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# Mesalazine and thymoquinone attenuate intestinal tumour development in $Msh2^{loxP/loxP}$ Villin-Cre mice

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## ABSTRACT

**Objective** Lynch syndrome is caused by germline mutations in DNA mismatch repair genes leading to microsatellite instability (MSI) and colorectal cancer. Mesalazine, commonly used for the treatment of UC, reduces MSI in vitro. Here, we tested natural compounds for such activity and applied mesalazine and thymoquinone in a  $Msh2^{loxP/loxP}$  Villin-Cre mouse model for Lynch syndrome.

**Design** Flow cytometry was used for quantitation of mutation rates at a CA<sub>13</sub> microsatellite in human colon cancer (HCT116) cells that had been stably transfected with pIRESHyg2-enhanced green fluorescent protein/CA13, a reporter for frameshift mutations. Mice were treated for 43 weeks with mesalazine, thymoquinone or control chow. Intestines were analysed for tumour incidence, tumour multiplicity and size. MSI testing was performed from microdissected normal intestinal or tumour tissue, compared with mouse tails and quantified by the number of mutations per marker (NMPM).

**Results** Besides mesalazine, thymoquinone significantly improved replication fidelity at 1.25 and 2.5  $\mu$ M in HCT116 cells. In  $Msh2^{loxP/loxP}$  Villin-Cre mice, tumour incidence was reduced by mesalazine from 94% to 69% ( $p=0.04$ ) and to 56% ( $p=0.003$ ) by thymoquinone. The mean number of tumours was reduced from 3.1 to 1.4 by mesalazine ( $p=0.004$ ) and to 1.1 by thymoquinone ( $p<0.001$ ). Interestingly, MSI was reduced in normal intestinal tissue from 1.5 to 1.2 NMPM ( $p=0.006$ ) and to 1.1 NMPM ( $p=0.01$ ) by mesalazine and thymoquinone, respectively. Thymoquinone, but not mesalazine, reduced MSI in tumours.

**Conclusions** Mesalazine and thymoquinone reduce tumour incidence and multiplicity in  $Msh2^{loxP/loxP}$  Villin-Cre mice by reduction of MSI independent of a functional mismatch repair system. Both substances are candidate compounds for chemoprevention in Lynch syndrome mutation carriers.

## INTRODUCTION

Lynch syndrome (LS) also known as hereditary non-polyposis colorectal cancer (CRC) is the most common familial cancer syndrome that predisposes to early onset of CRC and other extracolonic malignancies. It is estimated that LS accounts for 3% of all CRCs.<sup>1</sup> The majority of germline mutations in LS families affects genes of the mismatch repair (MMR) system such as *Msh2* (60%), *Mlh1*

## Significance of this study

### What is already known on this subject?

- Lynch syndrome (LS) accounts for approximately 3% of colorectal cancer (CRC).
- In LS germline mutations of mismatch repair (MMR) genes predispose to early onset CRC with microsatellite instability (MSI).
- Mesalazine, widely used in UC for its anti-inflammatory properties, improves replication fidelity in MMR-deficient cancer cell lines.
- Thymoquinone is a natural compound from *Nigella sativa* with various anti-carcinogenic properties.

### What are the new findings?

- Similar to mesalazine, also thymoquinone, improves replication fidelity in MMR-deficient cancer cell lines.
- Mesalazine and thymoquinone reduce incidence and multiplicity of intestinal tumours in  $Msh2^{loxP/loxP}$  Villin-Cre mice.
- Both substances tested reduced MSI in *Msh2*-deficient epithelium.

### How might it impact on clinical practice in the foreseeable future?

- We propose mesalazine and thymoquinone as candidate compounds for chemoprevention in LS mutation carriers.

(30%) and *Msh6* (7%–10%).<sup>2</sup> Somatic hypermethylation of *Msh2* and microsatellite instability (MSI) are frequent events in LS CRCs.<sup>3</sup> In fact, MSI is found in virtually all Lynch tumours as a result of an accumulation of frameshift mutations at repetitive sequences due to polymerase slippage at microsatellites and MMR deficiency.<sup>4 5</sup> Microsatellites are present throughout the genome mostly in non-coding regions. When present in the coding region of tumour suppressor genes, frameshift mutations in such microsatellites cause protein truncation and loss of function, thereby promoting carcinogenesis.<sup>6 7</sup>

Mesalazine (5-aminosalicylic acid, 5-ASA), the active moiety of sulfasalazine,<sup>8</sup> and structurally related to aspirin, is used for treatment of active UC and maintenance of remission. In general, mesalazine is well tolerated, and most colitis patients take it for several years. Longstanding UC predisposes to

CRC. Case-control studies have pointed to possible chemopreventive effects of sulfasalazine and mesalazine in the setting of colitis.<sup>9</sup> The chemopreventive properties of mesalazine were subject of various *in vitro* studies.<sup>10</sup> Mesalazine exerts anti-inflammatory and chemopreventive effects by inhibition of nitric oxide synthase,<sup>11</sup> downregulation of cyclooxygenase (COX)-2,<sup>11</sup> reactive oxygen species scavenging<sup>12</sup> and inhibition of nuclear factor (NF)- $\kappa$ B pathway.<sup>13</sup> Furthermore, its involvement in c-myc expression,<sup>14</sup> epidermal growth factor receptor,<sup>15</sup> peroxisome proliferator-activated receptor (PPAR)- $\gamma$ <sup>16</sup> and Wnt/ $\beta$ -catenin<sup>17</sup> signalling by inhibition of p-21 activated kinase 1<sup>18</sup> has been reported. Mesalazine, but none of its derivatives, arrests MMR-deficient human colon cancer (HCT116) cells in the S-phase by activation of a replication checkpoint and improves replication fidelity in mononucleotide, dinucleotide and tetranucleotide repeats.<sup>12–22</sup> In this regard, mesalazine seems to be a good candidate for chemoprevention of LS. Also, certain natural compounds are thought to have chemopreventive properties. It is possible that specific natural compounds may also interfere with replication fidelity and may be candidate drugs for chemoprevention of LS.

Here, we tested certain natural compounds for their ability to improve replication fidelity *in vitro*. The Msh2<sup>loxP/loxP</sup> Villin-Cre mouse had been established to simulate intestinal carcinogenesis in LS for development of chemopreventive drugs.<sup>23</sup> In this transgenic mouse, the Msh2-loxP allele was crossed with the Villin-Cre transgene, achieving an intestinal-specific, conditional knockout of Msh2, where the Msh2 allele is excised by Cre-recombinase in Villin-expressing tissue. Msh2<sup>loxP/loxP</sup> Villin-Cre mice develop spontaneous intestinal neoplasia within 10 months, predominantly in the small intestine. Here, we also used this Msh2<sup>loxP/loxP</sup> Villin-Cre mouse to test whether mesalazine or thymoquinone (TQ) interferes with intestinal carcinogenesis *in vivo*.

