;190:412-6.
6. Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000;119:854-65.
7. Kallay E, Adlercreutz H, Farhan H, Lechner D, Bajna E, Gerdenitsch W, Campbell M, Cross HS. Phytoestrogens Regulate Vitamin D Metabolism in the Mouse Colon: Relevance for Colon Tumor Prevention and Therapy. *J Nutr* 2002;132:3490S-3493S.
8. Katchamart S, Williams DE. Indole-3-carbinol modulation of hepatic monooxygenases CYP1A1, CYP1A2 and FMO1 in guinea pig, mouse and rabbit. *Comp Biochem Physiol C Toxicol Pharmacol* 2001;129:377-84.
9. van Kranen HJ, van Iersel PW, Rijnkels JM, Beems DB, Alink GM, van Kreijl CF. Effects of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the ApcMin mouse. *Carcinogenesis* 1998;19:1597-601.
10. Kim DJ, Shin DH, Ahn B, Kang JS, Nam KT, Park CB, Kim CK, Hong JT, Kim YB, Yun YW, Jang DD, Yang KH. Chemoprevention of colon cancer by Korean food plant components. *Mutat Res* 2003;523-524:99-107.
11. Ntambi JM. Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. *J Lipid Res* 1999;40:1549-58.
12. Oshino N, Sato R. The dietary control of the microsomal stearyl CoA desaturation enzyme system in rat liver. *Arch Biochem Biophys* 1972;149:369-77.
13. Mezquita J, Pau M, Mezquita C. Several novel transcripts of glyceraldehyde-3-phosphate dehydrogenase expressed in adult chicken testis. *J Cell Biochem* 1998;71:127-39.
14. Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1999;1432:159-84.
15. Martinez ME, O'Brien TG, Fultz KE, Babbar N, Yerushalmi H, Qu N, Guo Y, Boorman D, Einspahr J, Alberts DS, Gerner EW. Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc Natl Acad Sci U S A* 2003;100:7859-64.
16. Babbar N, Gerner EW. Polyamines as modifiers of genetic risk factors in human intestinal cancers. *Biochem Soc Trans* 2003;31:388-92.
17. Phillips RW, Kikendall JW, Luk GD, Willis SM, Murphy JR, Maydonovitch C, Bowen PE, Stacewicz-Sapuntzakis M, Wong RK. beta-Carotene inhibits rectal mucosal ornithine decarboxylase activity in colon cancer patients. *Cancer Res* 1993;53:3723-5.
18. Shertzer HG, Senft AP. The micronutrient indole-3-carbinol: implications for disease and chemoprevention. *Drug Metabol Drug Interact* 2000;17:159-88.
19. Han X, Kazarinoff MN, Seiler N, Stanley BA. Rat colon ornithine and arginine metabolism: coordinated effects after proliferative stimuli. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G389-99.

Nederlandse Samenvatting

Voedselbereidingswijzen en de voedselsamenstelling zijn belangrijke exogene factoren die het risico op kanker in positieve of negatieve zin kunnen beïnvloeden. De meest consistente bevinding betreffende de voeding als determinant van kankerrisico is de associatie tussen consumptie van groenten en fruit en een verlaagd risico op verscheidene soorten kanker. Overtuigend epidemiologisch bewijs is er voor preventie van kanker van het maagdarm stelsel (mondholte, keel, slokdarm, maag, dikke darm) en kanker van de luchtwegen (long). Wereldwijd is longkanker de meest voorkomende vorm van kanker, gevolgd door borstkanker en op de derde plaats door dikke darmkanker. De consumptie van een aanzienlijke hoeveelheid gevarieerde groenten per dag (>400 g) kan de incidentie van longkanker en dikke darmkanker verlagen met 45, respectievelijk 40 procent (range 30-50). De manieren waarop groenten hun werking uitoefenen in het lichaam zijn gevarieerd. Uit de resultaten van experimentele studies blijkt dat groenten in staat zijn om specifieke biotransformatie enzymen in de cel te induceren dan wel te remmen, de cel te beschermen tegen oxidatieve stoffen en vrije radicalen, de inductie van geprogrammeerde celdood (apoptose) te stimuleren, enzovoorts. Deze processen gaan vaak gepaard met veranderingen in de expressie van genen. Echter, tot nu toe heeft het onderzoek hiernaar zich voornamelijk gericht op het meten van veranderingen in de expressie van biotransformatie genen in surrogaat weefsel zoals lymfocyten. Er zijn maar weinig studies gedaan waarin het effect van groenten of van bestanddelen van groenten op genexpressie veranderingen in doelorganen is onderzocht. Een nieuwe technologie die nu gebruikt kan worden om het effect te bepalen van een specifiek dieet op de expressie van talrijke genen of eiwitten in een enkel experiment is de microarray techniek. Deze methode kan helpen bij het identificeren van de mechanismen op het niveau van de genen die door groenten beïnvloed worden waardoor de in vorige studies geobserveerde verlaging van het kankerrisico mechanistisch onderbouwd kan worden.

