Biomarkers of quercetin-mediated modulation of colon carcinogenesis

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Abstract

Colorectal cancer (CRC) is hypothesized to be prevented by intake of fruits and vegetables that contain anti-carcinogenic compounds, including the flavonoid quercetin that is found in apples and onions. In this thesis, quercetin's mechanisms of cancer-preventive action were studied both *in vitro* and *in vivo*. The *in vitro* experiments were performed using the human Caco-2 cell line as a model for CRC, and quercetin stabilized by ascorbate in the culture medium. Unexpectedly, ascorbate-stabilized quercetin showed enhancement of cellular processes involved in CRC-development, including stimulated cell proliferation, reduced cell differentiation and enhancement of pathways that stimulate cell survival. Furthermore, transcriptomics showed that quercetin downregulated expression of genes involved in tumor suppression and phase II metabolism, and upregulated oncogenes. Comparison with Caco-2 cells exposed to quercetin in the absence of ascorbate showed the opposite, *i.e.* anti-carcinogenic effects by this flavonoid. This led to the hypothesis that quercetin-induced reactive oxygen species that eradicate tumor cells were scavenged by vitamin C, causing tumor cell survival. Without ascorbate, these reactive oxygen species may be responsible for anti-carcinogenic effects, pointing to beneficial effects of supposed adverse reactive intermediates.

Subsequently, the CRC-modulating potency of quercetin and its conjugate rutin were investigated in a rat model for CRC. Quercetin, but not its conjugate rutin decreased the tumor incidence, which was associated with the blood plasma levels of this anti-oxidant, but not reflected by the putative preneoplastic biomarker lesions, designated aberrant crypt foci. The combination of transcriptomics and proteomics showed that quercetin inhibited the potentially oncogenic mitogen-activated protein kinase (*Mapk*) pathway and enhanced expression of tumor suppressor genes, cell cycle inhibitors, and genes involved in xenobiotic metabolism. In addition, quercetin affected the energy production pathways, by increasing mitochondrial fatty acid degradation, and inhibiting glycolysis. This observation provided a new hypothesis pointing at another anti-carcinogenic mechanism for quercetin, based on an alteration in routes for energy metabolism, shifting them in favor of non-tumor like pathways like mitochondrial fatty acid degradation at the cost of the tumor-like glycolytic pathway for cellular energy supply.

Overall, the studies presented in the present thesis provided new hypotheses for the mode of action of quercetin as an anti-tumor agent, but it appeared that the

actual dose needed to exert this beneficial effect amounted to about 60 - 100 times the already relatively high prescribed dose for quercetin supplements. Therefore, it is concluded that health claims on the use of quercetin as an anti-cancer agent need better scientific support.

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Chapter 1

General introduction, aims and outline

Based on: "*Colon Cancer: Early Detection and Prevention by Food Compounds and Biomarkers of Preventive Effects Found by Employment of Microarrays*"
by Ashwin A. Dihal and Marjan J. van Erk. In: **Cancer Nanotechnology. Nanomaterials for Cancer Diagnosis and Theraypy**; editors: H.S. Nalwa and T. Webster, American Scientific Publishers, Los Angeles (2007), p. 31-42.
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1.1 Introduction

Colorectal cancer (CRC) is a highly frequent malignancy in the Western world and shows a risk that increases with higher age [1]. Worldwide, CRC takes the second place in cancer-related deaths with an estimated 530,000 people per year and is third when considering the yearly incidence of 1,000,000 people [2]. About 15% of patients inherit CRC in an autosomal-dominant fashion and may therefore develop familial adenomatous polyposis (FAP) or hereditary non-polyposis colon cancer (HNPCC) [3]. On the other hand, the remaining 85% of patients develop colon cancer without a genetic predisposition, *i.e.* sporadic CRC. Risk factors suggested in the aetiology of sporadic CRC include low physical activity, obesity, and dietary composition [4]. Indications that development of sporadic CRC is mainly caused by lifestyle and environmental factors - rather than genetic factors - are retrieved from immigrant and epidemiological studies as well as geographic differences in the incidence of CRC. Japanese and Mexican immigrants who have moved to the United States tend to show an increased incidence of CRC similar to that of US residents, within one to two generations [5;6]. Furthermore, the worldwide incidence of CRC varies up to twentyfold with the highest incidence in the United States and the lowest incidence in India, suggesting that environmental and dietary factors might be involved in CRC etiology [7]. Indeed, epidemiological studies do suggest that the incidence of CRC is inversely related with the intake of fruits and vegetables [8], dietary fibre [9], and flavonoids [10].

Flavonoids are plant secondary metabolites that have a common C6-C3-C6 structure with at least one hydroxyl group [11]. These polyphenolic compounds protect plants against phyotopathogens e.g. fungi and bacteria, and against UV-light. One of the major flavonoids is quercetin (**Figure 1A**) that is present in the human diet and exerts inhibitory activity in experimental colon carcinogenesis [12-18]. In nature, quercetin is glycosylated, e.g. as its conjugate rutin (**Figure 1B**) and can be found in several fruits and vegetables, including apples, onions, broccoli, tea, leeks, and blueberries [19-21].

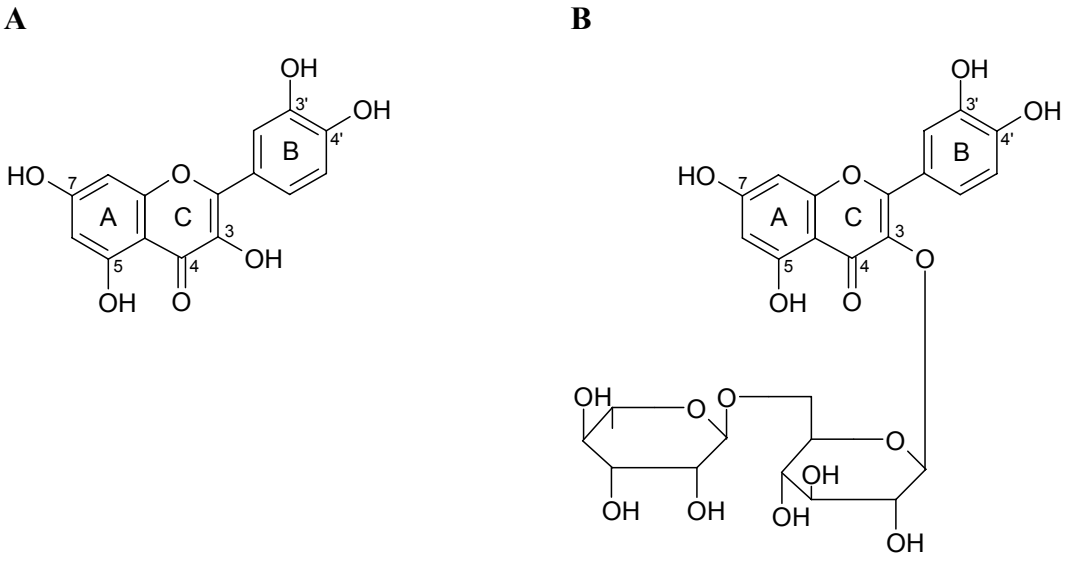

Fig. 1. Structural formula and atom numbering of quercetin (panel A) and its conjugate rutin (panel B) that contains a rhamno-glycosidic bond at the C-3 position of the C-ring.

After ingestion, rutin can be hydrolyzed to yield the aglycone quercetin by intracellular and membrane-bound ß-glycosidase in the small intestine [22]. Through the enterohepatic cycle quercetin can be metabolized in intestinal and liver cells. In the colon, quercetin glycosidic bonds can be hydrolyzed by local ß-glycosidase that is produced by enterobacteria, leading to colonic release and absorption of quercetin [23;24]. This absorption mechanism was demonstrated by means of a human supplementation study. Following ingestion of onions, peak plasma levels of quercetin were reached in less than 1 hour, whereas after supplementation with rutin capsules, peak plasma levels were reached after 9 hours [25]. These data indicate that quercetin glucosides, that are highly present in onions are absorbed from the small intestine. On the contrary, rutin is not absorbed from the small intestine, but hydrolyzed by colonic bacterial ß-glycosidases, leading to local release and absorption of quercetin. Thus, depending on the type of glycoside, quercetin can be deconjugated and absorbed either in the small or large intestine. Rutin is the main quercetin glycoside in tea [25] and shows relative low bioavailability when compared to quercetin aglycone [26-28]. In humans, quercetin aglycone shows relatively poor bioavailability [20], probably as a result of rapid metabolic conversion that leads to formation of glucuronidated, methylated, and sulphated metabolites with different biological activities [29;30]. It has been proposed that quercetin exerts anti-

carcinogenic properties both *in vitro* [31;32] and *in vivo* [12;18]. A possible mechanism involved is the scavenging of free radicals. This capacity is exerted by its phenolic hydroxyl groups, especially by the two neighboring hydroxyl groups present on the B-ring, alias the catechol moiety (**Figure 1A** and **1B**). Other proposed mechanisms of action include induction of apoptosis [18], inhibition of cell proliferation [13;31] and inhibition of protein kinase C, lipoxygenase [33], cyclooxygenase 2 [34;35], and p21-Ras [36].

Nowadays, quercetin is commercially available and claimed to be a beneficial food supplement. As its mechanisms of action in colorectal carcinogenesis have not yet been assessed in detail and no substantial literature regarding its safety in clinical trials is available, this health claim should be interpreted with care.

To identify biomarkers in quercetin-modulated experimental colorectal cancer, both *in vitro* and *in vivo* models are required. Hence, the background of these models, and of the healthy gut are discussed below.

1.2 Gut physiology

The mammalian gut consists of the small intestine (duodenum, jejunum, and ileum) and the large intestine, also known as the colon (divided into colon ascendens, colon transversum, colon descendens and sigmoid). To facilitate efficient uptake of nutrients from the lumen, the intestinal absorption area is enlarged by mucosal foldings. In the small intestine this absorption area is further increased by a combination of mucosal cavities called crypts and finger-like protrusions designated villi. The colon, on the other hand, contains larger crypts, but lacks villi and has a flat surface instead. The intestinal lumen is lined by an epithelial monolayer that functions as a barrier against microorganisms, amongst others. Nutrient absorption from the colon generally occurs less efficiently as compared to the small intestine as a result of a smaller absorptive surface area and a lower density of transporters [21].

In the large intestine, the base of the crypts contains stem cells that are able to proliferate continuously and are the source of epithelial cells [37]. After proliferation in the stem cell niche, the daughter cells migrate upwards and can differentiate into absorptive cells that encompass all enterocytes, secretory cells called Goblet cells that secrete protective mucins, and enteroendocrine cells that secrete hormones, including serotonin [3]. These different cell types finally reach the top of the villus, go into

apoptosis, and are shed in the lumen of the gut within 4 - 8 days [38]. The continuous process of proliferation, differentiation, and apoptosis that occurs along the crypt axis is strictly regulated, predominantly by *Wnt* cascade signaling [39], but can be disrupted as a result of successive genetic mutations [40]. These "genetic hits" occur in both tumor suppressor genes as well as oncogenes and finally give rise to colon carcinomas through the adenoma-carcinoma sequence. Main targets in this sequence of genetic mutations are the oncogene *K-ras* and the tumor suppressor genes Adenomatous polyposis coli (*Apc*), *Smad 4* and *Tp53* [41].

1.3 Experimental models for colorectal cancer

1.3.1 In vitro

Colon cancer cell lines are frequently used as a model to investigate effects of nutritional or other compounds on colon cancer. There are many advantages in using cell lines, including availability, growth rate, homogeneous cell population and reproducibility. Another important advantage is a reduction in the use of laboratory animals. However, cell lines are certainly not fully representative of the tumors they were derived from, since cells are adapted to culture conditions and fast-growing cells dominate the composition of the cell culture [42]. Many colon cancer cell lines are available to study the effects of food compounds on mechanisms involved in development of colon cancer. There are differences between these cell lines and obviously the choice of cell system may influence the results.

The human colon cancer cell lines Caco-2 and HT-29 are among the most frequently used colon cancer cell lines. Hence, usage of these cell lines enables one to easily compare obtained results to those from other studies. HT-29 and Caco-2 both contain a mutation in the *Apc* [43] and p53 [44] genes. In addition, Caco-2 cells harbor a mutation in the ß-catenin gene [45] that is frequently mutated in human colorectal tumors [46;47].

For cell differentiation experiments described in this thesis, the Caco-2 cell line was used because of its ability to differentiate spontaneously [48], in contrast to HT-29 cells that only differentiate upon exposure to inducers, including sodium butyrate [49], glucose deprivation [50] or glucose substitution for galactose in the culture medium [51]. Upon reaching confluency, Caco-2 cells differentiate into absorptive

cells, which is a time-dependent process that mimicks phenotypic changes that normal colonic epithelial cells undergo during migration along the crypt-villus axis *in vivo* [48]. Once differentiated, Caco-2 cells express the so-called brush border that is formed by a high density of microvilli. Apical brush border enzymes that are positively correlated with the differentiation grade of enterocytes and therefore extensively used as Caco-2 differentiation markers, include intestinal alkaline phosphatase and sucrase isomaltase [48;52].

1.3.2 In vivo

The most frequently used species for *in vivo* research on experimental colon carcinogenesis are mice and rats. Some of the main reasons for application of these rodents are: 1) similarity of CRC development when compared with human colon carcinogenesis, 2) ease of animal handling and 3) relatively low costs to conduct experiments. Furthermore, 4) the short gestation period has the advantage that experiments can be planned, performed, and repeated in a relatively narrow time window. In addition, 5) the option of using inbred animals or littermates can be advantageous as the genetic variability is low, and therefore variation in biological response is limited. In these rodents the occurrence of spontaneously developed colon tumors is lower than 1% [53;54]. This low natural CRC susceptibility is a limiting factor in conducting studies in colon carcinogenesis, but can be circumvented by induction of colon carcinogenesis. In general, two main methods for CRC-induction in rodents are frequently used, namely induction by carcinogen treatment, or by genetic mutations in tumor suppressor genes in genetically engineered rodents, especially mice.

1.3.2.1 Induction of colorectal carcinogenesis

The most frequently used genetically engineered mice are the *Apc*-mutant mice that carry a germ-line mutation in the *Apc* tumor suppressor gene. A major disadvantage of these mice is that they mainly develop (adeno-) carcinomas in the small intestine, whereas in humans the majority of tumors occur in the colon and rectum. Therefore, only carcinogen-induced animal models are discussed.

Commonly used chemicals that induce CRC among rats and mice are 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), N-methyl-N-nitrosurea (MNU), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM).

The most frequently used carcinogens are DMH and AOM. In 1963, DMH was accidentally discovered to act as a carcinogen in rodent colons by Laquer *et al*. [55]. These researchers hypothesized that the high incidence of amyotrophic lateral sclerosis (a degenerative disorder that affects neurons in the brain and the spinal cord) among inhabitants of the island Guam might be caused by consumption of cycad flour made of nuts from the plant *Cycad circinalis L.*. Testing this hypothesis on rats learned that cycad flour induced colonic adenocarcinomas in stead of neurological disorders. Additional analyses showed that cycasin, a methylazoxymethanol glucoside showing structural similarity with DMH, was the phytochemical in cycad flour responsible for the induction of colon carcinogenesis. In the course of time, it became clear that DMH is a pro-carcinogen that requires metabolic activation via azomethane, azoxymethane, methylazoxymethanol, methyldiazonium (CH_3-$N^{\bullet}\equiv N$) and the highly reactive carbonium ion ($H_3C^{\bullet}$) [56]. Methyldiazonium can alkylate DNA at the guanine base [57;58], or can be converted to a carbonium ion that methylates DNA, RNA and proteins [56].

Although these agents are not present in the human diet, they are used more frequently than PhIP to study colon carcinogenesis, because of the high potency to induce CRC. When compared to DMH, usage of AOM is more convenient to induce CRC, because of a higher potency on a molar basis [59]. AOM-induced tumors show microsatellite instability [60] and contain mutations in ß-catenin [61;62] and *K-ras* [63], as is the case with human colon tumors [41]. On the other hand, AOM-induced tumors partially lack similarity to human colon carcinogenesis, as they rarely contain mutations in *Apc* [60;64] and the p53 tumor suppressor genes [63]. Taken together, chemical induced carcinogenesis is a good model to study sporadic colon carcinogenesis, since genetic mutations that are induced by carcinogen administration in rodents at least partially mimics the start of the adenoma-carcinoma sequence that occurs in humans. Moreover, using a carcinogen has the advantage that it is possible to make a distinction between the (pre-)initiation phase and promotion phase, depending on the time of (nutritional) intervention, which is interesting from a mechanistic point of view.

1.4 Biomarkers for colon cancer

Despite prevention strategies and efforts to improve treatment of CRC, the annual incidence and mortality are still alarming. In the course of time, it has become evident that patients die mainly from incurable metastases - predominantly from secondary tumors in the liver - rather than from the primary colon tumor itself [54]. Therefore, prevention or inhibition of the development of CRC, makes detection at an early stage of utmost importance.

Based on the tendency of colon tumors to bleed, the fecal occult blood test (also known as the stool test), has been proposed as a method for CRC screening. The stool test has the advantage of being cheap, easy to perform, and noninvasive, but when positive, the results of this test still have to be confirmed by colonoscopy [65]. This invasive method is conducted with a flexible endoscope, which enables examination of the rectum, sigmoid, and descending colon where approximately two-thirds of colon (adeno-) carcinomas occur [65]. Flexible sigmoidoscopy has the major disadvantage of missing lesions that occur in the proximal colon. This is in contrast to full colonoscopy, which is considered the ideal CRC screening method. However, both methods have the disadvantage of inconvenience for subjects undergoing examination and the risk of colonic bleeding and perforation.

Given the limitations and inconvenience of available detection methods to date, an ideal surrogate would be biomarkers that can be obtained with very limited or no invasion, are easy to detect at low-costs, are highly accurate, and have the option of obtaining results on a very short notice. Such biomarkers should ideally predict development of CRC within a specified time interval and/or reveal the presence of (an) existing tumor(s) [66]. These biomarkers should be expressed at different levels in normal, premalignant, and tumor tissue, in order to detect a deviation from the healthy state. CRC-biomarkers can be developed at the DNA, RNA, protein, metabolite, or histological level and should be correlated with (pre-) neoplastic lesions occurring in the colon as a target organ. Furthermore, it should be possible to study the effects of nutrients on these biomarkers of CRC.

1.4.1 Histological markers

Aberrant crypt foci

Ever since her 1987 publication in *Cancer Letters* [67], Ranjana Bird is mentioned as the first researcher who described aberrant crypts as putative precursors of carcinogenesis in the colons of mice treated with the carcinogen azoymethane [68]. However, Abulkalam Shamsuddin had already described these crypt abnormalities that are related to colon carcinogenesis in a tandem series of papers published in 1981 [69-71]. In patients with colon cancer, the mucosa in the vicinity and remote from the tumor revealed the presence of distorted crypts showing hypercellularity, atypia, and dilated luminal openings [70]. In addition, patients suffering from Crohn's disease, who are generally recognized as prone to development of CRC, were also described to be carriers of the same colonic aberrations that were located in and nearby the inflamed mucosa [69]. In a rat model for colon carcinogenesis, these lesions were located adjacent to and remote from the colon tumors, as was the case in patients with colon carcinoma [71]. Strikingly, the colonic aberrations preceded colon tumors and were only detected among carcinogen-treated rats. Based on these findings, Shamsuddin postulated these distorted crypts to be the earliest identifiable precursors of colon cancer. In Bird's 1987 paper, colonic lesions appearing after carcinogen treatment were designated "aberrant crypts" [67].

Unlike Shamsuddin, who investigated colonic lesions in sectioned colons by light and transmission electronic microscopy, Bird described a relatively easy-to-perform assay in unsectioned colons that were first formalin-fixed and subsequently stained with an ink-like dye designated "methylene blue". This staining method provides an observer with a nonlaborious and rapid method for detection of aberrant crypt foci (ACF) by (stereo) light microscopy at low magnification (40 - 100x). ACF are generally recognized as foci showing single or multiple enlarged crypts, enlarged luminal openings, and thickened epithelium in comparison with the surrounding normal crypts [72], as depicted in **Figure 2**.

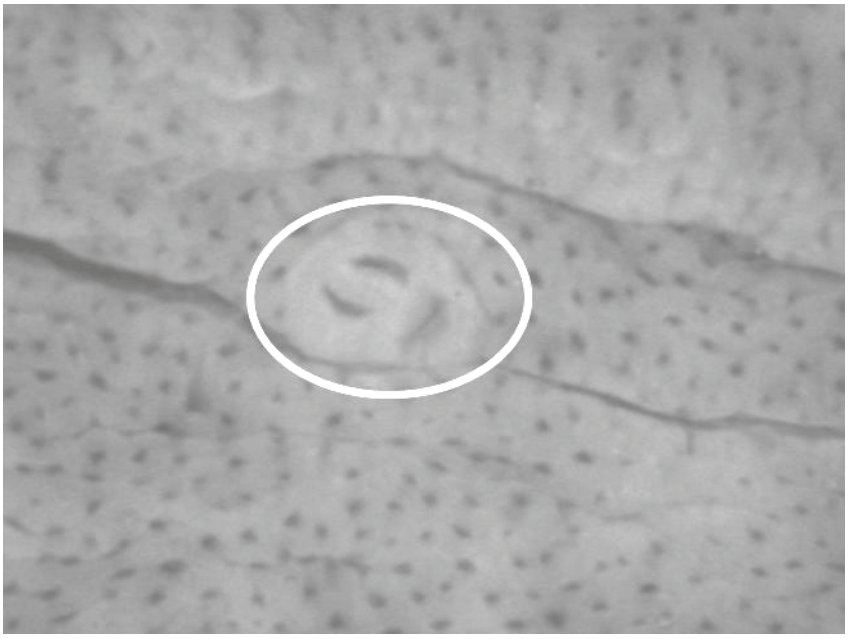

Fig. 2. Methylene blue stained ACF with three aberrant crypts that show a split enlarged lumen and thickened epithelium when compared to the surrounding healthy crypts. Magnification: 40x

Clinically, it has been suggested that ACF are associated with the presence of colon tumors [73;74]. In experimental colon carcinogenesis, however, the validity of ACF as reliable predictors of colon carcinogenesis is disputed [75-77].

ß-catenin accumulated crypts

Yasuhiro Yamada and others were the first to mention the appearance of ß-catenin-accumulated crypts (BCAC) in the colon of AOM-treated rats [62]. Unlike ACF, BCAC have no increased size, dilated lumen, or thickened epithelium when compared to healthy crypts (**Figure 3**).

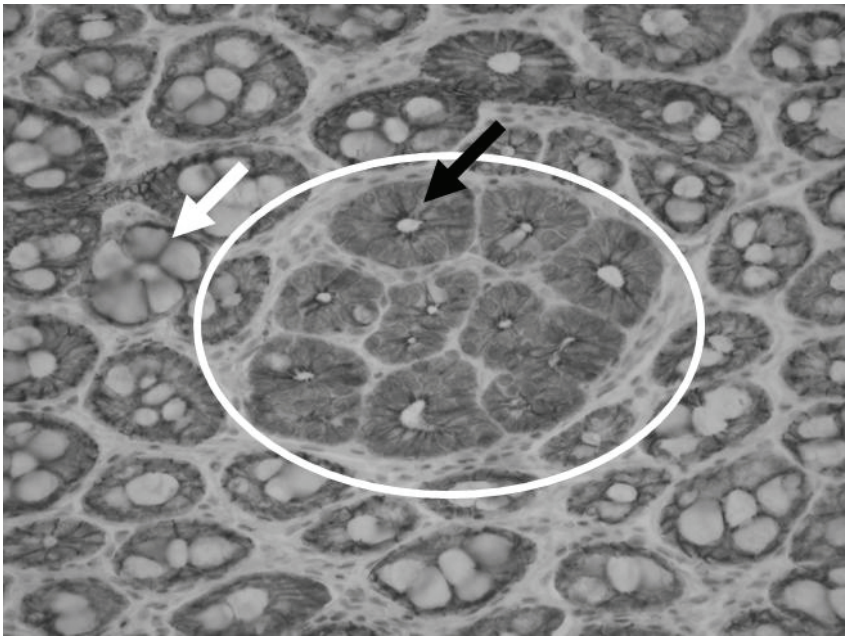

Fig. 3. Top view of a BCAC with 12 aberrant crypts. BCAC show ß-catenin immuno-reactivity in the cytoplasm (black arrow), whereas healthy crypts only show ß-catenin at the cell-cell contacts (white arrow). Magnification: 200x

In subsequent experiments, BCAC were shown to have higher proliferation rates and increased ß-catenin nuclear/cytoplasmic ratios when compared to ACF [78]. Furthermore, in the course of the carcinogenic process, BCAC number, diameter, and number of crypts per lesion showed a time-dependent increase. In addition, the number of BCAC increased towards the rectum, and was positively correlated with the occurrence of colon tumors, which is in contrast to ACF that showed an increase towards the proximal colon [79]. Taken together, these data indicate that BCAC might be better biomarkers for CRC than ACF. ß-catenin is a member of the adherence junction complex that is involved in signal transduction from the extracellular matrix toward the inner cell [80]. BCAC were shown to contain a mutation of the ß-catenin gene (*Ctnnb1*) in exon 3, which contains codons that are involved in degradation of the protein [61;62]. Cellular ß-catenin levels are controlled by, amongst others, the tumor suppressor gene *Apc*, which is part of the *Wnt* signaling pathway. In about 80% of human sporadic and hereditary colon cancers, mutations have been found in the *Apc* gene [81], which was initially discovered as the defect causing the hereditary cancer syndrome familial adenomatous polyposis (FAP) [82]. When the *Wnt* cascade reaction is inactive, ß-catenin is degraded by formation of a protein complex consisting of *Apc*, Axin, and glycogen synthase kinase 3ß (GSK3) [83]. Axin functions as a scaffold between *Apc*, GSK3, and ß-catenin thereby allowing cytoplasmic ß-catenin to be phosphorylated by GSK3 at serine and threonine residues

in its N-terminus. Phosphorylation of ß-catenin is required for its degradation by the proteasome pathway in order to maintain low cytoplasmic levels of the ß-catenin protein. On the contrary, in the presence of a *Wnt* signal, the cytoplasmic protein Dishevelled binds to Axin and consequently inhibits formation of the protein complex that phosphorylates ß-catenin [84;85]. As a result of this interference, ß-catenin phosporylation is disabled, leading to nuclear translocation of the protein where ß-catenin complexes with members of the T-cell factor family of DNA binding proteins and thus functions as a transcriptional co-activator [37]. Genes targeted by ß-catenin include c-*myc* and *cyclin D1*, both of which are involved in activation of cell proliferation and inhibition of apoptosis [86;87], suggesting that this pathway might be oncogenic if perturbed [61]. Indeed, when the *Apc* gene is mutated the *Apc* protein is almost always truncated [88], which prevents formation of the multiprotein complex that causes phosphorylation and concomitant degradation of ß-catenin. In addition, mutations in ß-catenin and Axin genes may lead to accumulation of the ß-catenin protein. Consequently, ß-catenin degradation is inhibited and results in its accumulation in the cytoplasm. Subsequently, stabilized ß-catenin migrates into the nucleus and will cause constitutive activation of the *Wnt* signaling pathway leading to increased cell proliferation decreased apoptosis, amongst others [89]. Therefore, accumulation of free ß-catenin is thought to be an early event in colon carcinogenesis [83], which makes ß-catenin-accumulated crypts potential predictors of colon carcinogenesis [79].

1.4.2 Molecular markers

Additional to histological markers, molecular markers for CRC development can be derived from molecular insights in the different stages of development of colon cancer. Colorectal carcinogenesis is a process with multiple steps: normal epithelium transfers into hyperproliferative epithelium and then further into adenoma, carcinoma, and eventually metastasis occurs. During this process, genetic alterations accumulate in genes like *Apc*, *Ctnnb1* (that encodes ß-catenin), *K-ras*, p53 and *c-myc* [90]. Other genes involved are cyclooxygenase 2 (COX-2), mismatch repair genes, and cell adhesion genes like matrix metalloproteases (MMPs) [91;92].

Apc mutations are found in many colon tumors and these mutations can lead to increased ß-catenin levels and activation of *Wnt* signaling, as described in the previous paragraph.

The preventive effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on colon cancer is thought to be related to its lowering effect on COX-2 levels. Expression of COX-2 is increased in colon cancer tissues compared to normal tissue [93], indicating the possible relevance of this gene in colon carcinogenesis. In addition, COX-2 plays a role in inflammation, and chronic inflammation is thought to play a role in colon cancer development [94].

Matrix metalloprotease (MMP) genes and tissue inhibitors of metalloproteases (TIMPs) play a role in cell adhesion and breakdown of the extracellular matrix. Therefore, these genes are thought to play a role in the process of metastasis development in colon carcinogenesis. High TIMP-1 [95;96] and MMP-7 [97] expression is correlated with tumor metastasis.

In the course of time, biomarker research has shifted from technologies that can analyze single markers one at a time to profiling methods that enable simultaneous measurement of a broad range of markers. These so-called “omics” techniques may facilitate the search for biomarkers for colorectal cancer (modified by nutritional compounds), since these techniques allow for holistic analyses of expression of gene transcripts (RNA), proteins and metabolites. Differential gene expression profiles at different stages of colon carcinogenesis have been described in several microarray studies [98-100]. Genes with differential expression levels at different stages of colon carcinogenesis could be considered as possible “new” biomarkers for disease progression. Examples include carbonic anhydrase genes and keratin genes. Subsequently, in-depth analysis of these potential biomarkers is needed to investigate the functions and mechanisms of these groups of genes in colon carcinogenesis.

Not only expression changes in single genes or proteins, but specifically, valuable biomarkers of carcinogenesis can be found by expression changes in cellular and gene ontology processes, as well as pathways, which may result in the identification of so-called “marker pathways” (e.g., signaling pathways that are perturbed like *Ras* and *Wnt* signaling or transcriptional regulation pathways). Specific pathway analysis tools using pathway maps or gene ontologies can be used for this approach.

In conclusion, application of the "omics" technologies may result in the elucidation of many more potential molecular markers, either as single genes or as sets of genes that interact in a pathway.

1.5 Microarray results from colon cancer studies

In recent years, the application of microarrays for analysis of gene expression in dietary modulation of colon carcinogenesis has increased. As early as in 1982, Leonard Augenlicht and others reported that arrays containing 400 cDNAs were applied to analyze differences between healthy colon and colon tumors in mice [101]. Subsequently, these authors expanded their microarrays to 4,000 cDNAs and applied scanning of arrays and digitizing of data to enable statistical analyses of gene expression in the healthy human colon mucosa, and in adenomas and carcinomas [102]. Ever since, comparison of gene expression between normal and pathological colon tissue is frequently carried out. In one such study, gene expression levels were determined after laser-captured microdissection of ACF, microadenomas, and adjacent normal-appearing colon epithelium from mice that were sensitive (A/J) or resistant (AKR/J) to development of CRC [103]. Cluster analysis of these colonic lesions revealed a set of genes expressed in both strains, indicating shared pathways in high- and low-risk backgrounds, whereas a number of genes were exclusively expressed in one strain. Within similar histological-based subclasses of ACF, originating from the different strains, differences were found in gene expression. As such, hyperplastic ACF in high-risk mice showed upregulation of genes that are part of the GTPase family, whereas in low-risk mice, hyperplastic ACF showed upregulation of genes involved in cell-cell adhesion, immune response, and anti-angiogenesis. These data suggest that different molecular mechanisms are involved in development of CRC as a result of different genetic backgrounds.

In a rat study with dietary heme, which has been hypothesized to increase CRC risk in humans, colonic cell proliferation was reported to be increased, as assessed by ^{3}H-Thymidine-incorporation into the DNA [104]. In this study, a novel rat colonic gene was reported to be downregulated, as assessed by microarrays. Subsequent identification revealed that this gene belongs to the pentraxin family of proteins, which are involved in immunological responses. As this pentraxin isoform was the first protein member discovered in the colon mucosa, it was designated "mucosal

pentraxin" (*Mptx*). Down-regulation of *Mptx* by dietary heme has been suggested to inhibit apoptosis in order to limit heme-induced cell loss.

In vitro studies have shown differential gene expression during differentiation of Caco-2 cells [48]. In the course of the differentiation process, genes involved in cell cycle progression and DNA synthesis, were downregulated, which was reflected by a decrease in cell proliferation. In addition, drug metabolism genes were up-regulated, which confirmed the observation that Caco-2 cells are able to metabolize xenobiotics in the course of the differentiation process.

Caco-2 cells exposed to quercetin showed that expression of genes involved in cell cycle, tumor suppression, apoptosis, as well as oncogenes, are modulated towards inhibition of colon cancer [105]. Inhibition of cell cycle-related genes was confirmed with a BrdU proliferation assay in quercetin-exposed cells, indicating that at least a part of microarray data are predictive for cellular physiology.

HT-29 cells exposed to 2.5 - 50 μM epigallocatechin-3 gallate, a tea anti-oxidant that belongs to the catechins, showed a dose-dependent decrease in development of spheroids, which are considered to be predictors of *in vivo* tumorigenicity [106]. This chemopreventive EGCG effect is realized by genes involved in cell proliferation, cell-cell contacts and cell-matrix interactions, as has been assessed using microarrays. Gene expression data for *c-FOS* (involved in cell proliferation) and *IQGAP2* (involved in signal transduction) were validated by applying Western blot. This approach showed that both gene and protein expression data were in agreement with one another.

In a clinical trial, healthy subjects and adenoma patients were fed either low (75 g/day) or high (300 g/day) doses of vegetables for 2 weeks and biopsied before and after dietary intervention [107]. Dedicated arrays containing genes involved in response to xenobiotics showed enhanced genetic mechanisms linked to inhibition of CRC, and included *PTGS2* (that encodes the COX-2 enzyme), *KI-67* (activation of proliferation), *c-FOS* (an oncogene), and *CHK1* (inhibition of cell cycle). However, adenoma patients receiving the low dose-diet, showed unfavorable expression of *AMACR* (involved in lipid metabolism), *PKCB1* (implicated in tumor-promotion of colon cells) and *CHK1*, suggesting enhancement of colon cancer. In a subsequent study with mice fed mixtures of vegetables, dedicated arrays revealed 17 differentially expressed genes involved in CRC, including *Sult1a1*, *Casp3*, *Stat1*, and *Oat* [108].

The above-mentioned data have in common that mechanisms involved in (nutritional modulation of) CRC can be picked up by application of microarrays, including tumor suppressors, oncogenes and genes involved in xenobiotic metabolism. In addition, microarrays can give more insight in the mechanisms involved in the development of CRC and as such generate novel biomarkers for development of colon cancer.

1.6 *Proteomics results from colon cancer studies*

Hitherto, application of proteomics in nutritional studies is limited when compared to transcriptomics [109]. In an *in vitro* study, the human pre-neoplastic cell line NCOL-I was exposed to the dietary flavonoids quercetin and flavone for 24 hr [110]. Proteomics analysis revealed that 73 proteins were altered by quercetin and 32 by flavone. Basically, both flavonoids decreased cell proliferation and induced cell differentiation. Only quercetin was shown to execute apoptosis, as determined by measurement of caspase 3 activity.This apoptosis inducing capacity was also demonstrated by proteomics as derived from the 73 altered proteins, including heat shock proteins and caspase substrates. These apoptosis inducing proteins were not found in the flavone-affected proteome, suggesting that proteomics is a powerful tool to pick up biological differences.

An *in vivo* rat survey into changes occurring in the distal colon of dimethylhydrazine treated rats showed formation of ACF and upregulation of prostaglandin synthesis [111]. In this study, proteomic analyses of the distal colons, performed by a combination of 2DE and MALDI-TOF, showed carcinogen-induced upregulation of carbonic anhydrase 2, creatin kinase and calreticulin, amongst others. Creatine kinase is involved in energy transduction in tissues with large energy demands, by catalyzing the transfer between creatine phosphate and ATP. The authors hypothesize that upregulated creatine kinase is associated with altered cellular metabolism involved in colorectal carcinogenesis.

Another biomarker study, in which mice were fed a vegetable mixture for 2 weeks, showed decreased expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and the mitochondrial ATP synthase oligomycin sensitivity conferral protein (*Oscp*), amongst the 6 identified colonic proteins that suggest a protective effect against development of CRC [112]. GAPDH protein expression is increased in human

CRC [113], but favourably altered by vegetable supplementation. At present, *Oscp* is not considered to be associated with colon cancer, but might be a novel biomarker. Additionally, a proteomics comparison between the SW480 cell line derived from a primary human colon tumor and the SW620 cell line derived from the lymph node metastasis in the same patient, showed 9 significantly altered proteins [114]. Both alpha enolase (a glycolytic enzyme) and triosephosphate isomerase (a glycolytic and glyconeogenic enzyme) were increased in the SW620 cell line, which was confirmed by Western blot analysis. The potency of these two cell lines to develop metastatic tumors *in vivo* was tested by injection in the spleen of mice. Strikingly, only SW620 cells caused metastases in the liver and peritoneum. Within the SW620-induced tumors, expression of alpha enolase and triosephosphate isomerise was higher in the metastasized tumors than in the primary splenic tumors. These data suggest that these two enzymes, identified by means of proteomics are indeed correlated with tumor metastasis.

In a clinical study, adenocarcinomas were compared with surrounding normal tissue, and showed that the energy metabolism was shifted from β-oxidation towards anaerobic glycolysis, in addition to decreased Na^+/K^+ exchange [115]. Increased ATP production via glycolysis might be used to overcome the relatively low ATP levels obtained by oxidative phosphorylation. In this way, tumor cells are thought to achieve an energetic favourable state, which contributes to cell survival [116].

Overall, the above mentioned reports suggest that proteomics can detect a variety of alterations, including cell proliferation, apoptosis, energy metabolism, and metastasis, all of which are associated with (nutritional modulation of) colorectal carcinogenesis.

1.7 Aims and outline of this thesis

The objective of this thesis was to identify histological and molecular biomarkers of quercetin-modulated colorectal carcinogenesis, by using both classical and omics-based technologies. This approach is thought to gain an insight into dietary modulation of CRC, using quercetin as a model compound. Based on previously described mechanisms of action of quercetin, it is hypothesized that quercetin will downregulate mechanisms involved in development of colorectal cancer.

The questions addressed to find answers supporting this hypothesis were:

1. Does quercetin affect proliferation, differentiation and phase II metabolism in Caco-2 cells?
2. Which molecular mechanisms in quercetin-exposed Caco-2 cells are associated with changes in the above mentioned parameters?
3. Does quercetin reduce colorectal carcinogenesis in rats and which histological biomarkers are predictive of this cancer-modulating potency?
4. Is the *in vivo* bio-availibility of quercetin involved in it's cancer-modulating potency?
5. Does quercetin - at the transcriptome and proteome level - protect the healthy rat colon from mechanisms involved in colorectal carcinogenesis?

To address these questions, the following experiments have been performed:

In chapter 2 and 3, the effects of quercetin at physiological concentrations relevant for the human colon is studied *in vitro* using the human CRC cell line Caco-2. Chapter 2 focussed on the effects of quercetin on cell proliferation, cell differentiation and phase II metabolism. The sequel, chapter 3, describes quercetin-mediated Caco-2 cell differentiation and associated cellular processes, at the transcriptome level.

In chapters 4 and 5, the effects of quercetin on colorectal carcinogenesis is studied in the colon of rats. In chapter 4, an experiment is described in which rats were first fed quercetin in the diet and subsequently treated with AOM. Quercetin's potency to modulate development of CRC was correlated with development of putative preneoplastic lesions, and the results were related to the bioavailability of this flavonoid. In chapter 5, the effect of dietary quercetin at the molecular level was studied in the healthy colon of non-AOM treated rats using both transcriptomics and proteomics. This technological approach was maintained to provide a near-holistic insight into molecular mechanisms evoked by quercetin that could explain its effect in AOM induced colorectal carcinogenesis. Finally, in chaper 6 the main findings of this thesis are summarized and discussed.