## MATERIALS AND METHODS

### Cells and compounds

HCT116-A2.1, an MMR-deficient MSI-reporter cell line for replication errors within a (CA)<sub>13</sub> repeat was grown in Iscove's Modified Dulbecco's Medium (Gibco/Invitrogen, Karlsruhe, Germany) containing 200  $\mu$ g/mL hygromycin B and 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) at 37°C, 5% CO<sub>2</sub> and full humidity.<sup>24</sup> Cinnamaldehyde (W228613, Sigma-Aldrich, St Louis, Missouri, USA), taurine (T0625, Sigma Aldrich) and TQ (274666, Sigma Aldrich) were dissolved in dimethyl sulfoxide.

### Flow cytometry-based assay for replication fidelity

Analysis of mutations was performed as previously described.<sup>22–24</sup> Briefly, 2500 non-fluorescent HCT116-A2.1 cells were sorted into 24-well plates using a FACSAria cell sorter equipped with FACSDiva Software (BD Biosciences, San Jose, USA). Cells were treated with various concentrations of compounds for 7 days, and enhanced green fluorescent protein (EGFP)-positive cells (M2-fractions) were analysed by flow cytometry.<sup>25</sup> Absolute cell counts were used as an indicator of cell growth.

### Mouse model

Four-week-old to 6-week-old female and male Msh2<sup>loxP/loxP</sup> Villin-Cre mice were housed at the Institute of Biomedical Research in specific pathogen-free conditions with 12 h light/dark cycles, offered chow and water *ad libitum* according to Austrian and European law, defined by the Good Scientific Practice guidelines of the Medical University Vienna (animal

ethics approval number: BMWF-66.009/0045-II/10b/2010).<sup>23</sup> Animals were weighed weekly and the amount of food intake was documented. Health status and body weight were monitored weekly. Weight loss of more than 20%, worsening condition and severe wounds were criteria for early euthanasia. One-hundred mice were randomly divided into five groups, 20 each, and treated with regular chow (control), mesalazine (5-ASA) or TQ (group 1: control; group 2: 5-ASA low; group 3: 5-ASA high; group 4: TQ low; group 5: TQ high) over a period of 43 weeks. The chow, a commercial rodent diet (C1000, Altromin, Lage, Germany) was mixed with mesalazine (Shire, Dublin, Ireland) at 500 mg/kg chow (5-ASA low) or 2500 mg/kg chow (5-ASA high), or TQ (274666, Sigma Aldrich, Germany) at a concentration of 37.5 mg/kg chow (TQ low) or 375 mg/kg chow (TQ high), respectively. Simultaneously, we housed an additional group of 30 untreated Msh2<sup>loxP/loxP</sup> Villin-Cre mice. This group was used to monitor tumour incidence after 20, 24, 34 and 39 weeks (see online supplementary figure S3). At the end of the experiment, mice were euthanased, the gut was dissected, flushed with phosphate buffered saline and neutral buffered formalin (10%), and coiled up into a Swiss roll.<sup>26</sup> Before paraffin embedding, the intestine was fixed in neutral buffered formalin for 24 h.

### Histology and immunohistochemistry

The paraffin-embedded Swiss rolls were cut in three layers (upper, middle, lower levels). In each level, five sections of 5  $\mu$ m thickness were collected, one for H&E staining, two sections mounted on polyethylene naphthalate (PEN) membrane slides and subsequently stained with Methyl Green for laser capture microdissection and two sections mounted on sialinated slides, left unstained and in paraffin-embedded state for subsequent histochemical examinations. These serial tissue sections were analysed for tumour multiplicity, incidence and size by two independent researchers who were blinded for the treatment group (BK and MP). Tumour size was scored as small, medium or large according to its visibility on 1, 2 or all 3 sections of the block (see online supplementary figure S1). Immunohistochemistry was performed using antibodies against Msh2 (IHC-00082, Bethyl Laboratories, Montgomery, Texas, USA; 1:250), Cre-recombinase (BIOT-106L, Covance, Princeton, New Jersey, USA; 1:100) and Ki-67 (ab15580, abcam, Cambridge, UK, 1:800) diluted in 10% normal goat serum (VECTOR S1000, Vector Laboratories, Peterborough, UK) or 2% horse serum (VECTOR S2000) and 3% bovine serum albumin (BSA) following standard protocols.<sup>27</sup> After removing paraffin from the heat-dried tissue slides, the sections were rehydrated in a descending ethanol row and water, followed by blocking of endogenous peroxidase with methanol +15% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval was performed by boiling the slides in citrate buffer (pH 6). Before antibody use, the tissue sections were blocked with 10% goat serum or 2% horse serum and 3% BSA. Staining was visualised by applying respective secondary antibodies and avidin-biotin-horseradish peroxidase complex (VECTASTAIN Elite ABC Kit, Vector Laboratories), 3,3'-diaminobenzidine (32750; Sigma-Aldrich, St Louis, Missouri, USA) and haematoxylin (5174, Merck, Darmstadt, Germany) for nuclear counterstaining.

For analysis of Ki-67 staining, a colour deconvolution plug-in (ImageJ, NIH, USA, V.1.45s) was run to split channels into a brown (Ki-67) and blue (haematoxylin) image. The brown images were converted into an eight-bit greyscale image, a threshold was set to represent Ki-67 positive cells only and the area fraction of positive cells in percentage was calculated.

### Laser capture microdissection and fragment analysis

Tissue sections mounted on PEN membrane slides (Leica Microsystems, Wetzlar, Germany) were dissected using the LMD6000 laser microdissection microscope (Leica) to obtain samples of normal mucosa and tumour tissue. DNA was extracted from the microdissected samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, The Netherlands) according to the instructions of the manufacturer.

To examine MSI in tissue samples, six different microsatellite loci as recommended by Kabbarah *et al.*<sup>28</sup> were amplified by PCR using the Multiplex PCR Kit (Qiagen), and fluorescent-labelled primers for the six microsatellite regions TG<sub>27</sub>, TA<sub>27</sub>, GA<sub>27</sub>, CT<sub>25</sub>CA<sub>27</sub>, A<sub>33</sub> and A<sub>27</sub>. PCR conditions were: 15 min at 95°C, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by 10 min at 72°C. 0.4 µL of the PCR product were added to 8.6 µL formamide and 0.4 µL GeneScan LIZ 500 size standard (Applied Biosystems, Life Technologies, Carlsbad, California, USA), followed by denaturation at 95°C. MSI from normal and tumour epithelium was compared with the respective tail DNA using GeneMapper software (Applied Biosystems). The major allele length of each sample was compared with tail DNA, and the number of mutations per marker (NMPM) was expressed for each sample. A shift of one base pair (bp) within a mononucleotide repeat and a 2-bp shift in a dinucleotide repeat were regarded as one mutation, respectively.

### TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. For visualisation of nuclei and mounting, Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (H-1200; Vector Laboratories) was used, and samples were analysed on an AxioImager fluorescence microscope (Zeiss, Jena, Germany).

### Statistical analysis

Continuous data are described by means and SDs in the case of normally distributed data, and by medians, minimum and maximum otherwise. Categorical data are described by frequencies and percentages. Mutation rates in HCT116-A2.1 were calculated as previously described using the 'Method of the mean' and the method of 'Maximum likelihood'.<sup>29</sup> Tumour incidence was analysed by Pearson's  $\chi^2$  test and exact logistic regression. Differences are described by relative risks (RR) and corresponding 95% CIs. Analysis of variance was used to compare tumours

per mouse, tumour multiplicity for all tumours, and individually for small, medium and large tumours, as well as NMPM, apoptotic cells and (percentage of) Ki-67 positive crypt cells in normal intestinal epithelium and tumours. Dunnett's two-sided comparison was used to determine level of significance. p Values were considered as statistically significant if  $\leq 0.05$ . Statistical analysis was performed using SPSS software V.19.0 and by SAS software (SAS Institute, Cary, North Carolina, USA). Weight curve data were calculated based on the mean of relative weight gain over time and compared with Dunnett's two-sided test.

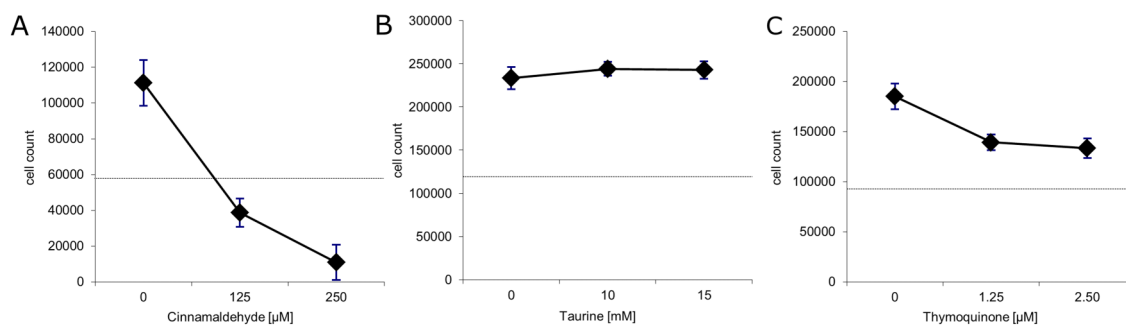
## RESULTS

### TQ improves replication fidelity

We previously found that mesalazine and certain mesalazine derivatives improve replication fidelity independent of a functional MMR system.<sup>20</sup> In a search for natural compounds, which may have similar properties, we tested cinnamaldehyde, taurine and TQ for their effect on replication fidelity within a CA<sub>13</sub> dinucleotide-repeat in MMR-deficient HCT116-A2.1 cells. Twenty-four hours after sorting 2500 EGFP-negative cells into 24-well plates, cinnamaldehyde, taurine and TQ were added at different concentrations for 7 days. Cinnamaldehyde strongly reduced cell growth (IC<sub>50</sub> 94 µM), while taurine did not affect cell growth (figure 1). No effect on replication fidelity was observed by treatment with cinnamaldehyde (at 125 or 250 µM) and taurine at (10 or 15 mM; table 1). TQ reduced cell growth at both concentrations tested (IC<sub>50</sub> 1.2 µM) and improved replication fidelity when used at a concentration of 2.5 µM (table 1). Therefore, we decided to use TQ and mesalazine for chemoprevention of intestinal neoplasia in Msh2<sup>loxP/loxP</sup> Villin-Cre mice.

### Mesalazine and TQ reduce intestinal tumour incidence and multiplicity

Msh2<sup>loxP/loxP</sup> Villin-Cre mice were genotyped (figure 2A) and characterised for expression of Cre-recombinase (figure 2B) and loss of Msh2 expression in intestinal epithelium (figure 2C). Cre-recombinase was expressed exclusively in intestinal epithelial cells, in which Msh2 expression was also lost. Mice were treated with two concentrations of mesalazine (5-ASA low and 5-ASA high) or TQ (TQ low and TQ high) for 43 weeks. Totally, four mice died within 20 weeks of treatment due to unrelated reasons (two in the untreated group, one each in the TQ high and the 5-ASA low groups). Overall, weight gain was regular except for the male population, in which treatment with 5-ASA high (p=0.002) or TQ high



**Figure 1** Effect of cinnamaldehyde, taurine and thymoquinone on cell growth. Twenty-four hours after sorting enhanced green fluorescent protein-negative human colon cancer HCT116-A2.1 into 24-well plates, cells were incubated with various concentrations of cinnamaldehyde (A), taurine (B) and thymoquinone (C). Cells were cultured for 6–7 days, and the total cell count was analysed by flow cytometry. Dose-dependent reductions were observed with cinnamaldehyde and thymoquinone, while taurine had no effect on cell growth. Data are shown as mean±SE of the mean.

**Table 1** Mutation rates at a (CA)<sub>13</sub> microsatellite in human colon cancer HCT116-A2.1 cells by two different computational methods

Treatment	Concentration	Method of the mean		Maximum likelihood	
		Control	Treated (%)	Control	Treated (%)
Cinnamaldehyde	125 $\mu$ M	6.1 $\pm$ 1.8	6.6 $\pm$ 2.4 (108)	5.2 $\pm$ 0.9	6.4 $\pm$ 1.4 (124)
	250 $\mu$ M		8.1 $\pm$ 3.9 (132)	5.2 $\pm$ 0.9	6.0 $\pm$ 2.4 (115)
Taurine	10 mM	2.9 $\pm$ 0.9	3.5 $\pm$ 1.0 (120)	2.8 $\pm$ 0.5	3.1 $\pm$ 0.5 (111)
	15 mM		4.5 $\pm$ 1.3 (154)	2.8 $\pm$ 0.5	2.7 $\pm$ 0.5 (98)
Thymoquinone	1.25 $\mu$ M	6.3 $\pm$ 1.8	5.2 $\pm$ 1.6 (82)	5.4 $\pm$ 0.9	4.8 $\pm$ 0.8 (89)
	2.50 $\mu$ M		3.0 $\pm$ 1.0* (47)	5.4 $\pm$ 0.9	2.5 $\pm$ 0.5* (46)
Mesalazine†	5 mM	6.0 $\pm$ 1.1	4.9 $\pm$ 1.4* (82)	4.8 $\pm$ 1.1	4.4 $\pm$ 2.1 (92)

Note: data ( $\times 10^{-4}$ ) are mean $\pm$ SE of mutation rates per microsatellite per generation.

