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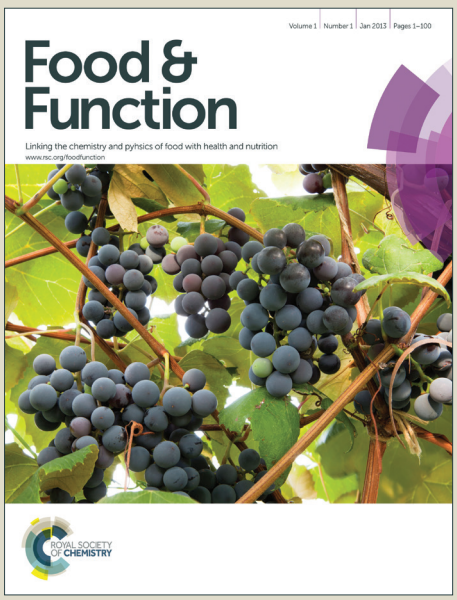

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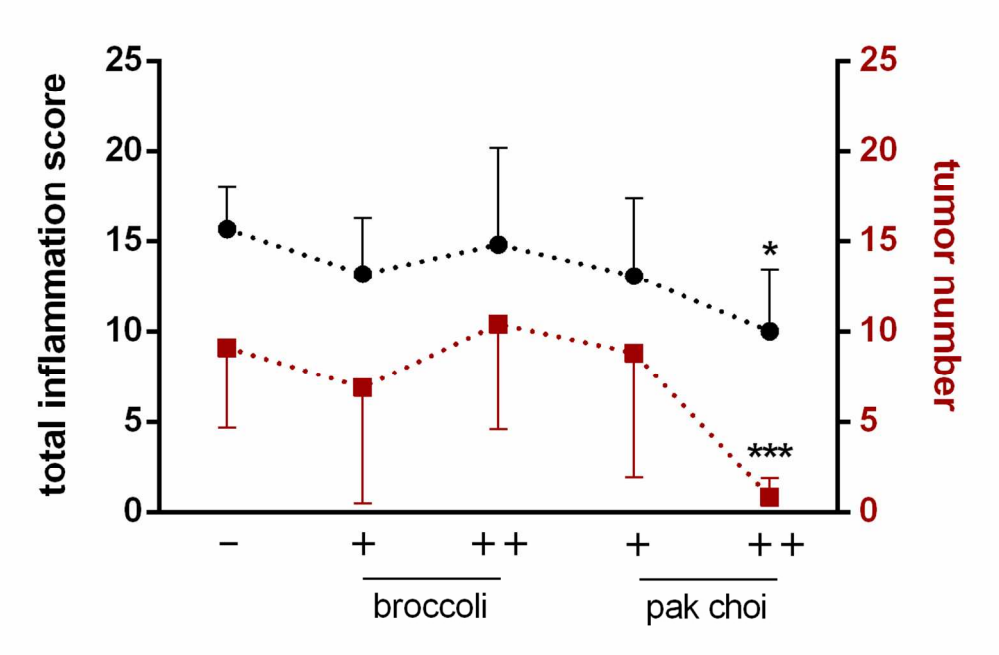

Feeding a glucosinolate-enriched pak choi diet reduced colitis and tumor numbers. No effects were observed by a glucosinolate-enriched broccoli diet.
112x74mm (300 x 300 DPI)

Glucosinolates from pak choi and broccoli induce enzymes and inhibit inflammation and colon cancer differently

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Running title: Glucosinolates of broccoli and pak choi in AOM/DSS-induced colon cancer

Key words: glucosinolates, broccoli, pak choi, Nrf2, AhR, inflammation, colon cancer

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Abstract

High consumption of *Brassica* vegetables is considered to prevent especially colon carcinogenesis. The content and pattern of glucosinolates (GSL) can highly vary among different *Brassica* vegetables and may, thus, affect the outcome of *Brassica* intervention studies. Therefore, we aimed to feed mice with diets containing plant material of the *Brassica* vegetables broccoli and pak choi. Further enrichment of the diets by adding GSL extracts allowed us to analyse the impact of different amounts (GSL-poor versus GSL-rich) and different patterns (broccoli versus pak choi) of GSL on inflammation and tumor development in a model of inflammation-triggered colon carcinogenesis (AOM/DSS model). Serum albumin adducts were analyzed to confirm the up-take and bioactivation of GSL after feeding the *Brassica* diets for four weeks. In agreement with their high glucoraphanin content, broccoli diets induced the formation of sulforaphane-lysine adducts. Levels of 1-methoxyindolyl-3-methyl-histidine adducts derived from neoglucobrassicin were highest in the GSL-rich pak choi group. In the colon, the GSL-rich broccoli and the GSL-rich pak choi diet up-regulated the expression of different sets of typical Nrf2 target genes like Nqo1, Gstm1, Srxn1, and GPx2. GSL-rich pak choi induced the AhR target gene Cyp1a1 but did not affect Ugt1a1 expression. Both, colitis and tumor number, were drastically reduced after feeding the GSL-rich pak choi diet while the other three diets had no effect. GSL can act anti-inflammatory and anti-carcinogenic but both effects depend on the specific amount and pattern of GSL within a vegetable. Thus, a high *Brassica* consumption cannot be generally considered to be cancer-preventive.

INTRODUCTION

The plant family of Brassicaceae consists of a large variety of common vegetables like broccoli, Brussels sprouts, cauliflower or pak choi, which contain characteristic secondary plant metabolites, the glucosinolates (GSL). Epidemiological studies suggest that a high consumption of *Brassica* vegetables may decrease the risk of developing colorectal cancer, however, results of these studies are inconsistent.[1-3] The composition and amount of GSL in *Brassica* vegetables highly depends on the species, variety, and the age of the plant as well as on environmental factors.[4] In general, most *Brassica* species contain a specific profile of less than twelve of the 132 known GSL.[5] The GSL composition might be the crucial factor for determining whether GSL prevent or promote cancer development. In fact, both carcinogenic and anti-carcinogenic effects of GSL present in broccoli were observed in animal studies.[6]

GSL are hydrolyzed by the enzyme myrosinase into bioactive metabolites like isothiocyanates (ITCs), nitriles, and indoles. Sulforaphane (SFN), the ITC derived from glucoraphanin (GRA; 4-methylsulfinylbutyl GSL) and commonly found in broccoli, has been most intensively studied and turned out to be a promising candidate for chemoprevention. Like other ITCs it activates the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2)[7, 8] and, thus, the expression of detoxifying phase II and antioxidant enzymes, which are generally considered to act cytoprotective. Promoters of Nrf2 target genes like NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutathione S transferase m1 (Gstm1), thioredoxin reductase 1 (TrxR1), sulfiredoxin (Srxn1), and the gastrointestinal glutathione peroxidase (GPx2) contain an electrophile responsive element, to which Nrf2 binds and, thus, activates gene expression. The exact mechanism has been reported elsewhere.[9-11]