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Chapter 2

Modulatory effects of quercetin on proliferation and differentiation of the human colon cancer cell line Caco-2

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Abstract

The effect of the dietary flavonoid quercetin was investigated on proliferation and differentiation of the human colon cancer cell line Caco-2. Confluent Caco-2 monolayers exposed to quercetin showed a biphasic effect on cell proliferation and a decrease in cell differentiation ($0.001 < P < 0.05$). During differentiation Caco-2 cells formed 5 phase II metabolites, of which the amount of 4'-O-methyl-quercetin-3'-O-glucuronide correlated with the differentiation grade ($r = 0.99$, $P < 0.003$).

The increment of cell proliferation at low quercetin concentrations and the decrease in cell differentiation are effects opposite to what would be expected for a functional food ingredient with anti-carcinogenic potential.

Introduction

When considered globally, colorectal cancer (CRC) takes the second place in cancer related deaths with an estimate of 492,000 people per year and is third when considering the yearly incidence of 945,000 people [1]. Under physiological conditions a limited number of stem cells are continuously dividing in the base of colonic crypts and are the source of epithelial cells [2]. Following division, daughter cells migrate upwards and undergo differentiation in the upper two-thirds of the crypt and finally undergo apoptosis when moving towards the top of the crypt, within 4-8 days [3]. In mammalian cells these continuous processes of proliferation, differentiation and apoptosis are under strict regulation, but can be disrupted as a result of successive genetic mutations [4]. These "genetic hits" occur in both tumor suppressor genes as well as oncogenes and finally give rise to colorectal carcinomas through a gradual series of well-characterized histo-pathological changes, also known as the adenoma-carcinoma sequence [5].

Risk factors suggested in CRC aetiology include genetic predisposition and dietary intake, such as high consumption of fat and low intake of fibres and flavonoids [6-8]. One of the flavonoids highly present in human diet is quercetin, which in nature is glycosylated and can be found in several fruits and vegetables. Major dietary sources of quercetin are amongst others onions, broccoli, leek and blueberries [9]. Following ingestion, natural quercetin derivatives can be deconjugated in the small intestine by cellular ß-glycosidase [10] or in the colon, by ß-glycosidase produced by colonic bacteria [11]. In humans, quercetin aglycone shows relatively poor bioavailability [12], probably as a result of rapid metabolic conversion, thereby forming glucuronidated, methylated and sulphated metabolites with different biological activities [13;14]. Quercetin has been proposed to exert anti-carcinogenic mechanisms both *in vitro* and *in vivo* [15;16]. A possible mechanism involved is scavenging of free radicals. This electron and/or hydrogen donating capacity is exerted by its phenolic hydroxyl groups, especially by the two neighboring hydroxyl groups present on the B-ring, also known as the catechol moiety. Other proposed mechanisms of action include induction of apoptosis and inhibition of cell proliferation, protein kinase C and lipoxygenase (reviewed in ref. [17]). Nowadays, quercetin is commercially available and claimed to be a beneficial food supplement. However, data regarding the benefits are relatively scarce and conflicting [18;19].

Aspects of cellular processes also involved in CRC, including proliferation and differentiation, can be studied *in vitro* using the Caco-2 model system, which is derived from a human colon adenocarcinoma [3]. Upon seeding, Caco-2 cells are able to proliferate towards confluency, thereby forming a monolayer including tight junctions, which maintain cell-cell contact and are essential in cellular polarization and differentiation [20]. When reaching confluency, Caco-2 cells show contact inhibition mediated cell cycle arrest and undergo spontaneous differentiation as a function of time. Differentiating Caco-2 cells express apical brush border enzymes, including sucrase isomaltase [21] and alkaline phosphatase (ALP) [22], which are positively correlated with the differentiation status of enterocytes. Another feature of differentiated Caco-2 cells is the resemblance with normal small intestinal cells, showing microvilli in a high density as well as drug absorption, metabolism and excretion [23].

Mechanisms of action evoked by nutrients with anti-carcinogenic properties are amongst others inhibition of cell proliferation and stimulation of cell differentiation as has been demonstrated with the flavonoid 2-phenyl-4H-1-benzopyran-4-one in the colon cancer cell line HT-29 [24], fish oil in the HT-29 and Caco-2 cell lines [25] and the short chain fatty acid butyrate in Caco-2 cells [26;27]. Based on proposed mechanisms involved in inhibition of carcinogenesis, the hypothesis of the present study was that quercetin should decrease cell proliferation and enhance cell differentiation in the Caco-2 cell model. Therefore, the aim of this study was to investigate the effects of physiological relevant concentrations of quercetin on proliferation and differentiation of Caco-2 cells. Furthermore, in order to assess whether the effect on cell proliferation and differentiation can be ascribed to quercetin and/or its metabolites, and whether the extent and type of quercetin metabolism is dependent on the differentiation status of Caco-2 cells, quercetin phase II metabolites were identified and quantified as a function of time.

Material and methods

Chemicals & Materials

Quercetin dihydrate and sodium ascorbate were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Dulbecco's modified eagle medium with

L-glutamine, 25 mM HEPES and 4.5 g/L D-glucose as well as supplements (100x MEM non-essential amino acids, gentamicin, L-glutamine (100x) and phosphate buffered saline (PBS) were purchased from Invitrogen™ Life Technologies (Breda, The Netherlands). Fetal calf serum was ordered from Cambrex (Verviers, Belgium). Flasks with a 75 cm^2 growth area, flat-bottomed 96-well plates, low protein binding membrane filters CA 0.22 µm and transwell® polycarbonate membrane inserts (12 wells/plate) were purchased from Corning Life Sciences (Cambridge, U.K.). PicoGreen® dsDNA quantitation kit was obtained from Molecular Probes (Leiden, The Netherlands). Dimethyl sulfoxide (DMSO) [Acros Organics] was purchased from Boom B.V. (Meppel, The Netherlands). The cell proliferation ELISA Bromo-2'-deoxyuridine (BrdU) colorimetric kit was ordered from Roche Diagnostics (Almere, The Netherlands). HPLC-grade acetonitrile and trifluoroacetic acid were purchased from Lab-Scan Ltd. (Dublin, Ireland) and Baker (Deventer, The Netherlands), respectively.

Quercetin stability in culture medium

Since quercetin has been reported to be unstable in an aqueous environment [18], its stability was tested in the presence of different ascorbate concentrations. To this end, 40 µM quercetin aglycone originating from a 200x DMSO stock solution was incubated at 37°C in 5% CO_2 and 95% humidity in combination with 0, 0.1, 1 or 10 mM of filter-sterilized sodium ascorbate in culture medium, in the absence of cells. The culture medium as needed for the rest of the experiment, further to be referred to as DMEM culture medium, consisted of Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) heat inactivated mycoplasma tested fetal calf serum, 2 mM L-glutamine (6 mM final concentration), 1% (v/v) MEM non-essential amino acids and 50 µg/ml gentamicin. Samples were taken in triplicate at t = 0, 24 and 48 hrs post-incubation, frozen in liquid nitrogen and kept at -80°C until further analysis. For precipitation of FCS in DMEM culture medium, samples were first diluted 2 fold with acetonitrile, vortexed vigorously and centrifuged at 13,000 rpm during 10 minutes. Finally, high performance liquid chromatography (HPLC) analysis of quercetin in the supernatant was performed according to a previously described method [28].

Cell culture

Caco-2 cells, originating from a human colorectal adenocarcinoma, were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). For subculturing, near-confluent monolayers were rinsed using PBS with 0.022% (w/v) EDTA, followed by treatment with 0.05% (w/v) trypsin in PBS with 0.022% (w/v) EDTA. Caco-2 cells of passage 40 and 41 were subcultured in T75 flasks in a 1 : 10 split ratio at 37°C in 5% CO_2 and 95% humidity, with DMEM culture medium.

Cell proliferation

Cell proliferation was measured as a marker for cytotoxicity induced by quercetin. Since 1 mM ascorbate was found to be non-toxic for Caco-2 cells and showed an acceptable grade of stability in culture medium, this ascorbate concentration was chosen as the optimal concentration for stabilization of quercetin in subsequent experiments. Caco-2 cells of passage 42 were seeded in duplicate in flat-bottomed 96-well plates at a density of 10,000 cells/well. After formation of a confluent monolayer on day 3 post-seeding, Caco-2 cells were exposed to 0 - 80 µM quercetin stabilized by 1 mM ascorbate. As controls, cells were exposed to 1 mM ascorbate in DMEM culture medium, including the solvent *i.e.* 0.5% (v/v) DMSO. Culture medium of all experimental conditions was completely changed every 24 hours and cell proliferation was measured every 24 hours during a 96 hour time period, using the Cell Proliferation ELISA, BrdU colorimetric kit. First, 96-wells plates were incubated with 10 µM BrdU to allow incorporation of this pyrimidine analogue instead of thymidine into the DNA of proliferating Caco-2 cells. Following a DNA denaturation step, for improvement of interaction with the BrdU antibody during the next step, cells were subsequently incubated with peroxidase (POD) labeled anti-BrdU. Finally, POD-labeled immune complexes were coloured by incubation with tetramethyl-benzidine and absorbances were measured with a Versamax tunable microplate reader at 370 nm, with a reference wavelength of 492 nm.

Cell differentiation

To asses the effect of quercetin on cell differentiation, near-confluent cells were subcultured at a split ratio of 1 : 10. To enable differentiation, cells of passage number 41 were seeded in triplicate at a density of ≈ 112,000 cells/cm^2 on polycarbonate membrane transwell® inserts (12 wells/plate) with a membrane diameter of 12 mm

(growth area 1.13 cm^2) and a 0.4 μm pore size. Upon reaching confluency on day 2 post-seeding (*i.e.* experimental day 0), the apical side of the Caco-2 monolayers was exposed to 0.5 ml of 40 μM quercetin stabilized by 1 mM filter-sterilized sodium ascorbate (40 μM quercetin). In addition, Caco-2 cells were exposed to DMEM culture medium with (control) and without (untreated) 1 mM sodium ascorbate, for analysis of possible ascorbate mediated effects on cell differentiation and of spontaneous differentiation, respectively. Both, untreated and control cells received culture medium with the quercetin solvent *i.e.* 0.5% (v/v) DMSO. Basolateral compartments were filled with 2.0 ml culture medium only. Caco-2 cells were exposed to quercetin in the apical compartment only, in order to mimic luminal exposure of the *in vivo* gut. Apical and basolateral culture medium of all conditions was completely changed every 24 hours. Caco-2 cells were allowed to undergo differentiation and cells were harvested on days 0 (*i.e.* prior to quercetin exposure), 3, 5, 7 and 10 post-confluency. First, monolayers were rinsed 2 times with ice cold PBS-only. Subsequently, membranes were cut out, taken up in 1 ml ice cold PBS in cryovials, snap frozen in liquid nitrogen and finally stored at -80°C until further analysis of ALP activity, double stranded DNA contents and protein amount.

Alkaline phosphatase activity

Activity of alkaline phosphatase (ALP), a marker for Caco-2 cell differentiation [20], was measured in triplicate on days 0, 3, 5, 7 and 10 post-confluency. First, cells on membranes were lysed by sonification for 3 x 20 seconds in ice-cold PBS. Subsequently, ALP activity was determined on a BM/Hitachi 911 using a colorimetric assay in which the release of p-nitrophenol out of the substrate p-nitrophenol phosphate is proportional to the ALP activity [29].

Trans epithelial electrical resistance

Trans epithelial electrical resistance (TEER) was measured as an additional marker for cell differentiation [20;30]. As the temperature is one of the factors influencing the TEER, culture medium in transwell® inserts was allowed to cool down to room temperature (20 ± 1°C), thereby avoiding temperature fluctuations in day-to-day measurement. TEER was measured in triplicate on days 0, 3, 5, 7 and 10 post-confluency, using a Millicel-ERS Volt Ohm meter (Millipore, Amsterdam, The

Netherlands). TEER values were calculated according to the following equation: TEER = R x filter area ($\Omega.cm^2$).

Picogreen® dsDNA quantification

To correct ALP for the amount of cells present in each well, double stranded DNA (dsDNA) - as a marker for cell number - was measured. First, 100 µl 200x diluted Picogreen® dsDNA quantification reagent was added to 100 µl of cell lysate in a flat-bottomed 96-well plate. Following a 5 minute incubation step, fluorescence of Picogreen® was determined at a wavelength of 530 nm after excitation at 485 nm. dsDNA was quantified according to a calibration curve of the λ-dsDNA standard in TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5).

Protein amount

Since ALP activity of differentiating Caco-2 cells has also been reported as ALP units/mg protein [3], protein amount of cell homogenates was measured according to Bradford [31].

Quercetin metabolism

In order to assess whether metabolism of quercetin is associated with the differentiation grade of Caco-2 cells, culture medium was sampled on days 1, 3, 5, 7 and 10 post-confluency from the apical and basolateral compartments, just before refreshing the culture medium. Thus, culture medium samples obtained on above mentioned time points reflect quercetin metabolism over the last 24 hours, as quercetin in culture medium was completely changed every 24 hours. Samples were snap-frozen in liquid nitrogen and stored at -80°C, until further analysis. Quercetin and its metabolites were detected on HPLC, with detection between 220 and 445 nm using a Waters 996 photodiode array detector. Quercetin and its phase II metabolites were characterized based on retention time, UV-vis (nm), LC-MS, and ^{1}H NMR characteristics as described previously and quantified assuming similar absorption coefficients of quercetin and its metabolites [32].

Statistics

Overall analysis of Caco-2 proliferation modulated by different quercetin concentrations, time of quercetin incubation, as well as the interaction between these

two parameters was performed using the 2-way ANOVA, incorporating incubation time, quercetin concentration, as well as the interaction between incubation time and quercetin concentration. Analysis of the effect of different quercetin concentrations on cell proliferation within each time point, as well as comparison of the effect of quercetin treatment on cell differentiation was performed using the Student's *t*-test. Correlations between cell differentiation data obtained by several methods, as well as the correlation between the differentiation grade and quercetin metabolite status were calculated with the Pearson's correlation coefficient. Differences were considered significant when $P < 0.05$.

Results

Quercetin stability

Stability of quercetin in the absence of cells was assessed over a 48 hour time period in the presence of 0, 0.1, 1 and 10 mM ascorbate. **Figure 1** depicts the stability of 40 μM quercetin in DMEM culture medium, in combination with 3 ascorbate concentrations. After 24 hours quercetin could not be detected in incubations with 0 or 0.1 mM ascorbate. Addition of 1 or 10 mM ascorbate on the other hand, resulted in stabilization of quercetin, showing 83% of the initial quercetin concentration for both conditions after 24 hours, declining to 23% and 73%, respectively after 48 hours. Based on these data, 1 mM ascorbate was used to stabilize quercetin, and culture medium was completely changed every 24 hours.

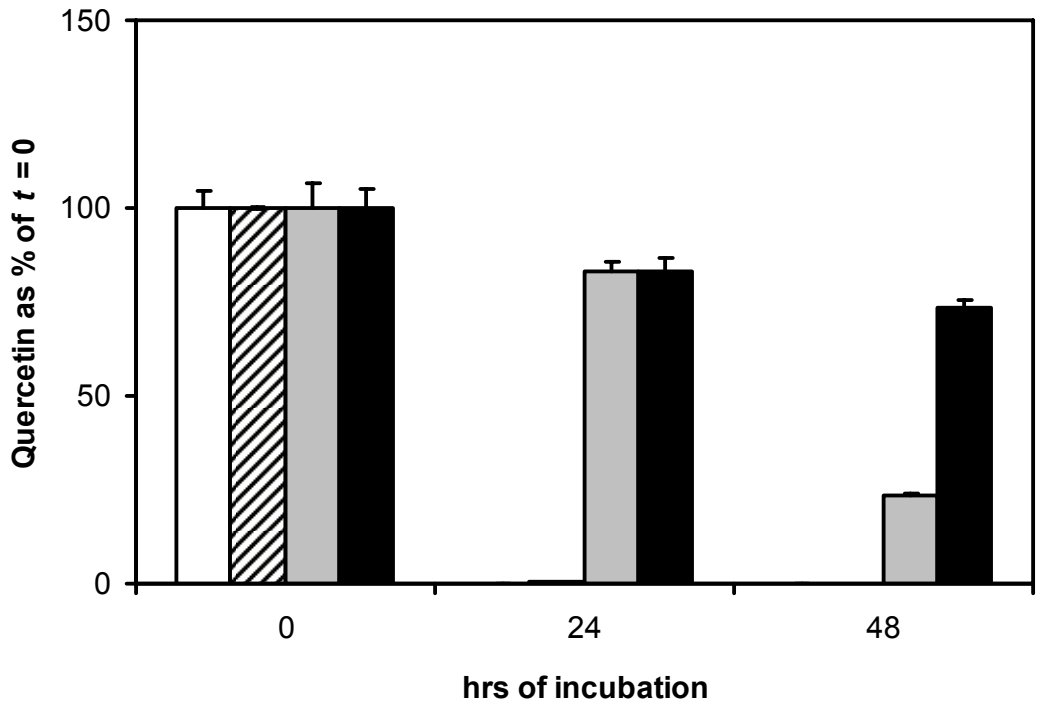

Fig. 1. Stability of 40 µM quercetin in culture medium in the absence (open bar) or presence of 0.1 mM (diagonally lined bar), 1 mM (grey bars) and 10 mM (black bars) of ascorbate, presented as percentage of $t = 0$. Values are given as mean ± standard deviation ($n = 3$).

Cell proliferation

The proliferation rate of Caco-2 cells was assessed during consecutive 24 hour time intervals, for a 96 hour time period following initial exposure to quercetin (figure 2A - D). Incubation with 1 mM ascorbate did not affect Caco-2 monolayer integrity, regardless of the quercetin concentration. When data of all 4 time points were considered, a significant inhibition on cell proliferation was observed with increasing quercetin concentrations ($P < 0.0001$) and with an increasing time of quercetin incubation ($P < 0.0001$). In addition, cell proliferation was inhibited significantly by the interaction between incubation time and quercetin concentration ($P < 0.002$). When looking at different time points individually, quercetin at 40 and 80 µM inhibited cell proliferation throughout the experiment, with the exception of 40 µM at $t = 24$ hours (figure 2A). Strikingly, a biphasic effect on cell proliferation could be seen at $t = 96$ hours (figure 2D), showing an increment of cell proliferation at quercetin concentrations up to 20 µM, followed by a decrease at higher concentrations.

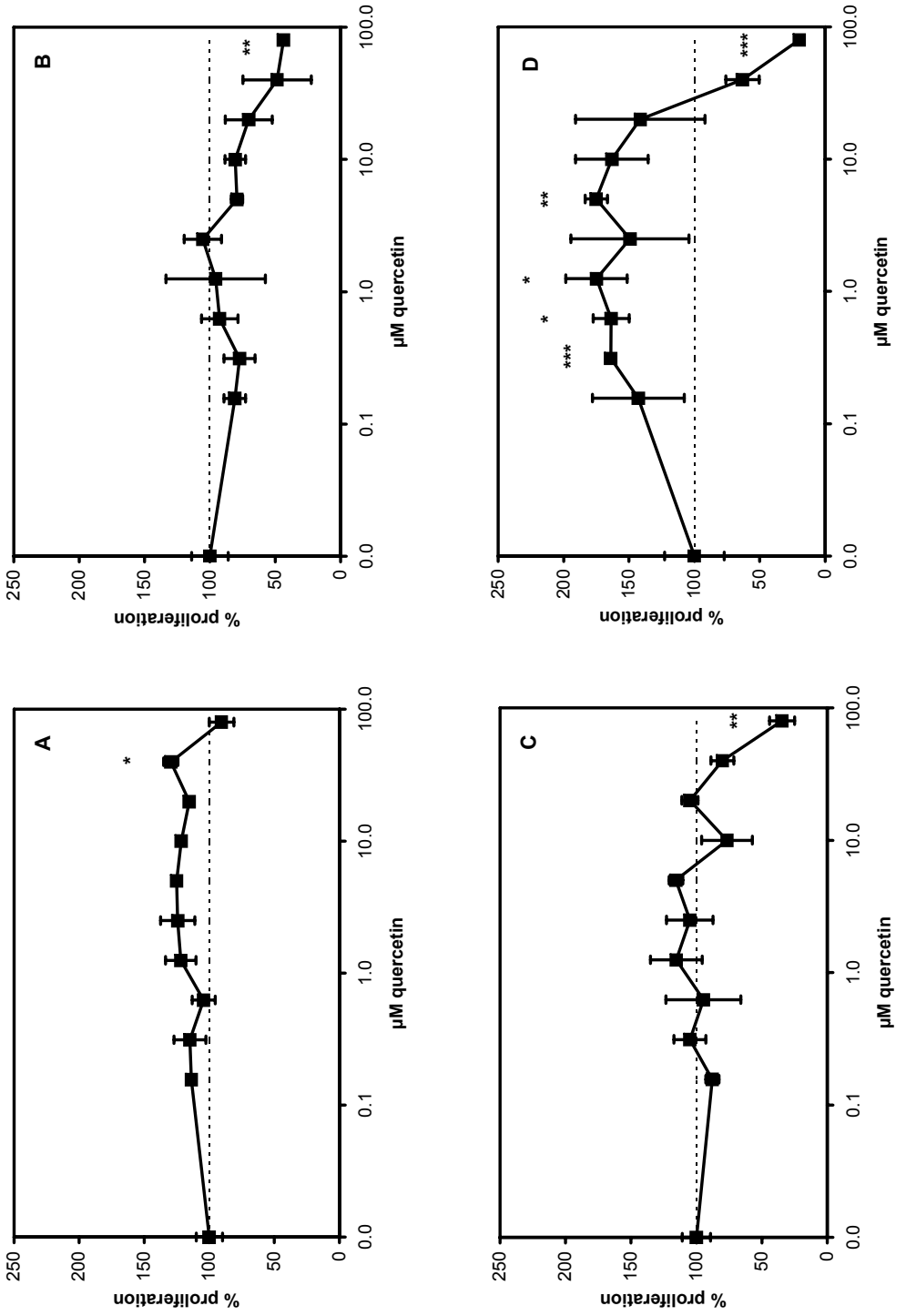

Fig. 2. Cell proliferation of Caco-2 cells incubated with 1 mM ascorbate stabilized quercetin, at $t = 24$ (panel A), $t = 48$ (panel B), $t = 72$ (panel C) and $t = 96$ hours (panel D) after initial exposure. For each time point separately, proliferation rate is presented relative to corresponding control cells treated with 1 mM ascorbate in combination with 0.5% DMSO (*i.e.* 100% proliferation). Values are given as mean ± standard deviation ($n = 2$).
* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Cell differentiation

Since alkaline phosphatase is one of the brush border membrane associated enzymes present on differentiated Caco-2 cells [20], activity of this enzyme was measured as a marker for cell differentiation. Previously, ALP activity of differentiating Caco-2 cells has been reported to be corrected for the number of cells present in culture, by measurement of mg protein [3]. Besides assessment of protein amount, in the present study dsDNA was measured as an alternative marker for cell number, since it could not be excluded that Caco-2 protein expression might differ between treatments and the amount of dsDNA per cell is likely to be more constant than protein expression. Absolute amounts of mg protein and ALP activity showed high correlations for quercetin ($r = 0.95$, $P < 0.05$), ascorbate ($r = 0.93$, $P < 0.02$) and DMEM ($r = 0.98$, $P < 0.003$) treated cells. In addition, corrected ALP activity expressed as ALP/µg dsDNA and ALP/mg protein correlated significantly with one another for all experimental conditions [$0.96 < r < 0.99$; $0.001 < P < 0.05$; protein data not shown]. From day 3 onwards, quercetin-treated cells showed a significantly lower increase in ALP activity per µg dsDNA, reaching a maximum of only 58% of ALP activity observed for ascorbate treated controls on day 10 post-confluency (**Figure 3**). In the course of the experiment, ALP activity of ascorbate treated control cells showed a similar increasing trend as untreated cells.

Trans epithelial electrical resistance, measured as an additional marker for cell differentiation [20;30], showed comparable differentiation grades relative to ALP units/µg dsDNA ($0.93 < r < 0.99$; $0.0006 < P < 0.05$) and to ALP units/mg protein ($0.89 < r < 0.97$; $0.007 < P < 0.05$) for different treatments (data not shown).

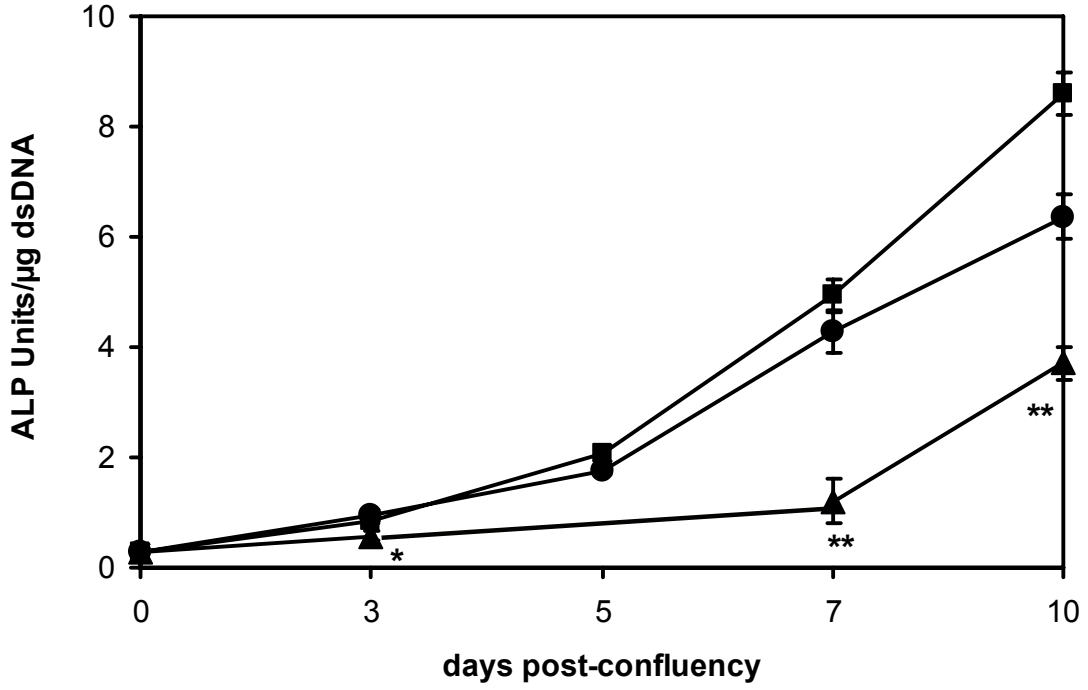

Fig. 3. Time dependent ALP activity in vehicle exposed cells (—■— : 0.5% DMSO), control cells (—●— : 1 mM ascorbate + vehicle) and treated cells (—▲— : 40 µM quercetin stabilized by 1 mM ascorbate). Values are given as mean ± standard deviation (n = 3). Data on day 5 for quercetin series not determined. * $P < 0.01$ and ** $P < 0.001$ when compared to ascorbate controls.

Quercetin metabolism

HPLC analysis was performed on apical and basolateral culture medium, in order to assess whether the quercetin-metabolizing capability of Caco-2 cells changes with the differentiation grade and to determine whether changes in cell differentiation are associated with changes in the amounts of quercetin or with (one of) its metabolites. To enable mutual comparison, per experimental day quercetin and its metabolites originating from the apical and basolateral compartments are expressed as percentage of the sum of peak areas.

Apical and basolateral levels of quercetin aglycone remaining after 24 hours of incubation, account for 48% and 39% of peak areas, respectively, on day 1 post-confluency, followed by a decrease in the course of the differentiation (**Figure 4A**). Quercetin was metabolized into 5 (double) phase II conjugates that were characterized as 4'-O-methyl-quercetin-3'-O-glucuronide (**figure 4B**), 3'-O-methyl-quercetin

(= isorhamnetin, **figure 4C**), 4'-O-methyl-quercetin-7-O-glucuronide (**figure 4D**), 3'-O-methyl-quercetin-4'-O-glucuronide (**figure 4E**) and 3'-O-methyl-quercetin-7-O-sulphate (**figure 4F**). Apical and basolateral amounts of 4'-O-methyl-quercetin-3'-O-glucuronide, as well as the sum of these amounts, were positively correlated with the differentiation grade of quercetin treated cells until day 7 ($r = 0.99$, $P < 0.003$), thereafter showing a slight decrease. Glucuronidated 4'-O-methylquercetin metabolites were found from day 3 onwards, without having detected 4'-O-methyl-quercetin itself throughout the experiment.

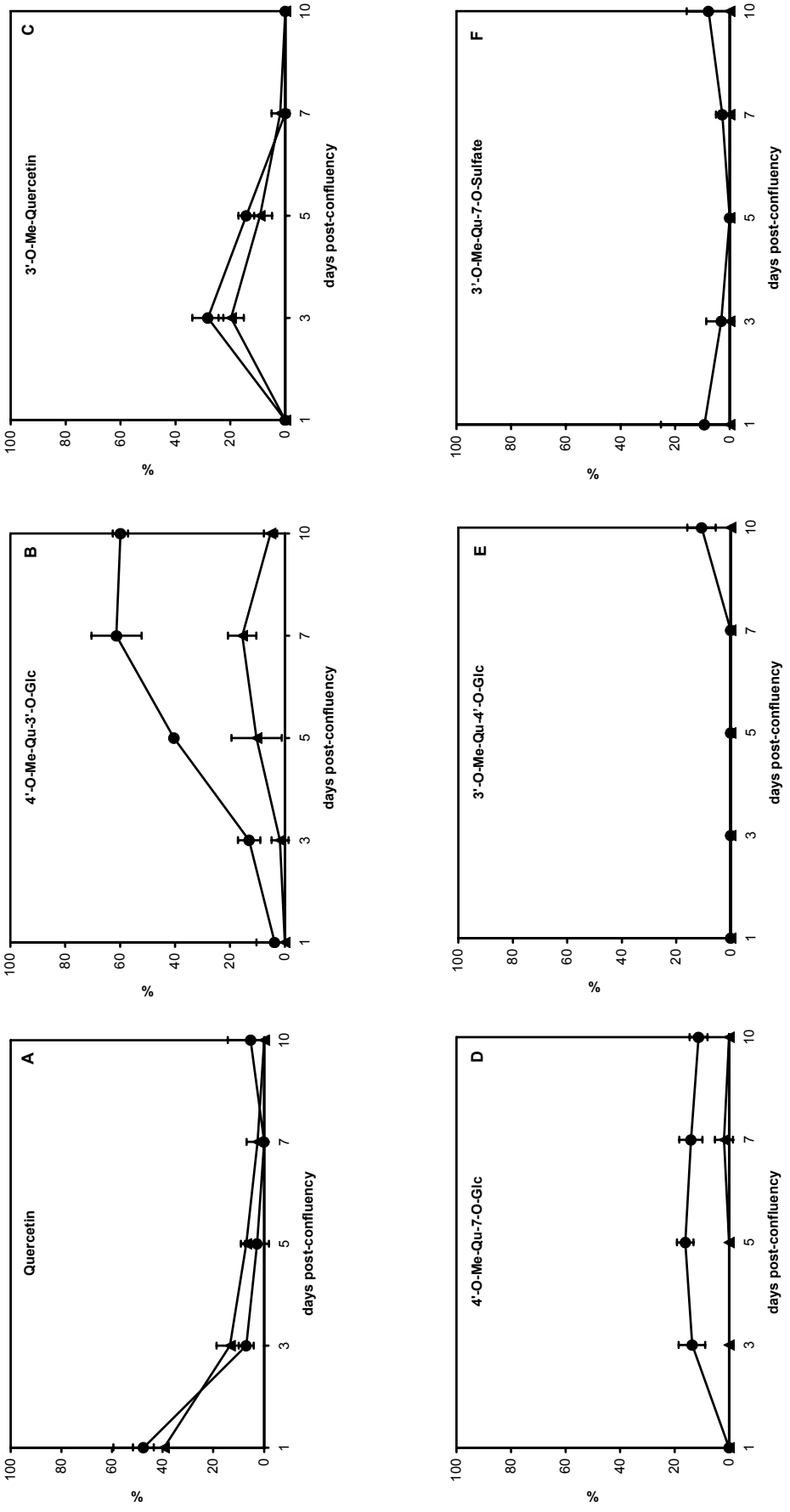

Fig. 4. Time course of quercetin and its metabolites originating from apical (dots) and basolateral (triangles) compartments, expressed as percentage of the sum of peak areas measured on each experimental day. Values are given as mean ± standard deviation ($n = 3$).

Discussion

Quercetin is a flavonoid of the flavonol type and believed to be beneficial for human health, based on amongst others its proposed ability to act as a scavenger of free radicals and its potency to inhibit cell proliferation of transformed epithelial cells [17;18]. In the present study we have analyzed the effect of quercetin on proliferation and differentiation of the human colon cancer cell line Caco-2. In addition, we have investigated whether the differentiation grade of Caco-2 cells is related to quercetin or its metabolites and to the extent of quercetin metabolism.

These experiments have been performed with quercetin concentrations up to 80 μM, based on epidemiological and human absorption data. In the Zutphen Elderly Study, the intake of quercetin in terms of aglycone has been estimated to be 16.3 mg per day [33]. Furthermore, absorption studies with subjects who underwent colectomy, show 76% recovery of orally administered quercetin aglycone in their ileostomy bag [12], which can be considered as a model compartment for the colon. Combining these data leads to the conclusion that on average 12.4 mg out of the 16.3 mg quercetin intake can reach the colon. When making the assumption that human colon volumes may vary between 0.5 - 1 L depending on dietary intake, quercetin concentrations of 40 - 80 μM in the colon are likely. Quercetin is commercially available and therefore intake of quercetin supplements may occur [34]. Ingestion of a 500 mg quercetin supplement would lead to an increment of approximately 40 times the physiological colon concentration, up to 2 - 3 mM, without scientific support regarding human safety in this concentration range.

Employing physiological relevant concentrations under optimized culture conditions, the results of the present study indicate that ascorbate-stabilized quercetin is an inhibitor of Caco-2 differentiation and modulates Caco-2 proliferation in a biphasic fashion. Furthermore, differentiating Caco-2 cells demonstrated glucuronidation, sulphation and methylation of quercetin aglycone. Quantification of these metabolites revealed that both apical and basolateral 4'-O-methyl-quercetin-3'-O-glucuronide correlated with the Caco-2 differentiation grade.

Based on mechanisms observed in human colon carcinoma cell lines exposed to nutrients with anti-carcinogenic properties, including flavonoids [24;35], fish and olive oil [25] and the short chain fatty acid butyrate [26;27], it was expected that quercetin would stimulate differentiation and decrease proliferation of Caco-2 cells. Interestingly, our experiments showed effects opposite to what would be expected for

a phytochemical with anti-carcinogenic potency. Previously, quercetin has been found to decrease proliferation [18;36-39], and enhance differentiation of human colon carcinoma cell lines [39]. The discrepancies in the effect of quercetin on cell differentiation observed between the present study and Wenzel *et al*. [39] might have been caused by usage of the HT-29 cell line in stead of Caco-2 cells. Moreover, comparison of their experimental protocol to that of the present study suggests that initial flavonoid exposure in a pre-confluent (40% confluency) stage of cell cultures might have contributed to the discrepancy in differentiation outcome, as compared to post-confluent experiments described in the present paper. Thus, the confluency stage of cell layers at initial exposure might be crucial for differentiation experiments, especially for Caco-2 cells, which at a post-confluent stage spontaneously differentiate into cells with small intestinal enterocyte-like features [3]. However, the confluency stage of Caco-2 cell cultures is probably not of importance for the outcome of proliferation experiments, since initial quercetin exposure of both pre-confluent [36] or confluent [38] Caco-2 cells resulted in inhibited cell proliferation. Strikingly, the above mentioned proliferation and differentiation experiments share a common experimental condition: all experiments were performed by exposing cells to quercetin for 2 - 3 days, without stabilization of the flavonoid [18;36-39]. Therefore, it is not clear whether these results are indeed caused by quercetin itself. Since our experiments have been performed with stabilized quercetin, it can be concluded that the observed effects are not caused by the flavonoids degradation products. Moreover, correction of ALP activity for the amount of cells present in transwell® inserts, indicated that inhibition of Caco-2 differentiation is not due to a quercetin mediated decrease in cell number.

Our data indicate that metabolism of quercetin is associated with the degree of cell differentiation. In the course of Caco-2 differentiation, the amount of quercetin aglycone remaining after 24 hours of incubation showed a decrease in favor of formation of its metabolites, predominantly of the differentiation dependent 4'-O-methyl-quercetin-3'-O-glucuronide. In a previous report the activity of the lipoxygenase enzyme, which is elevated in CRC [40], has been found to be inhibited most efficiently by quercetin when compared to its metabolites, regardless of the type or position of conjugation [14]. Thus, in terms of anti-carcinogenic potency quercetin seems to be more effective than its metabolites, when considering this particular mechanism in carcinogenesis. As in the present Caco-2 model, concentrations of

4'-O-methyl-quercetin-3'-O-glucuronide rose far above quercetin concentrations, this metabolite might have resulted into loss of quercetin's anti-carcinogenic effect.
It should be noted however, that these experimental conditions cannot be extrapolated to the *in vivo* gut. Following quercetin administration in the apical compartment, which stands as a model for the gut lumen, the flavonoid was metabolized by phase II enzymes. Consequently, Caco-2 cells were exposed to a mixture of quercetin and its metabolites during a 24 hour time period, until culture medium was changed. Obviously this condition is not likely to occur *in vivo*, as luminal quercetin and its metabolites will be excreted via faeces and/or eliminated from the gut lumen upon their absorption into plasma.
The presence of glucuronidated, methylated and sulphated quercetin conjugates in our experiment confirm the activity of UDP-glucuronosyltransferase (UDPGT), catechol-O-methyl transferase (COMT) and sulphotransferase, respectively in Caco-2 cells [23;41]. Murota *et al*. have described a time dependent increment of a metabolite described as "conjugated quercetin", present in both apical and basolateral compartments [23]. This quercetin conjugate might reflect 4'-O-methyl-quercetin-3'-O-glucuronide, of which formation has been shown to be differentiation dependent in our study.
The types of quercetin conjugation observed in the present study, *i.e.* methylation, glucuronidation and sulphation have also been described for human plasma after supplementation with quercetin or its conjugate rutin (quercetin-3-O-ß-rutinoside) [13;42]. Rutin is deconjugated in the intestinal tract and leads to local release of quercetin [11], as mimicked in our experiment by apical quercetin exposure of Caco-2 cells in transwell® inserts.

In the present paper, quercetin has been found to inhibit Caco-2 cell proliferation at concentrations ≥ 40 µM in the course of time, but strikingly only at t = 96 hours cell proliferation was increased at quercetin concentrations up to 20 µM. This biphasic effect on cell proliferation has been described before in experiments with the estrogen receptor (ER) positive human colon carcinoma cell lines HCT-116 and HT-29 and the human breast cancer cell line MCF-7, exposed to stabilized quercetin [19]. Assuming that the ER positive cell line Caco-2 [43;44] expresses higher ER levels as a function of the differentiation grade, biphasic cell proliferation in our study might be ER controlled as has been suggested for genistein mediated biphasic proliferation in Caco-2 [45] and MCF-7 cells [46].

Parallel to the iso-flavonoid genistein [45], in our experiments the ER-ligand and -agonist quercetin [47] at low concentrations probably acted as a phytoestrogen and stimulated cell proliferation through ER induced activation of intracellular protein tyrosine kinases. At high concentrations, however, quercetin leads to inhibition of cell proliferation, probably via inhibition of tyrosine kinases.

Since the mutagenic activity of quercetin has been reported in the late 1970's [48;49], the discussion whether quercetin is carcinogenic [19] or not [18;39] was stimulated. *In vivo* studies also show inconsistency: quercetin exerted both anti-carcinogenic [50] and pro-carcinogenic [51] activity in azoxymethane (AOM) induced colorectal cancer. Moreover, when administered alone, *i.e.* without AOM-induction of colorectal cancer, quercetin induced tumors in the ileal segment of the small intestine [52] and gave rise to putative preneoplastic lesions (focal areas of dysplasia) in the colon, in combination with increased colonic cell proliferation [53]. In line with the latter, enhanced cell proliferation was also found in the present *in vitro* model for colorectal cancer, but only at low quercetin concentrations. Together with the present findings, these observations indicate that the anti-carcinogenic effect of quercetin is still a matter of debate.