De hypothese die in dit proefschrift is onderzocht is dat een belangrijke bijdrage van de anticarcinogene effecten van groenten in de dikke darm en in de long wordt gevormd door het moduleren van de expressie van genen die betrokken zijn bij biologische en genetische processen relevant voor (chemische) carcinogenese. Het doel van dit proefschrift is om meer inzicht te verschaffen in de moleculaire mechanismen waardoor groenten hun anticarcinogene werking uitoefenen, door onderzoek te verrichten op het niveau van het genoom in de relevante doelcellen.

De opeenvolgende tumorstadia in de dikke darm worden beschreven in het Vogelstein model. Dit model beschrijft de ontwikkeling van dikke darmkanker door middel van een aantal opeenvolgende genetische veranderingen waarbij een aantal genen

zijn betrokken zoals APC (adenopolyposis coli), K-RAS, DCC and p53. Gedurende de afgelopen tien jaar zijn er aanvullende genetische veranderingen en specifieke moleculaire processen geïdentificeerd. Het is duidelijk geworden dat de belangrijkste genen van het Vogelstein model, intact dan wel gemuteerd, interacteren en een netwerk vormen en daarbij andere genen beïnvloeden. Deze genetische processen en de betrokken genen zijn mogelijke moleculaire doelwitten voor de bescherming tegen het ontstaan van dikke darmkanker door groenten.

Het onderzoek beschreven in **Hoofdstuk 2** is uitgevoerd om de genen te identificeren die *in vivo* gemoduleerd worden in humaan dikke darm weefsel door de inname van groenten te veranderen. In deze humane voedingsinterventie studie is het effect onderzocht van een halvering (= 75 g/dag) of verdubbeling (= 300 g/dag) van de mediane dagelijkse inname van groenten. Hiertoe consumeerden twintig vrouwelijke sporadische poliep patiënten en acht vrouwelijke gezonde vrijwilligers gedurende twee weken een mix van vier groenten (bloemkool, wortelen, erwten en uien) in plaats van de groenten die zij normaal gesproken zouden eten. Voor en na deze twee weken zijn stukjes darmweefsel geïsoleerd en onderzocht op genexpressie veranderingen met behulp van microarray analyse van 597 genen die betrokken zijn bij processen relevant voor carcinogenese. De twee onderzoeksgroepen zijn gekozen om te bekijken of het effect van de interventie verschillend is voor personen die een hoger risico lopen om dikke darmkanker te ontwikkelen ten opzichte van gezonde personen. Uit het onderzoek blijkt dat 52 verschillende genen gemoduleerd werden door de groenten, en door middel van literatuur onderzoek zijn 20 van deze 52 genen geïdentificeerd waarvan aannemelijk is dat ze een rol spelen bij het proces van (dikke darm) kanker. Zeven van deze 20 genen werden in zowel patiënten als gezonde proefpersonen gemoduleerd, altijd in dezelfde richting, en dertien genen werden alleen gemoduleerd in patiënten of in gezonde proefpersonen. Een verhoogde inname van groenten resulteerde in een afname van de expressie van genen die celproliferatie stimuleren en pro-carcinogenen activeren, en in een verhoging van de expressie van genen betrokken bij het stoppen van celgroei. Daarentegen, een verlaagde inname van groenten resulteerde in een afname van de expressie van genen betrokken bij het remmen van de celgroei en in een toename van de expressie van genen die cellulaire dedifferentiatie bevorderen en pro-carcinogenen activeren. Deze resultaten laten zien dat bijna alle genexpressie veranderingen mechanistisch verbonden zijn met cellulaire processen die een afname van het risico op dikke darmkanker verklaren door een hoge groenten inname en een toename van het risico op dikke darmkanker verklaren door een lage groenten inname. Verder lijkt het dat groenten in patiënten genen beïnvloeden die betrokken zijn in de latere stadia van het ontstaan van dikke darmkanker, terwijl in gezonde proefpersonen genen worden gemoduleerd die een rol spelen in de initiatie fase. Deze humane studie is de eerste studie waarin het effect van consumptie van groenten is onderzocht op genexpressie veranderingen in het doelorgaan, namelijk de dikke darm.