Changes are indicated in (%) treated versus control.

\* $p < 0.05$ .

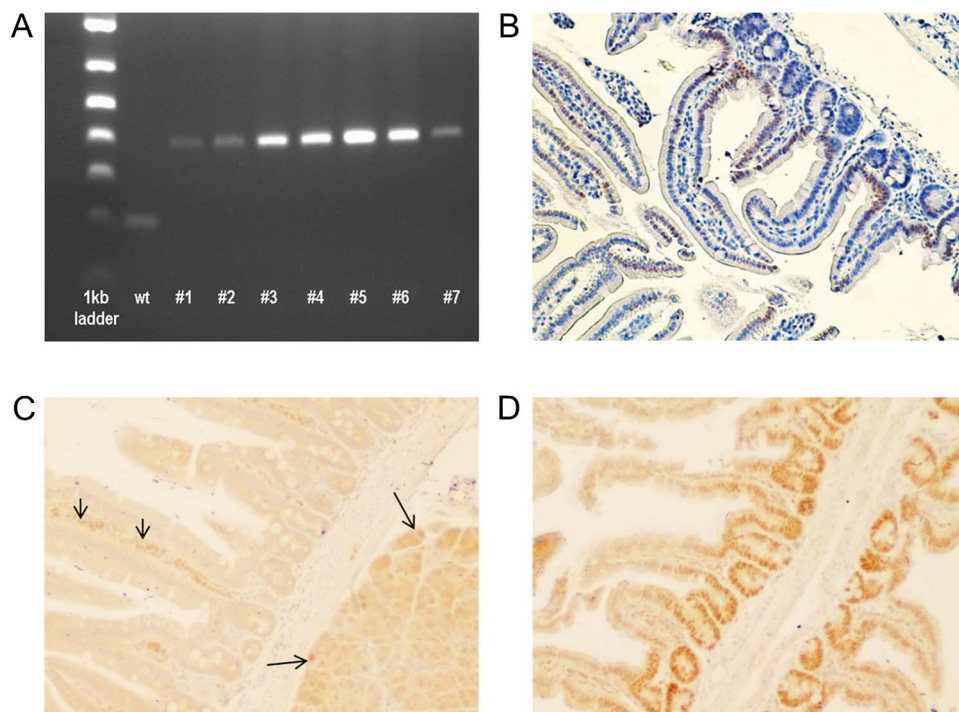
†Data from reference.<sup>22</sup>

( $p=0.007$ ) resulted in less weight gain compared with the untreated group (see online supplementary figure S2). This was likely due to a reduced food intake (about 5%–10% less) in the mesalazine and TQ treatment groups compared with the control group. A small group of control mice ( $n=7$ ) was euthanased every couple of weeks to evaluate tumour development over time. After 20, 24, 34 and 39 weeks of treatment, the small intestinal tumour incidence was 29%, 29%, 71% and 100%, respectively. Tumour multiplicity increased accordingly (see online supplementary figure S3). Both mesalazine and TQ reduced the incidence and multiplicity of tumours in the small intestine. Tumour incidence was reduced from 94% to 69% ( $p=0.04$ ; RR=0.73; 95% CI 0.58 to 0.93; 5-ASA low: 63%, 5-ASA high: 75%) by mesalazine and from 94% to 56% ( $p=0.003$ ; RR=0.60; 95% CI 0.44 to 0.80; TQ low: 58%, TQ high: 55%) by TQ (figure 3A). The difference between the compounds was not significant ( $p=0.25$ ). A dose relationship was observed only for TQ (figure 3A). Tumour multiplicity was reduced from mean 3.1 tumours per mouse to mean 1.4 by 5-ASA (combined  $p=0.004$ , 5-ASA low: 1.4,  $p=0.008$ ;

5-ASA high: 1.5,  $p=0.018$ ) and to mean 1.1 tumours per mouse by TQ (combined  $p < 0.001$ , TQ low: 1.4,  $p=0.008$ ; TQ high: 0.9,  $p < 0.001$ ) (figure 3B). These changes in small intestinal tumour numbers and multiplicity were independent of apoptotic events (see online supplementary figure S4) or any antiproliferative effects (see online supplementary figure S5) in normal and tumour tissue. Both compounds reduced the number of small-sized and medium-sized tumours, but had no effect on large tumour counts, or on small intestinal carcinomas (see online supplementary figure S6).

*Msh2*<sup>loxP/loxP</sup> Villin-Cre mice also displayed some single caecal adenomas and caecal carcinomas, while we observed no other colonic tumour (figure 3C). Both mesalazine and TQ showed a tumour reduction from 39% in untreated mice to 21% ( $p=0.19$ ; RR: 0.53, 95% CI 0.23 to 1.23) and to 31% ( $p=0.56$ ; RR: 0.79, 95% CI 0.38 to 1.67), respectively, which did not reach statistical significance. The present results suggest a chemopreventive effect of mesalazine and TQ on small intestinal and caecal tumorigenesis in *Msh2*<sup>loxP/loxP</sup> Villin-Cre mice independent of induction of apoptosis.

**Figure 2** Genotyping of conditional *Msh2* knock-out. (A) Genotyping of tail DNA for conditional knock-out of exon 12 of the *Msh2* gene. DNA from wildtype mice gave a 210 base pair product, whereas transgenic mice (#1–7) yielded a 405 base pair product. (B) Expression of Cre recombinase in nuclei of intestinal epithelial cells of *Msh2*<sup>loxP/loxP</sup> Villin-Cre mice. (C) *Msh2* expression is depleted in intestinal epithelial cells but not in pancreatic tissue and lamina propria (arrow) from *Msh2*<sup>loxP/loxP</sup> Villin-Cre mice (left). (D) Control mice (wildtype) express *Msh2* mainly in the crypts.