In conclusion, the present study indicates that ascorbate-stabilized quercetin modulates Caco-2 cell proliferation in a biphasic way and inhibits cell differentiation at physiological relevant concentrations. Biphasic modulation of cell proliferation occurred at a later time point and is probably dependent on the differentiation grade of Caco-2 cells and/or the presence of quercetin metabolites. Furthermore, quercetin aglycone is subject to intestinal phase II enzymes and metabolized into 4'-O-methyl-quercetin-3'-O-glucuronide, 3'-O-methyl-quercetin (= isorhamnetin), 4'-O-methyl-quercetin-7-O-glucuronide, 3'-O-methyl-quercetin-4'-O-glucuronide and 3'-O-methyl-quercetin-7-O-sulphate.

In general, proposed anti-carcinogenic mechanisms are not fully applicable to Caco-2 cells exposed to ascorbate-stabilized quercetin, probably as a result of flavonoid metabolism. To further elucidate the modulating effects of quercetin on cell proliferation and differentiation, transcriptome analysis will be performed to analyze which differentially expressed genes are involved in these processes.

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Chapter 3

Pathway and single gene analyses of inhibited Caco-2 differentiation by ascorbate-stabilized quercetin suggest enhancement of cellular processes associated with development of colon cancer

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Abstract

The aim was to investigate mechanisms contributing to quercetin's previously described effects on cell-proliferation and -differentiation, which contradicted its proposed anti-carcinogenic potency. In a 10-day experiment, 40 μM quercetin stabilized by 1 mM ascorbate reduced Caco-2 differentiation up to 50% ($P < 0.001$). Caco-2 RNA from days 5 and 10, hybridized on HG-U133A 2.0 Affymetrix® GeneChips®, showed 1,743 affected genes on both days ($P < 0.01$). All 14 Caco-2 differentiation-associated genes showed decreased expression ($P < 0.01$), including *intestinal alkaline phosphatase* that was confirmed technically (qRT-PCR) and functionally (enzyme-activity).

The 1,743 genes contributed to 27 affected pathways ($P < 0.05$) categorized under 6 gene ontology (GO) processes, including apoptosis and cell-cycle. Genes within these GO-processes showed fold changes that suggest increased cell survival and proliferation. Furthermore, quercetin downregulated expression of genes involved in tumor-suppression and phase II metabolism, and upregulated expression of oncogenes.

Gene expression changes mediated by ascorbate-stabilized quercetin were concordant with those occurring in human colorectal carcinogenesis (≈ 80 - 90%), but were opposite to those previously described for Caco-2 cells exposed to quercetin in the absence of ascorbate (≈ 75 - 90%).

In conclusion, gene expression among Caco-2 cells exposed to ascorbate-stabilized quercetin showed mechanisms contrary to what is expected for a cancer-preventive agent, and is associated with the presence of ascorbate. Whether this unexpected *in vitro* effect is relevant *in vivo*, remains to be elucidated.

Introduction

Quercetin is a phytochemical that belongs to the polyphenol group of anti-oxidants. This dietary compound is part of the flavonol subclass of flavonoids and can be found in glycosylated forms in fruits and vegetables, including curly kale, broccoli, blueberries and onions [1]. Following intake, conjugated quercetin can be hydrolyzed by ß-glycosidases present in the cytoplasm or on the membrane of small intestinal cells [2]. When absorbed from the small intestine, quercetin can be metabolized by phase II enzymes present in small intestinal cells and the liver. On the other hand, conjugated quercetin that reaches the colon can be hydrolyzed by bacterial ß-glycosidases [3], leading to colonic release and absorption of quercetin [4].
In the healthy colon, uptake of nutrients from the colon lumen is facilitated by foldings in the intestinal mucosa that enlarge the absorption area. The base of the colonic crypts contains a limited number of stem cells which after proliferation migrate towards the lumen and can differentiate into absorptive cells (enterocytes), goblet cells (responsible for secretion of protective mucins) or enteroendocrine cells (secretion of hormones, including serotonin) [5]. Within 4 - 8 days after migration from the base of the crypt, these different cell types reach the top of the villus, become apoptotic and are shedded in the gut lumen [6]. The continuous process of proliferation, differentiation and apoptosis that occurs along the crypt axis is strictly regulated. Once this equilibrium is perturbed, for example as a result of underlying gene mutations, colorectal tumors may develop through a gradual series of well-characterized histo-pathological changes, also known as the adenoma-carcinoma sequence [7].
The human derived colon cancer cell line Caco-2 is a well-accepted model to study cell proliferation, differentiation and apoptosis as a function of time [8]. When these cells proliferate toward a monolayer, contact inhibition occurs that leads to cell cycle arrest and spontaneous differentiation into absorptive cells [5]. This time-dependent process mimicks phenotypic changes that normal colonic epithelial cells undergo during migration along the crypt-villus axis *in vivo* [5]. A typical feature of differentiated Caco-2 cells is the brush border with a high density of microvilli. Brush border enzymes that are known to be positively correlated with the differentiation grade of enterocytes and therefore extensively used as Caco-2 differentiation markers, include *intestinal alkaline phosphatase* (*ALPI*) [6] and *sucrase-isomaltase* [8;9].

Quercetin is generally recognized as a compound that inhibits mechanisms involved in development of (colorectal) cancer, as demonstrated by *in vitro* [10;11] and *in vivo* [12;13] studies. However, data regarding its mechanisms of action are relatively scarce and the beneficial effect of quercetin on colorectal cancer is under debate [13;14]. In a previous study performed with Caco-2 cells, quercetin-treatment resulted in effects opposite to what would be expected for a phytochemical with anti-carcinogenic potency, including increased proliferation and inhibited differentiation [15]. The aim of the present study was to further investigate this unexpected effect in Caco-2 cells by characterizing quercetin-induced changes in cellular physiology and gene expression in the context of biological pathways and gene ontology (GO) processes.

Material and methods

Cell culture

The human colon cancer cell line, Caco-2, was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). For subculturing, near-confluent Caco-2 monolayers of passage 35 and 36 were seeded in a 1 : 10 split ratio in T75 flasks (Costar, Cambridge, U.K.) with DMEM culture medium, as described previously [15]. For differentiation experiments, Caco-2 cells of passage 37 were seeded (1 : 10 split ratio) in triplicate on polycarbonate membrane transwell® inserts (Corning Life Sciences, Cambridge, U.K.), with a membrane diameter of 75 mm (growth area 44 cm^2, 0.4 μm pore size) at a density of ± 40,000 cells/cm^2. After 2 days, cell cultures reached confluency, and this time point was considered experimental day 0.

Quercetin exposure

To prevent quercetin's unstability in culture medium [11], 40 μM quercetin (Sigma-Aldrich, Zwijndrecht, The Netherlands) out of a 200 x stock solution in DMSO (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared in DMEM culture medium shortly before cell exposure and stabilized by 1 mM (final concentration) sodium ascorbate (Boom B.V., Meppel, The Netherlands) [15]. This culturing condition is further referred to as "40 μM quercetin". From days 0 - 9, Caco-2 monolayers were exposed to 8 ml of 40 μM quercetin in the apical compartment only,

in order to mimic exposure as it would occur in the gut. As controls, cells were exposed to 8 ml of 1 mM sodium ascorbate, including the quercetin solvent (0.5% DMSO), a condition previously shown not to interfere with Caco-2 differentiation [15]. The latter culture condition is further referred to as "control". Basolateral compartments of both experimental conditions were filled with 12 ml DMEM culture medium. In the course of the experiment, culture medium in both compartments was refreshed every 24 hours.

Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured as a marker for Caco-2 cell differentiation [15]. As temperature fluctuations may influence the outcome of the TEER measurement, transwell® inserts were cooled down to room temperature (20 ± 1°C) prior to TEER measurement. On days 0, 3, 5, 7 and 10 post-confluency TEER was measured in triplicate, using a Millicel-ERS Volt Ohm meter (Millipore, Amsterdam, The Netherlands). TEER values were calculated according to the following equation: TEER = R x filter area ($\Omega.cm^2$).

Alkaline phosphatase

Activity of alkaline phosphatase, a Caco-2 differentiation marker, was determined on a BM/Hitachi 911 analyzer according to a colorimetric assay as described previously [6]. In brief, ALP converts the substrate p-nitrophenol phosphate into p-nitrophenol. Concomitantly, the time-dependent release of p-nitrophenol is proportional to the ALP activity. The activity of this enzyme was determined in the apical culture medium, since in previous studies intracellular ALP was shown to be correlated with its presence and activity in the apical culture medium [16;17]. ALP activity among quercetin-treated and control cells was first corrected for baseline ALP activity in DMEM culture medium. Subsequently, corrected ALP activity was normalized for the number of cells in culture, by measurement of total protein in the apical culture medium.

RNA extraction, cleanup and quality control

On days 5 and 10 post-confluency, cells were first rinsed with ice cold phosphate buffered saline without calcium or magnesium (Invitrogen™ Life Technologies, Breda, The Netherlands). Subsequently, cells were harvested in 2 x 0.75 ml of ice-

cold TRIzol® (Life Technologies, Paisley, U.K.), immediately frozen in liquid nitrogen and stored at -80°C for up to 2 months, until RNA isolation according to the TRIzol® protocol. Following isolation, total RNA was purified with RNeasy Midi columns (QIAGEN, Westburg, Leusden, The Netherlands), including a DNAse incubation step. Quality of purified total RNA was determined on a UV-VIS spectrofotometer (Shimadzu Benelux, 's Hertogenbosch, The Netherlands) by calculation of the A_{260}/A_{280} ratio (1.7 - 2.0) and confirmed on a Lab-on-a-Chip on the Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, California, U.S.A.) by determining the 28S/18S ribosomal RNA ratio (1.7 - 2.0).

RNA preparation for Affymetrix® GeneChip® arrays and hybridization

RNA originating from quercetin treated and control Caco-2 cells harvested on days 5 and 10, was used as an input for hybridization to HG-U133A 2.0 Affymetrix® GeneChip® arrays that comprise 22,215 probe sets, representing 14,500 well-characterized human genes.

For hybridization, the GeneChip® One-Cycle Eukaryotic Target Labeling Assay for expression analysis was performed, as described in the Affymetrix® GeneChip® Expression Analysis Technical Manual [18]. In brief, 2 µg of purified total RNA was reverse transcribed using a T7-Oligo(dT) Promoter Primer for synthesis of the first-strand complementary DNA (cDNA). Subsequently, double stranded cDNA was produced to serve as a template for the *in vitro* transcription. After cRNA fragmentation, meant for optimal hybridization efficiency, 10 µg of fragmented cRNA was hybridized to Affymetrix® GeneChip® HG-U133A 2.0 arrays and finally detected with the GeneChip® Scanner 3000 7G (Affymetrix® Inc., Santa Clara, California, U.S.A.).

Analysis of Affymetrix® GeneChip® array data

CEL files containing raw signal intensities, were imported in Rosetta Resolver 5.0 (Rosetta Inpharmatics LLC, Seattle, U.S.A.), applying data pre-processing (to estimate and reduce systematic errors) and error modeling (for improvement of accuracy of the present and absent calls for low replicate numbers). Genes with an "absent call" ($P \geq 0.065$ for HG-U133A 2.0 GeneChip® arrays) across all 8 arrays

(*n* = 7,285) were considered not to be affected by quercetin treatment and/or incubation time and were therefore excluded from further analyses (**Figure 1**).

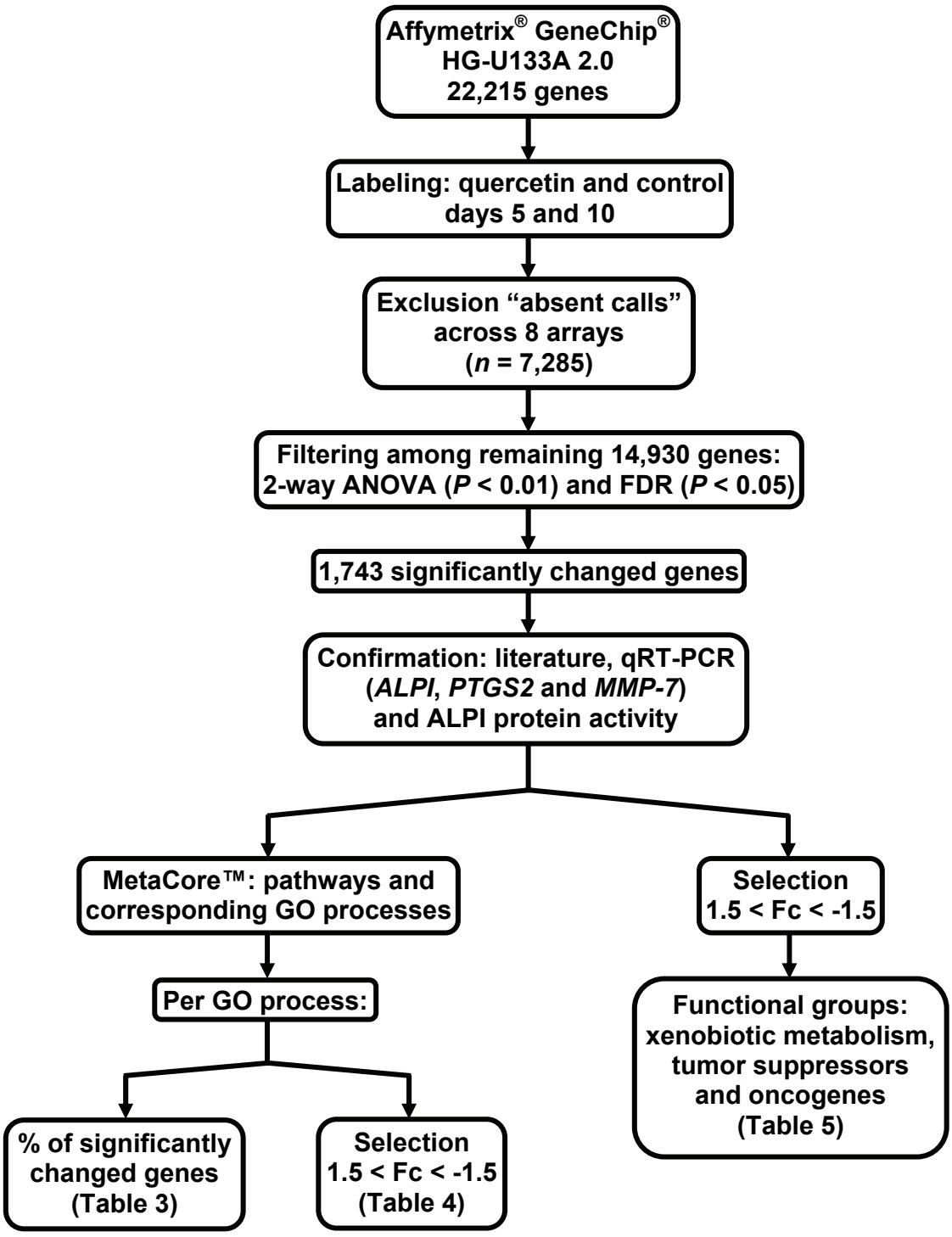

Fig. 1. Flow chart of statistical filtering of gene expression data analysis. FDR: False discovery rate; Fc: fold change.

Subsequently, normalized intensities originating from replicate Affymetrix® GeneChip® arrays (n = 2/group) were combined and fold changes (quercetin vs. control) were calculated for days 5 and 10. For selection of genes that changed upon quercetin-treatment in time, the remaining genes (n = 14,930) were analyzed by a combination of the 2-way ANOVA ($P < 0.01$), including error-weighting (to increase degrees of freedom) and the false discovery rate (FDR < 0.05) [19]. This analysis retrieved 1,743 significantly changed genes (≈ 8% of the initial number of genes). To interpret these differentially expressed genes at a higher biological level rather than the level of single genes only, data were loaded in the pathway mapping tool MetaCore™ (GeneGo Inc., St. Joseph, MI, U.S.A.). Significantly changed pathways were subsequently used to determine the relative contribution of significantly changed genes to pathway-associated gene ontology (GO) processes. To this end, different pathways categorized under identical GO processes were first grouped. Subsequently, within each set of grouped pathways, *i.e.* within each GO process, the number of significantly changed genes was expressed as percentage of the total number of genes. MIAME (Minimum Information About a Microarray Experiment) compliant data described in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number **GSE7259**.

Confirmation of Affymetrix® GeneChip® array expression data

To support the biological relevance of the statistically filtered dataset, a search was performed for the presence of previously described Caco-2 differentiation markers, regardless of the direction and/or magnitude of the fold change [8].

To confirm Affymetrix® GeneChip® array expression data and the biological relevance of the dataset obtained by statistical analyses, a subset of relevant genes, including the Caco-2 differentiation marker *intestinal alkaline phosphatase* (*ALPI*) [6], and the human CRC-associated genes encoding for cyclo-oxygenase 2 (COX-2) [20] and matrix metalloprotease 7 (MMP7) [21], was confirmed by means of real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Primers for real-time qRT-PCR were developed in Beacon Designer 4.02 (Premier Biosoft, Palo Alto, California, U.S.A.). Furthermore, the ALPI enzyme was confirmed functionally by measurement of its activity corrected for total protein in the apical culture medium [6].

Quantitative Real-Time Polymerase Chain Reaction

Per sample (n = 3/group) 200 ng total RNA was reverse transcribed into complementary DNA (cDNA) in an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands), using the iScript™ cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). This reaction was performed in a final volume of 20 µl containing 5% (v/v) of a Moloney murine leukaemia virus (MMLV) derived reverse transcriptase with RNAse inhibitor and 20% (v/v) of a 5x iScript™ Reaction Mix. Subsequent real-time qRT-PCR was performed either with the iQ™ SYBR® Green Supermix or the TaqMan® assay (**Table 1**).

The SYBR® Green assay was performed in a final volume of 25 µl containing 50% (v/v) of the 2x iQ™ SYBR® Green Supermix, 0.3 µM primermix with both forward and reverse primers (Biolegio, Nijmegen, The Netherlands) and 5 ng of cDNA. An initial denaturation step of 3 minutes at 95°C was followed by 45 cycles, each consisting of 94°C for 15 seconds, followed by annealing (temperatures given in **Table 1**) for 30 seconds and elongation at 72°C for 20 seconds. Subsequently, a melting curve was generated to ensure amplification of only the proper PCR product. This was performed by 80 cycles of 10 seconds each, starting at 54°C, increasing with 0.5°C per cycle up to 94°C and measuring the fluorescence.

The TaqMan® assay was carried out in a final volume of 25 µL with 57% (v/v) TaqMan® universal mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), ≈ 0.9 µM primer mix with both forward and reverse primers, ≈ 0.2 µM of the corresponding TaqMan® probe (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 50 pg of cDNA. After an initial denaturation step of 10 minutes at 95°C, 45 cycles were run, each of which consisted of 95°C for 15 seconds and 60°C for 60 seconds.

Real-time qRT-PCR reactions for both TaqMan® and SYBR® Green assays were performed in the iCycler iQ™ Real-Time PCR Detection System. Absolute copy numbers of the genes of interest were determined by linear regression from cDNA calibration curves of each gene. Expression data obtained by real-time qRT-PCR were normalized against the amount of total RNA used for each sample (200 ng), expressed as copies per microgram of RNA [22] and against the housekeeping genes *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *ß-actin* (*ACTB*) that were both unchanged upon quercetin treatment, according to the microarray data.

Table 1. Overview of primer sequences used for real-time quantitative RT-PCR

Gene symbol	Accession #	Assay	Primer sequence 5' → 3'		Amplicon length (bp)	Annealing Temperature (°C)
ALPI	NM_001631	SYBR® Green	Forward:	CAGTCCTCTGCTGTCTCC	76	64
			Reverse:	AAGCCTCTGCCTCATTGG		
PTGS2	NM_000963	SYBR® Green	Forward:	TGACAGTCCACCAACTTACAATG	119	65
(COX-2)			Reverse:	CCAAGGGAGTCGGGCAATC		
MMP7	NM_002423	SYBR® Green	Forward:	GGAACAGGCTCAGGACTATC	180	59
			Reverse:	ACATCTGGCACTCCACATC		
ACTB	NM_001101	TaqMan®	Forward:	CTGACTGACTACCTCATGAAGATCCT	86	-
			Reverse:	CTTAATGTCACGCACGATTTCC		
			Probe:	TACAGCTTCACCACCACGGCCGA		
GAPDH	NM_002046	TaqMan®	Forward:	AGCCTCAAGATCATCAGCAATG	107	-
			Reverse:	ACTGTGGTCATGAGTCCTTCCA		
			Probe:	CACCCCTGGCCAAGGTCATCCA		

qRT-PCR primers at a length of 18 - 26 bp were designed for either the SYBR® Green or TaqMan® assay. *ALPI*: alkaline phosphatase, intestinal; *PTGS2*: prostaglandin-endoperoxide synthase 2, encodes the Cyclooxygenase 2 (COX-2) enzyme; *MMP7*: matrix metalloprotease 7; *ACTB*: beta actin; *GAPDH*: glycerol-dehyde-3-hosphate dehydrogenase

Comparison with human colorectal carcinogenesis

Quercetin-induced fold changes in gene expression among Caco-2 cells were compared to fold changes previously described for human colorectal carcinogenesis. To this end, genes contributing to significantly changed pathways were selected based on an identical direction of fold change on both days 5 and 10 and a minimum up- or downregulation of 1.5 on both days (**Figure 1**). Gene expression data for human colorectal carcinogenesis were retrieved from PubMed articles, including microarray based studies, or from the Cancer Profiling Database Oncomine™ (http://www.oncomine.org/main/index.jsp).

In addition to MetaCore™ based analyses, the total set of 1,743 differentially expressed genes was evaluated for the presence of genes contributing to functional groups that are likely to be involved in nutritional modulation of colorectal cancer, *i.e.* tumor suppressor genes, oncogenes and genes encoding for enzymes involved in xenobiotic metabolism (**Figure 1**). These *in vitro* data were retrieved and compared with previously published data for human colorectal carcinogenesis, as described above.

Comparison to microarray data of Caco-2 cells exposed to quercetin without ascorbate

To determine whether effects on Caco-2 cells are caused by interference of ascorbate that is required for quercetin-stabilization, 2 comparisons were made with data from our previous report in which post-confluent Caco-2 cells were exposed to 5 and 50 μM quercetin for 48 hr, without ascorbate [10]. First, significantly affected genes as presented in **Table 2** (differentiation genes), **Table 4** (genes contributing to significantly changed pathways) and **Table 5** (single gene analysis) were compared to genes found in our previous study performed with quercetin only [10]. For the latter study, genes were included that were found to be present in at least 3 out of 4 microarrays per treatment group, showing an identical direction of fold change for both quercetin concentrations and at least one significant *P*-value of < 0.05, or in the case of data available for only one of two quercetin concentrations, showing a *P*-value of < 0.05.

Secondly, the list of 281 significantly changed genes from our previous study performed with quercetin only [10] was first filtered as mentioned above, and subsequently compared to a subset of 1,743 differentially expressed genes in the

present study performed with ascorbate-stabilized quercetin, retrieved after filtering on genes showing an identical direction of fold change on both days.

Statistics on "non-omics" parameters

Quercetin-induced effects on TEER, real-time qRT-PCR and ALPI activity were tested for significance with the Student's *t*-test and considered significantly changed when $P < 0.05$.

Results

Transepithelial electrical resistance

TEER was measured as an indicator of Caco-2 cell differentiation. In the course of the experiment, quercetin-treated cells showed a significantly lower increase in TEER values when compared to control cells (**Figure 2**). On day 10 post-confluency, TEER values for quercetin treated cells amounted to ≈ 50% of control cells. These results indicate that exposure of Caco-2 cells to 40 μM quercetin resulted in decreased cell differentiation.

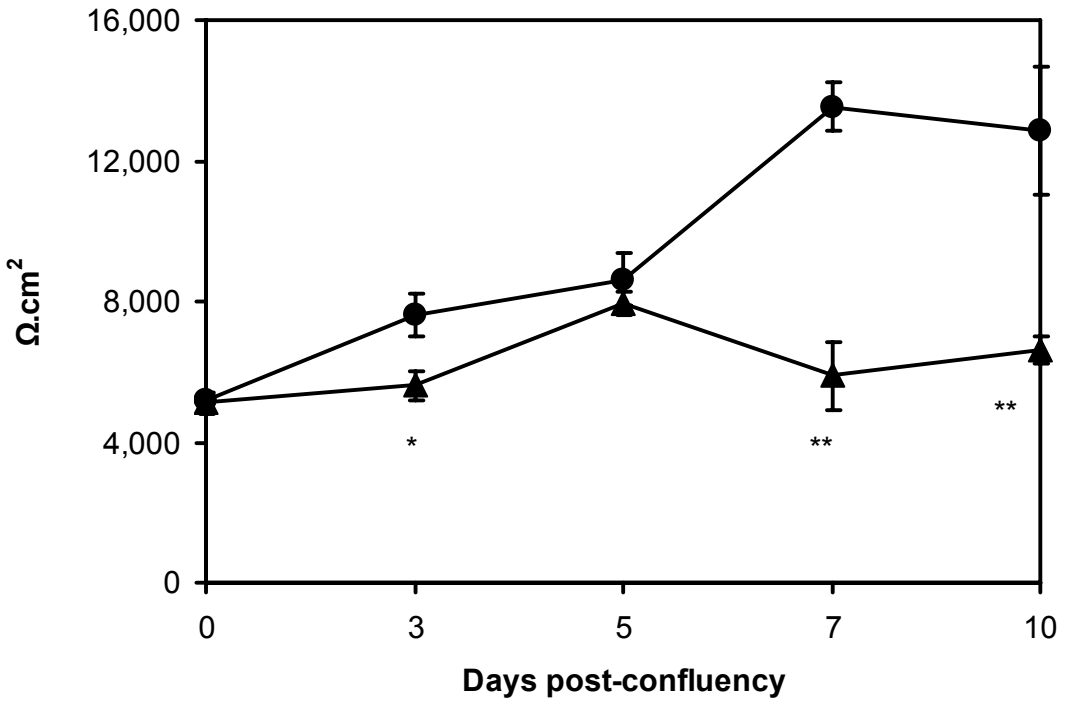

Fig. 2. Time dependent TEER values in 40 μM quercetin (triangles) and control cells (dots). Values are given as mean ± SD (n = 3/group). * $P < 0.01$ and ** $P < 0.001$ when compared to control cells.

Confirmation of decreased Caco-2 differentiation

To further investigate whether decreased Caco-2 cell differentiation could be supported by Affymetrix® GeneChip® array data, fold changes of Caco-2 differentiation related genes among the 1,743 significantly changed genes were compared with available Caco-2 literature. As can be seen in **Table 2**, mRNA expression of all 14 Caco-2 differentiation markers found among the significantly changed genes, including *intestinal alkaline phosphatase* (*ALPI*), *sucrase-isomaltase*, *dipeptidylpeptidase IV* (*DPP4* = CD26), *aminopeptidase N* and *P* that are expressed in the enterocyte brush border [5;8;23], were found to be decreased by quercetin. In addition, the main regulator of intestinal cell differentiation, *CDX2* [24], was near-significantly downregulated (fold change day 5: -2.0 and day 10: -1.2, $P < 0.03$). Decreased Caco-2 cell differentiation upon exposure to ascorbate-stabilized quercetin, as measured by the physiological parameter TEER, was thus confirmed by decreased expression of differentiation related genes among the statistically filtered data set.

Table 2. Expression data of genes that are positively correlated with Caco-2 cell differentiation and their behavior upon quercetin treatment, either with or without ascorbate stabilization.

				Fold change quercetin vs. control				
				+ ascorbate, + quercetin			- ascorbate, + quercetin	
Gene title (synonym)	**Gene symbol**	**Function**	**References**	**Day 5**	**Day 10**	***P* <**	**5 µM (*P*<)**	**50 µM (*P*<)**
Alkaline phosphatase, intestinal	*ALPI*	Cleavage of phosphate groups on DNA, RNA, (deoxy)-ribonucleosides and proteins	[5;6;23]	-2.1	-2.1	0.00609	1.5 (0.05)	1.5 (0.1)
Sucrase-isomaltase	*SI*	Digestion of sucrose and isomaltose in the intestine	[9]	-38.0	-3.8	2.3E-06	-	-
Calbindin 3 (Calbindin-D9K)	*S100G CALB3*	Vitamin D-dependent calcium binding	[8]	-4.6	-2.3	6.9E-12	-	-
Dipeptidylpeptidase IV (CD26)	*DPP4*	Proteolysis, immune response	[23]	-2.2	-1.4	0.00125	-	-
Glutaminase	*GLS*	Glutamine catabolism	[8]	-1.8	-1.9	0.00042	-	-
Hephaestin	*HEPH*	Copper and iron ion transport	[8]	-2.0	-1.2	9.6E-07	-	-
Transferrin	*TF*	Secreted Fe^{3+} transport protein	[9]	-7.2	-1.9	0.00007	-	-
Aquaporin 3	*AQP3*	H_2O transport	[8]	-6.4	-2.6	4.0E-13	-	-

Table 2, continued

				Fold change quercetin vs. control				
				+ ascorbate, + quercetin			- ascorbate, + quercetin	
Gene title (synonym)	**Gene symbol**	**Function**	**References**	**Day 5**	**Day 10**	***P* <**	**5 µM (*P*<)**	**50 µM (*P*<)**
Fatty acid binding protein 6, ileal (Gastrotropin)	*FABP6*	Lipid metabolism and negative regulation of cell proliferation	[39]	-2.2	-2.5	0.00697	-	-
Alanyl (membrane) aminopeptidase (Aminopeptidase N, Aminopeptidase M, Microsomal aminopeptidase, CD13, p150)	*ANPEP*	Cell differentiation and development	[23]	-4.4	-1.4	1.9E-08	-	-
Membrane-bound aminopeptidase P (XNPEP2)	*XPNPEP2*	Proteolysis by metalloexo-peptidase activity	[23]	-6.9	-3.0	0.00002	-	-
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	*ACE2*	Proteolysis by carboxypeptidase activity and zinc ion binding	[23]	-3.5	-1.6	0.00972	-	-
Apolipoprotein B	*APOB*	Lipid metabolism	[8]	-3.6	-1.6	1.1E-16	-	-
Apolipoprotein M (G3A)	*APOM*	Membrane lipid metabolism, lipid	[8]	-3.7	-2.0	0.00116	-1.4 (0.5)	-1.4 (0.01)

All 14 Caco-2 cell differentiation related genes were significantly downregulated by 40µM quercetin stabilized by 1 mM ascorbate. References indicate articles in which these genes have been reported as being positively correlated with Caco-2 cell differentiation. For comparison of quercetin-mediated effects in the absence of ascorbate, in the last two columns data are shown from Caco-2 cells exposed to 5 or 50 µM quercetin only, as described in our previous report [10].

Confirmation of Affymetrix® GeneChip® array expression data

In addition to the above mentioned literature-based confirmation, decreased mRNA expression of the Caco-2 differentiation marker *ALPI* as indicated by Affymetrix® GeneChip® arrays was confirmed both technically - by means of real-time qRT-PCR - and functionally by measurement of ALPI enzyme activity. As can be seen in **Figure 3A**, *ALPI* expression was significantly decreased on days 5 and 10 as determined with Affymetrix® GeneChip® arrays, as quantified by real-time qRT-PCR corrected for both total RNA concentration and the housekeeping genes *GAPDH* and *ß-actin*, and as determined with ALPI enzyme activity corrected for the number of cells in culture. In addition, since decreased cell differentiation is a hallmark of (colon) cancer [25], the possibility of differential expression of the colon cancer associated genes encoding for cyclo-oxygenase 2 (COX-2) [20] and matrix metalloprotease 7 (MMP7) [21] was also investigated by means of real-time qRT-PCR. Fold changes of these genes as obtained with Affymetrix® GeneChip® arrays compared to fold changes obtained with real-time qRT-PCR after correction for *GAPDH*, *ß-actin* and total RNA, were also in agreement with one another (**Figures 3B** and **C**).

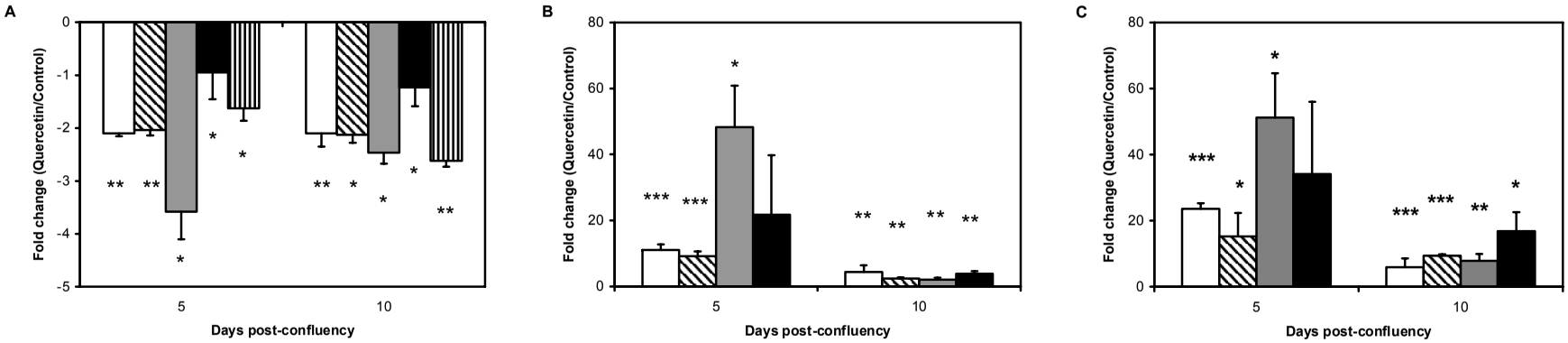

Fig. 3. Fold changes (quercetin/control) of *ALPI* (panel A), *PTGS2*, which encodes the COX-2 enzyme (panel B) and *MMP7* (panel C) on days 5 and 10, determined by measurement by Affymetrix® GeneChip® arrays (white bars), by real-time qRT-PCR corrected for the house keeping genes *GAPDH* (diagonally lined bars), *ß-actin* (grey bars) or for total RNA (black bars), and measurement of *ALPI* activity in the apical culture medium corrected for the number of cells (panel A, vertictally lined bars, expressed as µmol nitrophenol/minute/mg protein). Values are presented as mean ± SD, $n = 3$/group for real-time qRT-PCR and $n = 2$/group for Affymetrix® GeneChip® arrays. * $P < .0.05$; ** $P < .001$; *** $P < 0.001$

Biological pathways and corresponding GO processes

Since Affymetrix® GeneChip® array expression data were confirmed with additional techniques, this reliable gene expression data set was subsequently used for further analyses. In order to visualize and to interpret the statistically filtered set of 1,743 significantly changed genes at a higher biological level, this data set was loaded in the MetaCore™ software, in order to find affected biological pathways and their corresponding GO processes. **Table 3** shows an overview of significantly changed pathways ($n = 27$, $P < 0.05$) and GO processes. When the number of significantly changed genes within each GO process was expressed as percentage of the total number of genes within the very same GO process, the following percentages were found: cell cycle: 31%, apoptosis and cell death: 31%, transcription: 29%, protein kinase cascade: 26%, proteolysis: 25%, small GTPase mediated signal transduction: 24% and unknown: 30%. Within the specified GO processes showing the highest percentage of differentially expressed genes, *i.e.* "cell cycle", and "apoptosis and cell death", the most significantly changed pathways are "Role of anaphase promoting complex (APC) in cell cycle regulation" ($P = 0.0004$) and "Role of inhibitor of apoptosis proteins (IAP) in apoptosis" ($P = 0.035$), respectively (**Table 3**). In the "Role of anaphase promoting complex (APC) in cell cycle regulation" pathway, fold changes in gene expression suggest increased cell proliferation, as depicted in **Figure 4**. Among the six above mentioned GO processes (GO process "unknown" was excluded) affected by significantly changed pathways, 48 genes showed a combination of an identical direction of fold change and a minimum up- or downregulation of 1.5 on both days (**Table 4**). As can be seen in the GO processes "apoptosis and cell death", quercetin-treatment probably resulted in inhibition of apoptosis, as suggested by upregulation of apoptosis inhibitors, e.g. *BIRC3* and downregulation of apoptosis inducers, e.g. *TNFSF10*.

Table 3. Overview of significantly ($P < 0.05$) changed pathways after analysis of 1,743 significantly changed genes

Pathway	Pathway *P*-value	GO process	No. of significantly changed genes in pathway	Total no. of genes in pathway	% Changed in pathway	% Present on arrays
Androstenedione and testosterone biosynthesis and metabolism *p.2*	0.00000	Unknown	14	28	50.0	77.8
Androstenedione and testosterone biosynthesis and metabolism *p.3*	0.00002	Unknown	15	36	41.7	89.3
Role of APC in cell cycle regulation	0.00040	Cell cycle	17	54	31.5	87.0
Chromosome condensation in prometaphase	0.00044	Cell cycle	15	45	33.3	66.7
AP1 activation by TRAF proteins signaling pathway	0.00092	Apoptosis	12	34	35.3	97.1
AP1 activation by MADD proteins and c-FLIP/ c-RAF-1 related pathway	0.00299	Apoptosis cell death	10	29	34.5	100.0
Estradiol metabolism	0.00415	Unknown	7	17	41.2	88.2
Nucleocytoplasmic transport of CDK/Cyclins	0.00540	Cell cycle	8	22	36.4	95.5
Role SUMO in p53 regulation	0.00540	Cell cycle	8	22	36.4	95.5
Sister chromatid cohesion	0.00547	Cell cycle	13	46	28.3	63.0
Folic acid metabolism	0.00936	Unknown	6	15	40.0	100.0
Methionine-cysteine-glutamate metabolism	0.01332	Unknown	6	16	37.5	100.0

Table 3, continued

Pathway	Pathway *P*-value	GO process	No. of significantly changed genes in pathway	Total no. of genes in pathway	% Changed in pathway	% Present on arrays
Aspartate and asparagine metabolism	0.01455	Unknown	5	12	41.7	91.7
Transition and termination of DNA replication	0.01604	Cell cycle	10	36	27.8	94.4
Role of Akt in hypoxia induced HIF1 activation	0.02037	Proteolysis	12	48	25.0	91.7
Rac2 regulation pathway	0.02255	Unknown	4	9	44.4	100.0
P53 signaling pathway	0.02584	Transcription	11	44	25.0	95.5
Role ASK1 under oxidative stress	0.03124	Transcription	8	29	27.6	86.2
Role of IAP-proteins in apoptosis	0.03543	Apoptosis ,cell death	9	35	25.7	88.6
TRAF proteins signaling network	0.03543	Apoptosis, cell death death	9	35	25.7	100.0
Lipoprotein metabolism II. HDL metabolism	0.04057	Unknown	6	20	30.0	95.0
Glutathione metabolism	0.04189	Unknown	9	36	25.0	77.8
Catecholamine metabolism	0.04209	Unknown	12	53	22.6	83.0

Table 3, continued

Pathway	Pathway *P*-value	GO process	No. of significantly changed genes in pathway	Total no. of genes in pathway	% Changed in pathway	% Present on arrays
Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis	0.04527	Unknown	8	31	25.8	96.8
dATP/dITP metabolism	0.04665	Unknown	11	48	22.9	91.7
Role 14-3-3 proteins in cell cycle regulation	0.04835	Cell cycle	7	26	26.9	96.2
dGTP metabolism	0.04910	Unknown	9	37	24.3	91.9

Per pathway, the corresponding GO process is indicated and the number of genes obtained by statistical filtering (column "No. of significantly changed genes in pathway") is expressed as percentage (column "% Changed in pathway") of the total number of genes present in that particular pathway (column "Total no. of genes in pathway"). The column "% Present on arrays" represents the fraction of genes present on the HG-U133A 2.0 GeneChip® arrays relative to the total number of genes present in that particular pathway.