Om de bevindingen van de humane studie te bevestigen en mechanismen verder op te helderen zijn muizenstudies uitgevoerd waarbij eenzelfde aanpak en techniek is gebruikt. Hoewel muizen vaak gebruikt worden om het effect van een dieet op het ontwikkelingsproces van kanker te bestuderen, is het aantal studies waarin het effect op genexpressie is bestudeerd beperkt. Deze zijn meestal uitgevoerd in combinatie met toediening van een carcinogeen en beperkt tot het analyseren van een klein aantal genen. De muizenstudies die beschreven zijn in dit proefschrift geven daarom veel informatie over het effect van een specifiek groentedieet op de expressie van biologisch relevante genen in doelweefsel. Om dit te bewerkstelligen is een muizen cDNA microarray gemaakt die dezelfde genen bevat als de cDNA microarray voor de humane studie.

De muizenstudie beschreven in **Hoofdstuk 3** is een nabootsing van de humane studie. In deze muizenstudie is de afhankelijkheid van de dosis van een mix van groenten op genexpressie veranderingen in de dikke darm van de muis bestudeerd. Hiervoor werden C57Bl.6 vrouwelijke muizen verdeeld in vier groepen (7 muizen per groep) die elk gedurende twee weken een ander dieet kregen, namelijk een dieet zonder groenten, met 10%, 20% of met 40% van de groentenmix. De groentenmix bestond uit dezelfde combinatie en verhouding als de groentenmix gebruikt in de humane studie, namelijk bloemkool, wortelen, erwten en uien (30%, 30%, 30% en 10% natgewicht, respectievelijk). De resultaten van deze studie laten een dosis afhankelijkheid zien van het effect van groenten op genexpressie, echter, deze relatie is over het algemeen niet lineair. Bijna alle genexpressie veranderingen vonden plaats in de hoogste dosis (40%) groep. In totaal werden er 39 genen gemoduleerd, waarvan er 17 een rol kunnen spelen in de ontwikkeling van dikke darmkanker, zoals beschreven in de literatuur. Het effect op 15 van deze 17 genen is ten gunste van een verlaging van het kankerrisico. Een dieet met een hoog gehalte aan groenten moduleert op een positieve manier de expressie van genen die betrokken zijn bij verschillende processen, zoals remming van de vorming van carcinogenen, toename van DNA herstel capaciteit, inductie van apoptose, en in een afname van celgroei en tumor invasie. Verschillende nieuwe genen zijn geïdentificeerd waarvoor tot nu toe nog geen modulatie door groenten werd beschreven. Slechts één gen wordt op gelijke wijze gemoduleerd in zowel de humane groenten studie als in de muizen groenten studie, namelijk stearoyl-CoA desaturase (SCD). Echter, beide studies laten zien dat een dieet met een hoge inname van groenten genen moduleert in het voordeel van een afname van het dikke darmkanker risico via een aantal dezelfde processen, namelijk remming van celproliferatie en detoxificatie van pro-carcinogenen.

Vervolgens is met behulp van de proteomics benadering het effect van verschillende doses van de groentenmix op eiwit veranderingen in de dikke darm van dezelfde muizen onderzocht. (**Hoofdstuk 4**) Door middel van 2D gel elektroforese is de expressie van eiwitten bepaald. Dertig eiwitten kwamen verschillend tot expressie tussen de verschillende dieetgroepen, en zes daarvan konden geïdentificeerd worden met massa

spectrometrie (MLRN, CAH-1, HMG-1, PAP3, GAPDH en OSCP). Met uitzondering van OSCP zijn de expressieveranderingen van deze eiwitten in overeenstemming met een lager risico op het ontstaan van dikke darmkanker bij een hogere groenten inname. De eiwitten zijn betrokken bij verschillende processen zoals celgroei, celdifferentiatie en apoptose. Alleen GAPDH is geïdentificeerd in zowel de eiwitexpressie studie in muizen als de genexpressie studie in muizen, echter de waargenomen modulatie-richting is tegengesteld. Geen overeenkomsten werden gevonden tussen de eiwitexpressie studie in muizen en de genexpressie studie in proefpersonen.

Naast de hoeveelheid groenten is ook onderzocht of het soort groenten van belang is voor het induceren van genexpressie veranderingen. De studie beschreven in **Hoofdstuk 5** is uitgevoerd om het effect van vier individuele groenten, namelijk bloemkool, wortelen, erwten en uien, op de expressie van genen in het dikke darmweefsel van vrouwelijke C57BL/6 muizen te onderzoeken. In deze studie werden in totaal 18 genen gemoduleerd door één of meerdere groenten. Bloemkool moduleerde het grootste aantal genen (10), gevolgd door de uien (7) en de wortelen (7), erwten induceerden de minste genexpressie veranderingen (3). Met behulp van literatuur gegevens kan onderbouwd worden dat tien van de 18 genen waarschijnlijk een rol spelen in de ontwikkeling van dikke darmkanker. Zeven van deze tien genen worden op een dussdanige manier gemoduleerd dat een verlaging van het kankerrisico te verwachten is. De potentie van de individuele groenten om genen te moduleren is in overeenstemming met een afname van het kankerrisico lijkt gelijk, hoewel de betrokken processen meestal verschillend zijn. Genen betrokken bij de regulatie van celgroei en het handhaven van homeostase werden door alle vier de groenten beïnvloed. Bloemkool, wortelen en erwten moduleerden genen betrokken bij apoptose; genen gemoduleerd door uien spelen een rol bij het behouden van DNA stabiliteit en inductie van celdifferentiatie; en erwten, naast het verhogen van de expressie van een apoptose gen, induceerden de expressie van een angiogenese remmend gen. Hoewel bloemkool de meeste genen moduleerde, is van het merendeel van deze genen op dit moment niet bekend of ze betrokken zijn bij kankerbeschermende processen.