### Mesalazine and TQ reduce MSI in Msh2-deficient epithelium

As mesalazine and TQ reduce the mutation rate at microsatellites in vitro, we intended to measure microsatellite mutations from intestinal epithelial cells and tumour tissue after microdissection. After PCR amplification of a panel of previously established murine microsatellite markers, frameshift mutations were

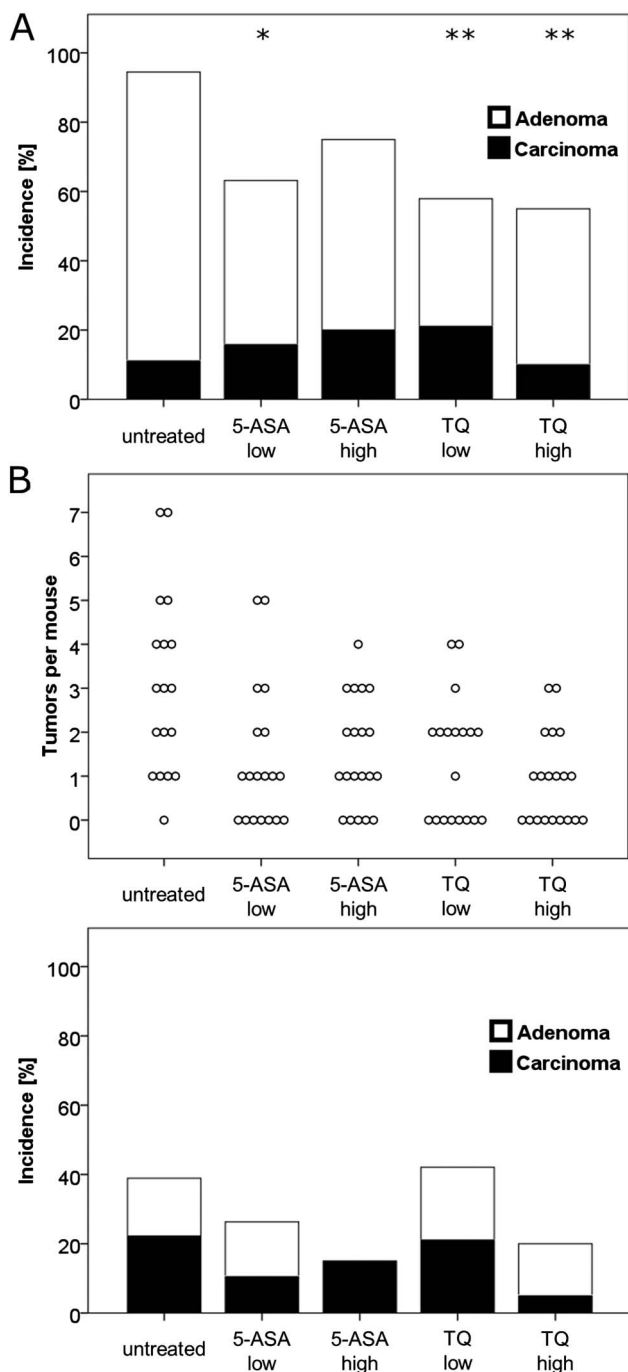
quantified as NMPM in comparison with tail DNA (figure 4A). Frameshift mutations were already observed in normal small intestinal enterocytes as they lack Msh2 (figure 2). The most sensitive marker to the treatment was CT<sub>25</sub>CA<sub>27</sub> (table 2). In normal small intestinal epithelium, a consistent reduction in NMPM was observed in all treatment groups, which was significant for 5-ASA high, TQ-low and TQ-high (figure 4B). In tumour tissue, the frequency of mutations in untreated mice was significantly higher than in normal tissue (tumour:  $2.4 \pm 0.12$  NMPM normal:  $1.49 \pm 0.09$  NMPM;  $p < 0.001$ , table 2). A significant reduction within tumour tissue was observed only by TQ ( $2.4 \pm 0.12$  NMPM to  $1.47 \pm 0.39$  NMPM;  $p = 0.016$ ) (figure 4C, table 2).

### DISCUSSION

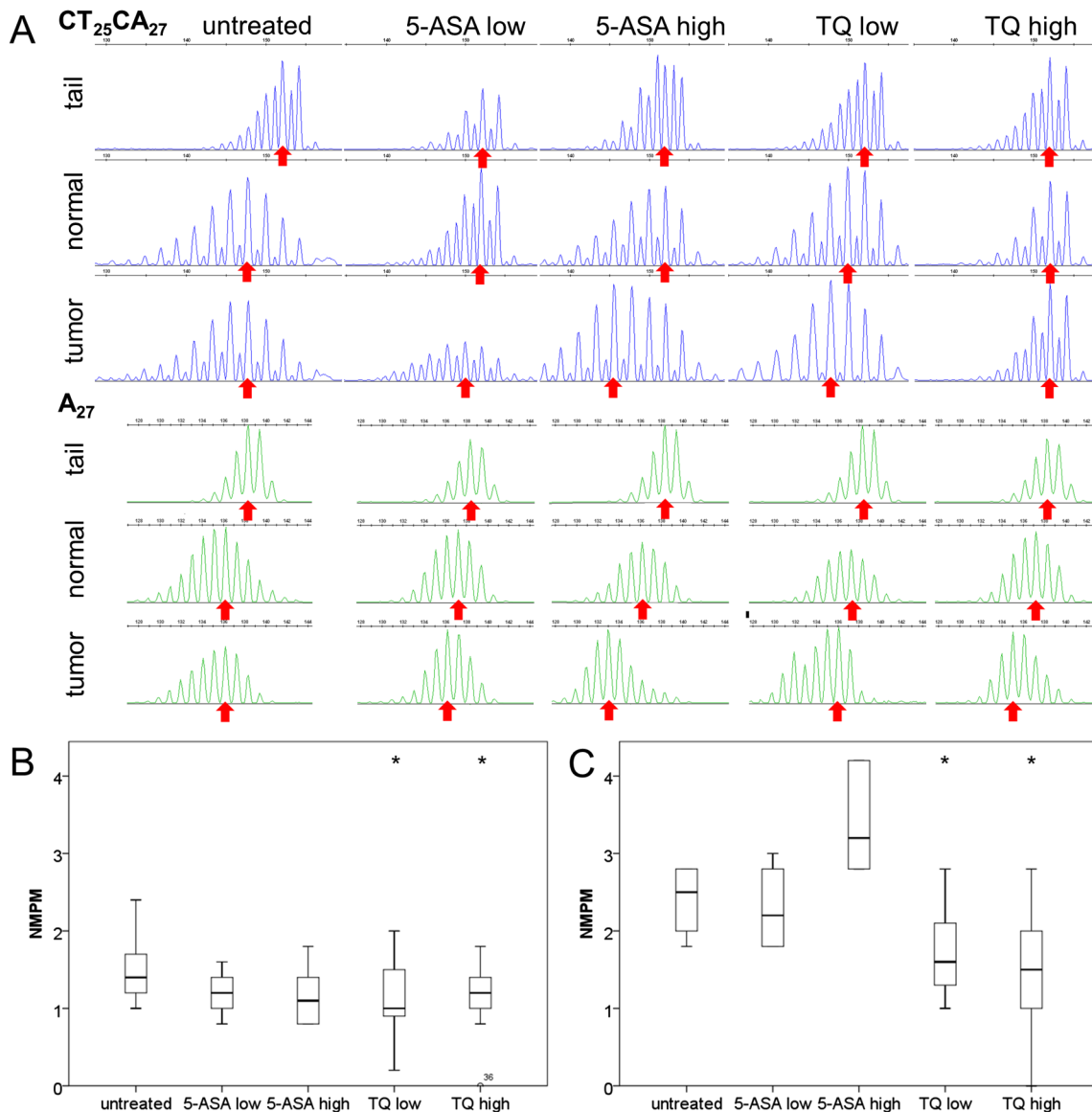
LS is caused by germline mutations in certain MMR genes, which, when the according wildtype allele is lost, result in MMR deficiency and MSI. An MMR-independent improvement of replication fidelity is an ideal mechanism for chemoprevention of LS as it lowers the mutation rate and delays the progression to cancer. Mesalazine has been shown to improve replication fidelity in vitro.<sup>12 19 20 22</sup> Here, we identified similar properties of TQ, an extract from *Nigella sativa*, and further advanced both compounds to in vivo tests in a LS mouse model with intestine-specific loss of Msh2. Both mesalazine and TQ reduced tumour incidence and multiplicity in this Msh2<sup>loxP/loxP</sup> Villin-Cre mouse. As hypothesised, the prevention of intestinal carcinogenesis was accompanied by a reduction of frameshift mutations in intestinal epithelial cells, a result of improved replication fidelity independent of the presence of Msh2. Such anti-mutation effect of mesalazine was not observed in tumour tissue, indicating that the chemopreventive properties of this compound can act only before tumour initiation. Low-dosed mesalazine was also more effective than at high dosage, a finding that warrants further investigation. One explanation could be elevated intracellular oxidative stress induced by mesalazine's activity on mitochondrial transmembrane potential.<sup>30</sup>