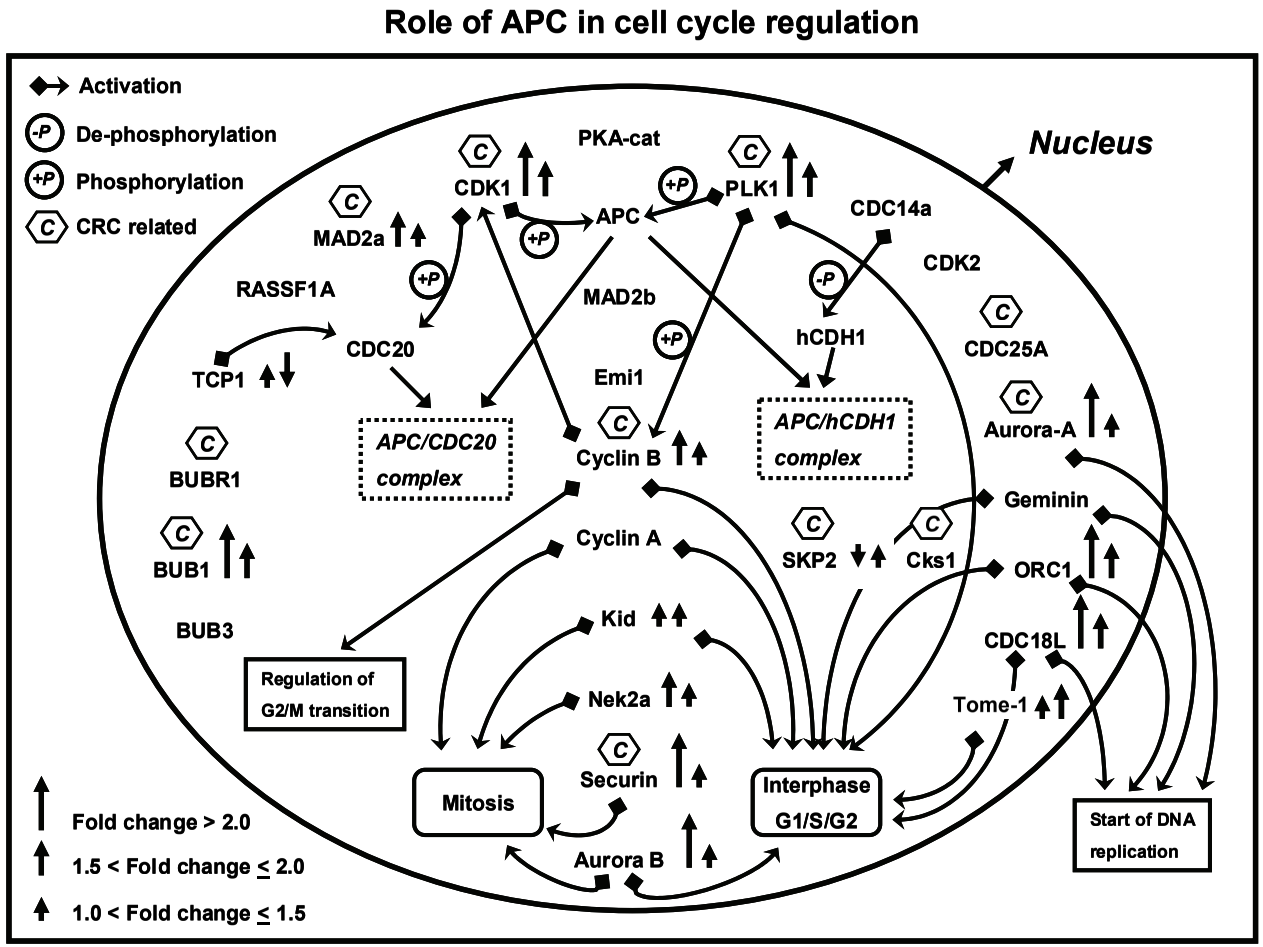

Fig. 4. Pathway entitled "Role of Anaphase promoting complex (APC) in cell cycle Regulation", including visualization of fold changes and colorectal cancer related genes expressed in the nucleus (adapted from MetaCore™). Per gene, the two neighboring arrows indicate fold changes on day 5 (left arrow) and day 10 (right arrow). Abbreviations used and synonyms: APC: Anaphase promoting complex; Aurora-A (= *STK6* in mice, *STK15* in humans): *Serine/threonine kinase 6*; *BUB1*: *Budding uninhibited by benzimidazoles 1*; *PLK1*: *Polo-like kinase 1*; *CDC*: *Cell division cycle*; *CDC18L* = *CDC6*; *CDK1* = *CDC2*; Kid = *KIFF22*: *Kinesin family member 22*; *MAD2a* = *MAD2L1*: *Mitotic arrest-deficient 2*; *ORC1*: *origin recognition complex subunit 1;* Securin = *PTTG1*: *Pituitary tumor-transforming 1* (= Tumor transforming protein 1); Tome-1 = *CDCA3*: *Cell division cycle associated 3*.

Table 4. Selection of genes contributing to GO processes affected by 27 significantly changed pathways

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
Cell Cycle	*BUB1 budding ninhibited by benzimidazoles 1 homolog (yeast)*	*BUB1*	1.8	2.2	↑	[40] *S*	Yes	-	-1.5 (0.05)	Yes
	Citron (rho-interacting, serine/threonine kinase 21)	*CIT*	1.5	1.9	-	-	-	-	-	
	Antigen identified by monoclonal antibody Ki-67	*MKI67*	1.9	2.2	↑	[40] *S*	Yes	-	-	
	HRAS-like suppressor 3	*HRASLS3 (HREV107)*	1.8	1.5	↑	[40] *S*	Yes	1.3 (0.02)	1.3 (0.05)	No
	Aurora kinase B	*AURKB (AIM-1, STK12, ARK2)*	2.1	1.7	↑	[41]	Yes	-	-	

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Kinesin family member 23	*KIF23*	1.5	1.9	-	-	-	-		
	CDC6 cell division cycle 6 homolog (S. Cerevisiae)	*CDC6 (CDC18)*	1.6	2.9	↑	[40] *S*	Yes	-4.1 (0.01)	-	Yes
	Discs, large homolog 7 (Drosophila)	*DLG7 (DLG1)*	1.6	2.2	↑	[42]	Yes	-	-	
	Polo-like kinase 1	*PLK1*	1.8	2.1	↑	[43] *S*	Yes	-1.5 (0.02)	-1.7 (0.01)	Yes
	Annexin A1	*ANXA1*	4.9	5.8	↑	[28]	Yes	-1.3 (0.1)	-0.3 (0.05)	Yes
	Cyclin D2	*CCND2*	2.9	7.4	↑	[40] *S*	Yes	-	-	
	G0/G1switch 2	*G0S2*	-2.7	-2.5	↑	[40] *S*	No	-	-	

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
Apoptosis, cell death	*Egl nine homolog 3 (C. Elegans)*	*EGLN3*	2.9	4.2	-	-		-	-	
	Pleckstrin homology-like domain, family A, member 1	*PHLDA1*	2.0	1.6	↑	[44]	Yes	-	-	
	Baculoviral IAP repeat-Containing 3	*BIRC3*	2.8	1.5	↑	[45]	Yes	-	-	
	Pleckstrin homology-like domain, family A, member 2	*PHLDA2*	1.7	3.9	-	-		-	-	
	Immediate early response 3	*IER3*	2.6	5.3	-	-		-	-	
	Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	3.9	10.8	↑	[46] *P*	Yes	-	-	
	Epithelial membrane protein 3	*EMP3*	8.0	3.4	-	-		-1.2 (0.5)	-1.2 (0.05)	Yes

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Transforming growth factor, beta 1 (Camurati-	*TGFB1*	4.1	2.8	↑	[47]	Yes	-1.4 (0.01)	-1.4 (0.5)	Yes
	Epithelial membrane protein 1	*EMP1*	2.3	5.2	↓	[43] *S*	No	-	-	
	BCL2-like 14 (apoptosis facilitator)	*BCL2L14*	-3.4	-1.7	-	-		-	-	
Transcription	*v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)*	*MAFF*	1.7	2.7	↑	[40] *S*	Yes	-	-	
	RAD54 homolog B (S. Cerevisiae)	*RAD54B*	1.7	2.2	-	-		-	-	

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Tribbles homolog 3 (Drosophila)	*TRIB3*	1.9	2.2	-	-		-	-	
	MCM4 minichromosome maintenance deficient 4 (S. Cerevisiae)	*MCM4 (CDC21, CDC54)*	1.7	2.2	↑	[49] *O*	Yes	-	-	
	Zinc finger protein 165	*ZNF165*	1.6	2.3	↑	[50]	Yes	-1.6 (0.05)	-1.5 (0.01)	Yes
	Activating transcription	*ATF3*	2.0	3.9	↑	[40] *S*	Yes	-	-	
	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	*MYBL2 (BMYB)*	1.8	1.8	↑	[40] *S*	Yes	-	-	
	Heat shock 70kDa protein	*HSPA1A,*	2.6	1.6	↑	[26]	Yes	-	-	

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	General transcription factor IIH, polypeptide 1, 62kDa	*GTF2H1*	-2.7	-1.5	↑	[49]	No	-	-	
	WW domain containing transcription regulator 1	*WWTR1*	-2.5	-1.6	-	-		-	-	
Protein kinase cascade	*Serine/threonine kinase 17b (apoptosis-inducing)*	*STK17B*	2.6	2.2	-	-		-	-	
Proteolysis	*Mucosa associated lymphoid tissue lymphoma translocation gene 1*	*MALT1*	2.1	2.2	↑	[40] *S*	Yes	-	-	
	Oxidized low density lipoprotein (lectin-like)	*OLR1*	2.3	2.5	-	-		1.1 (0.5)	1.7 (0.05)	No
	Matrix metallopeptidase 7 (matrilysin, uterine)	*MMP7*	23.6	5.9	↑	[21] *P*	Yes	-	-	
	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	*ACE2*	-3.5	-1.6	-	-		-	-	

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	X-prolyl aminopeptidase (aminopeptidase P) 2,	*XPNPEP2*	-6.9	-3.0	-	-		-	-	
	Macrophage stimulating 1 (hepatocyte growth factor-like)	*MST1*	-2.6	-1.5	-	-		-	-	
	Transmembrane protease, serine 6	*TMPRSS6*	-4.4	-2.5	-	-		-	-	
	Macrophage stimulating, pseudogene 9	*MSTP9*	-2.6	-1.5	-	-		-	-	
	Myosin IA	*MYO1A*	-4.0	-1.6	-	-		-	-	
	Meprin A, alpha (PABA peptide hydrolase)	*MEP1A*	-4.7	-1.5	↑	[51] *P*	No	-1.3 (0.5)	-1.2 (0.05)	No

Table 4, continued

GO process	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Cathepsin O	*CTSO*	-5.0	-2.0	-	-		-1.1 (1)	-1.7 (0.05)	No
	B-factor, properdin	*BF (CFB)*	-2.6	-2.1	↑	[44]	No	1.4 (0.01)	1.1 (0.5)	Yes
Small GTPase mediated signal transduction	*RAB3B, member RAS oncogene family*	*RAB3B*	3.1	3.4	-	-		-	-	

Genes with an identical direction of fold change on both days 5 and 10 and a minimum of 1.5 up- or downregulation on both days (n = 48), contributing to significantly changed pathways.

O gene expression data are only accessible via the Cancer Profiling Database Oncomine™ (http://www.oncomine.org/main/index.jsp)

P differential expression determined at the protein level

S gene expression data are only accessible via the online supplementary data of the corresponding articles

The column "In agreement" indicates whether the direction of gene expression changes in Caco-2 cells exposed to ascorbate-stabilized quercetin is in agreement with the direction of gene expression changes occurring in human colorectal carcinogenesis.

For comparison of quercetin-mediated effects in the absence of ascorbate, data are shown from Caco-2 cells exposed to 5 or 50 µM quercetin only, as described in our previous report [10]. The column "Opposite effect" indicates whether the direction of fold change as found by ascorbate-stabilized quercetin is reversed when the Caco-2 cells are exposed to quercetin only, without ascorbate.

Gene analysis by functional groups

In addition to analyses based on the MetaCore™ software, the total set of 1,743 significantly changed genes was evaluated for functional groups that are involved in nutritional modulation of colorectal cancer, *i.e.* tumor suppressor genes, oncogenes and xenobiotic metabolism. Genes involved in xenobiotic metabolism showed a tendency towards decreased expression of phase II metabolism genes (**Table 5**). Differentially expressed genes in the functional group "tumor suppressor genes and oncogenes" show a decreased expression of tumor suppressor genes and increased expression of oncogenes. As can be seen in the miscellaneous functional group, quercetin upregulated expression of heat shock proteins, which is a phenomenon also observed in human colorectal carcinogenesis [26].

Comparison with human colorectal carcinogenesis

Gene expression changes categorized by GO processes and functional groups were compared with changes reported to occur in human colorectal carcinogenesis. Comparison by GO processes showed that in total, 28 out of 48 genes (≈ 58%) could be compared to available literature on human colorectal carcinogenesis (**Table 4**). Within these 28 genes, 22 (≈ 79%) showed a direction of fold change that is concordant with fold changes reported for human colorectal carcinogenesis.
For gene expression changes categorized by functional groups, in total 15 out of 26 (≈ 58%) genes could be compared to available literature on human colorectal carcinogenesis (**Table 5**). Overall, 14 out of the 15 genes (≈ 93%), showed quercetin-mediated fold changes that were concordant with those occurring in human colorectal carcinogenesis.

Table 5. Overview of differentially expressed genes grouped by function

Functional group	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
Xenobiotic metabolism	*Cytochrome P450, family 1, subfamily A, polypeptide 1*	*CYP1A1*	12.5	5.6	↑	[52] *P*	Yes	1.2 (0.01)	11.4 (0.5)	No
	Cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	*CYP2B7P1*	-3.0	-2.4	-	-		-	-	
	Glutathione S-transferase A1	*GSTA1*	-2.3	-1.5	-	-		-	-	
	UDP glucuronosyltransferase 1 family, polypeptide A9	*UGT1A9*	1.5	1.9	-	-		-	-	
	UDP glucuronosyltransferase 1 family, polypeptide A10	*UGT1A10*	1.5	1.9	-	-		-	-	
	UDP glucuronosyltrans-	*UGT2B15*	-1.6	-2.1	↓	[48]	Yes	-	-	

Table 5, continued

Functional group	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Epoxide hydrolase 1, microsomal (xenobiotic)	*EPHX1*	-1.3	-1.9	↓	[53] *P*	Yes	-	1.3 (0.02)	Yes
	Epoxide hydrolase 2, cytoplasmic	*EPHX2*	-3.0	-1.9	↓	[49] *O*	Yes	-	-	
	Sulfotransferase family, cytosolic, 1C, member 1	*SULT1C1*	-2.6	-2.5	-	-		-	-	
	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	*SULT2A1*	-4.2	-2.6	-	-	-	-	1.3 (0.01)	Yes
	Flavin containing monooxygenase 1	*FMO1*	-2.4	-5.6	↓	[40] *S*	Yes	-	1.9 (0.05)	Yes

Table 5, continued

Functional group	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Flavin contai-nning mono-oxygenase 5	*FMO5*	-4.6	-1.8	↓	[43]	Yes	-	-	
	Glutathione peroxidase 2 (gastrointestinal)	*GPX2*	4.4	2.7	↓	[49] *O*	No	1.7 (0.02)	1.3 (0.05)	No
	ATP-binding cassette, sub-family A (ABC1), member 1	*ABCA1*	-4.2	-2.0	-	-		-	-	
Tumor	*TP53 activated protein 1*	*TP53AP1*	-2.9	-2.9	↓	[54] *O*	Yes	-	-	
suppressor genes and	*RAB3B, member RAS oncogene family*	*RAB3B*	3.1	3.4	-	-		-	-	
oncogenes	*v-myb myeloblastosis viral oncogene*	*MYBL2*	1.8	1.8	↑	[40] *S*	Yes	-	-	

Table 5, continued

Functional group	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	v-maf musculoapo-neurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	1.7	2.7	↑	[40] *S*	Yes	-	-	
	Epithelial membrane protein 1	*EMP1*	2.3	5.2	-	-		-	-	
	Transmembrane 4 L six family member 1	*TM4SF1 (TAAL6)*	1.5	2.6	↑	[55]	Yes	-1.4 (0.02)	1.4 (0.5)	Yes
	Rho GDP dissociation inhibitor (GDI) beta	*ARHGDIB (LY-GDI)*	2.8	2.5	↑	[56]	Yes	-	-	
Miscellaneous	*Heat shock 27kDa protein 1*	*HSPB1*	2.9	2.8	-	-		-	-	
	Heat shock 70kDa protein 1A, heat shock 70kDa protein 1B	*HSPA1A, HSPA1B*	2.6	1.6	↑	[26]	Yes	-	-	

Table 5, continued

Functional group	Gene title	Gene symbol (synonym)	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change in human CRC		In agreement	Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	Direction	Reference		5 µM (*P*<)	50 µM (*P*<)	
	Trefoil factor 2 (spasmolytic protein 1)	*TFF2 (SP)*	-7.4	-3.1	-	-		-	-	
	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	*CD24*	3.1	3.0	↑	[40] *S*	Yes	-	-	
	Frizzled homolog 2 (Drosophila)	*FZD2*	2.7	1.5	↑	[57] *P*	Yes	-	-	

Genes with an identical direction of fold change on both days 5 and 10 and a minimum of 1.5 up- or downregulation on both days, that are part of the 1,743 significantly changed genes.

O gene expression data are only accessible via the Cancer Profiling Database Oncomine™ (http://www.oncomine.org/main/index.jsp)

P differential expression determined at the protein level

S gene expression data are only accessible via the online supplementary data

The column "In agreement" indicates whether the direction of gene expression changes in Caco-2 cells exposed to ascorbate-stabilized quercetin is in agreement with the direction of gene expression changes occurring in human colorectal carcinogenesis.

For comparison of quercetin-mediated effects in the absence of ascorbate, in the last two columns data are shown from Caco-2 cells exposed to 5 or 50 µM quercetin only, as described in our previous report [10]. The column "Opposite effect" indicates whether the direction of fold change as found by ascorbate-stabilized quercetin is reversed when the Caco-2 cells are exposed to quercetin only, without ascorbate.

Comparison with Caco-2 cells exposed to quercetin without ascorbate

To determine whether the above mentioned adverse effects in Caco-2 cells are caused by possible interference of the anti-oxidant ascorbate that is required for stabilization of quercetin in the culture medium, a comparison was made with expression data from our previous report in which post-confluent Caco-2 cells were exposed to 5 and 50 μM quercetin for 48 hr without ascorbate [10]. First, significantly changed genes presented in tables 2, 4 and 5 were compared to data from our previous study [10]. Only two significantly changed differentiation related genes in the present study were also found in the previous study. *ALPI* expression was upregulated by quercetin in the absence of ascorbate, which was opposite to expression data obtained in the presence of ascorbate in the present study, where this gene was downregulated; *APOM* was downregulated in both conditions (**Table 2**). Similar analyses among genes contributing to the GO processes affected by the 27 significantly changed pathways showed that 12 genes could be compared among both data sets. In total, 8 out of these 12 genes (≈ 67%) showed an opposite direction of fold change when data were compared with and without addition of ascorbate (**Table 4**). In the absence of ascorbate, 7 of these 8 genes (≈ 88%) showed a direction of fold change that is contrary to that occurring in development of human CRC, and includes inhibition of cell cycle genes.

Among the genes grouped by function, 4 out of the 6 genes in the present study that were also found in the previous study (≈ 67%) showed an opposite direction of fold change when data were compared with and without addition of ascorbate (**Table 5**). In the absence of ascorbate, 3 of these 4 genes (= 75%) showed a direction of fold change that is contrary to that occurring in development of human CRC, and includes increased expression of genes involved in xenobiotic metabolism.

Overall, 13 out of the total number of 20 matching genes as described in tables 2, 4 and 5 (= 65%) showed an opposite direction of fold change when expression data obtained with ascorbate-stabilized quercetin were compared to those obtained with quercetin only. Furthermore, in the absence of ascorbate, 10 out of these 13 genes (≈ 77%) showed a direction of fold change that is contrary to that occurring in development of human CRC.

In the second type of analysis, the significantly affected genes from our previous study with quercetin only were compared to a subset within the 1,743 significantly changed genes (by ascorbate-stabilized quercetin) showing an identical direction of

fold change on both days. This comparison learned that 19 genes matched between the two datasets, of which 13 (≈ 68%) showed an opposite direction of fold change (**Table 6**).

Discussion

Epidemiological studies suggest that regular dietary intake of quercetin occurs at 16.3 mg/day, when expressed as aglycone [27]. Based on these data, the concentration of quercetin aglycone in the colonic lumen has been estimated at 40 μM [15], and as such provided an input to perform the present *in vitro* experiments at this physiological relevant concentration. In the present paper, quercetin at 40 μM reduced Caco-2 differentiation, which confirms our previous study conducted under identical experimental conditions [15]. Caco-2 differentiation was characterized using the TEER and *intestinal alkaline phosphatase*, the latter determined by Affymetrix® GeneChip® arrays, real-time qRT-PCR and enzyme activity. Furthermore, reduced Caco-2 differentiation was in accordance with the decreased expression of all differentiation related genes present in the statistically filtered dataset. In addition, ascorbate-stabilized quercetin near-significantly downregulated the intestine-specific caudal type homeo box transcription factor 2 (*CDX2*) which targets *sucrase isomaltase*, and is considered to be the main regulator of intestinal cell differentiation [24] and also downregulated in human colorectal cancer [28]. Importantly, the quercetin-mediated decrease in Caco-2 differentiation has been found with multiple techniques, which were all concordant. In addition, Affymetrix® GeneChip® array data indicated quercetin-induced upregulation of the human CRC-associated genes *PTGS2*, which encodes the COX-2 enzyme [20], and *MMP7* [21], both of which were confirmed by real-time qRT-PCR. Based on these findings, it is concluded that statistical filtering retrieved a reliable gene expression dataset that was therefore used for further analyses of quercetin-mediated biological effects in Caco-2 cells.

At the cellular level, (colorectal) carcinogenesis encompasses a decrease in cell differentiation, apoptosis and metabolism, and an increase in cell proliferation [25], as was also observed in the present study with quercetin exposed Caco-2 cells.

Table 6. Overview of significantly affected genes described in reference [10], after exposure to quercetin in the absence of ascorbate, in comparison to significantly affected genes after exposure to ascorbate-stabilized quercetin

			Fold change quercetin vs. control + ascorbate, + quercetin		Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
Gene title	**Gene symbol**	**Accession #**	**Day 5**	**Day 10**	**5 µM**	**50 µM**	
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	*ABCC3*	NM_003786	-2.4	-1.0	1.0	1.6	Yes
Aldehyde dehydrogenase 6 family, member A1	*ALDH6A1*	NM_005589	-2.4	-1.3	-1.5	-1.5	No
Branched chain keto acid dehydrogenase E1, beta polypeptide (maple syrup urine disease)	*BCKDHB*	NM_000056	-1.9	-1.0	1.9	1.7	Yes
CDC6 cell division cycle 6 homolog (S. cerevisiae)	*CDC6**	NM_001254	1.6	2.9	-4.1	-	Yes
Cytochrome P450, family 1, subfamily A, polypeptide 1	*CYP1A1***	NM_000499	12.5	5.6	1.2	11.4	No
Farnesyl-diphosphate farnesyltransferase 1	*FDFT1*	NM_004462	1.3	1.1	-1.8	-1.2	Yes
Galactosidase, alpha	*GLA*	NM_000169	1.6	1.1	-1.6	-1.3	Yes
High mobility group nucleosomal binding domain 4	*HMGN4*	NM_006353	1.4	1.1	-1.5	-1.7	Yes
Integrin, alpha 6	*ITGA6*	NM_000210	1.3	1.9	1.7	2.4	No

Table 6, continued

Gene title	Gene symbol	Accession #	Fold change quercetin vs. control + ascorbate, + quercetin		Fold change quercetin vs. control - ascorbate, + quercetin		Opposite effect
			Day 5	Day 10	5 µM	50 µM	
Kinesin family member 5B	*KIF5B*	NM_004521	1.1	1.3	-3.9	-3.6	Yes
Keratin 20	*KRT20*	NM_019010	1.8	4.4	2.0	2.0	No
MAD2 mitotic arrest deficient-like 1 (yeast)	*MAD2L1*	NM_002358	1.0	1.6	-1.7	-1.5	Yes
Methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	*MTHFD1*	NM_005956	1.4	1.0	-1.5	-1.8	Yes
N-myristoyltransferase 1	*NMT1*	NM_021079	1.4	1.1	-3.5	-2.9	Yes
Paraoxonase 3	*PON3*	NM_000940	-2.2	-3.3	-1.5	-2.0	No
RNA binding motif, single stranded interacting protein 2	*RBMS2*	NM_002898	2.0	1.3	-2.1	-1.4	Yes
Retinol binding protein 4, plasma	*RBP4*	NM_006744	-2.0	-2.5	-1.6	-2.0	No
Solute carrier family 2 (facilitated glucose transporter), member 3	*SLC2A3*	NM_006931	1.2	2.1	-1.9	1.2	Yes
Zinc finger protein 165	*ZNF165**	NM_003447	1.6	2.3	-1.6	-1.5	Yes

The column "Opposite effect" indicates whether the direction of gene expression fold changes as found by ascorbate-stabilized quercetin is reversed when the Caco-2 cells are exposed to quercetin only, without ascorbate. * Gene also present in Table 2; ** Gene also present in Table 3.

The most significantly changed pathway in relation to cell proliferation is the "Role of Anaphase promoting complex (APC) in cell cycle Regulation", comprising proliferation related genes that are mentioned hereafter. Proliferating cells express both *cyclin B1* (*CCNB1*) and *cyclin B2* (*CCNB2*) that complex with and activate c*yclin-dependent kinase 1* (*CDK1* = p34 = *CDC2*), which is required for cells to undergo mitosis [29]. *Cell division cycle 18* (*CDC18L = CDC6*) is involved in DNA replication during the cell cycle during the S-phase [10] suggesting that upregulation of this gene may contribute to increased cell proliferation. *Serine/threonine kinase 6* (*STK6*, = Aurora-A) is a cell cycle-regulated kinase that is involved in microtubule formation and increased in human colon cancer cell lines [30]. *PTTG1* (*Securin*) prevents segregation of chromosomes, is overexpressed in human colonic polyps and carcinomas at both the gene and protein level, and related to tumor invasion [31].
The most significantly changed pathway in relation to apoptosis is the "Role of IAP-proteins in apoptosis". Genes expressed in this pathway suggest decreased apoptosis and include *baculoviral IAP repeat-containing 3* (*BIRC3 = c-IAP2*), *baculoviral IAP repeat-containing 5* (*BIRC5* = Survivin), *cyclin B1* (*CCNB1*) and *cyclin-dependent kinase 1* (*CDK1 = CDC2*). *BIRC5* is known as an inhibitor of apoptosis and hypothesized to be lowly expressed in differentiated adult tissues, but overexpressed in the human colon cancer cell line HCT116 [32].
Moreover, ascorbate-stabilized quercetin caused upregulation of oncogenes and downregulation of tumor suppressor genes, which is a typical disequilibrium in (colorectal) carcinogenesis [7], and in contrast with quercetin's anti-carcinogenic potency. Quercetin also upregulated expression of heat shock proteins that prevent apoptosis, thus enabling cell survival, which is a mechanism also involved in development of human CRC [26].
Comparisons between fold changes in mRNA expression among quercetin exposed Caco-2 cells and fold changes reported for human colorectal carcinogenesis showed high similarity (≈ 80 - 90%), suggesting that ascorbate-stabilized quercetin in this *in vitro* model promotes processes involved in development of human CRC. Obviously, not all Caco-2 gene data could be compared to human CRC data, because of a lack of available literature. When assuming that fold changes for these genes are not restricted to quercetin-exposed Caco-2 cells, it can be hypothesized that these genes might be novel biomarkers for human colorectal carcinogenesis.

Quercetin downregulated expression of the majority of phase II enzymes, which is in contrast with its proposed anti-carcinogenic potency [33]. Proposed mechanisms involved in the anti-carcinogenic activity of dietary components *in vivo* comprise induction of detoxifying phase II enzymes that protect the colon mucosa against dietary carcinogens. Glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) are phase II detoxifying enzymes that metabolize xenobiotics via conjugation to glutathione, glucuronic acid or sulphate, respectively, thereby generating less active polar and water-soluble metabolites suitable for rapid excretion. Our *in vitro* data suggest that ascorbate-stabilized quercetin diminished this protective effect by downregulating gene expression of these enzymes.

Strikingly, in the current model quercetin stabilized by 1 mM ascorbate evoked effects that are contrary to what would be expected for a compound with anti-carcinogenic potency. A possible explanation is that quercetin-induced oxygen radicals that have the ability to eradicate tumor cells, might have been annihilated by ascorbate, which is a hypothesis based on previous studies [34-36]. The dietary flavonoid flavone and the anti-cancer drug camptothecin are reported to evoke mitochondrial $O_2^{-\bullet}$ that induced apoptosis of the human colon cancer cell line HT-29 [34]. When these two agents were co-administered with 1 mM ascorbate, HT-29 cells demonstrated a reduction in apotosis, caused by a decrease in both $O_2^{-\bullet}$ induction and caspase 3-like activity. Additional evidence is provided by a study showing that intake of ascorbate supplements by patients with colorectal adenomas is associated with decreased apoptosis in the healthy rectal mucosa [35]. The authors hypothesized that intake of ascorbate supplements by patients with colorectal adenomas may be contra-indicated as ascorbate might scavenge reactive oxygen species (ROS) that are meant to induce apoptosis among aberrant cells. Supporting evidence for beneficial effects of ROS is also described for the potentially anti-carcinogenic polyunsaturated fatty acids (PUFA) that reduced Caco-2 cell proliferation, whereas co-administration of the very same PUFA with the anti-oxidants ascorbate (vitamin C) or α-tocopherol (vitamin E) resulted in increased Caco-2 cell proliferation [36]. In accordance, the hypothesis of ROS scavenging by vitamin C was supported by comparison of the data from the present study to data from our previous study, in which Caco-2 cells were exposed to quercetin without ascorbate, demonstrating the anti-carcinogenic potency of this flavonoid [10]. In the absence of ascorbate, ≈ 80% of the genes showed a

quercetin-mediated direction of fold change that is contrary to that occurring in development of human CRC, thus supporting this flavonoid's anti-carcinogenic potency. Furthermore, Caco-2 cells exposed to quercetin, in the absence of ascorbate, showed a dose-dependent increase of the TEER as a marker of cell differentiation [37], which provides additional evidence for quercetin's anti-carcinogenic potency. The above mentioned mechanisms found by quercetin-exposure in the absence of ascorbate, are in line for what would be expected for an anti-carcinogenic compound, but are opposite to mechanisms found when quercetin was co-administered with ascorbate. Flavonoids, including quercetin, induce expression of phase II enzymes in case of a high oxidative cellular state, e.g. in the presence of ROS and/or electrophiles [38]. A mechanistic explanation for the opposite effects found in the present study might be scavenging of quercetin-induced ROS by ascorbate, which probably resulted in a lower cellular oxidative state and consequently in reduced expression of genes encoding for phase II metabolism enzymes. Together with the present data, it can be hypothesized that oxidative stress induced by dietary quercetin is a mechanism contributing to its anti-carcinogenic potency. Most likely, both induction and maintenance of ROS are required for eradication of tumor cells by quercetin, pointing to a beneficial effect of supposed adverse reactive oxygen species.

In conclusion, exposure of Caco-2 cells to ascorbate-stabilized quercetin at the physiological relevant concentration of 40 μM resulted in decreased expression of genes involved in cell differentiation, apoptosis, tumor suppression and phase II metabolism, and upregulated expression of oncogenes and genes involved in cell proliferation. These findings indicate that treatment of Caco-2 cells with ascorbate-stabilized quercetin leads to stimulation in stead of inhibition of mechanisms also involved in (human) colorectal carcinogenesis. Under which conditions this unexpected *in vitro* effect mediated by a supposed cancer preventive food ingredient would also be relevant for the *in vivo* situation, and to what extent the observation is due to the possibility that in the *in vitro* model quercetin acts by mechanisms dissimilar from its actual physiological mechanism, remain to be elucidated.

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Chapter 4

Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats

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Abstract

The effect of the flavonoid quercetin and its conjugate rutin was investigated on (biomarkers of) colorectal cancer (CRC). Male F344 rats (n = 42/group) were fed 0, 0.1, 1 or 10 g quercetin/kg diet, or 40 g rutin/kg diet. Two wks after initial administration of experimental diets, rats were given two weekly subcutaneous injections with 15 mg/kg bodyweight azoxymethane (AOM). At wk 38 post-AOM, quercetin dose dependently ($P < 0.05$) decreased the tumor incidence, multiplicity and size, whereas tumor incidences were comparable in control (50%) and rutin (45%) groups. The number of aberrant crypt foci (ACF) in unsectioned colons at wk 8 did not correlate with the tumor incidence at wk 38. Moreover, at wk 8 post-AOM, the number and multiplicity of ACF with or without accumulation of ß-catenin were not affected by 10 g quercetin/kg diet. In contrast, another class of CRC-biomarkers, ß-catenin accumulated crypts, partially showed a decrease in extent of ß-catenin accumulation ($P < 0.05$).

After enzymatic deconjugation, plasma concentrations of 3'-O-methyl-quercetin and quercetin at wk 8 were inversely correlated with the tumor incidence at wk 38 (r = -0.95, $P \leq 0.05$). Rats supplemented with 40 g rutin/kg diet showed only 30% of (3'-O-methyl-) quercetin concentrations found in 10 g quercetin/kg diet treated rats ($P < 0.001$).

In conclusion, quercetin at a high dose, but not rutin, inhibited colorectal carcinogenesis among AOM-treated rats, which was not reflected by changes in ACF-parameters. The lack of protection by rutin is probably caused by its low bioavailability.

Introduction

Colorectal cancer (CRC) is a highly frequent malignancy in the Western world with a lifetime risk of approximately 5% [1]. Epidemiological studies suggest that intake of fruits and vegetables [2], dietary fibre [3] and flavonoids [4] are inversely correlated with the CRC incidence. One of the major flavonoid representatives in the human diet is quercetin, which in its natural form is glycosylated and can be found in fruits and vegetables, including blueberries, onions, curly kale, broccoli and leek [5]. Once ingested, conjugated quercetin can be released by intracellular and membrane-bound ß-glycosidases of small intestinal cells [6]. In the colon, on the other hand, quercetin glycosidic bonds can be hydrolyzed by local ß-glycosidases produced by bacteria, leading to colonic release and absorption of quercetin [7]. This is also the case with rutin (quercetin-3-O-ß-rutinoside), the major quercetin glycoside found in tea. Worldwide, CRC shows a high annual incidence (1,000,000 patients) and mortality (530,000) [8]. Therefore, biomarkers that reveal colorectal carcinogenesis at an early stage are urgently needed for prevention of colorectal tumors. Such biomarkers were first described in 1981 by Shamsuddin *et al*. [9;10] and designated "aberrant crypts" in 1987 by Bird [11]. However, the validity of these histo-pathological aberrations as predictors of colon carcinogenesis is still a matter of debate [12-14]. It has been suggested that histological lesions showing accumulation of ß-catenin in colonic crypts may be more relevant indicators of colorectal carcinogenesis [15]. In mice, these ß-catenin accumulated crypts (BCA-C) demonstrate increased cell proliferation and have been shown to be positively correlated with the development of colorectal tumors. Gene mutations in ß-catenin, amongst others, may prevent phosphorylation of the ß-catenin protein, which is required for its degradation and can lead to its cytoplasmic accumulation [16]. Consequently, cytoplasmic ß-catenin can migrate into the nucleus and may target cell proliferation, differentiation and apoptosis [1;17]. Therefore, accumulation of free ß-catenin is thought to be an early event in colorectal carcinogenesis [16].

Since quercetin supplements are commercially available and claimed to be health promoting, the aim of the present study was to investigate the effects of quercetin aglycone, *i.e.* the sugar-free flavonoid, and its major dietary source rutin on (biomarkers of) colorectal carcinogenesis. Based on previous rodent studies [18;19] it was hypothesized that quercetin might inhibit development of colorectal cancer in azoxymethane (AOM) treated rats.

Materials and methods

Experimental design

At 4 wks of age, 210 inbred male F344 rats (Charles River Laboratories, Inc., Sulzfeld, Germany) weighing 40 - 90 g were housed (2 per cage) in sawdust covered plastic cages with wire tops and diet and tap water *ad libitum*. Experimental protocols were approved by the Ethical Committee of Wageningen University and Research Centre. Rats were first acclimatized for 3 wk on a basal RM3 [E] FG SQC diet [20] (SDS Special Diets Services, Witham, England), subsequently allocated to 5 groups of 42 rats each and maintained on the control (RM3) diet, or enriched diets containing either 0.1, 1 or 10 g quercetin/kg diet or 40 g rutin/kg diet (Sigma, Zwijndrecht, The Netherlands). Experimental diets were produced monthly and analyzed for flavonoid contents by HPLC [21]. After 2 wk on the experimental diets, 30 rats from each group were injected subcutaneously with 15 mg/kg body wt of the carcinogen azoxymethane (AOM, NCI/MRI, Kansas City, U.S.A.) dissolved in a physiological salt solution, whereas the remaining 12 rats per group received the solvent only. One wk thereafter, injections were repeated, *i.e.* wk 0 post-AOM.

Interim autopsy

At eight wks post-AOM, 8 AOM-treated and 6 non-AOM treated rats per diet group were killed in the morning without overnight food deprivation. Rats were anesthetized by inhalation of 5% isoflurane combination with O_2 and N_2O (1 : 1). After collection of abdominal aortic blood, quercetin (-metabolites) in plasma of non-AOM treated rats were stabilized by 5.6 mmol/L ascorbate (Merck, Darmstadt, Germany) [20]. Following excision, colorectums were cut longitudinally and rinsed with 70% ethanol at 4 °C. Colorectums of AOM-treated rats were fixed between filter paper and stored in 70% ethanol at 4 °C prior to the ACF-counts and finally embedded in paraffin.

Final autopsy

At wk 38 post-AOM, rats were killed as described above, with modifications. Among tumor-bearing rats, the tumor-number and maximum diameter were recorded. Large tumors (diameter ≥ 5 mm) were removed and split diagonally [13]. The luminal part, as well as small tumors (diameter < 5 mm) were fixed in RNA*later*®, for mRNA

analysis to be reported elsewhere. Therefore, tumor-classification as being adenomas or carcinomas, was only assessed in large tumors.

Aberrant crypt foci in unsectioned colons

To visualize ACF, colorectums of AOM-treated rats killed at the interim autopsy were stained for 10 min with 0.1% (w/v) methylene blue dissolved in 70% ethanol. ACF were recognized as single or multiple enlarged crypts, with altered luminal openings and thickened epithelium, in comparison with surrounding normal crypts [11]. ACF-numbers and -multiplicity, *i.e.* the number of aberrant crypts per ACF were scored.

Quantification of quercetin (-metabolites) in plasma

Quercetin metabolites in pooled plasma samples of non-AOM rats killed at the interim autopsy were deconjugated by the *Helix pomatia* enzyme (Sigma, Zwijndrecht, The Netherlands) with ß-glucuronidase (7,500 kU/L) and sulfatase (~500 kU/L) activity [20]. Quercetin (-metabolites) were analyzed by HPLC with Coularray detection and quantified with calibration curves for quercetin, 3'-O-methyl-quercetin (isorhamnetin, Roth, Karlsruhe, Germany) and 4'-O-methyl-quercetin (tamarixetin, Extrasynthese, Genay, France). Plasma concentrations of rats fed 1 and 10 g quercetin/kg diet were published previously [20].