Indole GSL are supposed to rather exert negative effects. Their metabolites enhance the expression of certain cytochrome P450 enzymes like Cyp1a1, which, among others, catalyze the metabolic activation of pro-carcinogens.[12] Cyp1a1 expression is primarily mediated via activation of the aryl hydrocarbon receptor (AhR),[13] which binds to xenobiotic response elements as a heterodimer with its nuclear translocator (Arnt). The AhR pathway is

activated by environmental toxins like the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Also indole GSL are ligands for the AhR although they have a lower binding affinity. In contrast to xenobiotic ligands, AhR activation by natural ligands such as indole-3-carbinol (I3C) derived from glucobrassicin (GBS; indole-3-yl-methyl GSL) rather exerts anti-carcinogenic effects.[14, 15] However, *N*-methoxyindole-3-carbinole (NI3C), one hydrolysis product of neoglucobrassicin (nGBS; 1-methoxy-indole-3-yl-methyl GSL) clearly has genotoxic properties.[16-18] In addition, hydrolysis products of nGBS inhibited the SFN-mediated induction of the Nrf2 regulated enzymes GPx2 and Nqo1 in HepG2 cells.[19] Thus, different GSL may interfere with each other and may inhibit or enhance their effects.

Analysis of different types of tumors revealed an anti-carcinogenic function of GSL metabolites in several animal studies (as reviewed in [20]). Regarding colorectal cancer, oral administration of SFN decreased azoxymethane (AOM)-induced aberrant crypt foci formation in rats.[21] Furthermore, a SFN-supplemented diet,[22] I3C, as well as the GBS metabolite 3,3-di-indolylmethane (DIM)[23] reduced the formation of polyps in $Apc^{min/+}$ mice. In addition, I3C also counteracted the induction of colon cancer induced by heterocyclic aromatic amines in rats.[24, 25]

The role of *Brassica* vegetables during inflammation-associated colon carcinogenesis has been less well studied. In the AOM/DSS model,[26] oral application of DIM dose-dependently attenuated colitis and reduced colonic tumor formation.[27] In another study, SFN inhibited colon carcinogenesis and inflammation in mice under an adequate selenium supply and when given simultaneously with AOM, however, it acted pro-inflammatory under selenium restriction.[28] Accordingly, GSL and metabolites can not only interact among themselves but also with other food components like selenium.

As GSL effects highly depend on the specific composition within a vegetable we aimed to study a putative GSL interplay considering the food matrix by feeding mice *Brassica* vegetables instead of single purified substances. For this purpose, broccoli and pak choi diets with fundamentally different GSL patterns and levels (with and without GSL enrichment) were produced and given to healthy control as well as to AOM/DSS-treated mice. Diet-

induced effects on the induction of Nrf2 and AhR target genes and on colitis and tumor development were analyzed. We show that (i) a GSL-rich broccoli diet induced gastrointestinal Nrf2 targets most effectively, but did not affect colitis and colon carcinogenesis and (ii) that the GSL-rich pak choi diet strongly induced the AhR target gene Cyp1a1 in the colon, attenuated colitis, and reduced colonic tumor formation.

EXPERIMENTAL

Broccoli and pak choi diets

Mice were fed one out of five different diets that were: (1) a semisynthetic GSL-free diet (C1000, Altromin, Lage, Germany), (2) a GSL-poor broccoli diet with broccoli sprouts, (3) a GSL-rich broccoli diet enhanced with GSL extracted from broccoli seeds, (4) a GSL-poor pak choi diet with pak choi sprouts, and (5) a GSL-rich pak choi diet enhanced with GSL extracted from pak choi sprouts treated with 2 mM methyl jasmonate for 48 h to induce nGBS.[29] GSL-poor *Brassica* diets were produced by adding 1.2% (w/w) freeze-dried sprouts (12 days old) to the semisynthetic diet without any contact of the plant material with water to avoid hydrolysis of GSL by the plant's own myrosinase. The broccoli sprouts ´*Calabrese*´ (*Brassica oleraceae* var. *italica*) and pak choi sprouts ´Black Behi´ (*Brassica rapa* var. *chinensis*) were cultivated as described.[30] GSL-rich *Brassica* diets were obtained by adding respective purified GSL extracts to the GSL-poor diets. GSL analyses were performed in ten replicates as previously described[29, 31] using 100 mg of the respective diets. Dietary GSL contents are shown in table 1. Freeze drying and mixture of diet ingredients in a dried form was identified as the best way to preserve the endogenous myrosinase activity and the GSL content of the plant material. The powdered diets were stored at -80°C and freshly provided to the mice every other day. *Ex vivo* hydrolysis of the GSL diets for 15 min by adding water resulted in a complete loss of GSL, indicating that the plant-derived myrosinase was still intact.

Purified GSL extracts were obtained according to the following extraction protocol. Broccoli seeds (2 x 50 g) were homogenized in 200 ml 80% methanol, centrifuged (10,000 x

g, 4°C) and 2 x re-extracted in 150 ml methanol. Supernatants were loaded on columns packed with 60 ml DEAE-Sephadex A25 in 2 M acetic acid. Pak choi sprouts (2 x 25 g) were extracted with 70% methanol at 80°C for 10 min. Columns were preconditioned with 2 x 40 ml 6 M imidazole and washed with 2 x 40 ml ultra-pure water. After drop-wise loading of the plant extracts, the columns were washed with 2 x 30 ml of a formic acid-isopropanol-ultra-pure water mix (3:2:5) and 2 x with 20 ml ultra-pure water. GSL were eluted with 0.5 M potassium sulfate (in 3% isopropanol) into ethanol. For purification additional extraction rounds using methanol and ethanol were performed. GSL purification (> 98%) was verified by HPLC. A volume of 10 µl of purified GSL extracts was injected into a Dionex P680A HPLC-DAD system equipped with a narrow bore column (Acclaim TM120, 250 mm x 2.1 mm, 5 µm, RP18, Dionex). HPLC eluents for analysis of intact GSL in the purified extracts were A: 0.1 M ammonium acetate in ultrapure water and B: 40% acetonitrile containing 0.1 M ammonium acetate. The 43 min gradient was as follows: 0.5% B for 1 min, from 0.5% to 20% B for 7 min, 20% B for 2 min, from 20% to 50% B for 9 min, 50% B for 3 min, from 50% to 99% B for 6 min, a 5 min hold at 99% B, from 99% to 0.5% B for 3 min, and a 7 min final hold at 0.5% B. GSL were monitored at 229 nm.

Animal experiment and tissue sampling

Ten-week-old male C57BL/6J mice (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions with free access to food and water. Animal experiments were performed in compliance with the German animal protection law (TierSchG). The mice were housed and handled in accordance with good animal practice as defined by FELASA (www.felasa.eu/guidelines.php) and the national animal welfare body GV-SOLAS (www.gv-solas.de/index.html). The animal welfare committees of the DIfE as well as the local authorities (Landesamt für Umwelt, Gesundheit und Verbraucherschutz, Brandenburg) approved all animal experiments.