De resultaten van de studie met individuele groenten in muizen kunnen vergeleken worden met de humane interventie studie (**Hoofdstuk 2**) en de muizenstudie met groentenmix (**Hoofdstuk 3**). De hoeveelheid van de groenten in de groep die de hoogste dosis (40%) van de groentenmix kreeg is gelijk aan de som van de individuele groenten. Hierdoor kan de bijdrage van de vier individuele groenten aan het gecombineerde effect van deze groentenmix bestudeerd worden. Elf genen werden in beide studies gemoduleerd en voor zeven hiervan kan de modulatie in de studie met groentenmix verklaard worden door het effect van een specifieke groente. Hoewel van de meeste van deze genen op dit moment niet bekend is of ze een rol spelen in preventieve processen tegen darmkanker, geeft de modulatie van deze genen door groenten in beide studies het belang van deze genen aan. Hun mogelijke rol in de ontwikkeling van dikke darmkanker moet echter verder

onderzocht worden. Twee genen werden gemoduleerd in zowel de humane interventie studie (**Hoofdstuk 2**) als in de studie met individuele groenten in muizen, namelijk ODC en SCD. De expressie van ODC was verlaagd in de groep die de hoogste dosering aan groenten ontving, in zowel patiënten als controles, en in de muizen die het bloemkool dieet of wortelen dieet kregen.

Naast preventie van dikke darmkanker is er vanuit epidemiologische studies bewijs voor een bescherming tegen longkanker door groenten en fruit. In het bijzonder zijn de donkergroene en de oranje-rode groenten, rijk aan β -caroteen en vitamine E, de groenten waarvoor het bewijs het meest overtuigend is. In tegenstelling tot de darm, zijn de longen geen orgaan waarmee de groenten na inname in direct contact komen. Daarom zijn de longen alleen onderhevig aan systemische blootstelling van groenten bestanddelen. Bovendien kunnen de groenten componenten die de longen bereiken vooraf gemetaboliseerd worden in de lever. De mechanismen waardoor groenten beschermen tegen de vorming van longkanker kunnen daarom anders zijn vergeleken met de dikke darm. Om het effect van groenten op genexpressie veranderingen in de long te onderzoeken, zijn de longen van de muizen uit de studies beschreven in de **Hoofdstukken 3 en 5** geanalyseerd (**Hoofdstuk 6**). In de longen van de muizen uit de studie met groentemix werden 18 genen gemoduleerd. Net als in de studie waarin het effect in de dikke darm is onderzocht (**Hoofdstuk 3**), traden de meeste genexpressie verschillen in de longen op tussen de groep muizen die de hoogste dosering groenten ontvingen en één van de andere groepen. Echter, slechts zeven van de 18 genen zijn waarschijnlijk betrokken bij processen die beschermen tegen longkanker (literatuuronderzoek), waarbij vervolgens de richting van modulatie niet altijd in het voordeel is van preventie van longkankerrisico. In de longen van de muizen in de studie met individuele groenten werden in totaal 11 genen gemoduleerd, waarvan er acht, zoals gevonden in literatuuronderzoek, een rol kunnen spelen in mechanismen die beschermen tegen longkanker. De meeste (6) genmodulaties vonden plaats in de groep die wortelen consumeerde. In de groep die erwten kreeg toegediend traden drie genmodulaties op en in de groep die bloemkool at twee genmodulaties. Uien waren in staat om slechts één gen te moduleren, namelijk CTSS. Echter, het verwachte effect op het risico op longkanker door de modulatie van dit gen door de groenten is verhogend, door onderdrukking van het immuunsysteem. Ook voor de andere genen die gemoduleerd worden door de individuele groenten is het te verwachten effect op processen die beschermen tegen longkanker niet altijd positief, zoals beschreven in de literatuur. De resultaten van dit onderzoek laten zien dat groenten in staat zijn genen te moduleren die betrokken zijn bij processen die beschermen tegen longkanker, maar dat de modulatie niet altijd in het voordeel lijkt van een afname van het longkanker risico. Interessant is echter dat de wortelen de meeste genexpressie veranderingen induceren. Het is vanuit epidemiologisch onderzoek bekend dat een hoge inname van wortelen geassocieerd is met een afname van het longkanker risico. De resultaten van de