Immuno-histochemistry

After alcohol-fixation and paraffin-embedding, immuno-histochemistry was performed on the middle of the distal colon, because of the highest ACF-frequency. Per rat, 35 colon-sections were cut *en face* at 4 µm [15], meant for Hematoxylin and Eosin staining (H&E; every 1st slide) as a reference for adjacent slides stained for total ß-catenin (every 2nd slide) and Ki67 (every 3rd slide). After blocking of endogenous peroxidase (30 min in 0.3% (v/v) H_2O_2 in methanol) and heat-induced antigen retrieval (15 min in 1 mmol/L EDTA-NaOH, pH 8.0 at 95 - 98 °C), sections were incubated with 25% (v/v) goat serum in PBS (15 min). Subsequently, sections were incubated with primary antibodies against total ß-catenin for 30 min (4,000 x PBS-dilution, rabbit polyclonal antibody) or the proliferation marker Ki67 for 10 min (200 x PBS-dilution, rabbit monoclonal antibody) Both antibodies were retrieved from Neomarkers, Inc., Fremont, California, U.S.A.. Sections were then incubated with the secondary PowerVision™ Poly horseradish peroxidase (HRP) labeled polyclonal

antibody (Immuno Vision Technologies, Brisbane, California, U.S.A.) for 30 min. HRP-activity was visualized by incubation with Vector® NovaRED™ (Vector Laboratories, Burlingame, California, U.S.A.) for 10 min. Finally, sections were weakly counterstained with hematoxylin for 45 s.

BCA-C and BCA-ACF were recognized as crypts or ACF, respectively, showing nucleic and/or cytoplasmic ß-catenin accumulation [15]. The number of lesions, multiplicity, and grade of ß-catenin accumulation was scored, the latter expressed as excessive ß-catenin staining intensity relative to normal crypts *i.e.* low, intermediate, or high. To distinguish ACF in unsectioned and *en face* sectioned colons, the term "*en face* ACF" is used for the latter.

In Ki67-stained slides, the number of positive and negative nuclei were scored per ACF and expressed as the proliferation index [22]. Large colorectal tumors (diameter ≥ 5 mm) were classified in H&E slides [13].

Statistics

The dose dependent effect of quercetin on ACF-multiplicity and tumor incidence was analyzed using the Cochran-Mantel-Haenszel test. Tumor incidences among different groups were compared with one another, using the Chi-Square test. Correlations between dietary quercetin and plasma concentrations of quercetin (-metabolites) were calculated using the Pearson's correlation coefficient. Quercetin mediated effects on CRC-precursors, assessed by immuno-histochemistry in rats fed 0 or 10 g quercetin/kg diet, were analyzed using the non-parametric Mann-Whitney U test to take account of unequal variances. Plasma concentrations of (3'-O-methyl-) quercetin were compared using the 1-way ANOVA, followed by the *t*-test with the overall error term to compare all groups with one another. Mean values are presented as means ± SD and differences were considered significant when $P \leq 0.05$ (2-tailed).

Results

Diets, animal weight, dietary intake and survival

The basal diet was shown to be free of quercetin and rutin. For AOM rats supplemented with 10 g quercetin/kg diet, the corrected dietary intake amounted to ≈ 900 mg quercetin/(kg body wt.d) at wk -2, declining to ≈ 450 mg quercetin/(kg body wt.d) at wk 38 post-AOM. Corrected dietary intake of 1 g/kg and 0.1 g

quercetin/kg diet treated non-AOM rats amounted to ≈ 10% and ≈ 1%, respectively, of the 10 g /kg treated group. AOM rats supplemented with 40 g rutin/kg diet demonstrated an intake of 3,500 mg rutin/(kg body wt.d) at wk -2, declining to 1,700 mg rutin/(kg body wt.d) at wk 38. In the course of the experiment, the corrected dietary intake was comparable between AOM- and non-AOM rats fed quercetin or rutin.

In total, 4 AOM-treated rats were in poor health or moribund as a result of colon tumors (2 rats), a zymbal gland tumor (1 rat) and a small intestinal tumor (1 rat), most likely caused by AOM-toxicity. As these rats were killed 3 - 4 months before completion of the study, they were excluded from further analyses to ensure proper time-matched comparisons between groups.

Aberrant crypt foci in unsectioned colons

All AOM-treated rats killed at week 8 post-AOM (n = 8/group) developed ACF that were mainly found in the distal colon. Quercetin lacked an effect on ACF, since ACF-numbers in control rats (45.8 ± 20.0) were comparable to rats fed 0.1 (59.4 ± 18.3), 1 (42.3 ± 13.8), or 10 g quercetin/kg diet (49.6 ± 17.8). In addition, ACF-numbers among rutin treated rats (42.9 ± 14.9) were comparable with the controls. Furthermore, quercetin lacked an effect on the ACF-multiplicity (multiplicity range: 1 - 8), even when ACF were classified as small (≤ 3 aberrant crypts/focus) and large (≥ 4 aberrant crypts/focus) [23;24] [data not shown].

ß-catenin accumulation

Two sub-populations of *en face* ACF were found that showed either ß-catenin immuno-reactivity restricted to the cell membrane, as for the normal adjacent crypts (**Figure 1A**), or immuno-reactivity in the nucleus and/or cytoplasm (**Figure 1D**). The second class of putative CRC biomarkers - ß-catenin accumulated crypts (**Figure 1G**) - did not show an enlarged crypt lumen or thickened epithelium relative to the surrounding normal crypts, in contrast to (ß-catenin accumulated) ACF.

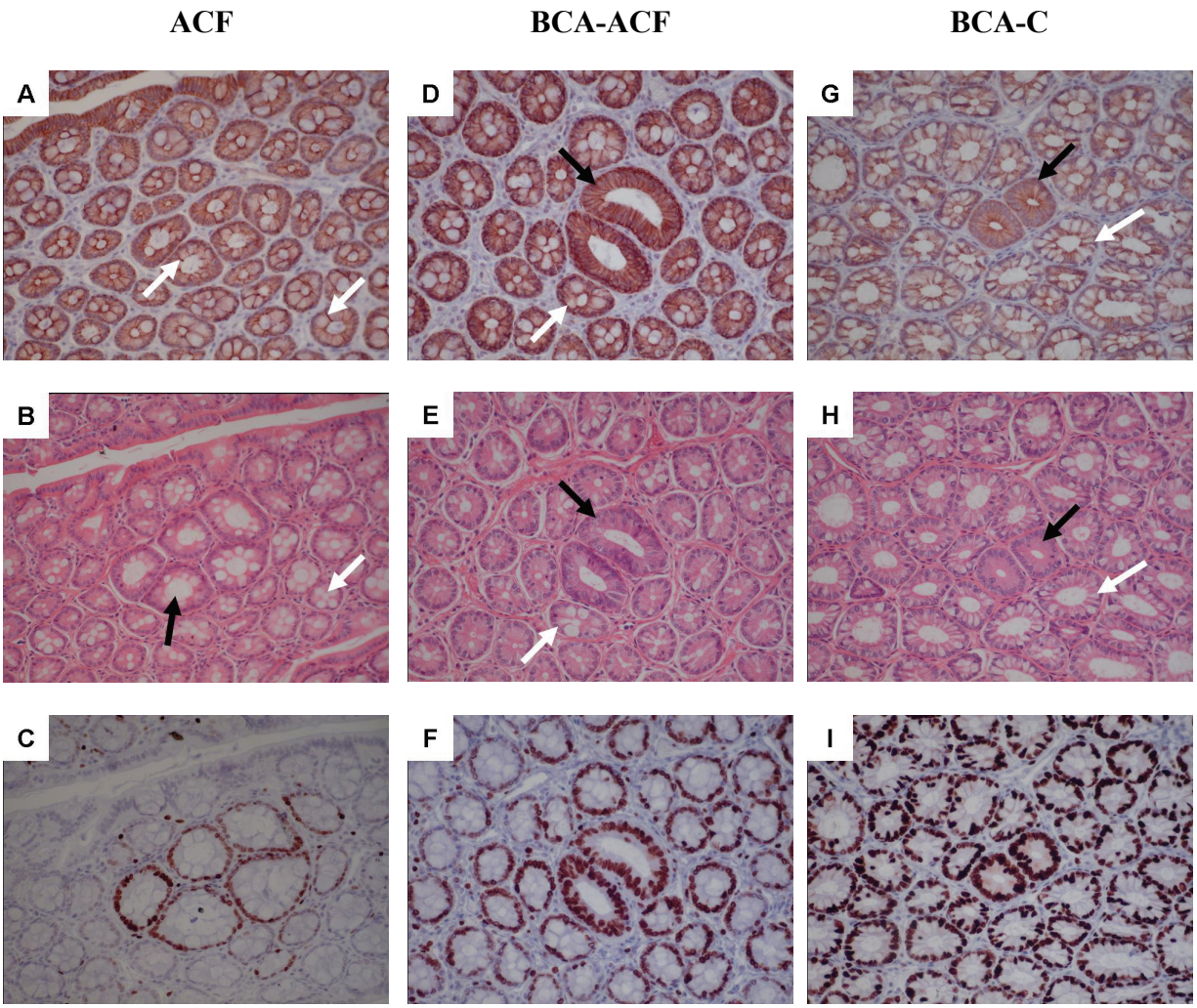

Fig. 1. ß-catenin, hematoxylin & eosin and Ki67 staining in *en face* oriented aberrant crypt foci (ACF), ß-catenin accumulated aberrant crypt foci (BCA-ACF) and ß-catenin accumulated crypts (BCA-C) in colons of AOM-treated rats killed at wk 8 post-AOM (200 x magnification).
(**A**) ß-catenin staining in "normal" ACF is located at the cell surface, similar to healthy surrounding crypts (white arrows). (**B**) These ACF show enlarged crypts with wide luminal openings (black arrow) when compared to healthy surrounding crypts (white arrow) in combination with (**C**) a single layer of Ki67 positive nuclei in the crypts. (**D**) In BCA-ACF, ß-catenin staining occurred in both the cytoplasm and nuclei (black arrow), whereas surrounding healthy crypts showed ß-catenin staining located at the cell surface (white arrow). (**E**) BCA-ACF showed enlarged crypts (black arrow) relative to the healthy surrounding crypts (white arrow), with (**F**) multiple layers of Ki67 positive nuclei. (**G**) BCA-C showed accumulation of ß-catenin in both the cytoplasm and nucleus (black arrow) in contrast to normal cells that showed ß-catenin staining located at the cell membrane (white arrow). (**H**) BCA-C lack typical morphological features of ACF, including a split enlarged lumen, are similar in size (black arrow) in comparison with the surrounding normal mucosa (white arrow) and show (**I**) multiple layers of Ki67 positive nuclei.

When compared to control rats, the number of BCA-C was not affected by 10 g quercetin/kg diet. However, BCA-C numbers with an intermediate grade of ß-catenin accumulation were significantly ($P < 0.05$) lower among rats fed 10 g quercetin/kg diet (**Table 1**).

Table 1. Grade of ß-catenin accumulation in putative biomarkers of colorectal cancer among rats fed 0 or 10 g quercetin/kg diet

		Grade of ß-catenin accumulation		
	g quercetin /kg diet	**Low**	**Intermediate**	**High**
BCA-ACF	**0**	2.0 ± 0.0	1.5 ± 1.0	1.0 ± 0.0
	10	1.3 ± 0.5	1.3 ± 0.5	1.0 ± 0.0
BCA-C	**0**	3.8 ± 1.7	4.9 ± 2.1	2.0 ± 0.0
	10	3.3 ± 1.8	2.4 ± 1.2 *	1.0 ± 0.0

Values are means ± SD, $n = 8$/group. * Different from 0 g quercetin/kg diet, $P < 0.05$.

When compared to controls, the number of *en face* ACF, regardless of the ß-catenin accumulation status, was not affected by treatment with 10 g quercetin/kg diet (0 vs. 10 g quercetin/kg diet: 10.3 ± 4.4 vs. 10.5 ± 6.7, $n = 8$/group). The same was true for ACF with (1.4 ± 1.1 vs. 1.8 ± 1.3) and without (9.0 ± 4.1 vs. 9.0 ± 6.2) ß-catenin accumulation, which is in line with observations in methylene blue stained ACF in unsectioned colons (45.8 ± 20 vs. 49.6 ± 17.8).
Multiplicities of ACF, BCA-ACF and BCA-C were not affected by treatment with 10 g quercetin/kg diet relative to controls, even when a distinction was made between small (≤ 3 aberrant crypts/focus) and large (≥ 4 aberrant crypts/focus) lesions [data not shown]. This result is in agreement with methylene blue stained ACF in unsectioned colons.

Proliferation

The proliferation index within the total number of *en face* ACF, regardless of the ß-catenin accumulation status, decreased significantly ($P < 0.05$) from 99.0 ± 0.5%

(n = 5/group) in control rats to 80 ± 16.5% in 10 g quercetin/kg diet treated rats. In ACF without ß-catenin accumulation, a decrease in proliferation index approached significance (P = 0.060) from 98.7 ± 1.1% in control rats to 79.8 ± 18.5% in 10 g quercetin/kg diet treated rats, as was the case among the relatively low number of ACF with ß-catenin accumulation (99.8 ± 0.4% to 82.1 ± 18.9%, P = 0.07).

Colorectal tumors

At 38 wk post-AOM, non-AOM rats fed the control diet or diets supplemented with quercetin or rutin, did not demonstrate colorectal tumors. The tumor incidence, *i.e.* the number of tumor bearing rats expressed as percentage of the total number of rats, amounted to 50% in AOM-only rats and was dose dependently ($P < 0.05$) decreased by dietary quercetin (0 vs. 10 g quercetin/kg diet: $P < 0.05$, **Table 2**). The dose dependent decrease in tumor incidence found among quercetin treated rats at wk 38 did not correlate with the ACF-numbers scored in unsectioned colons obtained at wk 8. Both the tumor multiplicity, *i.e.* the mean number of tumors per rat after exclusion of tumor negative rats, and the tumor diameter showed a significant dose dependent decrease with higher quercetin concentrations (both: r = -0.98, $P < 0.05$).
The tumor incidence, multiplicity and size were not affected by 40 g rutin/kg diet.

Plasma concentrations of quercetin (-metabolites)

Following enzymatic hydrolysis, plasma concentrations of quercetin and 3'-O-methyl-quercetin at week 8 (**Table 3**) correlated linearly with dietary concentrations of quercetin (both: $r \geq 0.99$, $P < 0.01$). Interestingly, rats fed the 40 g rutin/kg diet, which equals ≈ 20 g quercetin aglycone/kg diet, had only ≈ 30% of both quercetin and 3'-O-methyl-quercetin relative to rats maintained on 10 g quercetin/kg diet ($P <$ 0.001).
Tumor incidences in quercetin-fed rats at wk 38 were negatively correlated with plasma concentrations of both quercetin and 3'-O-methyl-quercetin after enzymatic deconjugation at wk 8 (r = -0.95, $P \leq 0.05$). Plasma concentrations of tamarixetin (4'-O-methyl-quercetin) were below the detection limit for all flavonoid supplemented groups.

Table 2. Tumor data from control, quercetin and rutin groups, obtained at wk 38 post-AOM

	Quercetin (g/kg diet)				Rutin (g/kg diet)
	0	**0.1**	**1**	**10**	**40**
Rats, *n*	22	22	22	20	20
Tumor bearing rats, *n*	11	9	8	4	9
Tumor incidence[1], %	50	41	36	20 *	45
Tumors, *n*	17	13	11	4	12
Tumor multiplicity[2], *n*	1.55 ± 0.93	1.44 ± 1.01	1.38 ± 0.74	1.00 ± 0.0	1.33 ± 0.50
Tumor size, overall[2], mm	5.94 ± 3.83	5.85 ± 4.22	5.64 ± 3.98	5.00 ± 1.83	5.92 ± 3.73
Small (< 5 mm) tumors, % of total	47	46	45	50	50
Large (≥ 5 mm) tumors, % of total	53	54	55	50	50
Adenoma, % of large tumors	11	-	50	-	50
Carcinoma, % of large tumors	89	86	33	100	67

Values are means ± SD, or %. * Different from control, $P < 0.05$

[1] Quercetin mediated a dose dependent decrease in tumor incidence: $P < 0.05$.

[2] Tumor multiplicity and size were inversely associated with increasing dietary quercetin (both: $r = -0.98$, $P < 0.05$).

Table 3. Plasma concentrations of (3'-O-methyl-) quercetin in non-AOM rats fed quercetin or rutin, killed at the interim autopsy

	Quercetin (g/kg diet)			Rutin (g/kg diet)
	0.1	**1**	**10**	**40**
Total quercetin	0.85 ± 0.13 [a]	7.66 ± 0.35 [b]	37.44 ± 5.06 [c]	13.01 ± 0.40 [d]
Total 3'-O-methyl-quercetin	1.74 ± 0.21 [a]	15.70 ± 1.22 [b]	59.91 ± 12.73 [c]	17.88 ± 0.96 [b]

Values are presented as µmol/L and means ± SD, n = 5/group. Within data series, means without a common letter differ from one another ($0.001 < P < 0.05$).

Discussion

In the present paper quercetin aglycone and its glycoside rutin were investigated for their potency to modulate (biomarkers of) colorectal carcinogenesis. The results of the present study indicate that quercetin, but not rutin is able to reduce AOM-induced colorectal carcinogenesis when administered as of the pre-initiation phase. Furthermore, methylene blue stained ACF in unsectioned colons at wk 8 did not correlate with the CRC incidence at wk 38, even when discriminating between small and large ACF. This finding is in contrast to previous studies, which indicated that especially large ACF (≥ 4 crypts/focus) are reliable CRC-biomarkers [23;24]. The lack of a correlation between the number of methylene blue stained ACF and the tumor incidence is described before [12-14]. Wijnands *et* a*l*. investigated the effect of 40 g rutin/kg diet in the similar AOM-model and mentioned comparable tumor incidences in controls and rutin treated rats [13], as was the case in the present study. As no relationship was found between ACF and the tumor incidence, it was hypothesized that a specific ACF-subclass might be related to colorectal cancer. Therefore, ACF were immuno-histochemically classified into lesions with and without accumulation of the CRC related protein ß-catenin. However, neither of these ACF-subclasses was influenced by quercetin. Taken together, ACF cannot be considered general biomarkers for colorectal cancer.

Another putative CRC-biomarker analyzed, is the ß-catenin accumulated crypt (BCA-C). Among BCA-C, quercetin evoked a significant decrease in ß-catenin accumulation at an intermediate grade, whereas none of the ACF-related parameters were affected. Although quercetin only partially affected BCA-C related parameters,

these results suggest that the BCA-C might be a better biomarker for colorectal cancer than ACF. Previous evidence that BCA-C, but not ACF are reliable biomarkers in AOM-induced colorectal carcinogenesis, is provided by amongst others Hata *et al*. [15]. These authors described increasing ACF-numbers, but decreasing tumor numbers towards the proximal colon. In contrast, occurrence of BCA-C and colorectal tumors increased towards the rectum and were highly correlated with one another. Quercetin not only dose dependently decreased the incidence of colorectal tumors, but also reduced the tumor multiplicity and size of AOM-induced colorectal tumors. The quercetin mediated decrease in tumor incidence is in line with Deschner *et al*. who fed AOM-treated CF1 mice with 1 - 20 g quercetin/kg diet for 50 wk [18]. In the present study, the protective effect evoked by quercetin might be caused by decreased cell proliferation, as was indicated by the dose dependent decrease in tumor size and the significant decrease in proliferation index in ACF. Another mechanism involved might be induction of apoptosis by quercetin [19].

Rats were supplemented with 40 g rutin/kg diet (≈ 20 g quercetin aglycone/kg diet), in order to compensate rutin's low bioavailability [25;26]. Therefore, calculated concentrations of quercetin (-metabolites) in the 40 g rutin/kg diet treated group should be ≈ 2 times higher in comparison with rats maintained on 10 g quercetin/kg diet, which is in contrast to our findings. Rutin supplemented rats showed only ≈ 30% of both quercetin and 3'-O-methyl-quercetin concentrations as found in rats maintained on 10 g quercetin/kg diet. Limiting factors involved in bioavailability of the quercetin moiety in rutin are probably intra-colonic quercetin degradation and limited colonic absorption [7], the latter as a result of a relatively low absorptive area in the colon [5].

In the present study, corrected quercetin intake by rats supplemented with the lowest quercetin concentration of 0.1 g quercetin/kg diet amounted to 4.5 - 9.0 mg quercetin/(kg body wt.d) in the course of the experiment. Linear extrapolation of this dietary intake by rats to a human weighing 60 kg would equal daily supplementation with ≈ 0.3 - 0.5 gram quercetin, which approximates daily intake of one quercetin supplement of 500 mg. The CRC incidence was significantly decreased in AOM-treated rats fed 10 g quercetin/kg diet, which after extrapolation to a human weighing 60 kg would be equal to supplementation with 30 - 50 gram quercetin aglycone per day. This amount is a ≈ 2,000 - 3,000 times increase of the regular dietary quercetin intake that has been estimated to be 16.3 mg/d when expressed as aglycone [27]. One

of the major quercetin sources in the human diet is rutin [7] that in the present experiment seems to be the inactive quercetin isoform with respect to reduction of colorectal carcinogenesis. Thus, when assuming similarity in AOM-induced development of colorectal cancer in the rat and colorectal carcinogenesis in humans, inhibition of human colorectal carcinogenesis cannot be achieved by regular dietary intake of conjugated quercetin and neither by intake of supplements containing quercetin aglycone. The latter, because quercetin's beneficial effect might only occur at non-realistic high concentrations equalling daily intake of 60 - 100 quercetin pills of 500 mg each.

In conclusion, inhibition of the tumor incidence by quercetin at wk 38 was not associated with the ACF-number or -multiplicity at wk 8, nor with the ACF-classification based on the absence or presence of ß-catenin accumulation. Therefore, the aberrant crypt focus is considered not to be a reliable biomarker for colorectal cancer. On the other hand, ß-catenin accumulation in ß-catenin accumulated crypts was partially decreased by quercetin. Therefore, BCA-C might be more relevant CRC biomarkers than (ß-catenin accumulated) ACF, but require further research. In contrast to quercetin aglycone, rutin lacks anti-carcinogenic activity, probably as a result of its low bioavailability.

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Chapter 5

Transcriptome and proteome profiling of colon mucosa from quercetin fed F344 rats point to tumor preventive mechanisms, increased mitochondrial fatty acid degradation and decreased glycolysis

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Quercetin has been shown to act as an anti-carcinogen in experimental colorectal cancer (CRC). The aim of the present study was to characterize transcriptome and proteome changes occurring in the distal colon mucosa of rats supplemented with 10 g quercetin/kg diet for 11 weeks. Transcriptome data analyzed with Gene Set Enrichment Analysis showed that quercetin significantly downregulated the potentially oncogenic mitogen-activated protein kinase (*Mapk*) pathway. In addition, quercetin enhanced expression of tumor suppressor genes, including *Pten, Tp53* and *Msh2*, and of cell cycle inhibitors, including *Mutyh*. Furthermore, dietary quercetin enhanced genes involved in phase I and II metabolism, including *Fmo5, Ephx1, Ephx2* and *Gpx2*. Quercetin increased PPARα target genes, and concomitantly enhanced expression genes involved in mitochondrial fatty acid degradation. Proteomics performed in the same samples revealed 33 affected proteins, of which 4 glycolysis enzymes and 3 heatshock proteins were decreased. A proteome-transcriptome comparison showed a low correlation, but both pointed out towards altered energy metabolism.

In conclusion, transcriptomics combined with proteomics showed that dietary quercetin evoked changes contrary to those found in colorectal carcinogenesis. These tumor-protective mechanisms were associated with a shift in energy production pathways, pointing at decreased glycolysis in the cytoplasm towards increased fatty acid degradation in the mitochondria.

Introduction

Quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the major flavonoids in the human diet and belongs to the subclass of flavonols. In nature, quercetin is present in glycosylated forms in fruits and vegetables, including onions, curly kale, broccoli, leek and blueberries [1]. Quercetin may function as an anti-oxidant, resulting from the electron and/or hydrogen donating capacity of its phenolic hydroxyl groups [2]. It has been hypothesized that scavenging of free radicals might be one of the mechanisms involved in protection against oxidative stress-associated chronic diseases, including cardiovascular diseases, diabetes and cancer [2;3]. Indeed, epidemiological studies suggest that high intake of flavonoids, including quercetin, may protect against coronary heart disease [4]. In addition, quercetin has been shown to be protective in models for diabetes [5] and cancer of the brain [6], breast [7], prostate [8], lung [9] and the colorectum [9;10].

Previously, dietary quercetin has been described to reduce azoxymethane (AOM) induced colorectal carcinogenesis in rats [11], especially in the distal colon (unpublished data), which is also the preferred site of development of colorectal cancer (CRC) in humans [12]. The aim of the present study was to investigate the molecular effects of dietary quercetin in the distal colon mucosa of rats, to elucidate mechanisms by which this anti-oxidant may protect against colorectal carcinogenesis. This approach models the effects of quercetin in the distal colon of humans that consume commercially available quercetin supplements.

A combination of transcriptomics and proteomics was used to obtain a holistic insight into quercetin-mediated effects, since proteins are frequently expressed at levels and in isoforms that cannot always be predicted from mRNA analysis.

Materials & Methods

Animals, treatment and autopsy

Experimental protocols were approved by the Ethical Committee of Wageningen University and Research Centre. At the age of 4 weeks, eight male inbred F344 rats (Charles River Breeding Laboratory, Someren, The Netherlands), weighing 50 ± 6 g (mean ± SD) entered the study and were housed (2 per cage) in sawdust covered plastic cages with wire tops and diet and tap water *ad libitum*. During the 3 weeks of adaptation, rats were fed an RM3 [E] FG SQC rat breeder diet (SDS Special Diets

Services, Witham, England) that was shown to be free of quercetin (-conjugates) [11]. The dietary experiment was subsequently initiated (experimental week 0) by feeding the rats either the control RM3 diet (n = 4/group), or the RM3 diet enriched with 10 g quercetin/kg diet (n = 4/group) for a time period of 11 wk (quercetin obtained from Sigma, Zwijndrecht, The Netherlands). During autopsy, the colorectums were cut open longitudinally, followed by separation of the distal colon, situated in between the fish-bone like structured colon mucosa and the anus. Mucosa of the distal colon was rinsed with 70% ethanol at 4°C, subsequently scraped with sterilized glass slides and fixed in RNA*later*® (Ambion®, Cambridgeshire, U.K.) according to the manufacturer's protocol.

RNA isolation and cleanup

After thawing colon mucosa scrapings on ice, total RNA was isolated with the TRIzol® Reagent (Life Technologies, Paisley, U.K.). Following centrifugation of the TRIzol® homogenate, 3 distinct phases are found: a colorless upper aqueous phase (containing RNA), an interphase (containing DNA and proteins) and a reddish organic phase (containing proteins). Total RNA, present in the aqueous upper phase, was isolated and purified with the NucleoSpin® RNA II kit (Machery-Nagel, Düren, Germany), including a DNAse incubation step to digest possible traces of DNA. Stability of total RNA was checked by a 1 hr incubation step at 37°C, followed by denaturing agarose gel electrophoresis and ethidium bromide staining, showing sharp ribosomal RNA bands. Subsequent analysis on the Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, California, U.S.A.) indicated high quality total RNA (28S/18S ribosomal RNA ratios ~2.0) that was suitable for hybridization experiments.

Affymetrix® GeneChip® arrays

RNA, meant for hybridization on Affymetrix® GeneChip® arrays, was processed according to the GeneChip® One-Cycle Eukaryotic Target Labeling Assay [13]. cRNAs were hybridized to Affymetrix® GeneChip® Rat 230-2.0 arrays, containing 31,042 probe sets that cover 28,000 well-substantiated rat genes. Finally, probe set intensities were detected with the GeneChip® Scanner 3000 7G (Affymetrix® Inc., Santa Clara, California, U.S.A.).

Protein isolation

After centrifugation of the TRIzol® homogenates and removal of the RNA containing aqueous phase for transciptomic purposes, the organic (reddish) protein containing phase of the very same TRIzol® processed samples was isolated and processed for protein isolation. Per sample, 50 µL of the organic phase was first washed with acetone (-20°C) and precipitated by a 10 min centrifugation step at 10,000 g, for a total of three times. The protein pellet was subsequently dissolved in 100 µL 0.1% (w/v) RapiGest™ SF reagent (Waters, Milford, Massachusetts, U.S.A.) dissolved in 50 mM ammonium bicarbonate, pH 7.0 (Merck, Darmstadt, Germany). This mixture was subsequently sonified with an Ultrasonic Disruptor Sonifier® II (Branson Ultrasonics Corp., Danbury, Connecticut, U.S.A.) at an energy setting of 70%, for a maximum of 5 min until the pellet was dissolved. During sonification, samples were cooled in water at 20°C. The resulting protein solution was enzymatically digested by addition of gold grade trypsin (Promega, Madison, Wisconsin, U.S.A.) at 0.1 g/L and overnight incubation at 37°C. To terminate the trypsin reaction and to break down the RapiGest™ SF reagent into volatile compounds, 10 µL of 500 mM HCl was added (final concentration: 30 - 50 mM HCl, pH < 2) and incubated for 45 min at 37°C. Desalting of samples, in order to prevent ion suppression in the ion source of the mass spectrometer, was performed with zip tips according to the manufacturer's instructions (Millipore, Molsheim, France).

Samples were then processed for proteomics-analyses, performed by a combination of two mass spectrometry techniques. Matrix-assisted laser desorption/ionization (MALDI) Fourier transform ion cyclotron resonance mass spectrometry (FT-MS) was applied for accurate measurement of peptide masses at a resolution of less than 1 ppm and corresponding signal intensities at a dynamics of four to five orders of magnitude. In addition, MALDI time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) was used for peptide identification and linked to FT-MS retrieved peptide signal intensities [14], as outlined below.

MALDI-FT-MS

Onto an anchorchip target plate (600/384 anchorchip with transponder plate; Bruker Daltonics, Bremen, Germany), 0.5 µL of a solution containing 10 mg dihydroxy-benzoic acid (DHB) matrix (Bruker Daltonics, Bremen, Germany) per mL 0.1% (v/v)

TFA in water was spotted, followed by immediate mixing with 0.5 µL of the above described desalted peptide solution. Subsequently, the mixture was allowed to dry at ambient temperature. The measurements were performed by MALDI-FT-MS (Apex Q 9.4 Tesla equipped with a combi-source, Bruker Daltonics, Bremen, Germany). For each measurement 200 scans were summed up and for each scan, ions generated by 10 laser shots were accumulated in a mass range of 800 - 4,000 *m*/*z*. To align the mass spectra of different samples, an internal calibration was performed on cytokeratin 19 (Type I keratin KA19) peptide masses, present in all samples (1029.59386; 1041.60509; 1104.5466; 1122.57894; 1197.5858; 1204.599; 1329.70081; 1537.73149; 1628.71547; 1760.90984; 1859.94971; 1879.9395 and 2498.33608). Subsequently, peak intensities were retrieved using a peak finding algorithm that determines the highest peak intensity within a 3 ppm window at both sides of each *m/z* value present above a signal to noise ratio (S/N) of > 4, as described by Titulaer *et al.* [15].

MALDI-TOF/TOF-MS

Trypsin-mediated peptide samples were run on an Ultimate 3000™ Nano and Capillary LC System (Dionex, Sunnyvale, California, U.S.A.) and fractionated by a monolithic column (200 µm I.D. x 150 mm, 3 µm, Dionex, Sunnyvale, CA, U.S.A.). Fractionation was performed by eluting 5 µL of quercetin and control samples at a flow of 2 µL/min, with a 45 min gradient from 0 to 64% of acetonitrile (ACN) using solution A [0.05% (v/v) trifluoroacetic acid (TFA) in nanopure water] and solution B [80% (v/v) ACN, 20% (v/v) nanopure water and 0.04% (v/v) TFA; 0 - 1 min: 0%, 1 - 4 min: 10%, 4 - 35 min: 50%, 35 - 35.1 min: 80%, 35.1 - 37 min: 80%, 37 - 37.1 min: 0% and 37.1 - 45 min 0%].

The flow-through containing the fractions was spotted on Prespotted Anchor Chip plates (Bruker Daltonics, Bremen, Germany) using a Probot™ Micro Fraction Collector (LC Packings/Dionex, Sunnyvale, California, U.S.A.). Subsequently, the spots were identified by the Ultraflex™ I MALDI-TOF/TOF-MS (Bruker Daltonics, Bremen, Germany).

For MS/MS identification, peptides were matched in the Mascot search engine (www.matrixscience.com, Matrix Science, London, U.K.) to the MSDB rat database with the following settings: semi-Trypsin digestion, a +/- 200 ppm peptide mass tolerance and a fragment mass tolerance of +/- 0.5 Da.

Transcriptomics data processing and analyses

The data described in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number **GSE 7479**. Transcriptome signal intensities were normalized using the GCRMA algorithm, and signal intensities of < 10 were substituted by 10. Subsequently, intensities were converted into mean-centered ^{2}log ratios per probe set, and genes showing a signal of 10 in all samples were excluded (n = 10,893). Transcriptomics data were analyzed on differential expression of either individual genes or of gene sets. For the first analysis, genes were considered to be significantly affected by quercetin when at least three out of four quercetin samples showed a minimum up- or downregulation of 1.6 (^{2}log 0.7) relative to the average intensities of controls. This was combined with the restriction that the average fold change should be more than 2 times the standard deviation of the control samples.

In the second type of analysis, the full transcriptome dataset was analyzed using the Gene Set Enrichment Analysis 2.0 (GSEA) tool [16]. In brief, GSEA first makes a ranking list based on fold changes in gene expression. Subsequently, by using a pre-defined set of associated genes based on prior biological knowledge, GSEA calculates whether this set is mainly enriched at the top or bottom of the fold change-based ranking list, or randomly distributed [16]. Gene sets applied were part of GSEA's Molecular Signature Database, including the curated (functional-based) C2 database that contains gene sets of metabolic and signaling pathways [17] and the C4 (cancer neighborhood) database containing co-expressed genes in the neighborhoods of ≈ 400 cancer related genes [18]. Additionally, GSEA analyses were performed with self-created gene sets derived from microarray-based toxicology and cancer literature data, referred to in Table 2. Gene sets were permutated 1,000 times in order to calculate the proportion of false positives, expressed as the false discovery rate (FDR). Gene sets were considered to be significantly changed by dietary quercetin when showing a combination of $P < 0.05$ and FDR < 0.3.

Mass spectrometry data processing and analyses

FT-MS intensities were normalized to the overall average, to enable sample-to-sample comparison. To enable calculation of fold changes (quercetin/control) in signal intensities among identified peptides, data obtained by MALDI-FT-MS (for measurement of signal intensities) and MALDI-TOF/TOF (for peptide identification)

were matched within in a +/- 3 ppm mass tolerance window. Among FT-MS data, absent peptide signal intensities as a result of a cut off at S/N > 4, were substituted by 50% of the lowest signal intensity found for that particular peptide, to enable calculation of fold changes. For subsequent analyses, proteins were included with at least 2 identified peptides. Proteins were considered to be significantly affected by quercetin when all identified peptides showed an identical direction of fold change (either 100% of the peptides upregulated or 100% of the peptides downregulated), combined with an average peptide fold change showing a minimum up- or down-regulation of 1.3.

Comparison of proteomics with transcriptomics data

To determine if and to what extent proteome fold changes could be compared to transcriptome fold changes, identifiers of significantly changed proteins were linked to gene symbols by data retrieval from MartView [19], ExPASy [20], Ensembl [21] and NCBI [22]. Thus retrieved gene symbols were linked to Affymetrix probe set IDs to obtain the mRNA fold changes. In the proteomics-transcriptomics comparison, only genes with ≥ 1 probe set(s) showing a minimum up- or downregulation of 1.3 were included.

Results

Body weights and dietary intake

Quercetin supplemented and control rats showed comparable body weights ranging from 132 ± 4 g vs. 134 ± 4 g, respectively at week 0, increasing to 325 ± 13 g vs. 309 ± 14 g at week 11. In addition, dietary intake was also comparable between quercetin and control rats ranging from 15.5 ± 0.4 g/d vs. 16.1 ± 1.9 g/d, respectively at wk 0, to 16.9 ± 0.0 g/d vs. 15.7 ± 1.0 g/d at wk 11. In the course of the experiment, rats fed the 10 g quercetin/kg diet showed a corrected dietary intake of 900 mg quercetin/kg body wt/day at wk 1, decreasing to 520 mg quercetin/kg body wt/day at wk 11.

Single gene analyses

Transcriptomics data were analyzed in two ways; the first analysis was based on filtering of genes of which at least 3 out of 4 quercetin rats showed a minimum up- or downregulation of 1.6 when compared to the average of controls, in combination with

the criterion that the fold change was at least 2 times the standard deviation of controls. In total, 207 genes were significantly affected by quercetin, of which 42 were downregulated and 165 were upregulated (**Appendix of this chapter, Table 1**). The top 10 downregulated genes among annotated genes were found with *Plcd3* (Phospholipase C, delta 3 [predicted]; fold change: -3.2) and *Anxa1* (Annexin A1; fold change: -3.1), as can be seen in **Table 1**. On the other hand, the top 10 of genes upregulated by quercetin included the antigen binding *LOC500183* (Similar to IG Kappa chain V-V region K2 precursor; fold change: 4.4) and *Mgll* (Monoglyceride lipase; fold change: 3.4) that encodes a serine hydrolase which converts monoglycerides into glycerol and fatty acid (FA).

GSEA analyses

The second analysis was performed with GSEA by using the complete Affymetrix® GeneChip® dataset. Quercetin affected 57 gene sets that could be classified in 13 self-defined categories based on biological function, namely inhibition of carcinogenesis, metabolism, energy metabolism, redox homeostasis, xenobiotic metabolism, immune reaction, nucleotide modification, protein modification, translation, cell-cell interaction, amino acid metabolism and miscellaneous (**Table 2**).

In view of its anti-carcinogenic potency, a noteworthy effect evoked by quercetin is downregulation of the potentially oncogenic mitogen-activated protein kinase *(Mapk* or *Erk1*/*2*) pathway that showed the highest significance in the category "Inhibition of carcinogenesis" ($P << 0.001$, FDR < 0.05, **Table 2**). This gene set includes the *Ras* proto-oncogene associated genes *Sos1* (Son of sevenless homolog 1 (Drosophila); fold change: -1.3), *Shc1* (Src homology 2 domain-containing transforming protein C1; fold change: -1.2) and *Grb2* (growth factor receptor bound protein 2; fold change: -1.3), as depicted in **Figure 1A**. In addition to reduction of these potentially oncogenic genes, quercetin significantly upregulated the gene set entitled "Tumor suppressors" ($P < 0.05$, FDR < 0.05, **Figure 1B**). Upregulated tumor suppressor genes include *Pten* (phosphatase and tensin homolog deleted on chromosome 10; fold change: 1.2), *Tp53* (tumor protein 53; fold change: 1.5) and *Msh2* (DNA mismatch repair protein, alias MutS homolog 2; fold change: 1.2). Another significantly upregulated gene set in this category is "Cell cycle inhibition" ($P < 0.05$, FDR < 0.3) that encompasses genes involved in inhibition of progression through the cell cycle, including *Mutyh* (mutY

homolog [*E. coli*]; fold change: 1.3) that is overexpressed in all quercetin rats, when compared to controls (**Figure 1C**).