To induce colon cancer, mice received 10 mg AOM/kg body weight (Sigma-Aldrich, Steinheim, Germany) dissolved in saline (Sigma-Aldrich) by intraperitoneal injection. One

week later, mice obtained drinking water containing 1% dextran sulphate sodium (DSS, 36-50 kDa, MP Biomedicals, Illkirch, France) for 7 days to induce colitis.[26] Control mice received saline and drinking water without DSS. Respective diets were fed for 4 weeks via racks starting one week before AOM application until one week after DSS withdrawal (Fig. 1A). Mice of the inflammation groups were killed at the end of week four. Mice of the tumor groups were treated identically but received the semisynthetic diet until week nine after DSS withdrawal (Fig. 1A). In total we had 20 different experimental groups, including ten inflammation (mice per group, n = 10) and ten tumor groups (n = 12). The ten groups were further subdivided into the five different feeding groups (semisynthetic, GSL-poor and GSL-rich broccoli and GSL-poor and GSL-rich pak choi) with and without AOM/DSS treatment.

Mice were anesthetized with isoflurane and blood was withdrawn with heparinized capillaries by puncture of the retro-orbital plexus. Plasma was obtained after centrifugation of the blood for 10 min (3,000 x *g*, 4°C). Anesthetized animals were sacrificed by cervical dislocation. Tissue sampling was performed as reported.[28] Briefly, for enzyme activities and mRNA analyses, the proximal 2 cm of the colon were snap-frozen and stored at -80°C. Inflammation was scored in the transversal and distal parts of the colon fixed as a Swiss roll. Tumors were analyzed in the entire longitudinally opened colon, stretched on filter paper and fixed in 4% neutral-buffered formalin.

Inflammation score and tumor analysis

Severity of colitis was assessed by using an established scoring system[28, 32] including the disease activity index (DAI)[33] and histological parameters (Fig. S1). The DAI was based on changes in body weight, visible fecal blood, and diarrhea (Fig. S1A). These parameters were monitored daily in all animals (with and without AOM/DSS) from the beginning of DSS treatment until one week after DSS withdrawal. The maximum DAI score was nine. The total inflammation score, analyzed in animals of inflammation groups only, consisted of the DAI, changes in colon macroscopy, and histological parameters (Fig. S1B). Histological parameters were analyzed using 2-µm hematoxylin and eosin (H&E) stained sections of

colon Swiss rolls (Fig. S1C). The highest total inflammation score was 21.5 and was also given to mice that died or had to be killed according to brake-off criteria before finishing the experiment. For tumor analysis the fixed colon was stained with 0.1% methylene blue. Number of tumors was counted and tumor size was measured using a stereo microscope (SZH10, Olympus, Japan). All analyses were performed in a blinded fashion.

Serum albumin adducts

Adducts of reactive metabolites of nGBS with histidine, τ*N*-(1-methoxy-3-indolylmethyl)-histidine [τ*N*-(1-MIM)-His] and GRA with lysine, N^6-({[3-(methylsulfinyl)propyl]amino}carbonothioyl)lysine (SFN-Lys) were analyzed after enzymatic digestion of serum albumin with pronase E as described.[34] In brief, serum albumin was isolated from blood plasma by adding an equal volume of saturated ammonium sulfate solution to precipitate globulins. The serum albumin content was determined with a BCA Protein Assay Kit (Thermo Fischer Scientific) after desalting with Amicon centrifugal filter tubes (30 kDa mass cut-off). Isotope-labeled standards, 4 pmol of 1-MIM-His (τ*N*-(1-MIM)-[$^{15}N_3$]) and 60 nmol of SFN-[$^{13}C_6{}^{15}N_2$]Lys, were added to an aliquot of 1 mg serum albumin. It was digested with 0.34 mg pronase E in 50 mM potassium phosphate buffer (pH7.4) for 18 h. Adducts were extracted via solid phase extraction on Chromabond C18ec-columns (Macherey-Nagel, Düren, Germany). Extracts were re-uptaken in water and methanol (1:1, v/v, 0.1% formic acid) and subjected to ultraperformance liquid chromatography coupled with tandem mass spectrometry in the positive electrospray ionization mode (UPLC-ESI-MS/MS).

Enzyme activities

Aliquots of ground tissue (20 mg) were homogenized in 500 µl homogenization buffer (100 mM Tris-HCl, 300 mM KCl, 0.1% Triton X-100, pH 7.6) containing 4 µl of protease inhibitor cocktail III (Calbiochem, Bad Soden, Germany) using a tissue lyzer (Qiagen, Hilden, Germany) for 2 x 2 min at 30 Hz. Homogenates were centrifuged (21,000 x *g*, 15 min, 4°C) and the supernatant was used for further analysis. Protein content was assessed according

to Bradford.[35] Nqo1 and TrxR activities were measured with standard procedures optimized for estimation in a microplate reader as described.[28]

RNA isolation and quantitative real-time PCR

Total RNA from the proximal colon of mice without AOM/DSS treatment was isolated using Trizol and reversely transcribed as reported.[36] Real-time PCR was performed using SYBR Green I (Invitrogen, Karlsruhe, Germany) in a Mx3005P™ qPCR system (Stratagene, Amsterdam, Netherlands) as described.[36] The annealing temperature was 60°C for all PCR reactions. Mouse primer (Sigma-Aldrich) sequences (forward and reverse) were: β-actin (5´-CACTGCCGCATCCTCTTCCT-3´ and 5´-GATTCCATACCCAAGAAGGAAGGC-3´), Hprt1 (5´-GCAGTCCCAGCGTCGTG-3´ and 5´-GGCCTCCCATCTCCTTCAT-3´), Cyp1a1 (5´-CTTCATTCCTGTCCTCCGTTACCT-3´ and 5´-GGATGTGGCCCTTCTCAAATGTC-3´), Nqo1 (5´-ATGTACGACAACGGTCCTTTCCAG-3´ and 5´-GATGCCACTCTGAATCGGCCA-3'), Gstm1 (5'-AGCTCATCATGCTCTGTTACAACC-3' and 5'-AATCCACATAGGTGACCTTGTCCC-3'), Gpx2 (5'-GTGCTGATTGAGAATGTGGC-3' and 5'-AGGATGCTCGTTCTGCCCA-3'), Srxn1 (5'-AGCCTGGTGGACACGATCCT-3' and 5'-TGCTGGTAGGCTGCATAGCG-3'), Ugt1a1 (5'-TCATAGCACCTGAAGCCTCAATACAC-3' and 5'-TAAAGGCAGTCCGTCCAAGTTCC-3'). mRNA expression was normalized to the geometric mean of the two reference genes β-actin and Hprt1.

Statistics

Significance was tested by 1-way or 2-way analysis of variance (ANOVA), Student's t-test (GraphPad Prism®, version 5.0, San Diego, CA) or Fisher's exact test (SPSS®, version 20, IBM, Armonk, New York) as indicated in the figure legends. A p-value <0.05 was considered statistically significant.