studie met individuele groenten in muizen bevestigen dat de consumptie van wortelen van belang zijn bij het moduleren van het longkanker proces. Bij het vergelijken van de genmodulaties in de long studie met die van de dikke darm studies (**Hoofdstuk 3, 4 en 5**) zijn er acht genen die in beide studies gemoduleerd worden, namelijk EFHU1, G1P2, GAPDH, SULT1A1, SEPP1, HPGD, TOP2A en BNIP1. Echter, de modulatie was tegengesteld (behalve voor GAPDH). Naast overeenkomstige genen werden ook verschillende genen gemoduleerd in beide organen. Het aantal genen gemoduleerd in de long is kleiner en ook zijn deze genen niet in duidelijke mate betrokken bij kankerbeschermende mechanismen. Opvallend is wel dat in de long de wortelen de grootste potentie hebben om genen te moduleren, terwijl in de dikke darm de individuele groenten eenzelfde potentie hebben om genen te moduleren in het voordeel van dikke darmkanker preventie.

Over het geheel genomen verschaffen de onderzoeken beschreven in dit proefschrift veel informatie over het effect van groenten op de veranderingen in genexpressie in doelorganen waar het kankerrisico wordt verlaagd door een verhoogde inname van groenten. In het bijzonder ondersteunen de studies betreffende de genexpressie veranderingen in de dikke darm de hypothese die in dit proefschrift is onderzocht. In de dikke darm moduleren de groenten veel genen die betrokken zijn bij biologische en genetische processen leidend tot preventie van dikke darmkankerrisico. Genen waarvoor al een relatie met groenten en/of kanker bekend is werden gemoduleerd, maar ook 'nieuwe' genen werden beïnvloed waarvoor deze relatie nog niet bekend is. Opvallend is dat de dosis groenten die nodig is om genen te moduleren in het voordeel van een afname van het kankerrisico relatief hoog is in muizen. Dit ondersteunt de aanbeveling dat mensen veel groenten moeten consumeren om het risico op kanker te verlagen. Het bewijs voor modulatie van genen in beschermende processen tegen longkanker zijn minder duidelijk vergeleken bij de resultaten uit de dikke darm studies. Bijzonder interessant en veelbelovend is echter de rol van de wortelen in het moduleren van genen in het voordeel van een afname van het longkanker risico. De resultaten van de longstudie voorzien echter in te weinig informatie om de hypothese te behouden of te verwerpen. Meer onderzoek is nodig waarin de bijdrage van specifieke genen en processen door verschillende groenten wordt onderzocht.

Verscheidene genen zijn interessant voor vervolgonderzoek. In het bijzonder SCD, omdat de modulatie van dit gen in drie onafhankelijke studies in zowel de mens als de muis is waargenomen. Daarnaast is de modulatie van COX-2 door de groenten in de humane studie zeer belangrijk, vanwege de rol van COX-2 in dikke darmkanker. Behalve specifieke genen, zijn ook specifieke processen van bijzondere interesse. In zowel de humane als de muizenstudies zijn genen gemoduleerd die een rol spelen bij remming van de celgroei. Betrokken processen die hierbij de overhand hebben zijn het polyamine

biosynthese proces en apoptose. Door gebruik te maken van genetisch gemodificeerde muizen kan het effect van groenten en de bijdrage van een specifiek gen op verschillende processen bestudeerd worden. Daarnaast kunnen relatief nieuwe technieken zoals RNA interference en laser capture microdissectie een bijdrage leveren aan het optimaliseren van genexpressie onderzoek. De resultaten verkregen in de studies in dit proefschrift geven nieuwe aanknopingspunten voor vervolgonderzoek en kunnen leiden tot de ontwikkeling van functionele voeding en/of voedingssupplementen die beter beschermen tegen het ontstaan van kanker.