Table 1. Top 20 of affected genes in the distal colon mucosa of rats fed 10 g quercetin/kg diet

Gene Title	Gene symbol	Affymetrix ID	Fold change (quercetin/control)	GO Biological Process Description
Top 10 most downregulated genes				
Transcribed locus	-	1392097_at	-3.8	-
Phospholipase C, delta 3 (predicted)	*Plcd3_predicted*	1381747_at	-3.2	Lipid metabolism, signal transduction, intracellular signaling cascade
Annexin A1	*Anxa1*	1394451_at	-3.1	Regulation of cell proliferation, lipid metabolism, cell motility, inflammatory response, cell cycle, signal transduction, cell surface receptor linked signal transduction, insulin secretion, arachidonic acid secretion
TSC22 domain family 3	*Tsc22d3*	1380777_at	-3.0	Anti-apoptosis, fluid secretion, regulation of transcription, DNA-dependent
Tropomyosin 1, alpha	*Tpm1*	1379936_at	-3.0	Regulation of muscle contraction, muscle development, regulation of heart contraction

Table 1, continued

Gene Title	Gene symbol	Affymetrix ID	Fold change (quercetin/control)	GO Biological Process Description
Basic helix-loop-helix domain containing, class B2	*Bhlhb2*	1379483_at	-3.0	Negative regulation of transcription, DNA-dependent, nervous system development, entrainment of circadian clock, regulation of neuronal synaptic plasticity
Myosin VC (predicted)	*Myo5c_predicte*	1380067_at	-3.0	-
Transcribed locus	-	1396955_at	-2.9	-
Period homolog 2 (Drosophila)	*Per2*	1368303_at	-2.9	Regulation of transcription, DNA-dependent, signal transduction, rhythmic behavior, circadian rhythm, rhythmic process. transcription
Transcribed locus	-	1381646_at	-2.8	-
Top 10 most upregulated genes				
Peroxiredoxin 6	*Prdx6*	1367969_at	2.2	Lipid catabolism, response to reactive oxygen species
Progressive ankylosis homolog (mouse)	*Ank*	1369249_at	2.2	Skeletal development, transport, phosphate transport, sensory perception of sound, locomotory behavior, regulation of bone mineralization
Kinesin family member C1	*Kifc1*	1376185_at	2.3	Microtubule-based movement
Solute carrier family 22, member 3	*Slc22a3*	1387189_at	2.3	Transport, ion transport, cation transport. organic cation transport

Table 1, continued

Gene Title	Gene symbol	Affymetrix ID	Fold change (quercetin/control)	GO Biological Process Description
Transcribed locus	-	1373229_at	2.5	-
Plasminogen activator, tissue	*Plat (t-PA)*	1367800_at	2.5	Protein modification, proteolysis, blood coagulation, platelet-derived growth factor receptor signaling pathway
Nuclear receptor subfamily 1, group D, member 1	*Nr1d1*	1370816_at	2.8	Regulation of transcription, DNA-dependent, circadian rhythm
Angiotensin 1 converting enzyme	*Ace*	1387791_at	2.9	Response to hypoxia, proteolysis, blood pressure regulation, carbohydrate metabolism
Monoglyceride lipase	*Mgll*	1388644_at	3.4	Proteolysis, lipid metabolism, aromatic compound metabolism, inflammatory response
Similar to IG Kappa chain V-V region K2 precursor, similar to NGF-binding Ig light chain	*LOC500180, LOC500183*	1387902_a_at	4.4	-

Genes affected by 10 g quercetin/kg diet selected on the criteria of ≥ 3 quercetin samples with a minimum up- or downregulation of 1.6 when compared to the average of controls, combined with a fold change of ≥ 2 times the standard deviation of controls.

In the category "Metabolism", the most significantly upregulated gene set contained upregulated target genes of PPARα (peroxisome proliferator-activated receptor α, $P << 0.001$, FDR < 0.001, **Table 2** and **Figure 1D**). This gene set includes upregulated genes involved in fatty acid degradation, including *Hmgcs2* (3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2; fold change: 1.7), *Acaa1* (Acetyl-Coenzyme A acyltransferase 1; fold change: 1.3) and *Acads* (Acetyl-Coenzyme A dehydrogenase, short chain; fold change: 1.2). This effect is more pronounced in the upregulated gene set "Fatty acid catabolism" and especially in "Mitochondrial fatty acid beta oxidation", both of which are involved in fatty acid degradation, and showed the highest significance ($P << 0.001$, FDR < 0.001) in the category "Energy metabolism" (**Table 2** and **Figure 1E**). Genes involved in this set include *Slc25a20* (solute carrier family 25 = mitochondrial carnitine/acylcarnitine translocase, member 20; fold change: 1.5) and *Acadvl* (acyl-Coenzyme A dehydrogenase, very long chain; fold change: 1.4).

Furthermore, quercetin treatment led to upregulation of genes encoding for enzymes involved in phase I and II metabolism that encompass enzymes involved in metabolism of xenobiotics. Among phase I enzymes, *Fmo5* (flavin containing monooxygenase 5; fold change: 1.2), *Ephx1* (epoxide hydrolase 1, microsomal; fold change: 1.7) and *Ephx2* (epoxide hydrolase 2, cytoplasmic; fold change: 1.2) were upregulated (**Figure 1F**), as was the case with the phase II enzymes *Gpx2* (glutathione peroxidase 2; fold change: 1.5) and *Comt* (catechol-O-methyltransferase; fold change: 1.6), depicted in **Figure 1G**.

Table 2. Overview of significantly affected gene sets found by GSEA analysis of gene expression in the distal colon mucosa of rats fed 10 g quercetin/kg diet

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Inhibition of carcinogenesis							
Mapk (= Erk1/2) pathway	The mitogen-activated protein kinase (*Mapk*) pathway is regulated by *Raf*, *Mos*, and *Tpl-2*	0.000	0.05	31	39	[17]	Down
DNA damage signaling	Genes involved in reconstruction of a continuous two-stranded DNA molecule without mismatches	0.000	0.05	57	56	[17]	Up
B-cell receptor complexes	Antigen binding to B cell receptors activates protein tyrosine kinases, such as the Src family, which ultimate activate MAP kinases	0.000	0.05	31	39	[17]	Down
Up in trichostatin A treated hepatoma cell lines	Up-regulated in more than one of several human hepatoma cell lines by 24-hour treatment with the	0.001	0.18	23	52	[17]	Up
DNA repair	Cancer related genes involved in DNA repair	0.009	0.05	21	76	[17]	Up
Caspase activation	Intracellular signaling in caspase activation, usually followed by apoptosis	0.009	0.16	11	55	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Up in TNF-treated HeLa cells	Upregulated genes in tumor necrosis factor (TNF) stimulated (6 hr) HeLa cells in which NF-kappaB is inhibited by doxycycline	0.016	0.08	67	36	[49]	Down
CD40 pathwaymap	Genes related to CD40 signaling	0.017	0.24	28	43	[17]	Down
Tumor suppressors	Genes involved in suppression of tumors	0.032	0.05	20	50	[17]	Up
Cell cycle inhibition	Genes that inhibit cell proliferation	0.033	0.30	34	26	[48]	Up
Tnfrsf1b neighborhood	Genes in the neighborhood of tumor necrosis factor receptor superfamily, member 1b (*Tnfrsf1b*)	0.035	0.14	31	35	[18]	Up
Metabolism							
PPARα targets	PPARα regulated genes	0.000	0.00	66	44	[36]	Up
Bile acid biosynthesis	Genes involved in synthesis of bile acids	0.000	0.01	19	32	[17]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Cholesterol metabolism	Decomposition of cholesterol	0.000	0.02	25	24	[48]	Up
PPARα, 5 day stimulation	Upregulated PPARα target genes in the rat liver following administration of a PPAR agonist for 5 days	0.000	0.20	292	33	[50]	Up
Glucose metabolism	Metabolism of glucose	0.016	0.21	12	58	[48]	Up
PPARα, 6hr stimulation	Upregulated PPARα target genes in the rat liver following administration of a PPAR agonist for 6 hrs	0.019	0.28	75	33	[50]	Up
Energy metabolism							
Mitochondrial fatty acid (FA) beta oxidation (FA catabolism)	Fatty acid oxidation (FA catabolism) in mitochondria of cells that utilize free fatty acids as an energy source	0.000	0.01	21	52	[17]	Up
Mitochondrion	Mitochondrial genes	0.000	0.01	200	48	[48]	Up
Peroxisome	Intracellular microbodies that contain peroxidase, catalase and allied enzymes	0.004	0.12	38	50	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Fatty acid metabolism	Lipid catabolism in cells that utilize free fatty acids as an energy source	0.001	0.23	55	8	[17]	Up
Acyltransferase activity	Enzymes that catalyse the acyl group transfer of acyl Co-A	0.004	0.14	40	40	[48]	Up
ATPase activity	Enzymes that catalyse ATP hydrolysis which is usually coupled with another function e.g. Ca^{2+} transport across a membrane	0.005	0.20	39	33	[48]	Up
Xenobiotic metabolism							
Phase I metabolizing enzymes	Genes encoding for phase I enzymes	0.018	0.06	49	35	[51-53]	Up
Phase II metabolizing enzymes	Genes encoding for phase II enzymes	0.047	0.08	43	47	[51;52]	Up
Redox homeostasis							
Oxidoreductase activity	Enzymes catalyzing oxidoreduction reactions during which the substrate that is oxidized, is regarded as a hydrogen donor	0.000	0.04	162	47	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Electron transport	Transport of electrons from a reduced substrate to molecular oxygen	0.000	0.09	84	36	[48]	Up
Ion transporter activity	Transporters of ions across energy-transducing cell membranes	0.012	0.14	11	27	[48]	Up
Metal ion binding	Genes encoding for enzymes involved in binding of metal ions	0.028	0.18	16	50	[48]	Up
Immune reaction							
Acute-Phase response	Early local inflammatory reaction to insult or injury that consists of increased inflammatory humoral factors	0.000	0.01	14	21	[48]	Up
LAIR pathway	The local acute inflammatory response (LAIR) is mediated by activated macrophages and mast cells or by complement activation	0.002	0.16	10	28	[17]	Up
Down in 5 endothelial cell lines	Genes down-regulated by interferon-gamma that mediates cell-cell interactions among primary endothelial cells of the colon, dermis, iliaca, aorta and lung	0.004	0.04	40	63	[17]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
IL-12 pathway	Antigen-presenting dendritic cells and macrophages secrete IL-12, which induces Th1 cell differentiation	0.006	0.28	14	50	[17]	Down
Nucleotide modification							
DNA repair	Genes that reconstruct damaged DNA, leading to continuous two-stranded DNA without mismatches	0.000	0.06	32	34	[48]	Up
RNA binding	Proteins that bind to RNA molecules	0.001	0.21	85	57	[48]	Up
Transcription	Synthesis of messenger RNA	0.001	0.08	28	45	[48]	Up
Transcription from RNA polymerase II promoter	Polymerase responsible for synthesizing messenger RNA	0.005	0.07	11	29	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Endonuclease activity	Enzymes capable of hydrolyzing highly polymerized dsDNA by splitting phosphodiester linkages, catalyzing endonucleolytic cleavage	0.028	0.20	16	44	[48]	Up
Protein modification							
Cysteine protease inhibitor activity	Inhibition of endopeptidases which have a cysteine involved in the catalytic process	0.002	0.06	12	50	[48]	Up
Proteasome	Multisubunit complex involved in the degradation of the majority of cytosolic and nuclear proteins in eukaryotic cells	0.002	0.03	16	50	[17]	Up
Aminopeptidase activity	Exopeptidases that act on the free N terminus end of a polypeptide, liberating a single amino acid residue	0.008	0.15	16	38	[48]	Up
Serine-type endopeptidase activity	Endopeptidases containing at the active site a serine residue involved in catalysis	0.016	0.20	38	32	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Translation							
Protein biosynthesis	Translation from mRNA into proteins	0.000	0.10	100	53	[48]	Up
Ribosome	Genetic translation of transcripts as well as	0.004	0.26	64	48	[48]	Up
Translation initiation factor activity	Translational initiation for global protein synthesis	0.014	0.17	14	20	[17]	Up
Cell-cell interaction							
Microtubule-based movement	Motor-protein-mediated motility of vesicles and organelles along microtubules that are part of the cytoskeleton and composed of the protein tubulin	0.014	0.18	19	42	[48]	Up
Cellular defense response	Defense response mediated by cells, e.g. by cell-cell and cell-matrix interactions	0.015	0.14	35	20	[48]	Up
Cell communication	Genes involved in cell-to-cell communication	0.035	0.27	14	29	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Amino acid metabolsim							
Valine, Leucine and Isoleucine degradation	Valine, Leucine and Isoleucine degradation	0.000	0.00	27	59	[17]	Up
Histidine metabolism	Metabolism of histidine, an essential amino acid required for the production of histamine	0.004	0.01	17	59	[17]	Up
Miscellaneous							
G-alpha-q pathway	G-alpha-q (GAQ) activates phospholipase C, resulting in calcium influx and increasing protein kinase C activity	0.000	0.00	30	43	[17]	Up
Isomerase activity	Enzymes that catalyze geometric or structural changes within a molecule to form a single product	0.000	0.02	34	74	[48]	Up
Intrinsic prothrombin activation pathway	The intrinsic prothrombin activation pathway is activated by traumatized blood vessels and induces	0.000	0.11	12	10	[17]	Up
Microsome	Enzymatic activity in microsomes that are formed from the endoplasmic reticulum	0.004	0.14	44	43	[48]	Up

Table 2, continued

Geneset	Description	*P*-value	FDR q-value	Total no. of genes	% affected genes	Reference	Quercetin effect
Down by caloric restriction	Downregulated in the gastrocnemicus of aged (30-months) mice subjected to caloric restriction	0.06	0.30	16	38	[17]	Up
Neurotransmitter secretion	Secretion of neurotransmistters	0.014	0.14	12	75	[48]	Down

Significantly affected gene sets analyzed in GSEA grouped by self-defined categories based on biological function. Within each category gene sets are sorted on decreasing significance. The number of affected genes is expressed as percentage of the total number of genes within each gene set. The column "Quercetin effect" describes the ratio of quercetin/control. FDR: false discovery rate.

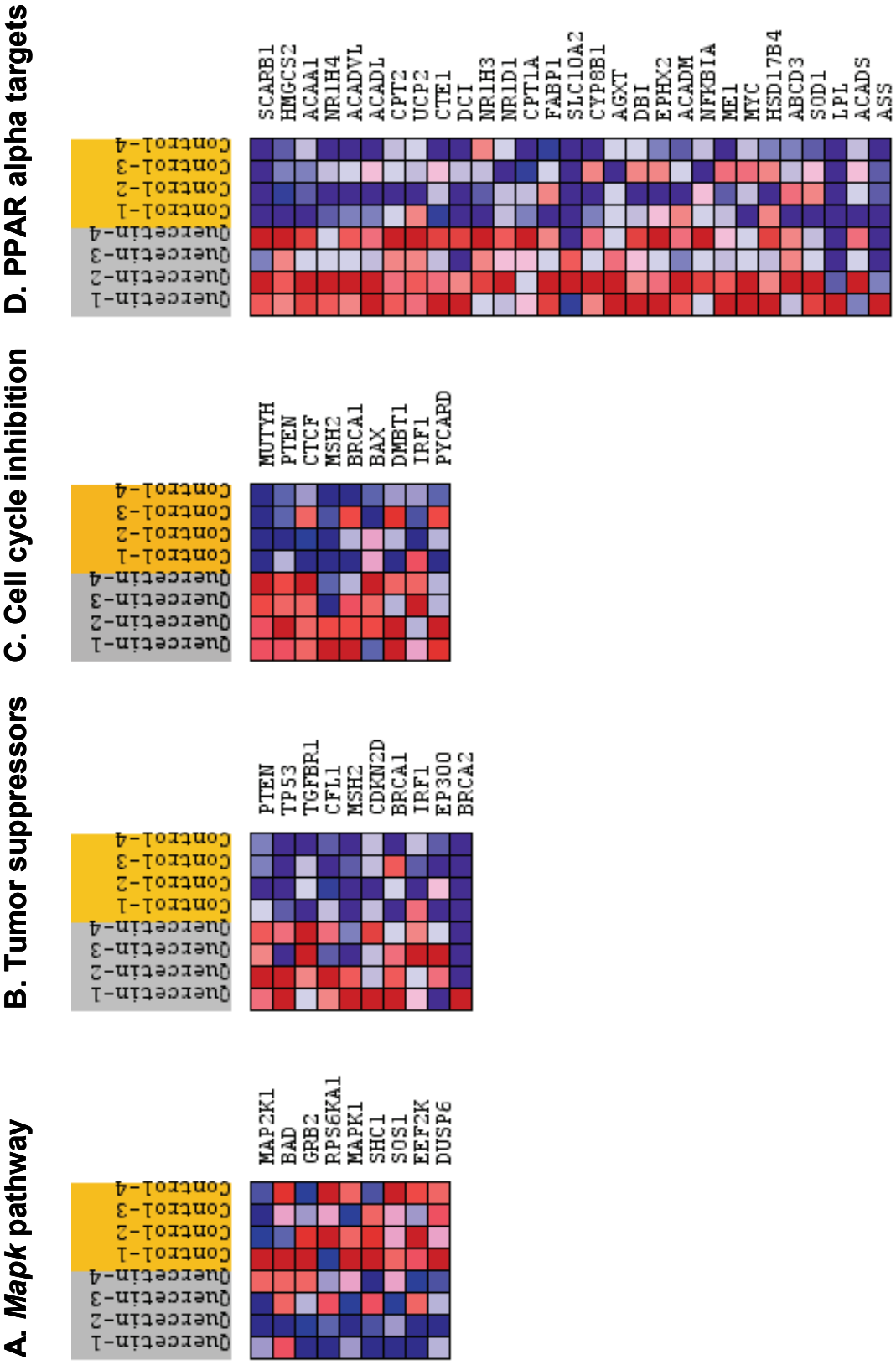
Mass spectrometry data

MALDI-TOF/TOF-MS identification in quercetin and control samples retrieved 624 distinct peptide masses. In total, 244 out of 624 peptides (≈ 39%) were also found to be present among the 8,531 unique MALDI-FT-MS retrieved masses, within a 3 ppm mass tolerance window; these 244 peptides were associated with 135 proteins (**Figure 2**). Subsequent filtering on proteins with at least two identified peptides retrieved 191 unique peptides (≈ 78% of the 244 overlapping peptides). Additional filtering on proteins with peptides showing an identical direction of fold change and a minimum average up- or downregulation of 1.3, retrieved 71 unique peptides (≈ 29% of the 244 overlapping peptides), associated with 33 unique proteins. In total, 3 proteins were upregulated and 30 downregulated. Four glycolysis related enzymes were identified, all of which were down-regulated, including fructose-bisphosphate aldolase A (fold change: -1.9 ± 0.7) and glyceraldehyde-3-phosphate dehydrogenase (-2.4 ± 2.2) as can be seen in **Table 3** (detailed peptide information available from **Appendix Table 2**). In addition, three heatshock proteins were downregulated by quercetin (fold changes -1.6 to -2.4), as well as proteins involved in cell-cell contacts and the cytoskeleton, including annexin A2 that showed a 2-fold downregulation.

In total, 13 out of the 33 proteins affected by quercetin (≈ 40%) are potentially (but not exclusively) localized in mitochondria, according to the gene ontology "cellular component" (*Atp5f1, Ckb, Ckmt1, Cs, Gapdh, Hba, Hmgcs2, Hspcb, Pkm2, Tuba1, Tuba6, Uba52* and *Uqcrc2*).

Comparison of proteomics and transcriptomics

Gene filtering on ≥ 1 genes showing a minimum up- or downregulation of 1.3 allowed 12 proteins and genes to be compared with one another (**Table 4**). In total, only 2 out of these 12 hits showed a fold change in identical direction at both the protein and gene level, namely *Anxa2* (Annexin A2) and *Ckb* (Creatine kinase B-type).


A. Mapk pathway
B. Tumor suppressors
C. Cell cycle inhibition
D. PPAR alpha targets
MAP2K1
BAD
GRB2
RPS6KA1
MAPK1
SHC1
SOS1
EEF2K
DUSP6
PTEN
TP53
TGFBR1
CFL1
MSH2
CDKN2D
BRCA1
IRF1
EP300
BRCA2
MUTYH
PTEN
CTCF
MSH2
BRCA1
BAX
DMBT1
IRF1
PYCARD
SCARB1
HMGCS2
ACAA1
NR1H4
ACADVL
ACADL
CPT2
UCP2
CTE1
DCI
NR1H3
NR1D1
CPT1A
FABP1
SLC10A2
CYP8B1
AGXT
DBI
EPHX2
ACADM
NFKBIA
ME1
MYC
HSD17B4
ABCD3
SOD1
LPL
ACADS
ASS
Quercetin-1
Quercetin-2
Quercetin-3
Quercetin-4
Control-1
Control-2
Control-3
Control-4

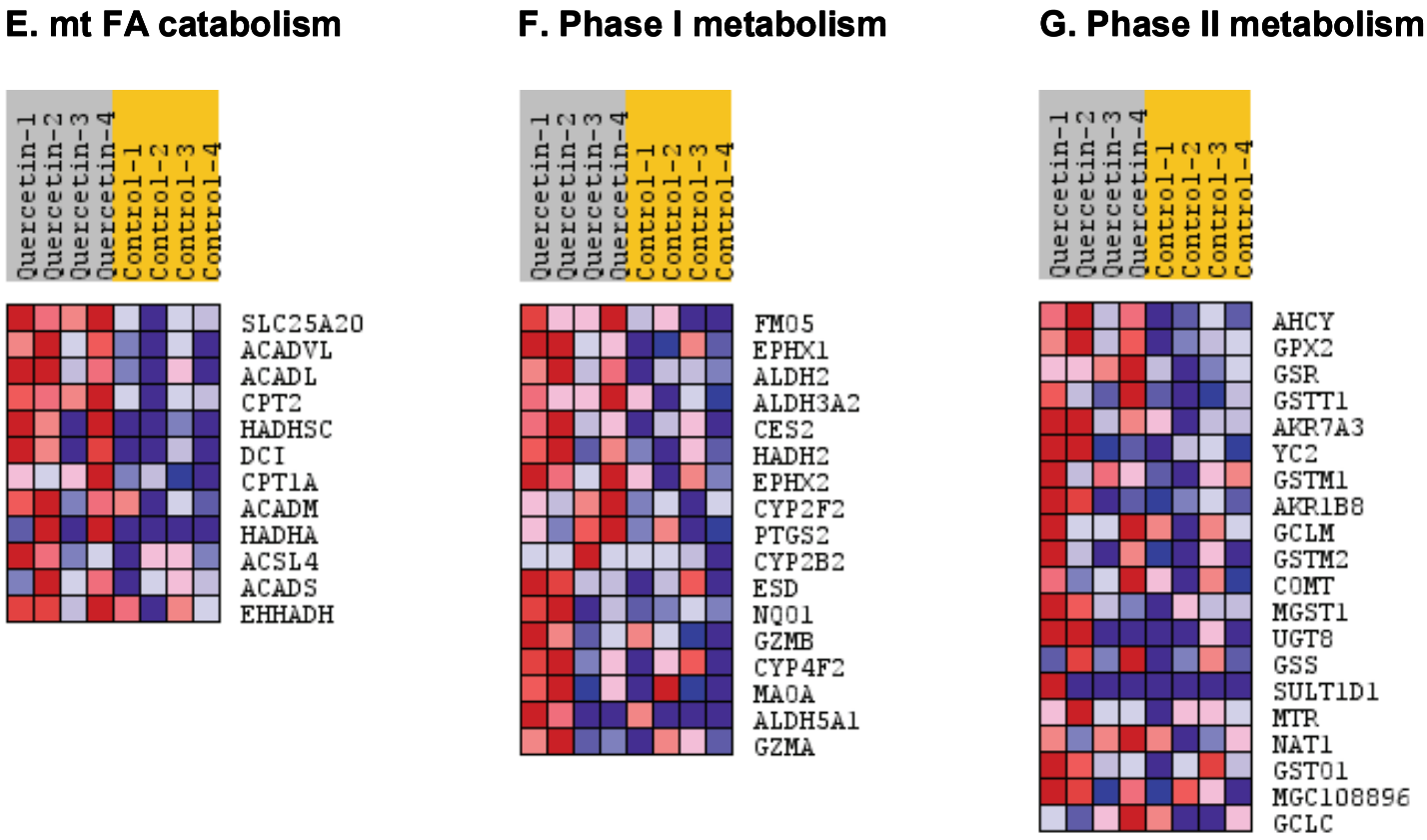


Fig. 1. Heatmaps showing the enriched parts of the significantly affected gene sets *Mapk* pathway (panel A), tumor suppressors (panel B), cell cycle inhibition (panel C), PPARα targets (panel D), mitochondrial fatty acid metabolism (panel E), and phase I and II metabolism (panels F and G, respectively. Red blocks indicate relatively high gene expression, and blue blocks relatively low gene expression. *Mapk*: mitogen-activated protein kinase; PPAR: peroxisome proliferator-activated receptor; mt: mitochondrial; FA: fatty acid.

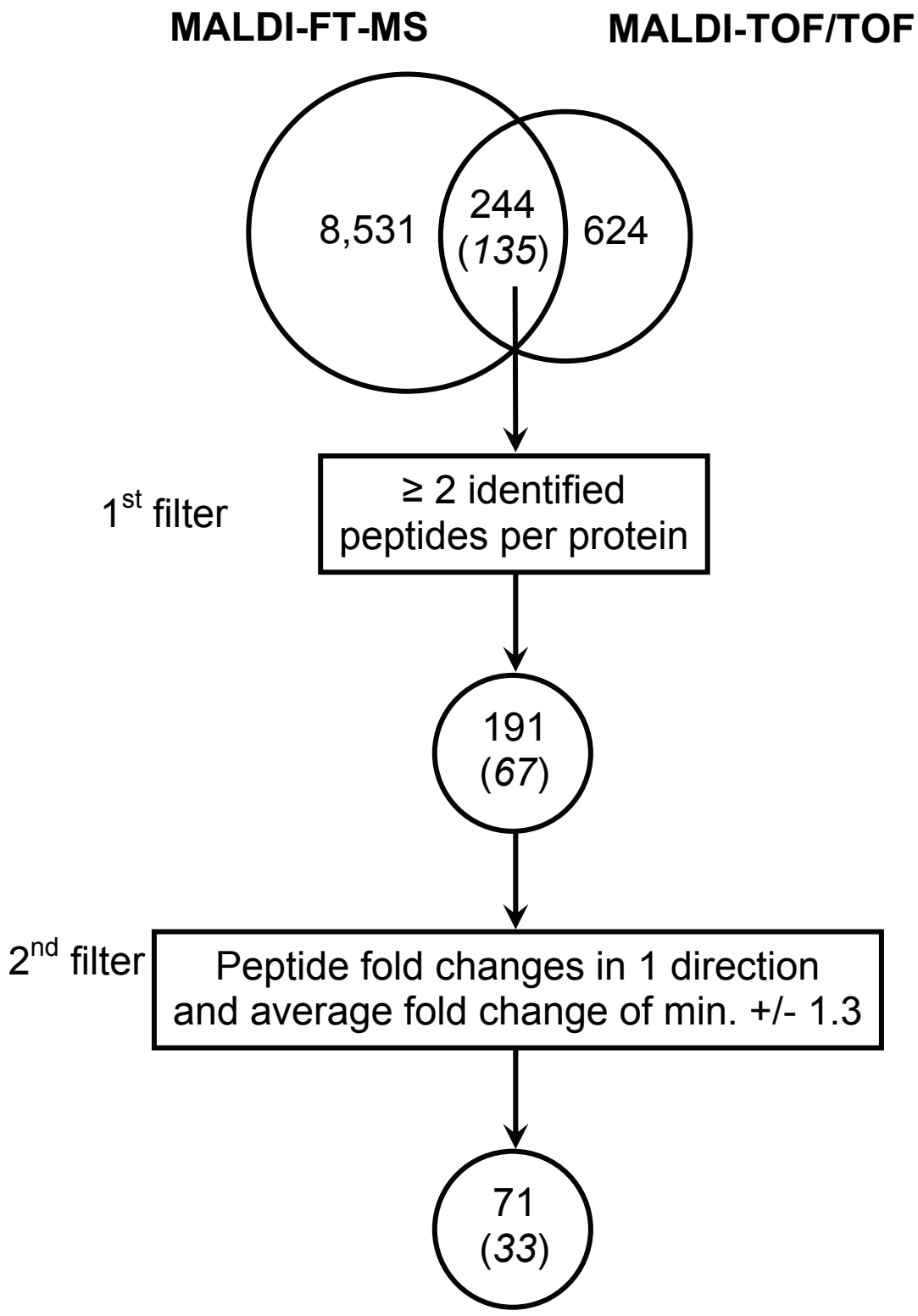


Fig. 2. Overview of proteomics data filtering. Numbers in circles indicate the number of unique peptide masses and italic numbers between brackets indicate the number of proteins associated with these peptide masses.

Table 3. **Significantly affected proteins in the distal colon mucosa of rats fed 10 g quercetin/kg diet.**

Protein description (synonym)	Protein accession	Sequence coverage % (% matched peptides)	No. of peptides	Mean fold change ± SD	GO biological process description
Glycolysis					
Alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	A23126	13 (100)	3	-1.5 ± 0.6	Glycolysis
Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase)	AAH64440	15 (100)	3	-1.9 ± 0.7	Glycolysis, fructose metabolism
Pyruvate kinase isozyme M2 (EC 2.7.1.40)	A26186	8 (100)	2	-1.7 ± 0.1	Glycolysis
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)	G3P	16 (100)	3	-2.4 ± 2.2	Glycolysis, apoptosis, glucose metabolism
Heatshock proteins					
Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	HSP7C	92 (50)[A]	2	-2.4 ± 0.5	Regulation of progression through cell cycle, protein folding
Heat shock protein 86 (Heat shock protein 1, alpha)	Q91XW0	12 (50)	2	-1.6 ± 0.1	Protein folding
Heat shock protein HSP 90-beta (HSP 84)	AAB23369	4 (100)	2	-1.6 ± 0.1	Protein folding
Cytoskeleton and cell-cell contact					
Transgelin 2	Q5XFX0	13 (100)	2	-1.5 ± 0.3	Muscle development
Tubulin alpha-1 chain (Alpha-tubulin 1)	AAH62238	13 (100)	4	-2.4 ± 1.0	Microtubule-based process, protein polymerization

Protein description (synonym)	Protein accession	Sequence coverage % (% matched peptides)	No. of peptides	Mean fold change ± SD	GO biological process description
Tubulin alpha-6 chain (Alpha-tubulin 6)	AAH78829	14 (80)	5	-2.7 ± 1.0	Microtubule-based process, protein polymerization
Tubulin alpha-8 chain (Alpha-tubulin 8)	AAH79185	7 (100)	2	-1.6 ± 0.2	Microtubule cytoskeleton organization and biogenesis
Keratin 21, type I, cytoskeletal	A40452	8 (100)	2	-1.6 ± 0.4	
Actin, alpha cardiac (Alpha-cardiac actin)	ACTC	7 (100)	2	2.4 ± 1.1	Regulation of muscle contraction
Annexin A2 (Lipocortin II) (Calpactin I heavy chain) (Chromobindin 8) (p36) (Protein I)	ANXA2	13 (100)	2	-1.9 ± 0.0	Angiogenesis, skeletal development
Profilin-1 (Profilin I)	CAA65655	21 (100)	2	-2.6 ± 1.9	Actin cytoskeleton organization and biogenesis
Dynein heavy chain, cytosolic (DYHC) (Cytoplasmic dynein heavy chain) (MAP1C)	DYHC	< 1 (100)	2	3.5 ± 3.0	Microtubule-based movement
Krebs cycle					
Citrate synthase	Q8VHF5	5 (100)	2	-1.5 ± 0.6	Main pathways of carbohydrate metabolism, tricarboxylic acid cycle
Kinase activity					
Creatine kinase B-type	AAA40933	6 (100)	2	-2.0 ± 0.9	Phosphocreatine metabolism
Creatine kinase, ubiquitous mitochondrial precursor (EC 2.7.3.2) (U- MtCK) (Mia-CK) (Acidic-type mitochondrial creatine kinase)	S17189	12 (75)	4	-2.2 ± 1.2	Phosphocreatine biosynthesis

Protein description (synonym)	Protein accession	Sequence coverage % (% matched peptides)	No. of peptides	Mean fold change ± SD	GO biological process description
Protein modification					
Polyubiquitin [Fragment]	Q63654	25 (100)	2	-1.5 ± 0.1	Ubiquitin-dependent protein catabolism
Ubiquitin / ribosomal protein S27a, cytosolic [validated]	I52328	25 (100)	2	-1.5 ± 0.1	Protein biosynthesis. protein modification
Ubiquitin / ribosomal protein L40, cytosolic [validated]	I65237	25 (100)	2	-1.5 ± 0.1	Protein biosynthesis
Mitochondrial import stimulation factor L subunit (14-3-3 protein epsilon)	AAC52676	9 (100)	2	-1.7 ± 0.6	Protein targeting, signal transduction
Electron transport and binding					
Ubiquinol cytochrome c reductase core protein 2	Q5XIR3	7 (100)	2	-2.2 ± 0.6	Electron transport, proteolysis
Hemoglobin subunit alpha-1/2 (Hemoglobin alpha-1/2 chain) (Alpha-1/2- globin)	AAP13984	34 (100)	2	1.4 ± 0.3	Oxygen transport
Na^+/K^+-exchanging ATPase alpha-1 chain [validated] (EC 3.6.3.9)	A24639	7 (100)	6	-1.8 ± 0.7	Potassium and sodium ion transport. ATP hydrolysis coupled proton transport
H^+-transporting two-sector ATPase (EC 3.6.3.14) chain b precursor, mitochondrial - rat	A35340	10 (100)	2	-1.3 ± 0.3	ATP synthesis coupled proton transport. proton transport

Protein description (synonym)	Protein accession	Sequence coverage % (% matched peptides)	No. of peptides	Mean fold change ± SD	GO biological process description
Miscellaneous					
Alcohol dehydrogenase 1 (EC 1.1.1.1)	A26468	8 (66)	3	-1.4 ± 0.3	Alcohol metabolism
Corticosteroid 11-beta-dehydrogenase isozyme 2 (EC 1.1.1.-) (11-DH2)	AAH87023	13 (100)	3	-1.4 ± 0.4	Glucocorticoid biosynthesis. cell-cell signaling
UDP-glucose 6-dehydrogenase (EC 1.1.1.22) (UDP-GlcDH) (UDPGDH)	BAA28215	6 (100)	2	-1.4 ± 0.1	UDP-glucose metabolism
IgE-binding protein	A54889	35 (100)	4	-2.7 ± 2.5	Protein binding, extracellular matrix organization and biogenesis
Hydroxymethylglutaryl-CoA synthase precursor (EC 4.1.3.5)	A35865	8 (100)	2	-1.7 ± 0.6	Cholesterol biosynthesis, ketone body biosynthesis
Transketolase (EC 2.2.1.1) (TK)	AAA18026	6 (67)	3	-1.8 ± 0.6	Regulation of growth

Significantly affected proteins that meet the criteria of all peptide fold changes headed in one direction and an average peptide fold change of at least +/- 1.3. Sequence coverages of significantly changed peptides are expressed as percentage of the complete protein sequence and were retrieved from Mascot, with the following settings: MSDB database, 0 missed cleavages and 0.5 ppm mass tolerance window (performed on 22 January 2007). Percentages of matched peptides, given between brackets, indicate the fraction of peptides that could be matched applying filter criteria as described in the M&M section.

[A] Sequence coverage based on homology with the protein “collecting duct water channel, renal” (I51905).

Table 4. Comparsion between proteomics and transcriptomics fold changes in the distal colon mucosa of rats fed 10 g quercetin/kg diet.

Protein description (synonym)	Protein accession	Proteomics fold change ± SD	Gene symbol	Gene Title (synonym)	Affymetrix IDs	Gene fold change	Average gene fold change ± SD	In agreement
Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase)	AAH64440	-1.9 ± 0.7	*Aldoa*	Aldolase A	1367617_at 1388340_at	1.2 1.8	1.5 ± 0.4	No
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)	G3P	-2.4 ± 2.2	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	1367557_s_at	1.3	1.3	No
Creatine kinase B-type	AAA40933	-2.0 ± 0.9	*Ckb*	Creatine kinase, brain	1380830_at	-1.3	-1.3	Yes
Heat shock protein HSP 90-beta (HSP 84)	AAB23369	-1.6 ± 0.1	*Hspcb*	Heat shock 90kDa protein 1, beta	1375335_at 1375336_at	1.2 1.5	1.3 ± 0.2	No
Tubulin alpha-1 chain (Alpha-tubulin 1)	AAH62238	-2.4 ± 1.0	*Tuba1, Tuba6*	Tubulin, alpha 1/ Tubulin, alpha 6	1367579_a_at	1.4	1.4	No
Tubulin alpha-6 chain (Alpha-tubulin 6)	AAH78829	-2.7 ± 1.0	*Tuba1, Tuba6*	Tubulin, alpha 1/ Tubulin, alpha 6	1367579_a_at	1.4	1.4	No

Protein description (synonym)	Protein accession	Proteomics fold change ± SD	Gene symbol	Gene Title (synonym)	Affymetrix IDs	Gene fold change	Average gene fold change ± SD	In agreement
Annexin A2 (Lipocortin II) (Calpactin I heavy chain) (Chromobindin 8) (p36) (Protein I)	ANXA2	-1.9 ± 0.0	*Anxa2*	Annexin A2	1367584_at 1392250_at	-1.1 -1.6	-1.3 ± 0.4	Yes
Profilin-1 (Profilin I)	CAA65655	-2.6 ± 1.9	*Pfn1*	Profilin 1	1367605_at	2.2	2.2	No
Hemoglobin subunit alpha-1/2 (Hemoglobin alpha-1/2 chain) (Alpha-1/2- globin)	AAP13984	1.4 ± 0.3	*Hba1, Hba-1*	Hemoglobin alpha, adult chain 1 / hemoglobin alpha 2 chain	1370239_at 1370240_x_at 1388608_x_at	-1.6 -1.5 -1.6	-1.5 ± 0.0	No
Na^+/K^+-exchanging ATPase alpha-1 chain [validated] (EC 3.6.3.9)	A24639	-1.8 ± 0.7	*Atp1a1*	ATPase, Na^+/K^+ transporting,	1367585_a_at 1371108_a_at	1.2 1.8	1.5 ± 0.4	No
Hydroxymethylglutaryl-CoA synthase precursor (EC 4.1.3.5)	A35865	-1.7 ± 0.6	*Hmgcs2*	3-hydroxy-3-methylglutaryl -Coenzyme A synthase 2	1370310_at	1.7	1.7	No

Protein description (synonym)	Protein accession	Proteomics fold change ± SD	Gene symbol	Gene Title (synonym)	Affymetrix IDs	Gene fold change	Average gene fold change ± SD	In agreement
Transketolase (EC 2.2.1.1) (TK)	AAA18026	-1.8 ± 0.6	*Tkt*	Transketolase	1386859_at	1.4	1.4	No

Proteomics and transcriptomics comparisons, after filtering on significantly changed proteins and associated probe set IDs with ≥ 1 IDs with a minimum up- or downregulation of 1.3. Column "In agreement" indicates agreement in direction of proteomics and transcriptomics fold changes.

Discussion

Quercetin is a flavonoid present in several fruits and vegetables and generally recognized as an anti-carcinogenic phytochemical endowed with anti-oxidant capacity [2]. Based on previous studies showing quercetin's chemopreventive potency in azoxymethane-induced colorectal carcinogenesis [10;11], the present study was performed to obtain an insight into quercetin's mechanisms of action in the healthy distal colon. Both transcriptomics and proteomics were employed as hypothesis-generating methods to obtain a near-holistic overview of quercetin-mediated effects at both the mRNA and protein level. To date, combined transcriptomics and proteomics studies are performed in separate samples within the same treatment group due to different isolation protocols, which makes this approach subject to sample-to-sample variation [23;24]. In the present study, however, a TRIzol®-based method was developed that enabled analysis of both the transcriptome and proteome within the very same sample, thus by-passing the sample-to-sample variation. The novelty of the approach as described here concerns nano-LC preparation of the proteome directly out of the organic (reddish) phase, thereby reducing unwanted pre-filtering of the proteome, as is the case with 2D-gels.

Transcriptomics in combination with proteomics suggests that quercetin affected mechanisms involved in protection against (colorectal) carcinogenesis by upregulation of tumor suppressor and proliferation inhibiting genes, and shifted genes involved in energy metabolism from glycolysis towards fatty acid metabolism. As such, quercetin significantly downregulated the potentially oncogenic *Mapk* (= *Erk1/2*) pathway that is constitutively activated in human colorectal cancer [25]. MAP kinases are activated upon growth factor mediated stimulation of the proto-oncogene *Ras* via *Grb2, Shc1* and *Sos1*. Stimulated *Ras* activates *Raf-1*, resulting in phosphorylation and activation of *Mapk* kinase 1 (*Mapkk1* or *Mek1*) and *Mapk*, respectively [26]. Subsequently, activated *Mapk* migrates to the nucleus and phosphorylates transcription factors, including *Elk-1*, that activate cell growth. Therefore, constitutive activation of *Mapk* will result in cellular transformation. Conversely, inhibition of the *Mapk* pathway has been described to reduce growth of colon tumors in rodents [27]. Hence, it can be hypothesized that inhibition of this potentially oncogenic pathway by quercetin, encompassing decreased expression of

Grb2, *Shc1* and *Sos1*, amongst others, might be a contributing factor in its potency to reduce colorectal carcinogenesis.