RESULTS

Daily intake and systemic availability of GSL

Mice were fed the control and *Brassica* diets for 4 weeks. GRA, glucoiberin, and glucoerucin were the most abundant GSL in both broccoli diets, whereas progoitrin and GNA dominated in the pak choi diets (bold printed in table 1). nGBS, which was hardly detectable in the broccoli diets and in the GSL-poor pak choi diet, was markedly increased in the GSL-rich pak choi diet. Without AOM/DSS treatment, no significant differences were found in mean food intake and body weights between the different feeding groups. The average daily GSL intake per mouse was 1.93 µmol with the GSL-poor broccoli and 2.79 µmol with the GSL-poor pak choi diet (Table 1). The daily intake of GSL was about 6-fold higher in the GSL-enriched groups (12.39 and 19.32 µmol with the GSL-rich broccoli or pak choi diet, respectively).

Adducts with serum albumin can be used as biomarkers for the systemic availability of the reactive metabolites of GSL. τ*N*-(1-MIM)-His adducts, specific for nGBS, were detected in all animals receiving a *Brassica* diet, but not in any animal on the semisynthetic diet. Their level was very high in the GSL-rich pak choi group, but low in the GSL-poor pak choi group and in both broccoli groups (Fig. 1B). SFN-Lys adducts were only detectable in mice on the two broccoli diets (Fig. 1C).

During DSS treatment food intake and mean GSL intake were temporarily reduced due to the acute colitis (Table 1). Consequently, lower amounts of τ*N*-(1-MIM)-His and SFN-Lys adducts were found in AOM/DSS-treated mice (Fig. 1B and C).

Induction of Nrf2 and AhR target genes by *Brassica* diets

Feeding GSL-rich broccoli significantly increased Nqo1 activity and tended to increase TrxR activity in the colon (Fig. 2). The GSL-rich pak choi diet enhanced Nqo1 activity to a similar extend (Fig. 2A) but did not affect TrxR activity (Fig. 2B). Basal activity of Nqo1 was significantly enhanced in AOM/DSS-treated mice, and could have masked an increase by the GSL diets.

To further characterize the effects of feeding the *Brassica* diets we analyzed mRNA expression of genes known to be regulated via Nrf2 (Gstm1, Nqo1, Gpx2, Srxn1) or AhR (Cyp1a1, Ugt1a1). In addition, we analyzed the expression of cytokines (IL-6, IL-10) and cell cycle regulators (p21, p27, Cdk2, cyclin A2), which remained unaffected by the *Brassica* diets (data not shown). Cyp1a1 mRNA was highly increased by the GSL-rich pak choi diet, while all other diets had no effect (Fig. 3A). However, effects could not be confirmed for another AhR target gene, namely Ugt1a1 (Fig. 3B). The Nrf2 targets Gstm1 and Gpx2 were only induced by the GSL-rich broccoli diet (Fig. 3C and E). mRNA levels of Nqo1 (Fig. 3D) nicely reflected activity levels (Fig. 2A). Both GSL-rich diets significantly enhanced Nqo1 expression although the GSL-rich broccoli diet was more potent. The Nrf2 target gene Srxn1 was only up-regulated in the GSL-rich pak choi group (Fig. 3F). In summary, AhR and Nrf2 target genes were induced in the colon by the GSL-rich pak choi and broccoli diets, respectively, indicating that these diets distinctly modulated the enzymatic repertoire of the colon.

The GSL-rich pak choi diet attenuated DSS-induced colitis and tumor development, whereas the GSL-rich broccoli diet did not

Mice without AOM/DSS treatment showed no signs of inflammation, whereas they were unexpectedly severe in AOM/DSS-treated mice. Severity of colitis was assessed by the total inflammation score (Fig. S1) including the DAI and histological parameters. Only the GSL-rich pak choi diet significantly attenuated the severity of colitis in comparison to the control group (Fig. 4A). The other three *Brassica* diets did not show any effect. This could be confirmed and, thus, strengthened in the tumor groups, in which only non-invasive parameters of the DAI (Fig. S1A) were scored during the DSS phase (Fig. 4B). Thus, enrichment of the pak choi diet with GSL clearly had an anti-inflammatory effect.

In the AOM/DSS model, the tumor load of the colon is causally determined by the severity of colitis.[26] Also herein, tumor incidence and multiplicity (Fig. 4C) were interrelated to the inflammation score. Both were dramatically decreased in mice fed the GSL-rich pak choi

diet while the other *Brassica* diets again had no effect. In contrast, tumor size was completely unaffected by any of the *Brassica* diets (Fig. 4D). Thus, enrichment of the pak choi diet with GSL exerted an anti-carcinogenic effect most probably due to an anti-inflammatory action.

DISCUSSION

The enrichment of *Brassica* diets with GSL was successful regarding the systemic availability of the reactive GRA metabolite SFN and the reactive nGBS metabolite(s) (1-methoxy-3-indolylmethyl)-ITC and/or (1-methoxy-3-indolylmethyl)-sulfate, as indicated by specific serum albumin adducts in the plasma (Fig. 1B and C). Functional effects of *Brassica* diets were proven by the induction of Nrf2 and AhR target genes in the colon. Nqo1 is a target of both transcription factors, Nrf2 and AhR.[37] Accordingly, its mRNA expression as well as its activity was enhanced by both GSL-rich diets (Fig. 2A and 3D). Therefore, more specific Nrf2 and AhR target genes were analyzed. As expected, the Nrf2 targets Gstm1 (Fig. 3C) and Gpx2 (Fig. 3D) were only induced in the GSL-rich broccoli group, whereas Srxn1 expression was only induced in the GSL-rich pak choi group (Fig. 3F). The AhR target Cyp1a1, but not Ugt1a1, was up-regulated upon feeding GSL-rich pak choi (Fig. 3A and B). Taken together, the GSL-rich broccoli diet clearly enhanced Nrf2 target genes, whereas the GSL-rich pak choi diet specifically induced the AhR target Cyp1a1 and, to a smaller extent, Nrf2-specific targets. Nqo1, inducible by both, Nrf2 and AhR, was enhanced by both diets. The identification of the responsible GSL within the different diets needs to be further investigated. Of particular interest is to answer the question whether Nrf2 effects can be counteracted by AhR as observed in HepG2 cells previously.[19] Nevertheless, it is obvious that depending on their GSL pattern and content *Brassica* diets have diverse effects on enzyme regulation in the colon, the part of the intestine where cancer was induced and analyzed.