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Liefs Simone

Curriculum Vitae

Simone Gerty Jeannette van Breda werd geboren op 2 januari 1977 te Hulsberg. Na het behalen van het Atheneum bèta diploma aan het Sintermeerten College te Heerlen in juni 1995, heeft ze enkele maanden geneeskunde gestudeerd aan de Katholieke Universiteit Leuven in België. In december 1995 begon zij haar studie Gezondheidswetenschappen met als afstudeerrichting Milieugezondheidkunde aan de Universiteit Maastricht. Tijdens deze studie werkte zij als student-onderzoeker bij de capaciteitsgroep Gezondheidsrisico Analyse en Toxicologie (GRAT). De studie werd afgesloten met twee stage-onderzoeken, waarvan de eerste is uitgevoerd bij TNO voeding te Zeist, divisie Toxicologie. Vervolgens werd een tweede stage uitgevoerd bij de capaciteitsgroep GRAT aan de Universiteit Maastricht. In maart 2000 behaalde ze haar doctoraal diploma. Vanaf april 2000 was Simone werkzaam als assistent in opleiding bij de capaciteitsgroep GRAT aan de Universiteit Maastricht waar het onderzoek dat beschreven is in dit proefschrift werd uitgevoerd. Naast het behalen van de certificaten 'Stralingshygiëne, deskundigheidsniveau 5b' en 'Proefdierkunde op grond van art.9 van de Wet op de Dierproeven' heeft zij verscheidene modules van de Postdoctorale Opleiding Toxicologie gevolgd om voor erkenning als toxicoloog in aanmerking te komen. Vanaf januari 2005 zal Simone werkzaam zijn als post-doc bij de capaciteitsgroep GRAT aan de Universiteit Maastricht.

List of Publications

Articles

- 1 Van Maanen, J.M., Albering, H.J., Van Breda, S.G., Curfs, D.M., Ambergen, A.W., Wolffenbuttel, B.H., Kleinjans, J.C. and Reeser, H.M. (1999) Nitrate in drinking water and risk of childhood diabetes in The Netherlands. *Diabetes Care*, 22, 1750.
- 2 Van Maanen, J.M., Albering, H.J., de Kok, T.M., Van Breda, S.G., Curfs, D.M., Vermeer, I.T., Ambergen, A.W., Wolffenbuttel, B.H., Kleinjans, J.C. and Reeser, H.M. (2000) Does the risk of childhood diabetes mellitus require revision of the guideline values for nitrate in drinking water? *Environ Health Perspect*, 108, 457-61.
- 3 Nia, A., Maas, I., Van Breda, S., Curfs, D., Kleinjans, J., Wouters, E. and Van Schooten, F.J. (2000) Applicability of induced sputum for molecular dosimetry of exposure to inhalatory carcinogens: 32P-postlabeling of lipophilic DNA adducts in smokers and nonsmokers. *Cancer Epidemiol Biomarkers Prev*, 9, 367-72.
- 4 Van Maanen, J.M., Albering, H.J., de Kok, T.M., Van Breda, S.G., Curfs, D.M., Vermeer, I.T., Ambergen, A.W., Wolffenbuttel, B.H., Kleinjans, J.C. and Reeser, H.M. (2000) Nitraat in drinkwater en risico voor type I diabetes in kinderen in Nederland. *Nederlands Tijdschrift voor Geneeskunde*, 144, 1272-1276.
- 5 Van Delft, J.H., Van Agen, E., Van Breda, S.G., Herwijnen, M.H., Staal, Y.C. and Kleinjans, J.C. (2004) Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling. *Carcinogenesis*, 25, 1265-1276.
- 6 Van Breda, S.G., Van Agen, E., Van Sanden, S., Burzykowski, T., Kienhuis, A.S., Kleinjans, J.C. and Van Delft, J.H. (2004) Dose dependent effect of vegetables on gene expression in colon mucosa of C57BL6 female mice. *Submitted*.
- 7 Van Breda, S.G., Van Agen, E., Van Sanden, S., Burzykowski, T., Kleinjans, J.C. and Van Delft, J.H. (2004) Gene expression modulation by specific vegetables in colon mucosa of C57BL6 female mice. *Submitted*.
- 8 Van Breda, S.G., Van Agen, E., Van Sanden, S., Burzykowski, T., Kleinjans, J.C. and Van Delft, J.H. (2004) Gene expression modulation in lung cancer preventive pathways by vegetables in C57BL6 female mice. *Submitted*.
- 9 Van Breda, S.G., Van Agen, E., Engels, I., Moonen, E.J., Kleinjans, J.C. and Van Delft, J. (2004) Altered vegetable intake affects pivotal carcinogenesis pathways in colon mucosa from adenoma patients and controls. *In press, Carcinogenesis*.
- 10 Breikers, G., Van Breda, S.G., Bouwman, F.G., Van Herwijnen, M.H., Renes, J.W., Mariman, E.C., Kleinjans, J.C. and Van Delft, J.H. (2004) Identification of

proteins differentially expressed in colon mucosa of C57BL6 female mice by altered vegetable intake. *In preparation*.

- 11 Van Delft, J.H., Van Agen, E., Van Breda, S.G., Herwijnen, M.H., Staal, Y.C. and Kleinjans, J.C. (2004) Comparison of supervised clustering methods to discriminate genotoxic from non-genotoxic carcinogens by gene expression profiling. *Submitted*.