Quercetin upregulated expression of tumor suppressor genes that function as gate-keepers in carcinogenesis, including *Pten*, *Tp53* and *Msh2*. *Pten* is hypothesized to inactivate the *Mapk* pathway by dephosphorylation of *Shc1*, resulting in downregulation of *Grb2* recruitment and ultimately in inhibition of *Mapk* [28]. Furthermore, inactivating mutations in *Tp53* and *Msh2* are well-known to be involved in colorectal carcinogenesis [29], suggesting that upregulation of these wildtype genes by quercetin is a beneficial effect.

Additionally, quercetin enhanced expression of genes involved in inhibition of cell proliferation, including *Mutyh*. The protein encoded by this base excision repair gene removes adenines mis-incorporated into the DNA opposite to guanine during DNA replication, and *Mutyh* mutations are associated with hereditary colorectal cancer [30]. Furthermore, single gene analysis showed decreased expression of *Anx1* that encodes the proliferation stimulating annexin 1 protein (= p35), suggesting decreased cell proliferation [31]. Reduced cell proliferation as suggested by transcriptomics corroborates our previous findings, in which 10 g quercetin/kg diet was described to decrease cell proliferation in putative precursors of colorectal cancer, aberrant crypt foci, in carcinogen treated rats [11].

Quercetin increased expression of genes involved in phase II metabolism, which is a mechanism to protect the colon mucosa from (dietary) carcinogens by production of water-soluble metabolites suitable for rapid excretion [32]. The phase I enzymes *Fmo5* , *Ephx1* and *Ephx2* are downregulated in human colon cancer [33-35], but upregulated by dietary quercetin, suggesting a beneficial effect.

Single gene analysis showed overexpression of *Mgll* that converts monoglycerides into glycerol and fatty acids, which is the first step in fatty acid degradation that may occur in the peroxisomes or mitochondria [36]. Most likely, quercetin-induced fatty acid degradation preferentially occurred in the mitochondria, as suggested by the most significantly upregulated energy metabolism gene set entitled “mitochondrial fatty acid catabolism”. PPARα regulates mitochondrial fatty acid ß-oxidation by activation of free fatty acids into fatty acyl-CoA derivatives. Subsequently, activated fatty acyl-CoAs are imported into the mitochondria, meant for β-oxidation into acetyl-CoA that can be used as an energy source in the Krebs cycle [36].

Proteomics data revealed a quercetin-mediated decrease in cytokeratin 21, transketolase, creatine kinase, b-type and annexin 2, all of which are increased in human CRC [37-40], suggesting a beneficial effect evoked by quercetin. Furthermore, three heat shock proteins *Hspa8*, *Hspca* and *Hspcb* were decreased by quercetin, but previously described to be overexpressed in human colorectal cancer [35;41]. Upregulated heat shock proteins prevent apoptosis, thus enabling cell survival, which is a mechanism involved in development of CRC [42], but counteracted by quercetin in the present study.

When combined with proteomics, transcriptomics data suggest that quercetin shifted the cellular energy metabolism from glycolysis in the cytoplasm towards fatty acid degradation in the mitochondria. Conversely, cancer cells generally exhibit increased glycolysis and mitochondrial dysfunction, as initially described by Warburg in 1930 [43]. Additionally, in human colon tumors the particular increase in glycolysis combined with decreased fatty acid degradation as an energy source, was described previously [44;45]. Therefore, it can be speculated that the quercetin-mediated glycolysis-to-fatty acid degradation shift in energy pathways could be an additional underlying mechanism involved in quercetin's potency to inhibit AOM-induced colorectal carcinogenesis [10;11]. Increased fatty acid catabolism, in addition to decreased expression of a key regulator of glycolysis, was also found in our previous microarray study of the rat lung, in which effects of quercetin at the same dietary concentration was analyzed after chronic exposure for 41 weeks [46]. Based on these findings, it can be concluded that increased fatty acid catabolism and decreased glycolysis are not organ-specific, but probably general mechanisms evoked by dietary quercetin in rats.

Comparison between transcriptomics and proteomics yielded a low correlation between quercetin induced changes in mRNA and protein expression. One explanation for this discrepancy might be that quercetin evoked effects by mechanisms independent from mRNA expression, but (partially) regulated at the protein level. Another possible explanation might be that mass spectrometry as performed in the present study probably detects the most abundant proteins, since no pre-filtering was performed as applied in 2D-gels. This was supported by the observation that 70% of the transcripts associated with the significantly affected proteins was present among the top 5% of transcripts with the highest signal intensities. The difficulty of proteomics to detect proteins with low expression levels

might explain why proteomics failed to detect the majority of genes found to be differentially expressed by transcriptomics. The low correlation in a proteomics-transcriptomics comparison has been described before in rat livers [23] and indicates a general, rather than organ-specific phenomenon.

In a study performed with the human colon cancer cell line Caco-2, quercetin beneficially altered expression of genes involved in proliferation, tumor suppression, xenobiotic metabolism and, interestingly, of genes contributing to the *Mapk* pathway [47], as was the case in the present study. Probably these genes are general targets of quercetin and contribute to its chemopreventive action in the colon.

In conclusion, transcriptomics and proteomics data suggest that quercetin enhanced several mechanisms involved in prevention of (colorectal) carcinogenesis, including inhibition of the *Mapk* pathway, in addition to enhanced expression of tumor suppressor genes, cell cycle inhibitors, and of genes involved in xenobiotic metabolism. Moreover, quercetin exerted a shift in pathways for energy supply, from cytoplasmic glycolysis towards mitochondrial fatty acid degradation. Previous studies pointed at a possible role for a shift in cellular pathways for energy supply with reduced glycolysis and increased fatty acid degradation, which might be an additional mechanism underlying the quercetin-mediated inhibition of colorectal carcinogenesis.

Notes

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Appendix transciptomics

Table 1. Significantly affected genes in the distal colon mucosa of rats supplemented with 10 g quercetin/kg diet

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Transcribed locus	-	1392097_at	-3.8	-
Phospholipase C, delta 3 (predicted)	*Plcd3_predicted*	1381747_at	-3.2	Lipid metabolism, signal transduction, intracellular signaling cascade
Annexin A1	*Anxa1*	1394451_at	-3.1	Regulation of cell proliferation, lipid metabolism, cell motility, inflammatory response, cell cycle, signal transduction, cell surface receptor linked signal transduction, insulin secretion, arachidonic acid secretion
TSC22 domain family 3	*Tsc22d3*	1380777_at	-3	Anti-apoptosis, fluid secretion, regulation of transcription, DNA-dependent
Tropomyosin 1, alpha	*Tpm1*	1379936_at	-3	Muscle contraction, regulation of muscle contraction, muscle development, regulation of heart contraction
Basic helix-loop-helix domain containing, class B2	*Bhlhb2*	1379483_at	-3	Negative regulation of transcription, DNA-dependent, nervous system development, entrainment of circadian clock, regulation of neuronal synaptic plasticity

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Myosin VC (predicted)	*Myo5c_predicted*	1380067_at	-3	-
Transcribed locus	-	1396955_at	-2.9	-
Period homolog 2 (Drosophila)	*Per2*	1368303_at	-2.9	Regulation of transcription, DNA-dependent, signal transduction, rhythmic behavior, circadian rhythm, rhythmic process, transcription
Transcribed locus	-	1381646_at	-2.8	-
Similar to KARP-1 binding protein 1	*LOC500726*	1377541_at	-2.8	-
Similar to RIKEN cdna 4631427C17	*RGD1564895_predicted*	1397410_at	-2.8	-
Thioredoxin domain containing 12 (endoplasmic reticulum)	*Txndc12*	1379023_at	-2.7	Electron transport
Vacuolar protein sorting 37B (yeast) (predicted)	*Vps37b_predicted*	1392156_at	-2.7	-
Similar to SLIT-ROBO Rho gtpase-activating protein 1	*RGD1566260_predicted*	1391633_at	-2.7	-
Basic helix-loop-helix domain containing, class B2	*Bhlhb2*	1381121_at	-2.7	Negative regulation of transcription, DNA-dependent, nervous system development, entrainment of circadian clock, entrainment of circadian clock, regulation of neuronal synaptic plasticity, regulation of neuronal synaptic plasticity, regulation of transcription

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
ATPase, Ca++ transporting, plasma membrane 1	*Atp2b1*	1380195_at	-2.7	Transport, cation transport, calcium ion transport, metabolism, ion transport
AT hook, DNA binding motif, containing 1 (predicted)	*Ahdc1_predicted*	1380858_at	-2.6	-
Similar to modulator of estrogen induced transcription	*RGD1307526*	1381175_at	-2.6	-
Ash1 (absent, small, or homeotic)-like (Drosophila) (predicted)	*Ash1l_predicted*	1381291_at	-2.6	Dna packaging, regulation of transcription, DNA-dependent, transcription from rna polymerase ii promoter, cell-cell signaling, chromatin modification
Tropomyosin 1, alpha	*Tpm1*	1395794_at	-2.6	Regulation of muscle contraction, muscle development, regulation of heart contraction
Oxysterol binding protein-like 2	*Osbpl2*	1378814_at	-2.6	Steroid metabolism, transport, lipid transport, steroid metabolism
LIM domain only protein 7	*LMO7*	1381798_at	-2.6	Cell-cell adhesion, actomyosin structure organization and biogenesis
Transcribed locus	-	1373759_at	-2.6	-
Muscleblind-like 2 (predicted)	-	1382497_at	-2.5	-
5'-nucleotidase, cytosolic II-like 1 (predicted)	*Col10a1*	1398122_at	-2.5	Skeletal development, phosphate transport, cell adhesion

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Phosphatidylinositol glycan, class A	*Piga*	1396561_x_at	-2.5	Biosynthesis
Similar to Myosin VI	*RGD1560646_predicted*	1390277_at	-2.5	-
Transcribed locus	-	1385986_at	-2.5	-
Similar to RIKEN cdna C330005L02	-	1381036_at	-2.5	-
Similar to MAPK-interacting and spindle-stabilizing protein (predicted)	*RGD1311455_predicted*	1392133_at	-2.5	-
Similar to mkiaa1737 protein (predicted)	*RGD1309492_predicted*	1386311_at	-2.5	-
Eukaryotic elongation factor-2 kinase	*Eef2k*	1389999_at	-2.5	Translational elongation, protein amino acid phosphorylation
Transcribed locus	-	1397944_at	-2.5	-
Atpase, Ca++ transporting, plasma membrane 1	*Atp2b1*	1394714_at	-2.5	Transport, cation transport, calcium ion transport, metabolism, ion transport
Similar to mkiaa1757 protein (predicted)	*RGD1310433_predicted*	1395895_at	-2.4	-
Similar to diacylglycerol kinase, delta 130kda isoform 1	*RGD1563309_predicted*	1392186_at	-2.4	-
Similar to HIV-1 Rev binding protein	*LOC363266*	1393488_at	-2.4	Regulation of gtpase activity
Dual specificity phosphatase 6	*Dusp6*	1382778_at	-2.4	Regulation of progression through cell cycle, inactivation of mapk activity, protein amino acid dephosphorylation, cell differentiation

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Similar to T-cell activation protein phosphatase 2C (predicted)	-	1375498_at	-2.4	-
Similar to Expressed sequence AW547186	-	1396340_at	-2.4	-
Glioma tumor suppressor candidate region gene 1 (predicted)	-	1380124_at	-2.3	-
Vinculin (predicted)	*Vcl_predicted*	1375538_at	-2.3	Cell adhesion, metabolism, lamellipodium biogenesis, regulation of cell migration
Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)	*Mll5*	1377321_at	-2.3	Regulation of transcription, DNA-dependent
Matrin 3	*Matr3*	1382522_at	-2.3	-
Cryptochrome 1 (photolyase-like)	*Cry1*	1392640_at	-2.3	Negative regulation of transcription, negative regulation of circadian rhythm, dna repair
Similar to RIKEN cdna C330005L02	-	1380084_at	-2.3	-
Serine/arginine-rich protein specific kinase 2 (predicted)	*Srpk2_predicted*	1378431_at	-2.3	Protein amino acid phosphorylation
Mitogen activated protein kinase 1	*Mapk1*	1383997_at	-2.3	*Mapkkk* cascade, nuclear translocation of mapk, protein amino acid phosphorylation, protein amino acid phosphorylation, induction of apoptosis, chemotaxis, response to stress, cell cycle, signal transduction, signal transduction, intracellular signaling cascade, protein kinase cascade, synaptic transmission, organ morphogenesis, phosphorylation, positive regulation of transcription

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Transcribed locus	-	1394779_at	-2.3	-
-	-	1392368_at	-2.3	-
Transducin (beta)-like 1X-linked receptor 1 (predicted)	*Tbl1xr1_predicted*	1380606_at	-2.3	-
Similar to hypothetical protein FLJ12661 (predicted)	-	1375497_at	-2.3	-
Transcribed locus	-	1379089_at	-2.3	-
Tetraspanin 8	*Tm4sf3*	1381125_at	-2.3	Protein amino acid glycosylation, negative regulation of blood coagulation
Similar to RNA-binding protein Musashi2-S	-	1381260_at	-2.3	-
Syntaxin 7	*Stx7*	1397565_at	-2.3	Intracellular protein transport, post-golgi vesicle-mediated transport, vacuole organization and biogenesis, synaptic vesicle exocytosis, synaptic vesicle exocytosis, vesicle-mediated transport
Dishevelled associated activator of morphogenesis 1 (predicted)	*Daam1_predicted*	1398184_at	-2.3	Cell organization and biogenesis, actin cytoskeleton organization and biogenesis
Dynactin 2	*Dctn2*	1391061_at	-2.3	Microtubule-based process
Transcribed locus	-	1393570_at	-2.3	-
Aconitase 2, mitochondrial	*Aco2*	1378945_at	-2.3	Generation of precursor metabolites and energy, tricarboxylic acid cycle, citrate metabolism, metabolism

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Stromal interaction molecule 1 (predicted)	*Stim1_predicted*	1384873_at	-2.3	Cell adhesion, positive regulation of cell proliferation, negative regulation of progression through cell cycle
Neuronal PAS domain protein 2 (predicted)	*Npas2_predicted*	1398024_at	-2.3	Regulation of transcription, DNA-dependent, signal transduction, central nervous system development, circadian sleep/wake cycle, locomotor rhythm
Serine protease inhibitor, Kazal type 1	*Spink1*	1387193_a_at	-2.3	-
Similar to Transcription factor 7-like 2 (HMG box transcription factor 4) (T-cell-specific transcription factor 4) (TCF-4) (htcf-4)	*LOC683733*	1379815_at	-2.3	-
Similar to ortholog of human amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 19 (ALS2CR19)	*LOC301455*	1396763_at	-2.3	-
Phosphoinositide-3-kinase, class 2, beta polypeptide (predicted)	*Pik3c2b_predicted*	1381576_at	-2.2	Intracellular signaling cascade
Sprouty protein with EVH-1 domain 1, related sequence	*Spred1*	1392788_at	-2.2	Inactivation of mapk activity, development, regulation of signal transduction
Ubiquitin specific protease 33	-	1391770_at	-2.2	Ubiquitin-dependent protein catabolism, ubiquitin cycle, protein deubiquitination
Similar to KIAA1217	*RGD1563437_predicted*	1397152_at	-2.2	-

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
HIV-1 Rev binding protein-like (predicted)	*Hrbl_predicted*	1397370_at	-2.2	Regulation of gtpase activity
Protein phosphatase 3, catalytic subunit, alpha isoform	*Ppp3ca*	1379175_at	-2.2	G1/s transition of mitotic cell cycle, protein amino acid dephosphorylation, protein import
Similar to bromo domain-containing protein disrupted in leukemia	-	1392214_at	-2.2	-
Ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	*Ube2e2*	1376438_at	-2.2	-
ATPase, Ca++ transporting, plasma membrane 1	*Atp2b1*	1397030_at	-2.2	-
CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase-like (predicted)	*Ctdspl_predicted*	1397137_at	-2.2	-
Bromodomain containing 4	*Brd4*	1380392_at	-2.2	-
PX domain containing serine/threonine kinase	*Pxk*	1397380_at	-2.2	Protein amino acid phosphorylation, intracellular signaling cascade
Calpain, small subunit 1	*Capns1*	1392341_at	-2.2	Proteolysis, positive regulation of cell proliferation
Microtubule associated monoxygenase, calponin and LIM domain containing 3	*RGD1306314_predicted*	1379999_at	-2.2	Electron transport, aromatic compound metabolism, metabolism
Dehydrogenase/reductase (SDR family) member 1	*MGC94370*	1391259_at	-2.2	Metabolism
Vacuolar protein sorting 26 (yeast)	*Vps26*	1381673_at	-2.1	Intracellular protein transport, retrograde transport, endosome to golgi, transport, intracellular protein transport, protein transport

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Similar to Serine/threonine protein kinase 24	*RGD1561742_predicted*	1398089_at	-2.1	-
Poliovirus receptor-related 2 (herpesvirus entry mediator B)	*Pvrl2*	1389899_at	-2.1	-
Component of oligomeric golgi complex 4 (predicted)	*Cog4_predicted*	1396663_at	-2.1	-
Neuroepithelial cell transforming gene 1	*Net1*	1381781_at	-2.1	-
Similar to sorbin and SH3 domain containing 1 isoform 3	*LOC686098*	1374263_at	-2.1	-
Dihydropyrimidinase-like 2	*Dpysl2*	1380728_at	-2.1	Nucleobase, nucleoside, nucleotide and nucleic acid metabolism, signal transduction, nervous system development, regulation of axon extension
Similar to mkiaa0376 protein (predicted)	*RGD1309570_predicted*	1380790_at	-2.1	-
Abhydrolase domain containing 6	*Abhd6*	1380332_at	-2.1	Aromatic compound metabolism
Bromodomain containing 1 (predicted)	*Brd1_predicted*	1380145_at	-2.1	Regulation of transcription, DNA-dependent
Forkhead box O1A	*Foxo1a*	1396965_at	-2.1	Blood vessel development, regulation of transcription, DNA-dependent, regulation of transcription from rna polymerase ii promoter, anti-apoptosis, insulin receptor signaling pathway, regulation of cell proliferation
Pam, highwire, rpm 1 (predicted)	*Phr1_predicted*	1390423_at	-2.1	Protein ubiquitination
Transcribed locus	-	1396559_at	-2.1	-
Fibulin 1 (predicted)	-	1393252_at	-2.1	Chitin metabolism

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Dynein, cytoplasmic, light intermediate chain 1	*Dncli1*	1399090_at	-2.1	Microtubule-based movement, mitotic spindle organization and biogenesis, regulation of centrosome cycle, small gtpase mediated signal transduction
Similar to RIKEN cdna 5033405K12 (predicted)	-	1380752_at	-2.1	-
Transcribed locus	-	1397999_at	-2.1	-
Dullard homolog (Xenopus laevis)	*Dullard*	1380874_at	-2.1	-
Loc499304	-	1372760_at	-2	-
GTP cyclohydrolase 1	*Gch*	1380230_at	-2	Tetrahydrobiopterin biosynthesis, induction of apoptosis, induction of apoptosis, biosynthesis
Similar to zinc finger protein 61	*LOC499094*	1378650_at	-2	Regulation of transcription, DNA-dependent
Similar to hypothetical protein dkfzp564d0478 (predicted)	*RGD1304793_predicted*	1397041_at	-2	-
Hypothetical LOC312654 (predicted)	-	1397624_at	-2	-
Similar to RIKEN cdna 2310005O14	*LOC498909*	1390491_at	-2	-
Elastin microfibril interfacer 2 (predicted)	*Emilin2_predicted*	1378346_at	-2	-
Testis-specific kinase 2	*Tesk2*	1394772_at	-2	Protein amino acid phosphorylation, apoptosis, male meiosis, intracellular signaling cascade, spermatogenesis, actin cytoskeleton organization and biogenesis, focal adhesion formation
Lymphoid nuclear protein related to AF4-like (predicted)	*Laf4l_predicted*	1394682_at	-2	-

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Transcribed locus	-	1392026_at	-2	-
Transcribed locus	-	1379181_at	-2	-
OCIA domain containing 1	*Ociad1*	1381408_at	-2	-
Lipin 2 (predicted)	*Lpin2_predicted*	1391378_at	-2	-
Similar to SPRY domain-containing SOCS box 4	-	1396863_at	-2	-
Transcribed locus	-	1383971_at	-2	-
Transcribed locus, strongly similar to XP_510753.1 PREDICTED: similar to Transcription elongation	-	1391031_at	-2	-
Similar to Nuclear receptor ROR-gamma (Nuclear receptor RZR-gamma) (Thymus orphan receptor)	*LOC368158*	1379833_at	-2	-
Macrophage erythroblast attacher	*Maea*	1377200_at	-2	Apoptosis, cell adhesion, development
Transcribed locus	-	1395047_at	-2	-
Tropomyosin 1, alpha	*Tpm1*	1390471_at	-2	Regulation of muscle contraction, muscle development, regulation of heart contraction
Transcribed locus	-	1395819_at	-2	-

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Dolichol-phosphate (beta-D) mannosyltransferase 1 (predicted)	*Dpm1_predicted*	1391372_at	-2	Protein amino acid glycosylation
-	-	1393662_at	-1.9	-
Heat shock protein 4	*Hspa4*	1394557_at	-1.9	Protein folding, response to unfolded protein, response to heat
Nuclear respiratory factor 1 (predicted)	*Nrf1_predicted*	1385617_at	-1.9	Carbohydrate metabolism, generation of precursor metabolites and energy, regulation of transcription, DNA-dependent, regulation
Transcribed locus	-	1383039_at	-1.9	-
SCY1-like 2 (S. Cerevisiae) (predicted)	*Scyl2_predicted*	1396581_at	-1.9	Protein amino acid phosphorylation
Ring finger protein 10	-	1398142_at	-1.9	Protein ubiquitination
Megakaryoblastic leukemia (translocation) 1 (predicted)	*Mkl1_predicted*	1394624_at	-1.9	Transcription, regulation of transcription, DNA-dependent, anti-apoptosis
Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	*Ppp2r2a*	1392741_at	-1.9	Protein amino acid dephosphorylation, signal transduction
Similar to cisplatin resistance-associated overexpressed protein (predicted)	-	1384615_at	-1.9	-
Similar to RIKEN cdna G430041M01	-	1382755_at	-1.9	-
UDP-glucose dehydrogenase	*Ugdh*	1384574_at	-1.9	UDP-glucose metabolism, glycosaminoglycan biosynthesis, udp-glucuronate biosynthesis, electron transport

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
A kinase (PRKA) anchor protein 11	*Akap11*	1392049_at	-1.9	Protein kinase cascade
Protein phosphatase 1 (formerly 2C)-like (predicted)	*Ppm1l_predicted*	1376122_at	-1.9	Mapkkk cascade, protein amino acid dephosphorylation, transmembrane receptor protein serine/threonine kinase signaling pathway
Phosphatase and actin regulator 2	*Phactr2*	1377881_at	-1.8	-
Zinc finger, FYVE domain containing 1 (predicted)	*Zfyve1_predicted*	1381708_at	-1.8	Vesicle-mediated transport
FYVE, rhogef and PH domain containing 3 (predicted)	*Fgd3_predicted*	1396518_at	-1.8	-
Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B	*Sema4b*	1381549_at	-1.8	Development
Transcribed locus, strongly similar to XP_535481.2 PREDICTED: similar to Dmx-like 2 isoform 1 [Canis familiaris]	*LOC315676*	1397755_at	-1.8	-
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) (predicted)	*Elavl1_predicted*	1391006_at	-1.8	Nuclear mrna splicing, via spliceosome, rna catabolism, development
Platelet derived growth factor, alpha	*Pdgfa*	1393494_at	-1.8	Regulation of progression through cell cycle, cell surface receptor linked signal transduction, cell-cell signaling, cell proliferation, organ morphogenesis, cell projection biogenesis, actin cytoskeleton organization and biogenesis, regulation of peptidyl-tyrosine phosphorylation
Ectonucleoside triphosphate diphosphohydrolase 4 (predicted)	*Entpd4_predicted*	1375728_at	-1.8	UDP catabolism

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) (predicted)	-	1376430_at	-1.8	Nuclear mrna splicing, via spliceosome, rna catabolism, development
Testis-specific kinase 2	*Tesk2*	1394863_at	-1.8	Protein amino acid phosphorylation, apoptosis, male meiosis, intracellular signaling cascade, spermatogenesis, actin cytoskeleton organization and biogenesis,
Similar to SH3 domain binding glutamic acid-rich protein like 2	*LOC501026*	1398084_at	-1.8	-
Transcribed locus	-	1382605_at	-1.8	-
Similar to novel protein	*RGD1560155_predicted*	1380399_at	-1.8	-
Similar to farnesyl diphosphate synthetase	*LOC501976*	1396962_at	-1.7	-
Transcribed locus	-	1379506_at	-1.7	-
Similar to nipsnap2 protein (Glioblastoma amplified sequence)	*LOC498174*	1382731_at	-1.7	-
RNA guanylyltransferase and 5'-phosphatase (predicted)	*Rngtt_predicted*	1396373_at	-1.7	Mrna capping, protein amino acid dephosphorylation
Metastasis suppressor 1 (predicted)	*Mtss1_predicted*	1382750_at	-1.7	Cell motility, actin filament organization, cell adhesion, transmembrane receptor protein tyrosine kinase signaling pathway, nervous system development, muscle development, microspike biogenesis, actin cytoskeleton organization and biogenesis, actin filament polymerization
F-actin capping protein beta subunit	*Capzb*	1381494_at	-1.7	Actin cytoskeleton organization and biogenesis

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
DEAH (Asp-Glu-Ala-His) box polypeptide 36 (predicted)	*Dhx36_predicted*	1385871_at	-1.7	-
E1A binding protein p300	*Ep300*	1378682_at	-1.7	Regulation of transcription, DNA-dependent
Nitrilase 1	*Nit1*	1398049_at	-1.7	Nitrogen compound metabolism
Mitogen activated protein kinase kinase kinase 12	*Map3k12*	1368886_at	-1.7	Protein amino acid phosphorylation, protein kinase cascade, jnk cascade, histone phosphorylation, peptidyl-serine phosphorylation, peptidyl-threonine phosphorylation, protein amino acid autophosphorylation
Transcribed locus	-	1383665_at	-1.7	-
Similar to TSC22 domain family protein 2 (TSC22-related-inducible leucine zipper protein 4)	*LOC499624*	1382171_at	-1.6	-
Transcribed locus	-	1379562_at	-1.6	-
Similar to flavoprotein oxidoreductase MICAL2 (predicted)	*Mical2_predicted*	1393860_at	-1.6	-
Ral GEF with PH domain and SH3 binding motif 2 (predicted)	*Ralgps2_predicted*	1396036_at	-1.6	Intracellular signaling cascade, small gtpase mediated signal transduction
Unknown (protein for MGC:72974)	*RGD:735147*	1376200_at	-1.6	Protein amino acid methylation
Similar to microfilament and actin filament cross-linker protein isoform a	*LOC362587*	1379153_at	-1.5	-

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Sperm associated antigen 9 (predicted)	*Spag9_predicted*	1394436_at	-1.4	Spermatogenesis
Methyltransferase like 7A	*Mettl7a*	1371479_at	1.6	-
Chemokine (C-X-C motif) ligand 10	*Cxcl10*	1387969_at	1.6	Cell motility, chemotaxis, inflammatory response, immune response, signal transduction, muscle development, positive regulation of cell proliferation, protein
Similar to Potential phospholipid-transporting atpase IF (atpase class I type 11B) (atpase IR)	*LOC361929*	1384002_at	1.6	Cation transport
Scaffold attachment factor B	*Safb*	1368058_at	1.6	Establishment and/or maintenance of chromatin architecture, regulation of transcription, DNA-dependent, transcription
Nucleolar and coiled-body phosphoprotein 1	*Nolc1*	1368032_at	1.6	Nucleolus organization and biogenesis, regulation of protein import into nucleus
X transporter protein 3	*RGD:621651*	1369705_at	1.6	Transport, neurotransmitter transport
Similar to Gem (nuclear organelle) associated protein 4	*LOC497958*	1374665_at	1.6	-
Aminoacylase 1	*Acy1*	1372170_at	1.7	Proteolysis, amino acid metabolism
-	-	1386306_at	1.7	-
Scavenger receptor class B, member 1	*Scarb1*	1386956_at	1.7	Cell adhesion
Glucose regulated protein, 58 kda	*Grp58*	1398788_at	1.7	Electron transport, protein import into nucleus, protein retention in er, signal transduction

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
NMDA receptor-regulated gene 1 (predicted)	*Narg1_predicted*	1372558_at	1.7	Angiogenesis, transcription, regulation of transcription, DNA-dependent
Poly A binding protein, cytoplasmic 4 (predicted)	*Pabpc4_predicted*	1371777_at	1.7	Nuclear mrna splicing, via spliceosome, rna processing, rna catabolism, protein biosynthesis, blood coagulation, response to pest, pathogen or parasite
Branched chain ketoacid dehydrogenase E1, alpha polypeptide	*Bckdha*	1370897_at	1.7	Metabolism, branched chain family amino acid catabolism
Discoidin domain receptor family, member 1	*Ddr1*	1370217_at	1.7	-
Transcribed locus, strongly similar to XP_576460.1 PREDICTED: similar to hypothetical protein PB402898.00.0 [Rattus norvegicus]	-	1398922_at	1.7	-
Similar to RIKEN cdna 1700027M01	*RGD1311815*	1376824_at	1.8	Protein modification
Retinoblastoma-associated factor 600	*Rbaf600*	1379025_at	1.8	Ubiquitin cycle
IQ motif containing gtpase activating protein 3 (predicted)	*Iqgap3_predicted*	1374902_at	1.8	Small gtpase mediated signal transduction
Monoglyceride lipase	*Mgll*	1375247_at	1.8	Proteolysis, lipid metabolism, aromatic compound metabolism, inflammatory response
Sortilin-related receptor, L(DLR class) A repeats-containing (predicted)	*Sorl1_predicted*	1393933_at	1.8	Lipid transport, receptor mediated endocytosis, cholesterol metabolism

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Sideroflexin 1	*Sfxn1*	1397740_at	1.8	Transport, cation transport, iron ion transport, erythrocyte differentiation
Sortilin-related receptor, L(DLR class) A repeats-containing (predicted)	*Sorl1_predicted*	1393626_at	1.8	Receptor mediated endocytosis
Monoamine oxidase A	*Maoa*	1377831_at	1.8	Electron transport, catecholamine metabolism, behavior, neurotransmitter catabolism, dopamine catabolism, catecholamine catabolism
4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (C. Elegans) (predicted)	*Nipsnap1*	1390454_at	1.9	-
Transcribed locus	-	1378185_at	1.9	-
Calmodulin 3	*Calm3*	1370873_at	1.9	Cell cycle, g-protein coupled receptor protein signaling pathway, g-protein coupled receptor protein signaling pathway
UDP-Gal:betaglcnac beta 1,4-galactosyltransferase, polypeptide 6	*B4galt6*	1387206_at	2	Carbohydrate metabolism, glycosphingolipid biosynthesis, glycosphingolipid biosynthesis, sphingolipid biosynthesis
Fucosyltransferase 2 (secretor status included)	*Fut2*	1371216_s_at	2	Carbohydrate metabolism, protein amino acid glycosylation, l-fucose catabolism
Branched chain aminotransferase 2, mitochondrial	*Bcat2*	1386928_at	2	Lactation, metabolism, branched chain family amino acid metabolism, branched chain family amino acid biosynthesis, branched chain family amino acid catabolism, amino acid biosynthesis

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Hexokinase 2	*Hk2*	1383519_at	2.1	Regulation of progression through cell cycle, glucose catabolism, glycolysis
Progressive ankylosis homolog (mouse)	*Ank*	1386344_at	2.1	Skeletal development, transport, phosphate transport, sensory perception of sound, locomotory behavior, regulation of bone
Peroxiredoxin 6	*Prdx6*	1367969_at	2.2	Lipid catabolism, response to reactive oxygen species
Progressive ankylosis homolog (mouse)	*Ank*	1369249_at	2.2	Skeletal development, transport, phosphate transport, sensory perception of sound, locomotory behavior, regulation of bone mineralization, regulation of bone mineralization
Kinesin family member C1	*Kifc1*	1376185_at	2.3	Microtubule-based movement
Solute carrier family 22, member 3	*Slc22a3*	1387189_at	2.3	Transport, ion transport, cation transport, organic cation transport
Transcribed locus	-	1373229_at	2.5	-
Plasminogen activator, tissue	*Plat*	1367800_at	2.5	Protein modification, proteolysis, blood coagulation, platelet-derived growth factor receptor signaling pathway
Nuclear receptor subfamily 1, group D, member 1	*Nr1d1*	1370816_at	2.8	Regulation of transcription, DNA-dependent, circadian rhythm
Angiotensin 1 converting enzyme	*Ace*	1387791_at	2.9	Response to hypoxia, proteolysis, blood pressure regulation, carbohydrate metabolism

Gene title	Gene Symbol	Affymetrix ID	Fold change (quercetin/control)	GO biological process description
Monoglyceride lipase	*Mgll*	1388644_at	3.4	Proteolysis, lipid metabolism, aromatic compound metabolism, inflammatory response
Similar to IG KAPPA CHAIN V-V REGION K2 PRECURSOR, similar to NGF-binding Ig light chain	*LOC500180*	1387902_a_at	4.4	-

Appendix proteomics

Table 2. Details of significantly changed proteins and associated peptides in the distal colon mucosa of rats supplemented with 10 g quercetin/kg diet

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Glycolysis						
Alpha-enolase (EC 4.2.1.11)	A23126	1804.9439	-2.2	-1.5 ± 0.6	R.AAVPSGASTGIYEALELR.D	*Eno1*
(2-phospho-D- glycerate						
hydro-lyase) (Non- neural enolase)		2047.0706	-1.1		K.FTATAGIQVVGDDLTVTNPK.R	
(NNE) (Enolase 1)		2192.0328	-1.1		K.AGYTDQVVIGMDVAASEFYR.A	
Fructose-bisphosphate aldolase A	AAH64440	1652.8312	-2.6	-1.9 ± 0.7	K.FSNEEIAMATVTALR.R	*Aldoa*
(EC 4.1.2.13) (Muscle-type aldolase)		2123.0913	-1.2		K.IGEHTPSSLAIMENANVLAR.Y	
		2258.0359	-1.8		K.YTPSGQSGAAASESLFISNHAY.- 2366	
Pyruvate kinase isozyme M2	A26186	1883.9034	-1.6	-1.7 ± 0.1	R.LNFSHGTHEYHAETIK.N	*Pkm2*
(EC 2.7.1.40)		2435.2816	-1.8		R.AATESFASDPILYRPVAVALDTK.G	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Glyceraldehyde-3-phosphate	G3P	1627.9529	-5.0	-2.4 ± 2.2	K.LVINGKPITIFQER.D	*Gapdh*
dehydrogenase (EC 1.2.1.12) (GAPDH)		2213.1092	-1.0		R.VIISAPSADAPMFVMGVNHEK.Y	
		2277.0379	-1.3		K.WGDAGAEYVVESTGVFTTMEK.A	
Heatshock proteins						
Heat shock cognate 71 kDa protein	HSP7C	1428.6841	-2.0	-2.4 ± 0.5	K.HWPFMVVNDAGR.P	*Hspa8*
(Heat shock 70 kDa protein 8)		1653.8318	-2.7		K.HWPFMVVNDAGRPK.V	
Heat shock protein 86 (Heat shock protein 1, alpha)	Q91XW0	1758.8405	-1.5	-1.6 ± 0.1	L.LSSGFSLEDPQTHANR.I	*Hspca*
		2255.9588	-1.7		K.HNDDEQYAWESSAGGSFTVR.T	
Heat shock protein HSP 90-beta	AAB23369	1782.9497	-1.5	-1.6 ± 0.1	K.HLEINPDHPIVETLR.Q	*Hspcb*
(HSP 84)						
		2255.9588	-1.7		K.HNDDEQYAWESSAGGSFTVR.A	
Cytoskeleton and cell-cell contact						
Transgelin 2	Q5XFX0	1215.6877	-1.3	-1.5 ± 0.3	R.TLMNLGGLAVAR.D	*Tagln2*
		1678.8217	-1.7		K.QMEQISQFLQAAER.Y	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Tubulin alpha-1 chain	AAH62238	1410.7739	-1.7	-2.4 ± 1.0	R.QLFHPEQLITGK.E	*Tuba1*
(Alpha-tubulin 1)						
		1701.9057	-3.6		R.AVFVDLEPTVIDEVR.T	
		1756.9632	-3.0		R.IHFPLATYAPVISAEK.A 1687	
		2330.0181	-1.4		R.AFVHWYVGEGMEEGEFSEAR.E 2436	
Tubulin alpha-6 chain	AAH78829	1410.7739	-1.7	-2.7 ± 1.0	R.QLFHPEQLITGK.E	*Tuba6*
(Alpha-tubulin 6)						
		1452.6609	-3.6		I.TNACFEPANQMVK.C	
		1701.9057	-3.6		R.AVFVDLEPTVIDEVR.T	
		1756.9632	-3.0		R.IHFPLATYAPVISAEK.A 1687	
		2330.0181	-1.4		R.AFVHWYVGEGMEEGEFSEAR.E 2436	
Tubulin alpha-8 chain	AAH79185	1410.7739	-1.7	-1.6 ± 0.2	R.QLFHPEQLITGK.E	*Tuba8*
(Alpha-tubulin 8)						
		2330.0181	-1.4		R.AFVHWYVGEGMEEGEFSEAR.E	
Keratin 21, type I, cytoskeletal	A40452	1444.7529	-1.3	-1.6 ± 0.4	K.TDLEIQIEELNK.D	*Krt20*
		2797.3606	-1.9		R.QLGNNVNVEVDAAPGLNLGEIMNEMR.Q	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Actin, alpha cardiac	ACTC	1515.7491	1.6	2.4 ± 1.1	K.IWHHTFYNELR.V 760	*Actc1*
(Alpha-cardiac actin)						
		1960.9109	3.1		K.YPIEHGIITNWDDMEK.I	
Annexin A2 (Lipocortin II) (Calpactin I heavy chain) (Chromobindin 8) (p36) (Protein I)	ANXA2	1908.8821	-1.9	-1.9 ± 0.0	R.AEDGSVIDYELIDQDAR.E	*Anxa2*
		2938.3853	-1.9		K.LSLEGDHSTPPSAYGSVKPYTNFDAER.D	*Anxa2*
Profilin-1 (Profilin I)	CAA65655	1616.9257	-1.2	-2.6 ± 1.9	K.TFVSITPAEVGVLVGK.D	*Pfn1*
		1639.7632	-3.9		R.DSLLQDGEFTMDLR.T	
Dynein heavy chain, cytosolic (DYHC)	DYHC	1369.7851	1.3	3.5 ± 3.0	R.FNALFVRPHIR.G	*Dync1h1*
(Cytoplasmic dynein heavy chain) (MAP1C)		2157.1450	5.6		K.AHQANQLYPFAISLIESVR.T	
Krebs cycle						
Citrate synthase	Q8VHF5	1167.6632	-1.9	-1.5 ± 0.6	R.VVPGYGHAVLR.K	*Cs*
		1762.8857	-1.1		K.GLVYETSVLDPDEGIR.F	
Kinase activity						
Creatine kinase B-type	AAA40933	1303.7256	-2.6	-2.0 ± 0.9	K.VLTPELYAELR.A	*Ckb*
		1671.8489	-1.3		K.TFLVWINEEDHLR.V	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Creatine kinase, ubiquitous mitochondrial precursor	S17189	1420.7107	-1.8	-2.2 ± 1.2	R.LYPPSAEYPDLR.K	*Ckmt1*
(EC 2.7.3.2) (U- MtCK) (Mia-CK)		1621.7527	-1.3		K.TTPTGWTLDQCIQTG.V	
(Acidic-type mitochondrial creatine kinase)		1645.7969	-4.0		K.SFLIWVNEEDHTR.V	
		2944.3920	-1.7		K.TVGMVAGDEETYEVFAELFDPVIQER.H	
Protein modification						
Polyubiquitin [Fragment]	Q63654	1067.6207	-1.5	-1.5 ± 0.1	K.ESTLHLVLR.L 347	*Ubb/Ubc*
		1787.9273	-1.4		K.TITLEVEPSDTIENVK.A	
Ubiquitin / ribosomal protein S27a,	I52328	1067.6207	-1.5	-1.5 ± 0.1	K.ESTLHLVLR.L 347	*Rps27a*
cytosolic [validated]		1787.9273	-1.4		K.TITLEVEPSDTIENVK.A	
Ubiquitin / ribosomal protein L40, cytosolic	I65237	1067.6207	-1.5	-1.5 ± 0.1	K.ESTLHLVLR.L 347	*Uba52*
[validated]		1787.9273	-1.4		K.TITLEVEPSDTIENVK.A	
Mitochondrial import stimulation factor L	AAC52676	1189.6609	-2.1	-1.7 ± 0.6	K.DSTLIMQLLR.D	*Ywhae*
subunit (14-3-3 protein epsilon)		1447.7097	-1.3		K.VAGMDVELTVEER.N	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Electron transport and binding						
Ubiquinol cytochrome c reductase core	Q5XIR3	1742.8496	-2.6	-2.2 ± 0.6	R.YENYNYLGTSHLLR.L	*Uqcrc2*
protein 2		2179.0566	-1.8		K.ITSEELHYFVQNHFTSAR.M	*Uqcrc2*
Hemoglobin subunit alpha-1/2 (Hemoglobin alpha-1/2 chain)	AAP13984	1572.7400	1.2	1.4 ± 0.3	K.IGGHGGEYGEEALQR.M	*Hba1*
(Alpha-1/2- globin)		1735.8649	1.6		K.TYFSHIDVSPGSAQVK.A	*Hba-1*
Na^+/K^+-exchanging ATPase alpha-1 chain	A24639	9825.316	-1.3	-1.8 ± 0.7	K.LSLDELHR.K	*Atp1a1*
[validated] (EC 3.6.3.9)		1135.5894	-1.6		R.YHTEIVFAR.T	
		1436.6573	-1.5		K.DMTSEELDDILR.Y	
		1584.8744	-1.8		R.AVFQANQENLPILK.R	
		1829.9239	-1.5		K.GVGIISEGNETVEDIAAR.L	
		2317.0156	-3.1		R.WINDVEDSYGQQWTYEQR.K	
H^+-transporting two-sector ATPase, chain b	A35340	1507.7032	-1.5	-1.3 ± 0.3	R.LDYHISVQDMMR.R	*Atp5f1*
precursor, mitochondrial (EC 3.6.3.14)		1840.9883	-1.1		R.LGLIPEEFFQFLYPK.T	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Miscellaneous						
Alcohol dehydrogenase 1	A26468	1090.5891	-1.7	-1.4 ± 0.3	K.INEAFDLLR.A	*Adh1*
(EC 1.1.1.1)						
		1471.7692	-1.3		R.GKPIHHFLSTSTF.S	
		2487.3282	-1.1		K.AAVLWEPHKPFTIEDIEVAPPK.A	
Corticosteroid 11-beta-dehydrogenase isozyme 2	AAH87023	1631.9056	-1.1	-1.4 ± 0.4	R.FLQNFFISHLLPR.A 1464	*Hsd11b2*
(EC 1.1.1.-) (11-DH2)		1944.0312	-1.8		R.GLGLMYFIHHYLPGGLR.R	
(11-beta-HSD2)		2745.3630	-1.3		R.ELLQAYGEDYIEHLHGQFLNSLR.M	
UDP-glucose 6-dehydrogenase	BAA28215	1294.6976	-1.3	-1.4 ± 0.1	R.MLKPAFIFDGR.R	*Ugdh*
(EC 1.1.1.22) (UDP-GlcDH) (UDPGDH)		2151.0676	-1.4		R.EQIVVDLSHPGVSADDQVSR.L	
IgE-binding protein	A54889	1287.6228	-1.1	-2.7 ± 2.5	K.GNDIAFHFNPR.F	*Lgals3*
		1298.7103	-1.3		K.IQVLVEADHFK.V	
		1469.7423	-2.0		R.QSAFPFESGKPFK.I	
		1677.8819	-6.4		K.VAVNDVHLLQYNHR.M	

Protein description (synonym)	Protein accession	Peptide mass (MH^+)	Fold change	Mean fold change ± SD	Peptide sequence	Gene symbol
Hydroxymethylglutaryl-CoA synthase	A35865	2397.1509	-2.1	-1.7 ± 0.6	K.VNFSPPGDTSNLFPGTWYLER.V	*Hmgcs2*
precursor (EC 4.1.3.5)		2626.3286	-1.3		K.DVGILALEVYFPAQYVDQTDLEK.F	
Transketolase (EC 2.2.1.1) (TK)	AAA18026	2020.0709	-1.7	-1.8 ± 0.6	K.ILATPPQEDAPSVDIANIR.M	*Tkt*
		2481.2004	-2.4		R.TSRPENAIIYSNNEDFQVGQAK.V	
		3419.5221	-1.3		R.MAAISESNINLCGSHCGVSIGEDGPSQM ALEDLA.M	

Chapter 6

Summary, conclusions and discussion

Summary

Colorectal cancer (CRC) is a common malignancy in the Western world and one of the leading causes of worldwide cancer-related morbidity and mortality. One of the hypothesized risk factors involved in development of CRC includes the dietary composition. A factor suggested to be preventive in development of (colorectal) cancer is a diet rich in fruits and vegetables. Important possible chemopreventive bioactive ingredients found in fruits and vegetables are flavonoids, amongst others, of which quercetin is one of the most studied. This flavonoid is commercially available as a supplement and claimed to be health-promoting. However, its mechanisms of action in colorectal carcinogenesis are poorly understood. An unbiased approach using the so-called "omics" techniques enables expression profiling of hundreds to thousands of proteins or genes and as such provide a near-holistic insight into quercetin-mediated effects on CRC, and may generate novel biomarkers.