Despite the significant up-regulation of Nrf2 targets by the GSL-rich broccoli diet in animals without AOM/DSS treatment it did not affect the severity of AOM/DSS-induced colitis

and tumor outcome (Fig. 4). This might be explained by the enhanced basal Nqo1 activity in AOM/DSS-treated mice, which may have masked the increase by the *Brassica* diets before AOM/DSS treatment (Fig. 2A). As shown in previous studies, Nrf2 is activated during the resolution of inflammation at least in lung.[38] Increased Nrf2 activity makes cells more resistant to oxidative and electrophilic stress.[39] Conversely, Nrf2 knockout mice are more susceptible to DSS-induced colitis[40] and AOM/DSS-induced colon carcinogenesis[41] than wild types. From our results we can only conclude that during the regeneration phase Nrf2 target genes were, if at all, only marginally enhanced by the GSL-rich broccoli diet, but might have been induced before AOM/DSS treatment (as shown for mice without AOM/DSS treatment). Nevertheless, feeding the broccoli diets neither changed total inflammation score nor DAI (Fig. 4A and B) significantly. It is well established that severity of colitis highly correlates with tumor development in the AOM/DSS model[26] and, thus, it does not astonish that also tumor numbers were not affected by the broccoli diets (Fig. 4C).

In contrast to the GSL-rich broccoli, GSL-rich pak choi substantially inhibited AOM/DSS-induced colitis and tumor development. Tumor incidence was reduced from 100% in the control or the GSL-poor pak choi group to 50% in the GSL-rich pak choi group. Tumor multiplicity was even more dramatically decreased (Fig. 4C). Thus, the enrichment of the GSL-poor pak choi diet with GSL (mainly progoitrin, GNA, and nGBS) had anti-inflammatory and anti-carcinogenic effects in the AOM/DSS model. Effects correlated with the high increase in Cyp1a1 expression in the colon of the GSL-rich pak choi group (Fig. 3A). The GSL-rich pak choi diet was the only diet that contained appreciable levels of indole GSL, which are well-known precursors of AhR ligands.[13, 42] Thus, Cyp1a1 up-regulation was considered to be caused by AhR activation. AhR knockout mice responded more sensitively to DSS-induced colitis[43-45] and spontaneously developed tumors in the cecum.[23] Vice versa AhR activation e.g. by TCDD reduced the severity of DSS-colitis.[46, 47] Also supplementation of a semisynthetic diet with the AhR ligand I3C attenuated the severity of colitis.[45] Thus, AhR activation as indicated by the up-regulation of the AhR target gene Cyp1a1 most probably was involved in the reduction of colitis. The GSL-rich pak choi diet is not only supposed to

activate the AhR pathway but might also activate Nrf2, which was inferred from the up-regulation of Nqo1 and Srxn1 mRNA expression (Fig. 3). Whether or not AhR and Nrf2 activation account for the anti-inflammatory effect of the GSL-rich pak choi diet and which of the transcription factors plays the major role needs to be further investigated.

An alternative mechanism for the chemopreventive effect of the GSL-rich pak choi diet is PXR activation. PXR is activated by a wide range of xenobiotics and among others regulates the expression of CYP3A enzymes. In HepG2 cells, CYP3A4 promoter activity can be significantly increased by the major pak choi GSL, GNA, progoitrin, and nGBS (own observation). Thus, PXR activity could also be increased *in vivo* by feeding the GSL-rich pak choi diet. Treatment of mice with the typical mouse PXR agonist pregnenolone-16 alpha-carbonitrile (PCN) reduced the severity of DSS-induced colitis.[48] Protective effects were only observed in wild type, but not in $PXR^{-/-}$ mice. DIM also dose-dependently attenuated colitis and reduced tumor numbers in the AOM/DSS model by suppressing nuclear translocation and DNA binding capacity of the NF-κB subunit p65.[27] In addition, $PXR^{-/-}$ mice expressed a higher amount of hepatic and intestinal NF-κB target genes.[49] Enhanced NF-κB target gene expression was counter-regulated by PCN treatment, which again was only observed in wild type mice. These data suggest that NF-κB signaling is inhibited following ligand-dependent PXR activation. Also in the colon of AOM/DSS-treated mice GSL of the GSL-rich pak choi diet might have interfered with NF-κB activation and, thus, contributed to the decrease in inflammation.

CONCLUSION

The present study clearly shows that GSL from *Brassica* vegetables cannot be generally considered to act anti-inflammatory and to prevent carcinogenesis. Effects depend on the model used, the environmental conditions, i.e. habits of food intake, the kind of *Brassica* vegetables with varying GSL content and pattern, and as known from the literature[50] the time point of starting GSL intervention. Further investigations are needed to understand the interactions of the GSL with each other and with other plant ingredients. In

addition, underlying mechanisms need to be further elucidated. At present, a diet rich in GSL and particularly GSL supplementation should be reflected critically and cannot be generally recommended.

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FIGURE LEGENDS

Fig. 1: Experimental design and protein adducts

Study design as indicated and described in the 'Experimental' section (A). Mice were fed a semisynthetic control diet (-) or one of four *Brassica* diets for four weeks (+ = GSL-poor; ++ = GSL-rich). Serum albumin adducts τ*N*-(1-MIM)-His (B) and SFN-Lys (C) were analyzed in the

plasma and expressed relative to serum albumin. Data are presented as box and whiskers (Tukey) with '+' indicating the mean (n ≥ 5). *p<0.05, ***p<0.001 versus control diet; $^{###}$p<0.001 versus respective GSL-poor diets; ^{xxx}p<0.001 versus respective -AOM/DSS group analyzed by 2way ANOVA, Bonferroni's Multiple Comparison Test.

Fig. 2: *Brassica* diets-induced changes in Nqo1 and TrxR activity

Nqo1 (A) and TrxR (B) activity was measured in lysates of the proximal colon of mice belonging to the inflammation groups and expressed as mU/mg protein. Data are presented as box and whiskers from min to max (n = 10). *p<0.05, **p<0.01 versus control diet; $^{##}$p<0.01 versus respective GSL-poor diets; ^{x}p<0.05, ^{xx}p<0.01, ^{xxx}p<0.001 versus respective -AOM/DSS group analyzed by 2way ANOVA, Bonferroni's Multiple Comparison Test.

Fig. 3: *Brassica* effects on mRNA levels of Nrf2 and AhR target genes

Cyp1a1 (A), Ugt1a1 (B), Gstm1 (C), Nqo1 (D), Gpx2 (E), and Srxn1 (F) mRNA expression was analyzed by qPCR in the colon of mice without AOM/DSS treatment (+ = GSL-poor; ++ = GSL-rich). Data were normalized to the geometric mean of the reference genes Hprt1 and β-actin and expressed relative to the control group as mean ± SD (n = 10). *p<0.05, **p<0.01, ***p<0.001 versus control diet; $^{###}$p<0.001 versus respective GSL-poor diets analyzed by 1way ANOVA, Bonferroni's Multiple Comparison Test. Symbols in brackets indicate analysis with Students *t-test*.