Aspirin and non-steroidal anti-inflammatory drugs (NSAID) may prevent colorectal carcinogenesis in familial adenomatous polyposis and sporadic CRC.<sup>31</sup> Such types of CRC are driven by adenomatous polyposis coli (APC) mutations and chromosomal instability. In LS, however, CRC is driven by MSI. In a large clinical trial, aspirin did not reduce the risk for intestinal neoplasia at the primary endpoint.<sup>32</sup> Such proposed effect was observed only after several years in a posthoc analysis.<sup>33</sup> Also, in vitro data on aspirin are conflicting: it does not improve replication fidelity,<sup>22</sup> but was reported to reduce the accumulation of microsatellite mutations in MMR-deficient cells by genetic selection.<sup>34</sup> Aspirin had no effect on intestinal tumorigenesis in Msh2<sup>loxP/loxP</sup> Villin-Cre mice.<sup>35</sup> Mesalazine has structural similarity to aspirin, but has almost opposite biological and clinical effect. Mesalazine improves replication fidelity at mononucleotide, dinucleotide and tetranucleotide repeats in vitro leading to reduction of MSI,<sup>12 13</sup> it increases enterocyte adherence and reduces intestinal permeability,<sup>18</sup> it is a rather weak COX-inhibitor, and arrests cells in S-phase rather than in G<sub>0</sub>/G<sub>1</sub>.<sup>19 36 37</sup> It is used as first-line treatment for UC, while aspirin and NSAIDs rather worsen colitis symptoms. Certain mesalazine effects even depend on the exact position of the amino group.<sup>12 20</sup> Lastly, mesalazine has minimal systemic effects and almost no adverse reactions.

TQ is the major phytochemical constituent of the volatile seed oil of *N sativa*, also called black cumin, which is widely used in herbal medicine. Studies have demonstrated



**Figure 3** Effect of mesalazine (5-ASA) and thymoquinone (TQ) on tumour incidence and multiplicity. (A) Both 5-ASA and TQ reduced tumour incidence in the small bowel of Msh2<sup>loxP/loxP</sup> Villin-Cre mice. The diagram shows the percentage of tumour-bearing mice in each group. (B) 5-ASA and TQ decrease tumour multiplicity in the small bowel. The diagram depicts the mice with their respective tumour burden. (C) 5-ASA and TQ high also showed a tendency to reduction of the incidence of caecal tumours. Significance is illustrated as \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 4** Mesalazine (5-ASA) and thymoquinone (TQ) reduce microsatellite instability (MSI) in microdissected tissue. (A) Raw data from a dinucleotide and a poly-A mononucleotide marker visualised with GeneMapper software. Major allele sizes of normal intestinal epithelium and tumour cells were compared with tail DNA. MSI was reduced by 5-ASA and TQ in Msh2-deficient epithelium. MSI quantification by number of mutations per marker (NMPM) in Msh2-deficient epithelium (B) and tumour cells (C). Significance was illustrated as \* $p < 0.05$ , \*\* $p < 0.01$ .

anti-inflammatory, antioxidant, anticarcinogenic and antiproliferative effects in various cancers, including CRC, where it induces apoptosis through suppression of NF- $\kappa$ B, activation of Akt, induction of the potent tumour suppressor PPAR- $\gamma$ , upregulation of p53 and p21<sup>WAF</sup> and inhibition of tumour angiogenesis.<sup>38</sup> In APC<sup>Min</sup> mice, TQ induced tumour-specific apoptosis.<sup>27</sup> In the Msh2<sup>loxP/loxP</sup> Villin-Cre mouse, we were unable to observe such increase in apoptosis both in normal and tumour tissue. One explanation might be that induction of apoptosis upon TQ might depend on Msh2. Interestingly, both compounds did not reduce proliferation of intestinal epithelial cells, but rather, increased the fraction of Ki-67-positive intestinal epithelial cells. This goes in line with mesalazine's induction of an S-phase check point, which increases the time of replication leaving more cells as Ki-67 positive.<sup>19</sup> The chemopreventive activity of TQ in this mouse model was superior to APC<sup>Min</sup> mice indicating that induction of apoptosis is not its main mechanism of action, but rather, improvement of

replication fidelity as shown in vitro, and by fragment analysis of microdissected tissue.