Abstracts

- 1 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Identification of mechanisms at the genome level for protection against colon cancer by vegetables. Proceedings of the 31st Annual Meeting of the European Environmental Mutagen Society (EEMS), Ghent, Belgium, September 2001. **Poster Award**.
- 2 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Identification of mechanisms at the genome level for protection against colon cancer by vegetables. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, Nijmegen, The Netherlands, November 2001.
- 3 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Modulation of gene expression by vegetables: identification of mechanisms for protection against colon cancer. Proceedings of the 1st Seminar of the European Graduate School, Maastricht, The Netherlands, November 2002. **Oral presentation**.
- 4 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Modulation of gene expression by vegetables: identification of mechanisms for protection against colon cancer. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, De Bilt, The Netherlands, January 2003. **Oral presentation**.
- 5 Van Breda, S.G., Van Agen, E., Kienhuis, A.S., Kleinjans, J.C. and Van Delft, J.H. Dose-response study of vegetables on gene expression in colon mucosa of C57BL6 female mice. 34th Annual Meeting of the Environmental Mutagen Society (EMS), Miami Beach, USA, May 2003. *Environmental and Molecular Mutagenesis*, 2003, 41: 210. **EMS Student Achievement Award**.
- 6 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Identification of mechanisms at the genome level for protection against colon cancer by vegetables. Proceedings of the 8th International conference

- on Mechanisms of Antimutagenesis and anticarcinogenesis (ICMAA-VIII) Pisa, Italy, October 2003. **Oral presentation.**
- 7 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Identification of mechanisms at the genome level for protection against colon cancer by vegetables. Proceedings of the 2nd Seminar of the European Graduate School, Maastricht, The Netherlands, October 2003. **Oral presentation.**
- 8 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Modulation of gene expression by vegetables in normal colorectal mucosa of sporadic colon adenoma patients and healthy controls. Proceedings of the Annual Meeting of the Netherlands Society of Gastroenterology (NVGE), Veldhoven, The Netherlands, March 2004. **Oral presentation.**
- 9 Van Delft, J.H., Van Agen, E., Van Breda, S.G., Van Herwijnen, M.H., Van Leeuwen, D.M., Staal, Y.C. and Kleinjans, J.C. Toxicogenomics approaches in genetic toxicology. Proceedings of the scientific meeting of the Netherlands Society of Toxicology, Veldhoven, The Netherlands, June 2004.
- 10 Van Delft, J.H., Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., and Kleinjans, J.C. Identification of mechanisms at the genome level for protection against colon cancer by vegetables. NUGO, Wageningen, The Netherlands, September 2004.
- 11 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Altered vegetable intake affects pivotal carcinogenesis pathways in colon mucosa from adenoma patients and controls. Proceedings of the 34th Annual Meeting of the European Environmental Mutagen Society (EEMS), Maastricht, The Netherlands, September 2004. **Oral presentation.**
- 12 Breikers, G., Van Breda, S.G., Bouwman, F.G., Van Herwijnen, M.H., Renes, J.W., Mariman, E.C., Kleinjans, J.C. and Van Delft, J.H. Identification of proteins differentially expressed in colon mucosa of C57BL6 female mice by altered vegetable intake. Proceedings of the 34th Annual Meeting of the European Environmental Mutagen Society (EEMS), Maastricht, The Netherlands, September 2004.
- 13 Van Breda, S.G., Van Agen, E., Engels, L.G., Moonen, E.J., Kleinjans, J.C. and Van Delft, J.H. Alteration in vegetable intake affects pivotal genetic pathways in colon carcinogenesis in sporadic colon adenoma patients and healthy controls. 12th United European Gastroenterology Week (UEGW). Prague, Czech Republic, September 2004. **Young Investigators Travel Award; Oral presentation.**

Abbreviations

2-DE	two-dimensional polyacrylamide gel electrophoresis
AC#	genbank accession numbers
ACF	aberrant crypt foci
ACTB	beta actin, cytoplasmic
AFP	alpha-fetoprotein
AICR	american institute for cancer research
ALDH1A1	aldehyde dehydrogenase family 1, subfamily A1
AMACR	methyl-CoA racemase alpha
APC	adenomatous polyposis coli
ATF3	activating transcription factor 3
ATP	adenosine triphosphate
BCL2	breakdown cluster lymphoma
BNIP1	BCL2/adenovirus E1B 19kDa-interacting protein 1
CAH-1	carbonic anhydrase I
CASP3	caspase 3, apoptosis related cysteine protease
CASP4	caspase 4, apoptosis-related cysteine protease
CASP7	caspase 7
CCNA2	cyclin A2
CCNG1	cyclin G1
CDH13	cadherin 13 H-cadherin
CDK	cyclin-dependent kinase
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)
cDNA	copy deoxyribonucleic acid
C-FOS	fos proto-oncogene
CHK1	checkpoint kinase-1
C-MYB	c-myb proto-oncogene
COX-2	cyclooxygenase-2
COXIV	cytochrome c oxidase subunit IV isoform 1
CRC	colorectal cancer
CTNNB1	catenin gene
CTSB	cathepsin B
CTSS	cathepsin S
Cy3	Cyanine 3
Cy5	Cyanine 5
CYP27B1	cytochrome P450 family 27, subfamily B, polypeptide 1.
CYP2C19	cytochrome P450 family 2, subfamily C, polypeptide 19