Fig. 4: Suppression of AOM/DSS-induced colitis and tumor development by the GSL-rich pak choi diet

(A) The severity of colitis was assessed by the total inflammation score (see Fig. S1) observed in mice of the inflammation groups (n = 10). (B) The disease activity index was determined in animals of the tumor groups (n = 12). Mice that died with severe symptoms of colitis during the DSS phase were evaluated with the maximum score. (C) Tumor incidence (indicated as % above the scatter dot blots) and multiplicity, and (D) tumor size were

analyzed 12 weeks after AOM application (n = 12). The removed colon was fixed in formalin and stained with 0.1 % methylene blue. Tumors were counted in a blinded fashion. Data are shown as scatter dot blot with mean (A-C) or as box and whiskers from min to max (D). *p<0.05, **p<0.01, ***p<0.001 versus control diet; ##p<0.01 versus respective GSL-poor diet analyzed by 1way ANOVA, Bonferroni's multiple comparison test. Tumor incidence was analyzed by Fisher's Exact Test with *p<0.05 versus all other feeding groups.

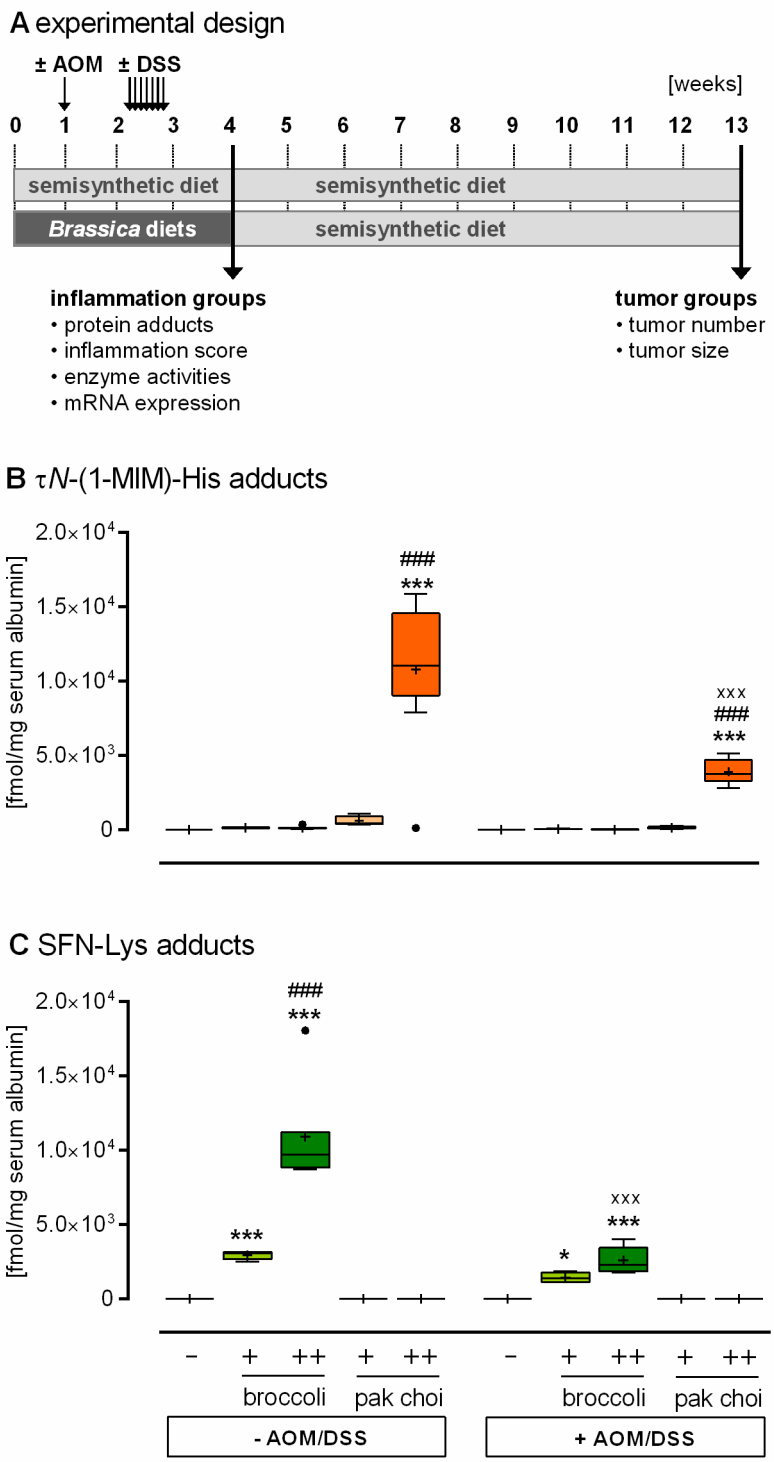
Fig. S1: Assessment of the severity of inflammation

(A) The disease activity index (DAI) was calculated in all mice after application of DSS and scored as indicated. The weight loss index[1] was calculated as the sum of days suffering from weight loss in the following categories: <5% (0), 5-10% (1), 10-15% (2), 15-20% (3) and >20% (4). (B) The total inflammation score consisted of the DAI, evaluation of macroscopical changes of the colon, and histological parameters. (C) H&E stained colonic Swiss rolls indicate the severity of mucosal loss ranging from mild to severe (arrows).

Table 1. Content of GSL in the diet [µmol/g diet] and / daily intake [µmol/d, n = 10]

Trivial name	Chemical name [GSL]	GSL-poor broccoli diet	GSL-rich broccoli diet	GSL-poor pak choi diet	GSL-rich pak choi diet
Methyl (thio/sulfinyl) alkyl					
Glucoiberin	3-Methylsulfinylpropyl	**0.15** / 0.48 ± 0.02	**1.12** / 3.61 ± 0.25	n.d.	n.d.
Glucoerucin	4-Methylthiobutyl	**0.08** / 0.26 ± 0.01	**0.54** / 1.76 ± 0.12	n.d.	n.d.
Glucoraphanin	4-Methylsulfinylbutyl	**0.28** / 0.89 ± 0.04	**1.82** / 5.91 ± 0.42	n.d.	n.d.
Glucoalyssin	5-Methylsulfinylpentyl	n.d.	0.02 / 0.06 ± 0.00	0.02 / 0.05 ± 0.00	0.04 / 0.12 ± 0.01
Alkenyl					
Gluconapin	3-Butenyl	n.d.	0.04 / 0.13 ± 0.01	**0.44** / 1.31 ± 0.11	**1.86** / 5.37 ± 0.40
Progoitrin	(2*R*)-2-Hydroxy-3-butenyl	0.02 / 0.07 ± 0.00	0.18 / 0.58 ± 0.04	**0.31** / 0.91 ± 0.08	**2.12** / 6.13 ± 0.45
Glucobrassicanapin	4-Pentenyl	n.d.	n.d.	0.10 / 0.30 ± 0.00	0.59 / 1.71 ± 0.13
Gluconapoleiferin	2-Hydroxy-4-pentenyl	n.d.	n.d.	0.03 / 0.08 ± 0.01	0.15 / 0.42 ± 0.03
Aromatic					
Gluconasturtiin	2-Phenylethyl	n.d.	n.d.	0.01 / 0.02 ± 0.00	0.03 / 0.09 ± 0.01
Indole					
Glucobrassicin	Indole-3-yl-methyl	0.04 / 0.11 ± 0.01	0.04 / 0.11 ± 0.01	0.01 / 0.04 ± 0.00	0.45 / 1.31 ± 0.10
4-Hydroxyglucobrassicin	4-Hydroxy-indole-3-yl-methyl	0.01 / 0.04 ± 0.00	0.04 / 0.12 ± 0.01	n.d.	0.03 / 0.08 ± 0.01
4-Methoxyglucobrassicin	4-Methoxy-indole-3-yl-methyl	0.02 / 0.07 ± 0.00	0.02 / 0.08 ± 0.01	0.02 / 0.05 ± 0.00	0.24 / 0.69 ± 0.05
Neoglucobrassicin	1-Methoxy-indole-3-yl-methyl	0.00 / 0.01 ± 0.00	0.01 / 0.03 ± 0.00	0.01 / 0.02 ± 0.00	**1.17** / 3.39 ± 0.25
total GSL [µmol/g diet]		**0.61**	**3.83**	**0.94**	**6.67**
total GSL intake of the control groups [µmol/d]		**1.93 ± 0.08**	**12.39 ± 0.87**	**2.79 ± 0.23**	**19.32 ± 1.42**
total GSL intake of the AOM/DSS groups [µmol/d]		**1.77 ± 0.16**	**11.25 ± 1.43**	**2.40 ± 0.13**	**18.26 ± 0.58**