The objective of this thesis was to identify histological and molecular biomarkers of quercetin-modulated colorectal carcinogenesis, by using both classical and omics-based technologies.

The effect of the dietary flavonoid quercetin was investigated on proliferation and differentiation of the human colon cancer cell line Caco-2, in order to characterize whether effects of quercetin on these colon cancer cells *in vitro* would be in line with its suggested anti-carcinogenic characteristics (**chapter 2**). Initially, quercetin in culture medium, in the absence of cells, was shown to be unstable under culture conditions. Combined stability and cytotoxicity experiments revealed that the addition of 1 mM of ascorbate resulted in quercetin stabilization for 24 hrs, without induction of cytotoxicity. Therefore, all *in vitro* experiments described in the present thesis were performed with quercetin stabilized by 1 mM ascorbate. Caco-2 cells were grown towards confluency, which is known to be the starting point of cellular differentiation [1], and subsequently exposed to physiologically relevant concentrations of 0 - 80 μM quercetin, stabilized by 1 mM ascorbate. Under these conditions quercetin exerted a biphasic effect on cell proliferation and reduced cell differentiation ($P < 0.05$). Upon differentiation, the amount of quercetin in the culture medium was reduced in the course of time, as a result of the metabolic activity of Caco-2 cells. This decrease of quercetin in the culture medium occurred in favor of formation of 5 phase II metabolites characterized as 4'-O-methyl-quercetin-3'-O-glucuronide, 3'-O-methyl-quercetin (= isorhamnetin), 4'-O-methyl-quercetin-7-O-glucuronide, 3'-O-methyl-

quercetin-4'-O-glucuronide and 3'-O-methyl-quercetin-7-O-sulphate. The amount of 4'-O-methyl-quercetin-3'-O-glucuronide correlated with the differentiation grade of the cells ($r = 0.99$, $P < 0.003$).

Strikingly, the increment of cell proliferation and the decrease in cell differentiation are effects opposite to what would be expected for a functional food ingredient with anti-carcinogenic potency. Hence, a second series of experiments was performed, to elucidate both molecular and cellular mechanisms associated with this unexpected effect pointing to pro- rather than anti-carcinogenic mechanisms (**chapter 3**). After a 10-day exposure to 40 μM quercetin stabilized by 1 mM ascorbate, Caco-2 differentiation was again significantly ($P < 0.001$) reduced up to 50%, in line with data described in chapter 2. RNA originating from Caco-2 cells harvested on days 5 and 10, and hybridized on HG-U133A 2.0 Affymetrix® GeneChips®, showed that quercetin affected 1,743 genes on both days ($P < 0.01$). Among these differentially expressed genes, 14 genes known to be positively correlated with Caco-2 differentiation, all showed decreased expression ($P < 0.01$), including intestinal alkaline phosphatase. This observation was confirmed both technically by real-time qRT-PCR and functionally by enzyme activity. In addition, increased expression of the colon cancer related genes *COX-2* and *MMP-7* found by microarrays, was confirmed by real-time qRT-PCR. These confirmations at both the mRNA and protein level indicated retrieval of a biologically relevant data set. To discover a possible interplay between these 1,743 differentially expressed genes, data were analyzed by the pathway mapping tool MetaCore™. Differentially expressed genes contributed to 27 significantly ($P < 0.05$) affected pathways categorized under 6 gene ontology (GO) processes, of which cell cycle, and apoptosis and cell death showed the highest enrichment. The most significantly changed pathways contributing to these GO processes are "Role of anaphase promoting complex (APC) in cell cycle regulation" ($P = 0.0004$) and "Role of inhibitor of apoptosis proteins (IAP) in apoptosis" ($P = 0.035$). Genes contributing to these GO processes showed fold changes that suggest increased cell survival and proliferation. In addition to pathway analysis, single gene analysis was performed, which revealed that quercetin downregulated expression of genes involved in tumor suppression (e.g. *Tp53AP1*) and xenobiotic metabolism (e.g. *Fmo5, Ephx1* and *Ephx2*), and upregulated oncogenes (e.g. *Rab3b* and *Maff*).

An *in vitro-in vivo* comparison learned that quercetin-mediated fold changes in Caco-2 gene expression were concordant with those occurring in human colorectal

carcinogenesis (≈ 80 - 90% concordance). *Ergo*, gene expression among quercetin-exposed Caco-2 cells showed mechanisms contrary to what is expected for a compound with anti-carcinogenic potency. A comparison with gene expression data obtained from Caco-2 cells exposed to 5 or 50 μM quercetin in the absence of ascorbate, showed that 75 - 90% of the above mentioned genes showed a direction of fold change that is contrary to that occurring in development of human CRC, which supports quercetin's anti-carcinogenic potency. These contradicting quercetin data found in the presence and absence of ascorbate, might have been caused by ascorbate-annihilation of quercetin-induced reactive oxygen species (ROS) that are able to eradicate tumor cells. This ascorbate-associated contrast was also reported for the human colon cancer cell line HT-29 exposed to flavone, another flavonoid [2]. When exposed to flavone only, HT-29 cells underwent apoptosis, whereas exposure to a combination of flavone and 1 mM ascorbate failed to execute apoptosis, thus interfering with this flavonoid's anti-carcinogenic potency. The authors have shown that ROS induced by flavone execute the apoptosis program and that ROS are scavenged by simultaneous addition of ascorbate, leading to failure of apoptosis-induction in HT-29 cells.

Whether the unexpected *in vitro* effects would be relevant for quercetin mediated modulation of colorectal carcinogenesis, was investigated in subsequent *in vivo* experiments performed with rats. The effect of quercetin aglycone (absorbed from the small intestine) [3;4] and its conjugate rutin (absorbed from the colon) [5] on the development of putative preneoplastic lesions (*i.e.* histological biomarkers) and of colorectal cancer, was studied using axozymethane (AOM) induced colon carcinogenesis in the rat as a model. The cancer-modulating potency of quercetin and rutin was related to their bioavailibility (**chapter 4**). Male F344 rats were fed 0, 0.1, 1 or 10 g quercetin/kg diet, or 40 g rutin/kg diet. Two weeks after initial administration of experimental diets, rats were given two subcutaneous injections with 15 mg AOM/kg bodyweight in two consecutive weeks. At wk 38 post-AOM, quercetin dose dependently ($P < 0.05$) decreased the tumor incidence, multiplicity and size. Compared to controls, the tumor incidence was significantly decreased at the highest concentration of 10 g quercetin/kg diet, whereas rutin showed no effect. The number of methylene blue-stained aberrant crypt foci (ACF) as putative preneoplastic colonic lesions in unsectioned colons at wk 8 post-AOM did not correlate with the tumor incidence at wk 38. Moreover, at wk 8 post-AOM, the number and multiplicity of

immunohistochemically discerned ACF with or without accumulation of the ß-catenin oncogene were not affected by 10 g quercetin/kg diet. Another class of putative CRC-biomarkers, ß-catenin accumulated crypts, that are completely different from ACF [6], were not affected in number, but partially showed a decrease in extent of ß-catenin accumulation ($P < 0.05$). This result suggested that ß-catenin accumulated crypts might be more relevant biomarkers for colorectal carcinogenesis. To determine whether bio-availibilty of these flavonoids is correlated with their cancer modulating potency, plasma concentrations of quercetin and its metabolites were measured after enzymatic deconjugation, for both quercetin and rutin fed rats. For rats exposed to increasing dietary levels of quercetin, plasma concentrations of both quercetin aglycone and 3'-O-methyl-quercetin at wk 8 were inversely correlated with the tumor incidence at wk 38 ($r = -0.95$, $P \leq 0.05$). Rats supplemented with 40 g rutin/kg diet, showed only 30% of the (3'-O-methyl-) quercetin concentrations found in 10 g quercetin/kg diet treated rats ($P < 0.001$). These plasma values point at a lower bioavailibilty of the quercetin moiety present in rutin, the more since on a molecular base the rutin diet contained a 2 times higher quercetin aglycone concentration when compared to the 10 g quercetin/kg diet.

In the present *in vivo* study, quercetin was found to exert a chemoprotective effect on colorectal carcinogenesis. In view of these results and the fact that quercetin is promoted as a cancer-preventive supplement, the next step was to elucidate what mechanisms are evoked by quercetin in the healthy colon. Effects of supplementation with 10 g quercetin/kg diet for 11 weeks were studied in the distal colon, since this site contained the majority of colon tumors in AOM-treated rats, and human colon tumors predominantly occur in the distal colon.

To obtain a near-holistic insight into quercetin-mediated effects, both transcriptomics and proteomics were applied within the same distal colon mucosa samples. Gene expression data were analyzed with the Gene Set Enrichment Analysis software tool that calculates a significance score for enrichment for pre-defined sets of genes involved in certain biological functions. The distal colon mucosa of rats indeed showed quercetin-induced changes in gene and protein expression levels of importance in mechanisms involved in inhibition of (colorectal) carcinogenesis. As such, quercetin decreased expression of the potentially oncogenic mitogen-activated protein kinase (*Mapk*) pathway. In addition, quercetin enhanced expression of genes involved in inhibition of the cell cycle, including *Mutyh*, of tumor suppressor genes,

including *Pten*, *Tp53* and *Msh2*, and of genes involved in xenobiotic metabolism, including *Fmo5*, *Ephx1*, *Ephx2* and *Gpx2*. Quercetin increased mitochondrial fatty acid degradation that is controlled by PPARα, which was also upregulated.
In a microarray study performed with Caco-2 cells exposed to quercetin in the absence of ascorbate, this flavonoid beneficially altered expression of genes involved in proliferation, tumor suppression, xenobiotic metabolism and, interestingly, of genes contributing to the *Mapk* pathway [7].
Proteomics was performed by a combinatorial approach consisting of FT-MS, for accurate masses and signal intensities that allow for calculation of fold changes, and MALDI-TOF/TOF, for peptide identification. Quercetin affected 33 proteins, including 4 glycolysis enzymes (e.g. *Gapdh* and *Pkm2*) and 3 heatshock proteins (*Hspa8*, *Hspca* and *Hspcb*) that were all decreased. In tumors, increased expression of heat shock proteins is found, which prevent cells to go into apoptosis [8], suggesting that quercetin beneficially reversed this effect.
Transcriptomics and proteomics showed a modest overlap at the level of individual genes and proteins. Both techniques pointed to inhibition of mechanisms involved in colorectal carcinogenesis and an effect on energy metabolism, indicating that transcriptomics and proteomics can be complementary to one another.
The combination of both techniques showed that quercetin decreased glycolysis in the cytoplasm and increased fatty acid degradation in the mitochondria. As tumor cells show an energetic balance that is mainly based on glycolysis and mitochondrial impairment [9], the shift in pathways for cellular energy supply evoked by quercetin is speculated to be a factor involved in its anti-carcinogenic potency.

Comparison of the *in vitro* and *in vivo* data described in the present thesis obviously show contradicting results with respect to quercetin's cancer-modulating potency. Previously, the dietary flavonoid flavone was shown to exert its tumor-inhibiting potency by induction of apoptosis via reactive oxygen species (ROS) produced in the mitochondria [2]. One could postulate that in the *in vitro* experiments described in this thesis, these ROS were probably scavenged by co-incubation of quercetin with the anti-oxidant ascorbate. As a result, the Caco-2 cells were able to survive and proliferate.

In the *in vitro* experiments, the ratio of 40 µM quercetin : 1 mM vit C amounted to 1 : 25, indicating an excess of ascorbate. However, *in vivo*, this ratio amounted to 10 g quercetin/kg diet : 8 mg vitamin C/kg diet, which equals 1250 : 1.

Obviously, vitamin C is not the only anti-oxidant present in the control diet, which will cause the ratio of quercetin vs. all oxidants to be lower than aforementioned. Nevertheless, the relative abundancy of dietary quercetin as maintained in the *in vivo* experiments, might have induced ROS, that in turn could eradicate tumor cells and thus contribute to this flavonoid's anti-carcinogenic potency, as hypothesized above using the *in vitro* data as an input.

Overall conclusions

From the studies described in this thesis, the following can be concluded:

1. Caco-2 cells exposed to ascorbate-stabilized quercetin showed enhancement of cellular processes involved in development of colon cancer, including increased cell proliferation and decreased cell differentiation, which was associated with formation of phase II metabolites of quercetin with sulphate, methyl and glucuronide moieties.
2. Molecular mechanisms underlying the above mentioned cellular processes involved in development of colon cancer are associated with the increased expression of oncogenes and apoptosis inhibiting genes, as well as downregulated the expression of tumor suppressor genes and genes involved in xenobiotic metabolism in Caco-2 cells exposed to ascorbate-stabilized quercetin. It is assumed that the enhancement of cellular mechanisms involved in development of colon cancer is caused by ascorbate-mediated scavenging of reactive oxygen species that are able to eradicate tumor cells.
3. *In vivo*, quercetin, but not rutin has been shown to inhibit AOM-induced colorectal carcinogenesis, without affecting ACF, which points to the conclusion that these putative pre-neoplastic lesions are no reliable biomarkers for colorectal carcinogenesis in these studies. Although not clear-cut, ß-catenin accumulated crypts might be more relevant biomarkers for colorectal carciogenesis.
4. Transcriptomics and proteomics confirmed quercetin's cancer-preventive mechanisms in the distal colon of non-AOM treated rats, at a relatively high dose of 10 g quercetin/kg diet administered to rats. Quercetin inhibited the potentially oncogenic *Mapk* pathway, and enhanced expression of tumor suppressor genes and genes involved in cell cycle inhibition and xenobiotic metabolism. Furthermore, quercetin evoked a shift in energy metabolism, from

glycolysis to fatty acid degradation, which is suggestive of an anti-carcinogenic mechanism [9].

5. The lack of an effect by rutin on AOM-induced colorectal carcinogenesis is related to its low bioavaility. This is important given the fact that most dietary quercetin supplements on the market actually contain rutin and not the quercetin aglycone.
6. The administration of the lowest dietary concentration at 0.1 g quercetin/kg diet to the rats would equal a human intake of 1 quercetin aglycone supplement of 500 mg per day, did not affect colorectal carcinogenesis in AOM-treated rats. In contrast, 10 g quercetin/kg diet significantly reduced colorectal carcinogenesis in AOM-treated rats, but would equal an unrealistic daily human intake of 60 - 100 quercetin pills of 500 mg each. Therefore, inhibition of CRC in humans by intake of quercetin supplements is most probably not feasible, and health claims on prevention of CRC by quercetin need better scientific support.

Discussion

Although quercetin in the present thesis was described to exert contradicting *in vitro* and *in vivo* results with respect to its mechanisms in modulation of colon cancer, both type of studies have in common that quercetin affects genes involved in cell proliferation, tumor suppressor genes, oncogenes and xenobiotic metabolism. Furthermore, the significant cancer-inhibiting and -preventive effects of quercetin in the *in vivo* study occurred at 10 g/kg diet, which after linear extrapolation to the human situation would be a ≈ 100-fold higher intake than prescribed for quercetin supplements. Hence, the health claim that quercetin may protect against colon cancer in humans needs better scientific support, and should take the dose levels required to achieve such effects into account.

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List of abbreviations

ACF	Aberrant crypt focus/foci
ACN	Acetonitrile
ALP(I)	(Intestinal) alkaline phosphatise
AOM	Azoxymethane
Apc	Adenomatous polyposis coli
APC	Anaphase promoting complex
BCA-ACF	ß-catenin-accumulated aberrant crypt focus
BCA(-)C	ß-catenin-accumulated crypt(s)
BrdU	Bromo-2'-deoxyuridine
COMT	Catechol-O-methyl transferase
cDNA	Complementary deoxyribonucleic acid
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
DHB	Dihydroxy-benzoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMH	Dimethylhydrazine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EDTA	Ethylene diamine tetra acetic acid
ER	Estrogen receptor
FA	Fatty acid(s)
FAD	Focal areas of dysplasia
FAP	Familial adenomatous polyposis
FDR	False discovery rate
FT-MS	Fourier transform ion cyclotron resonance mass spectrometry
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GSEA	Gene Set Enrichment Analysis

GST	Glutathione S-transferase
GO	Gene ontology
GSK3	Glycogen synthase kinase 3ß
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis proteins
LC-MS	Liquid chromatography coupled mass spectroscopy
MALDI-TOF	Matrix-assisted laser desorption/ionization - Time of flight
Mapk	Mitogen-activated protein kinase
MIAME	Minimum Information About a Microarray Experiment
MMLV	Moloney murine leukaemia virus
MMP	Matrix metalloprotease
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosurea
mRNA	Messenger ribonucleic acid
NMR	Nuclear magnetic resonance
NSAID	Nonsteroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PhIP	2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine
POD	Peroxidase
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S/N	Signal to noise ratio
SULT	Sulfotransferases
TE	Tris-EDTA
TEER	Trans epithelial electrical resistance
TFA	Trifluoroacetic acid
TIMP	Tissue inhibitors of metalloprotease
U(DP)GT	UDP-glucuronosyltransferase

Nederlandse samenvatting

Inleiding

In het algemeen wordt aangenomen dat groenten en fruit een gezondheidsbevorderende werking hebben, en onder andere beschermen tegen hart- en vaatziekten en bepaalde vormen van kanker. Een groep van voedingsingrediënten die deel uitmaakt van groenten en fruit en bijdraagt aan deze gezondheidsbevorderende werking, is de groep van anti-oxidanten. Een subklasse van deze anti-oxidanten betreft de categorie flavonoïden, waarvan quercetine een van de meest onderzochte is. Quercetine is op de lijst terechtgekomen van commerciële instellingen die voedingssupplementen produceren en aanprijzen als stoffen die beschermend zouden werken tegen onder andere kanker en hart- en vaatziekten. Bovendien wordt door deze bedrijven geclaimd dat de voedingssupplementen onschadelijk zouden zijn, omdat ze in de natuur voorkomen. Op grond van dergelijke claims is een groei van de inname van voedingssupplementen te verwachten. Echter, deze gezondheidsclaims zijn gebaseerd op een relatief gering aantal onderzoeken met behulp van celkweken en diermodellen en meestal niet gebaseerd op uitgebreide veiligheids- en effectiviteitsonderzoek bij de mens.

Doelstelling

Het onderzoek in dit proefschrift is gericht op het effect van quercetine op dikkedarmkanker, aangezien de gezondheidsclaims voor quercetine zich richten op de bescherming tegen onder andere kanker, waarvan dikkedarmkanker een van de meest voorkomende vormen van deze maligne aandoening is. Het doel van het onderzoek was om na te gaan of quercetine beschermend werkt op (de ontwikkeling van) dikkedarmkanker, welke mechanismen hieraan ten grondslag liggen en of er histologische en moleculaire indicatoren zijn, die geassocieerd zijn met deze werking van quercetine. Hiertoe zijn zowel cellijnen als ratten gebruikt als model voor dikkedarmkanker. De Caco-2 cellijn is afgeleid van een humane dikkedarmtumor (*in vitro* onderzoeksbenadering), terwijl met behulp van de kankerverwekkende stof azoxymethaan dikkedarmkanker is opgewekt bij ratten (*in vivo* onderzoeksbenadering).

Het effect van quercetine op de celdeling, differentiatie en fase 2 metabolisme van quercetine (hoofdstuk 2)

Omdat de eerste stap bestond uit het uitvoeren van experimenten met aan quercetine blootgestelde Caco-2-cellen, werd eerst nagegaan hoe stabiel quercetine is in het kweekmedium dat nodig is om de cellen in leven te houden. Gebleken is dat quercetine in het kweekmedium niet stabiel is en wordt afgebroken, maar dat dit proces geremd kon worden door toevoeging van vitamine C. De *in vitro* experimenten werden daarom uitgevoerd met vitamine C-gestabiliseerde quercetine, die iedere 24 uur werd ververst.

De Caco-2 cellijn werd blootgesteld aan quercetineconcentraties, die vergelijkbaar zijn met de inhoud van de menselijke dikkedarm. Uit deze studies bleek dat quercetine kankergerelateerde processen versterkte door stimulatie van de celdeling en remming van de celdifferentiatie. Verder bleek de Caco-2-cellijn quercetine te metaboliseren, wat bleek uit de tijdsafhankelijke afname van quercetine en de gelijktijdige opkomst van methyl-, sulfaat- en glucuronideconjugaten.

In het gebruikte *in vitro* model bleek de potentieel kankerremmende stof quercetine dus op een geheel onverwachte wijze te werken, door kankergerelateerde processen te versterken in plaats van te verminderen.

Moleculaire mechanismen die ten grondslag liggen aan de quercetine gemedieerde versterking van cellulaire processen betrokken bij het ontstaan van darmkanker (hoofdstuk 3)

Om na te gaan welke moleculaire mechanismen ten grondslag liggen aan het onverwachte versterkend effect van quercetine op cellulaire processen in het ontstaan van darmkanker, is gebruik gemaakt van transcriptomics. Deze techniek maakt het mogelijk om met behulp van microarrays de expressie van duizenden genen tegelijkertijd te bestuderen, zowel op individueel genniveau, als op het niveau van biologische routes (gerelateerde processen die tezamen een biologische functie vervullen). In deze studies bleek dat quercetine bij een fysiologisch relevante concentratie leidde tot verlaagde expressie van genen die betrokken zijn bij de differentiatie van Caco-2-cellen. Verder versterkte quercetine de biologische routes die de celdeling aansturen en ervoor zorgen dat cellen de geprogrammeerde celdood (apoptose) niet meer kunnen uitvoeren, waardoor er uiteindelijk meer cellen kunnen overleven. Quercetine bleek verder de expressie van genen te versterken die

betrokken zijn bij het ontstaan van tumoren (oncogenen) en de expressie van tumoronderdrukkende genen (tumor suppressors) te verlagen. Daarnaast bleek quercetine de expressie van genen te remmen, die de uitscheiding van lichaamsvreemde stoffen (xenobiotica) bewerkstelligen en zo bijdragen aan een verkorte blootstellingsduur aan kankerverwekkende stoffen.
Ook op het moleculair-biologische vlak bleek quercetine dus genen en biologische routes aan te sturen die cellulaire processen betrokken bij het ontstaan van darmkanker versterken in plaats van remmen.

Effecten van quercetine op het ontstaan van darmkanker in de rat (hoofdstuk 4)
Om na te gaan of de versterking van cellulaire processen betrokken bij het ontstaan van darmkanker door quercetine in de Caco-2 cellijn ook *in vivo* aan de orde is, is een proef met ratten uitgevoerd. De ratten werden in verschillende dieetgroepen ingedeeld, die verschillende quercetineconcentraties kregen toegediend. Omgerekend naar de mens kwamen de concentraties overeen met een inname van circa 1, 10 en 100 quercetinesupplementen van 500 mg per dag. Deze oplopende reeks is aangehouden om na te gaan bij welke quercetineconcentratie er sprake is van een significante beïnvloeding van de ontwikkeling van darmtumoren. Daarnaast is er ook een groep ratten meegenomen die op een rutinedieet zijn gezet, dat omgerekend circa twee keer meer quercetine bevatte dan het hoogste quercetinedieet. Rutine is een van de natuurlijk voorkomende varianten van quercetine en bestaat uit een quercetinemolecuul gekoppeld aan een suikergroep. Rutine is mogelijk interessant omdat dit via de dikkedarm wordt opgenomen, dus niet via de dunnedarm zoals met quercetine het geval is. De ratten zijn vervolgens gedurende twee opeenvolgende weken behandeld met een stof die dikkedarmtumoren induceert (azoxymethaan). Tijdens de secties die daarna zijn uitgevoerd, zijn mogelijke voorlopers van darmtumoren bestudeerd, de zogenaamde aberrant crypt foci, afgekort ACF (8 weken na azoxymethaanbehandeling) en het aantal tumoren (38 weken na azoxymethaanbehandeling). Quercetine bleek de vorming van het aantal darmtumoren te remmen. Dit effect werd versterkt met een hogere quercetineconcentratie in het dieet en bleek gepaard te gaan met een hogere concentratie in het bloedplasma. Alleen de hoogste quercetineconcentratie bleek het aantal tumordragende dieren significant te reduceren. Rutine bleek geen tumorbeschermende werking te hebben en dat was geassocieerd met relatief lage quercetineconcentraties in het plasma: onder de rutine

dieren werd circa eenderde van de quercetineconcentraties gemeten ten opzichte van quercetineconcentraties gemeten bij de dieren uit de hoogste quercetinegroep. Verder bleek het aantal ACF, als potentiële voorlopers van darmtumoren, niet in overeenstemming met het aantal dikkedarmtumoren. Omdat ACF alleen bij dieren voorkomen die met een kankerverwekkende stof zijn behandeld, bestond het vermoeden dat niet alle ACF, maar mogelijk bepaalde subpopulaties betrokken zijn bij het ontstaan van dikkedarmkanker. Op grond van deze hypothese is een onderscheid gemaakt tussen ACF met- en ACF zonder ophoping van het kankergerelateerde eiwit beta-catenine. Het aantal ACF met ophoping van beta-catenine bleek te laag om goede statistische analyses uit te voeren. Daarom is een ander klasse aan voorlopers van dikkedarmtumoren bestudeerd, namelijk de beta-catenine-geaccumuleerde crypten (BCAC), die totaal verschillend zijn van de ACF. Het aantal BCAC bleek niet beïnvloed te zijn door quercetine, maar de mate van ophoping van beta-catenine bleek deels gereduceerd te zijn in de quercetine-behandelde dieren. Dit zou mogelijk kunnen wijzen op een remmend effect op een van de processen betrokken bij het ontstaan van darmtumoren.
Uit deze *in vivo* proef bleek dus dat quercetine een beschermend effect heeft op de vorming van dikkedarmkanker en dat dit afhankelijk is van de biobeschikbaarheid van quercetine. Verder bleken ACF geen goede voorspellende biomarker te zijn om het beschermende effect van quercetine in een vroeg stadium aan te tonen, maar BCAC mogelijk wel.

Kankerpreventie door quercetine in de gezonde dikkedarm (hoofdstuk 5)
Quercetine bleek beschermend te werken in het model van darmkanker bij ratten. Op grond van de gezondheidsclaim dat quercetine preventief zou werken tegen kanker, was de volgende stap het bestuderen van de eventuele kankerpreventieve werking van quercetine in de gezonde darm. Hiertoe werden de ratten gedurende 11 weken op het quercetinedieet gezet dat in het voorgaande hoofdstuk de beste bescherming tegen dikkedarmkanker bleek te geven. Vervolgens is het slijmvlies van de dikkedarm bestudeerd met behulp van een combinatie van proteomics en transcriptomics, waarmee respectievelijk de expressie van honderden tot duizenden eiwitten en genen tegelijkertijd gemeten kan worden. In dit hoofdstuk is voor deze gecombineerde aanpak gekozen, omdat de genexpressie niet altijd voorspellend is voor de eiwitexpressie.

Uit deze studie bleek dat quercetine de expressie van tumorsuppressor-genen en genen betrokken bij de remming van de celdeling en uitscheiding van lichaamsvreemde stoffen versterkte. Verder bleek quercetine de genexpressie van een belangrijke biologische route voor het ontstaan van dikkedarmkanker, de mitogen-activated protein kinase (*Mapk*) pathway, te remmen. Bovendien stimuleerde quercetine de expressie van genen die betrokken zijn bij de vetzuurafbraak in de mitochondriën (energiecentrales van de cel) als energiebron.
Quercetine remde de expressie van eiwitten die betrokken zijn bij het ontstaansproces van dikkedarmkanker, waaronder de heatshock-eiwitten. Een ander effect van quercetine was de remming van de glycolyse, een andere energiebron van de cel. Ondanks het feit dat de uitkomsten van de transcriptomics- en proteomics-analyses een beperkte overlap vertoonden op individueel gen- en eiwitniveau, was er wel sprake van overeenkomende effecten op specifieke cellulaire mechanismen. Beide technieken wezen namelijk zowel op de remming van genen en eiwitten die betrokken zijn bij het ontstaan van dikkedarmkanker, als een effect op de energiebron van de cel. Quercetine veroorzaakte namelijk remming van de glycolyse in het cytoplasma en stimulering van de vetzuurafbraak in de mitochondriën. Deze quercetine-gemedieerde verandering in de verhouding tussen de twee verschillende energiebronnen blijkt tegengesteld te zijn aan de verhouding zoals die wordt gevonden in tumorcellen en suggereert een additioneel beschermend mechanisme op dikkedarmkanker.

Algemene discussie en conclusie

De *in vitro* experimenten toonden aan dat quercetine als potentieel kankerremmende stof geheel onverwacht enkele kankergerelateerde processen, zoals celdeling en celdifferentiatie, bleek te stimuleren. Deze effecten zijn nader onderzocht met behulp van genexpressie-onderzoek. Hiermee werd het versterkend effect van quercetine op cellulaire processen, betrokken bij het ontstaan van darmkanker, bevestigd. Dit tegengestelde effect is mogelijk te wijten aan het feit dat quercetine gestabiliseerd moest worden met vitamine C. Uit eerder onderzoek is gebleken dat voedingscomponenten met een vergelijkbare structuur als quercetine hun tumorremmende werking kunnen uitoefenen door vrije zuurstofradicalen te genereren, die in staat zijn tumorcellen te doden. Door toevoeging van vitamine C, die zijn anti-oxidantwerking uitoefent door vrije zuurstofradicalen weg te vangen, is het waarschijnlijk dat de quercetine-geïnduceerde vrije zuurstofradicalen door vitamine C

zijn weggevangen. Hierdoor zouden de tumorcellen overleven en hun kwaadaardige eigenschappen behouden.
Het *in vivo* onderzoek, toonde juist aan dat quercetine een kankerremmende en kankerpreventieve werking bezit. Het aantal tumordragende dieren bleek verlaagd te zijn door quercetine, zonder dat de ACF als veronderstelde voorlopers van darmtumoren van voorspellende waarde bleken te zijn. In de gezonde darm bleek quercetine eveneens mechanismen te remmen die betrokken zijn bij het ontstaan van darmkanker. Verder bleek quercetine een verschuiving te veroorzaken in de cellulaire routes voor energievoorziening, namelijk van de glycolyse naar de vetzuurafbraak. Deze verhouding is tegengesteld aan de energiehuishouding gerapporteerd voor tumorcellen.

Alleen bij de hoogste concentratie van omgerekend ca. 100 pillen van 500 mg per dag voor de mens heeft quercetine een significante remming van het aantal tumordragende dieren veroorzaakt. Omdat deze extreem hoge concentratie niet haalbaar is voor de mens, lijkt de gezonheidsclaim dat quercetine beschermend zou werken tegen darmkanker ongegrond. Er zijn eerst aanvullende humane studies nodig om dit te bevestigen, waarbij rekening gehouden moet worden met de mogelijk extreem hoge doseringen die benodigd zijn voor de beoogde gezondheidseffecten.

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Curriculum Vitae

Ashwin Ajay Dihal werd geboren op 6 februari 1972 te Paramaribo, Suriname. Na de basis school (Flu I) heeft hij de MULO (M.R. Dayanand school) gedaan. Na het behalen van zijn VWO diploma (Mr. Dr. J.C. de Miranda Lyceum) in 1991 is hij naar Nederland geëmigreerd om in datzelfde jaar de opleiding Scheikunde aan de Universiteit Leiden te volgen. Al snel stapte hij over naar de studie Biomedische Wetenschappen aan de Universiteit Leiden, die beter aansloot op zijn interesses. Voor de afronding van zijn studie heeft hij drie stages aan het Leids Universitair Medisch centrum gelopen, waarvan de eerste op de afdeling Pathologie onder begeleiding van Dr. E. de Heer, de tweede op de afdeling Maag,- darm- en leverziekten onder begeleiding van Dr. H.W. Verspaget en de hoofdvakstage op de afdeling Humane en Klinische Genetica in het Sylvius Laboratorium, begeleid door Dr. P. Devilee. In 1999 behaalde hij het doctoraal diploma en begon gelijk aan een onderzoeksbaan aan de Leidse tak van het Leiden/Amsterdam Centre for Drug Research. In het jaar daarop was hij werkzaam bij het ministerie van Volkshuisvesting, Ruimtelijke ordening en Milieu (VROM) en in 2001 als beleidsmedewerker aangesteld bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), om zich vervolgens weer te storten op het onderzoek. Het promotie onderzoek, zoals beschreven in dit proefschrift, werd in 2002 geïnitieerd en uitgevoerd bij TNO Kwaliteit van Leven te Zeist, Business Unit BioSciences in samenwerking met de afdeling Toxicologie van Wageningen Universiteit en Researchcentrum. Tijdens deze periode heeft Ashwin modules gevolgd voor de SMBWO erkenning tot Experimenteel Pathobioloog.

List of publications

Book chapter

Dihal AA and van Erk MJ. *Colon Cancer: Early Detection and Prevention by Food Compounds and Biomarkers of Preventive Effects Found by employment of Microarrays*. In: **Cancer Nanotechnology. Nanomaterials for Cancer Diagnosis and Theraypy**; editors: H.S. Nalwa and T. Webster, American Scientific Publishers, Los Angeles (2007), p. 31-42. ISBN: 1-58883-071-3

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Training and supervision plan

Overview of conferences and courses attended during PhD training

Confocal laserscan microscopy (UMC Utrecht)	2006
Transmission and immuno electronen microscopy (UMC Utrecht)	2006
Regulatory protein networks (UMC Utrecht)	2005
2nd International conference on Polyphenols and health (UC Davis), *U.S.A.*	2005
2nd Yearly meeting of the European Nutrigenomics Organisation (NuGO), *Italy*	2005
Nutritional and lifestyle epidemiology (VLAG)	2005
Workshop "Cell culture models of gut health" (NuGO), *Italy*	2005
1st Yearly meeting of the European Nutrigenomics Organisation (NuGO)	2004
International research conference on food, nutrition and cancer (AICR), *U.S.A.*	2004
Cancer Epidemiology (NIHES)	2004
Chemistry and biochemistry of antioxidants (VLAG)	2003
Practical Statistics for Microarray Data (Centre for Biostatistics)	2003
PhD symposium Netherlands organization for Toxicology (NVT)	2003
Dietetics (AMC)	2003
Towards comprehending scanned arrays; expression profiling (MGC)	2002
Oncogenesis and Tumor Biology (MGC)	2002
12th symposium of the Medical Genetics Centre (MGC)	2002
Thematic days of the Centre for Human Nutrigenomics (CHN)	2002

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