In this study, a substantial incidence of caecal tumours was found that had not been reported earlier.<sup>23 33</sup> We cannot rule out that such tumours were under-reported in the first place, but a change in tumour expression may also be caused by different housing conditions or different chow. The effects of both compounds on such caecal tumours were less pronounced than on small intestine and did not reach statistical significance as the total numbers were small. Contrary to the small intestine, mesalazine performed better than TQ, possibly due to different pharmacokinetics. As both compounds were part of the chow, translation of our data into the human setting is difficult, as a delayed-release delivery has not been tested. It is to say, that neither substances showed significant effect on large tumours of the small intestine, which have likely been the first to develop, presumably within the first couple of weeks of life before the mice were submitted to treatment. This finding

**Table 2** Number of mutations per individual microsatellite marker<sup>28</sup>

Microsatellite marker	Chromosome Gene Bank	Tissue	Untreated	5-ASA low	5-ASA high	TQ low	TQ high
TG <sub>27</sub>	10B5.1	SI epithelium	1.13 (0.09)	0.88 (0.08)	1.06 (0.06)	0.94 (0.10)	0.94* (0.06)
	AC098712	SI tumour	2.60 (0.31)	2.00 (0)	3.33 (0.99)	1.22 (0.22)	0.83 (0.17)
GA <sub>29</sub>	5G1	SI epithelium	1.56 (0.20)	1.06 (0.10)	1.33 (0.14)	1.24 (0.16)	1.18 (0.15)
	AC083948	SI tumour	3.40 (0.31)	3.20 (1.36)	5.00 (1.13)	1.56 (0.41)	1.67 (0.42)
CT <sub>25</sub> CA <sub>27</sub>	3A3	SI epithelium	1.19 (0.10)	0.88 (0.15)	0.61† (0.12)	0.81 (0.14)	0.94 (0.13)
	AC079442	SI tumour	2.40 (0.4)	2.40 (0.98)	5.33* (0.67)	1.88 (0.70)	1.17 (0.31)
A <sub>33</sub>	17E4	SI epithelium	1.38 (0.15)	1.18 (0.15)	0.89 (0.20)	0.94 (0.23)	1.06 (0.13)
	AC096777	SI tumour	1.10 (0.10)	1.60 (0.60)	1.67 (0.33)	1.00 (0.33)	1.17 (0.65)
A <sub>27</sub>	17E4	SI epithelium	2.19 (0.21)	1.94 (0.16)	1.89 (0.18)	1.81 (0.26)	1.65 (0.21)
	AC096777	SI tumour	2.50 (0.34)	2.40 (0.40)	3.83 (0.40)	2.78 (0.52)	2.50 (0.67)
All markers (NMPM)		SI epithelium	1.49 (0.09)	1.19 (0.06)	1.16* (0.08)	1.13* (0.13)	1.15* (0.09)
		SI tumour	2.40 (0.12)	2.32 (0.25)	3.24 (0.26)	1.73 (0.21)	1.47* (0.39)

Values represent mean (SEM).

\*p<0.05, †p<0.01.

5-ASA, 5-aminosalicylic acid; NMPM, number of mutations per marker; SI, small intestinal; TQ, thymoquinone.

suggests that both substances are more efficient in preventing cancer initiation than delaying its progression. Here, we demonstrate that improvement of replication fidelity can be achieved in vitro and in vivo, which results in deceleration of MSI. Importantly, the previously established mathematical model for calculation of mutation rates in vitro is generation-based and, therefore, a reduction of cell proliferation can be ruled out for being responsible for the observed improvement of replication fidelity.<sup>29</sup> Furthermore, similar cell lines revealed decreased replication fidelity upon polymorphonuclear neutrophils-induced stress paralleled by reduced cell proliferation.<sup>25</sup> Others have shown that mesalazine and TQ induce apoptosis dependent on dose, duration and type of cell line.<sup>39–40</sup> At the concentrations in our in vitro experiments, cell viability is maintained and induction of apoptosis has not been observed.<sup>22</sup> Furthermore, Msh2 is necessary for the induction of a G2/M checkpoint arrest after replication errors and for induction of DNA repair or apoptosis. The deletion of Msh2 in intestinal epithelial cells could be a reason for the resistance against apoptosis-inducing effects upon treatment with mesalazine and TQ.<sup>23 25 41</sup>

The clinical management of LS mutation carriers has been the subject of controversies, one of which is the regular use of aspirin at 600 mg per day for chemoprevention.<sup>33 42</sup> Also 5-fluorouracil treatment of LS tumours has been reconsidered.<sup>43</sup> LS mutation carriers bear an innate defect of their MMR machinery for which no cure is in sight. The deceleration of carcinogenesis through chemoprevention is complementary to future improvements in cancer surveillance, and thus, a great hope for LS mutation carriers. Our findings suggest that mesalazine and TQ are candidate compounds for chemoprevention of CRC in LS mutation carriers.

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**Contributors** (1) Conception and design of the study: CG, CC, GS. (2) Generation, collection, assembly, analysis and/or interpretation of data: BK, CC, ML, RE, MP, MM, TS, MK, WE. (3) Critical revision of the manuscript: BK, CC, ML, RE, MP, MM, TS, MHK, WE, VK, GS, CG.

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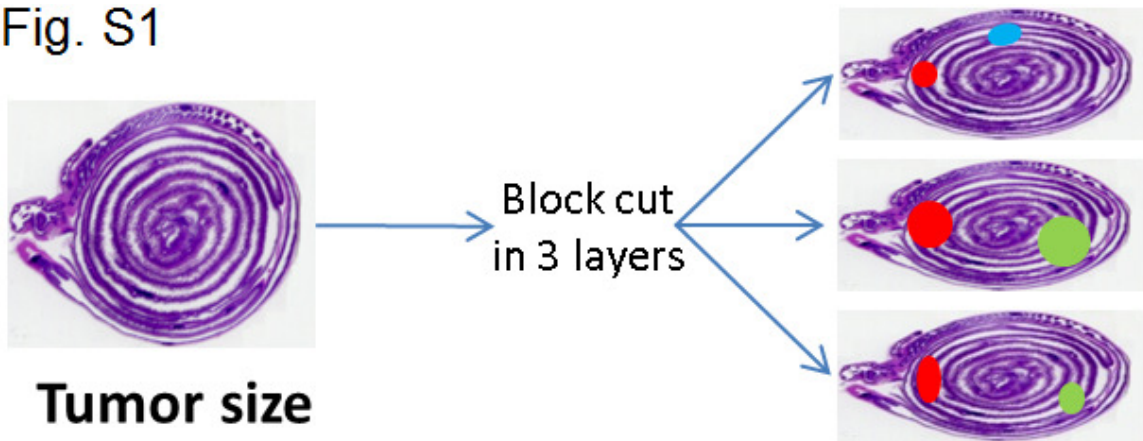
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## Supplementary Figures

**Supplementary Figure S1: Counting and sizing of tumours.** The paraffin block was cut in three layers and a tumour was rated in size according to the number of adjacent slides with presence of the same tumour (blue: small, yellow: medium, red: large).