532 Fig. 1



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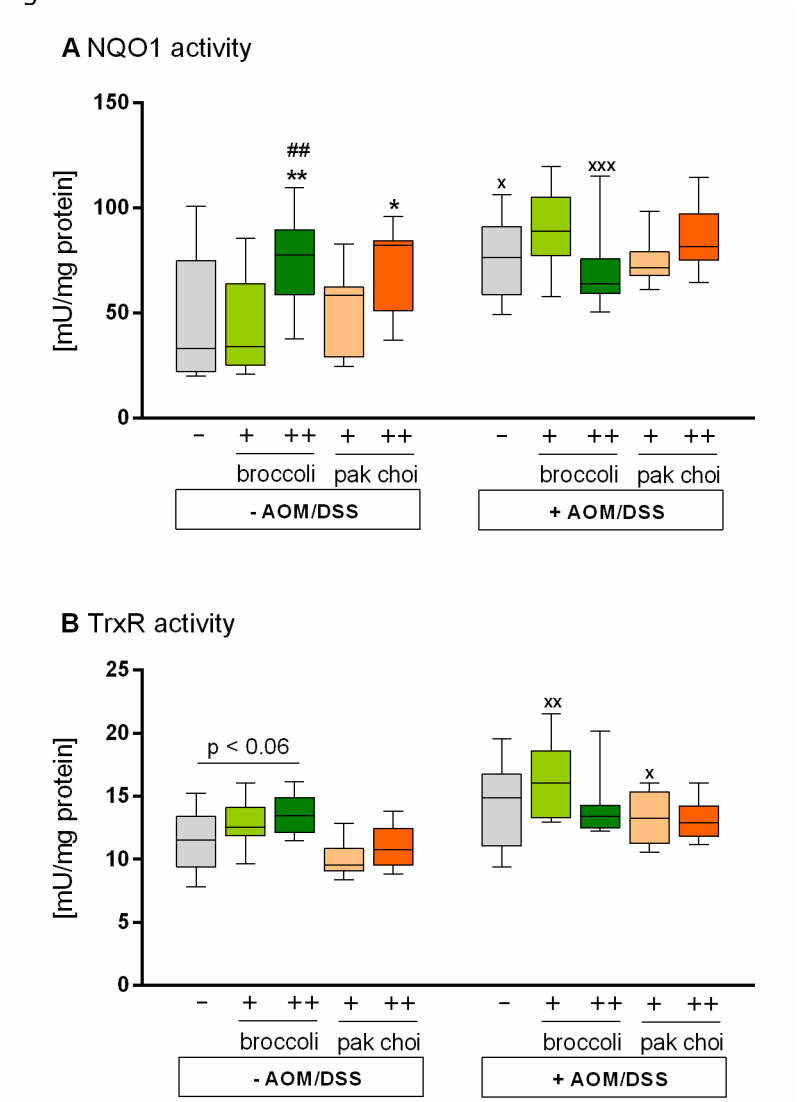
536 Fig. 2



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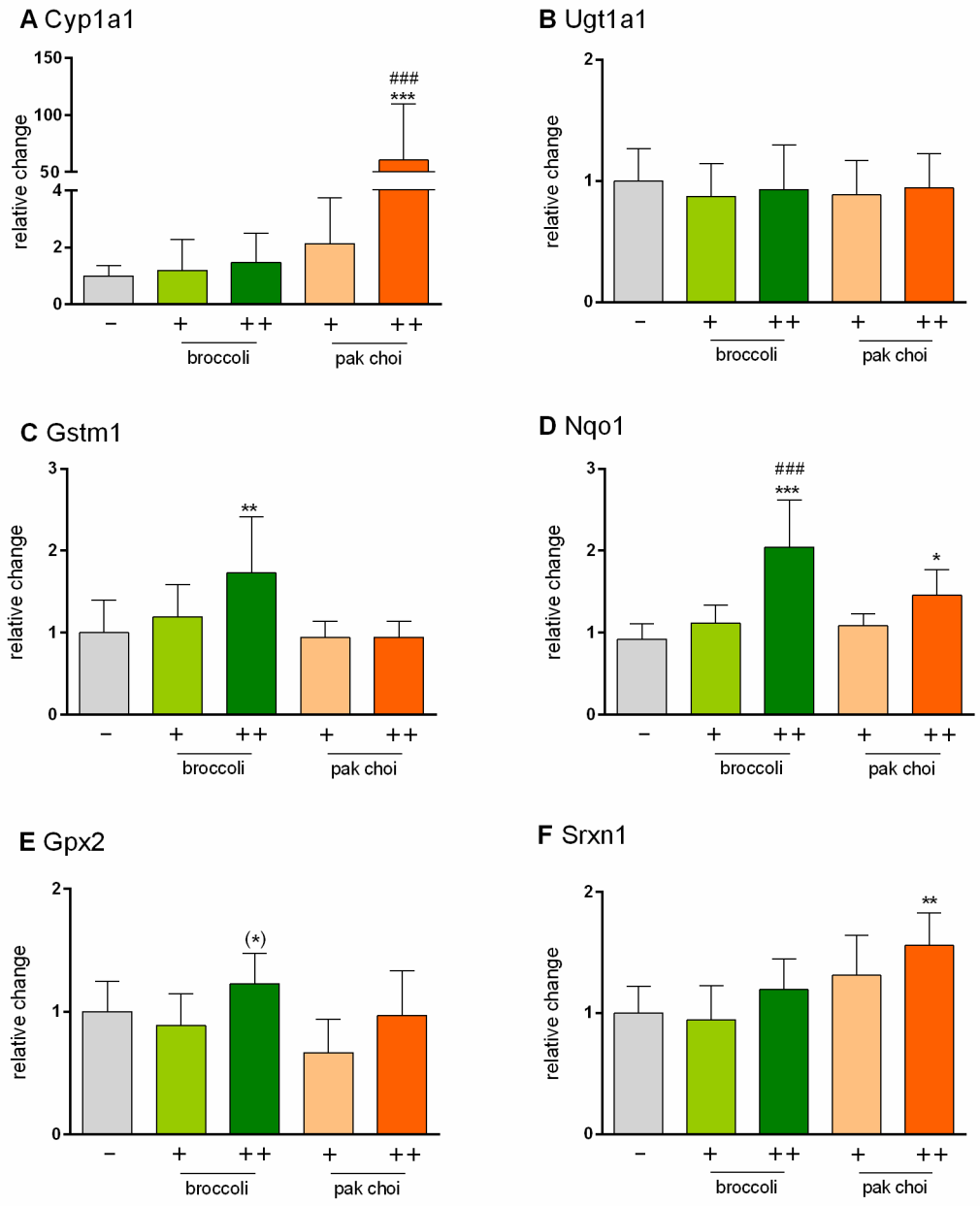
541 Fig. 3