CYP2C9	cytochrome P450 family 2, subfamily C, polypeptide 9
CYP2D6	cytochrome P450 family 2, subfamily D, polypeptide 6
CYP3A4	cytochrome P450 family 3, subfamily A, polypeptide 4
DCC	deleted in colorectal cancer
DNA	deoxyribonucleic acid
DNES	diffuse neuroendocrine system
DRA	down-regulated in adenoma
DTT	dithiothreitol
e.g.	<i>exempli gratia</i> (for example)
EASE	expression analysis systematic explorer
EFHU1	translation elongation factor EF-1 alpha-1 chain
ERBB	epidermal growth factor receptor
EST	expressed sequence tag
FAP	familial adenomatous polyposis
FCER1G	Fc receptor, IgE, high affinity 1, gamma polypeptide
FHIT	fragile histidine triad gene
G1P2	interferon, alpha-inducible protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUL	glutamate-ammonia ligase (glutamine synthase)
GRP	gastrin-releasing peptide
GSR	glutathione reductase 1
GSTA2	glutathione S-transferase, alpha 2 (Yc2)
HCA	heterocyclic aromatic amines
HGF	hepatocyte growth factor;
HGF/MET	hypocyte growth factor/metastasis proto-oncogene
HIF1A	hypoxia inducible factor 1, alpha subunit
HMG-1	high-mobility group protein 1
hMLH	human mutL homolog
HNPCC	hereditary nonpolyposis colorectal cancer
HPGD	hydroxyprostaglandin dehydrogenase 15 NAD
HPLC-UV	high performance liquid chromatography-ultraviolet
hPMS	human postmeiotic segregation
HSPD1	heat shock 60kDa protein 1 (chaperonin)
i.e.	<i>id est</i> (that is)
IGF-1	insulin-like growth factor 1
IGFBP3	insulin-like growth factor binding protein 3
IL18	interleukin 18
kDa	kilodalton
LDLR	low density lipoprotein receptor

LOH	loss of heterozygosity
MALDI-MS	matrix-assisted laser desorption ionization mass spectroscopy
MDM2	human mdm2-A
MLRN	smooth muscle isoform of myosin regulatory light chain 2
MMR	mismatch repair
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
MTHFR	5,10-methylenetetrahydrofolate reductase
MYBBP1a	MYB binding protein (P160) 1a
NCBI	national center for biotechnology information
NDF	new differentiation factor
NIA	national institute on aging
NSAIDs	non-steroidal anti-inflammatory drugs
NSCLC	non-small cell lung cancers
OAT	ornithine aminotransferase
ODC1	ornithine decarboxylase 1
OSCP	mitochondrial ATP synthase oligomycin sensitivity conferral precursor protein
PAP3	pancreatitis-associated precursor protein 3
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen gene
PKCB1	protein kinase C beta 1
PMP22	peripheral myelin protein
PPAR	peroxisomal proliferators-activated receptor
PRDX1	peroxiredoxin 1
PTEN	phosphatase and tensin homolog
PTGS2	prostaglandin-endoperoxide synthase 2
RAR	retinoic acid receptor
RASSF1A	tumor suppressor RASSF1 isoform A
RB	retinoblastoma
Reg	regenerating gene
RNA	ribonucleic acid
RRM1	ribonucleotide reductase M1 polypeptide
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RZPD	resource centre/primary database
SAGE	serial analysis of gene expression
SAT	spermidine/spermine N1-acetyl transferase 1
SCD2	stearoyl-Coenzyme A desaturase 2

SCF/KIT	stem cell factor/tyrosine kinase receptor-oncogene KIT 0
SCLC	small cell lung cancers
SDS	sodium dodecylsulfate
SE	standard error
SEM	standard error of the mean
SEPP1	selenoprotein P, plasma 1
SLC26A3	solute carrier family 26, member 3
SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
STAT1	signal transducer and activator of transcription 1
SULT1A1	sulfotransferase family 1A, phenol-preferring, member 1
TCF/Lef	T-cell factor/lymphoid enhancing factor
TGF-	transforming growth factor beta
TGFBR2	transforming growth factor beta receptor type II tumor suppressor gene
TGM2	transglutaminase 2, C polypeptide
TMSB10	thymosin, beta 10
TNFRSF6	tumor necrosis factor receptor superfamily, member 6
TOP2A	topoisomerase (DNA) II alpha 170 kDa
WCRF	world cancer research fund
wt	weight

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