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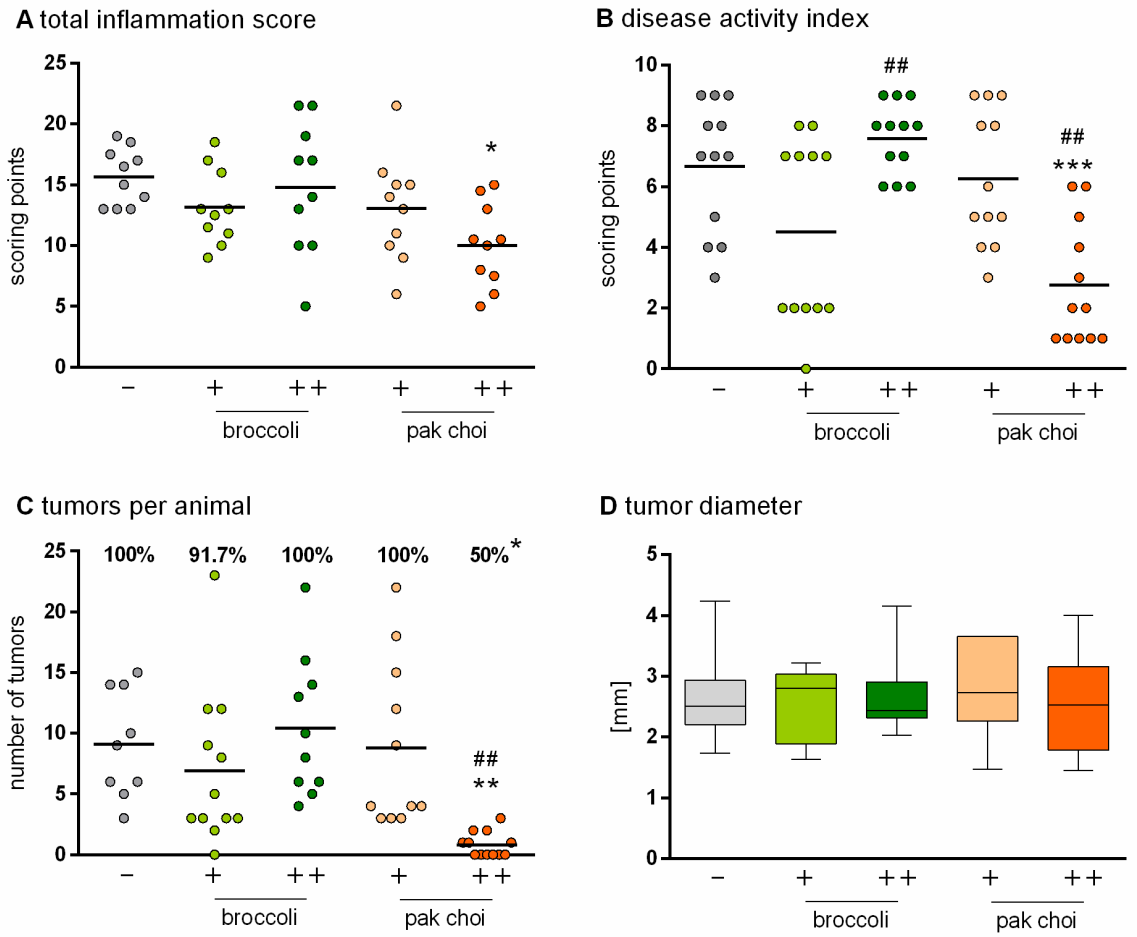
544 Fig. 4



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548 Fig. S1

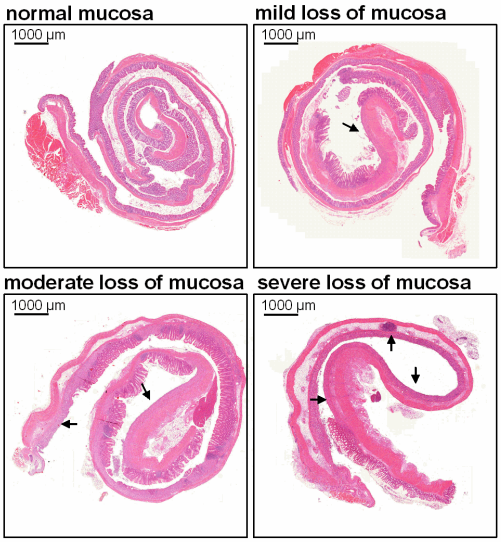
A Scoring of the disease activity index (DAI)

Weight loss index [1]	**Visible fecal blood** [days]	**Diarrhea** [days]	**Score (sum of 3 parameters)**
< 3	0	0	**0 (0)**
3-10	1-3	1-3	**1 (3)**
11-18	4-6	4-6	**2 (6)**
> 18	> 6	> 6	**3 (9)**

B Total inflammation score

Parameter	**Score**
DAI:	
- weight loss	0-3
- fecal blood	0-3
- diarrhea	0-3
Colon macroscopy (swelling and shortening):	0-3.5:
- no	0
- weak	1
- weak to moderate	1.5
- moderate	2
- moderate to strong	2.5
- strong	3
- very strong	3.5
Histological parameters:	
- edema of mucosa	no/yes (0/1)
- hemorrhage	no/yes (0/1)
- disturbed crypt architecture	no/yes (0/1)
- loss of mucosa	0-3
- inflammatory infiltration	0-3
	21.5

C H&E staining of colonic Swiss roles



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