Fig. S1



### Tumor size

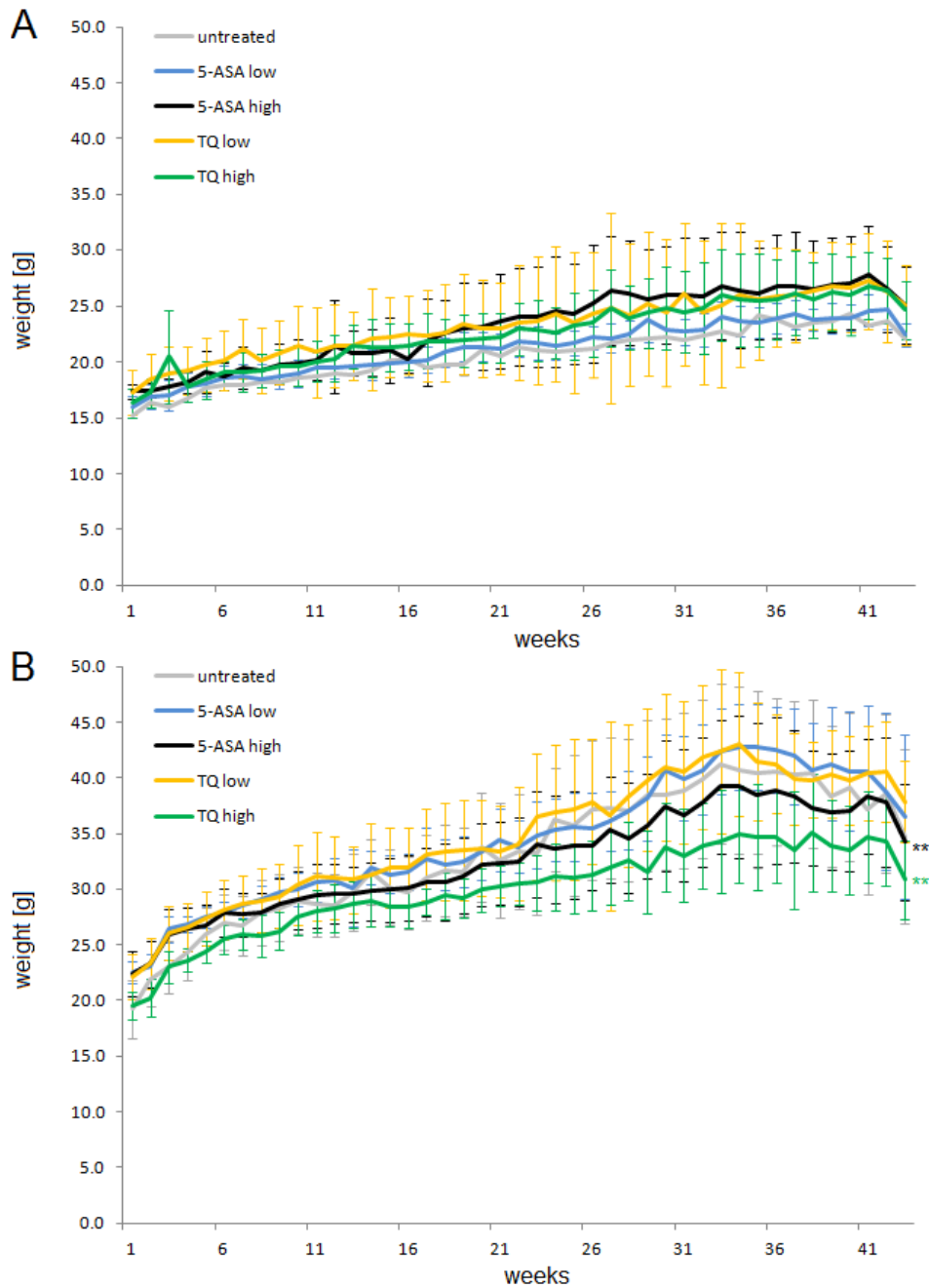
„small“ – visible in 1 slide

„medium“ – visible in 2 slides

„large“ – visible in all 3 slides

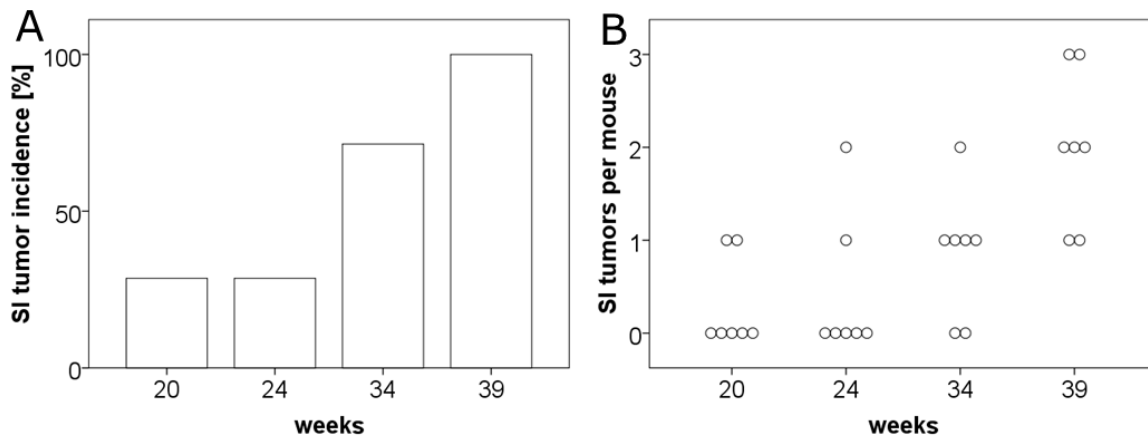
**Supplementary Figure S2: Body weight curves.** Body weight and standard deviation is blotted for each treatment group for female- (A), and male mice (B). In the male population, the 5-ASA high ( $p=0.002$ ) and the TQ high ( $p=0.007$ ) groups gained significantly less weight compared to the untreated group.

Fig. S2



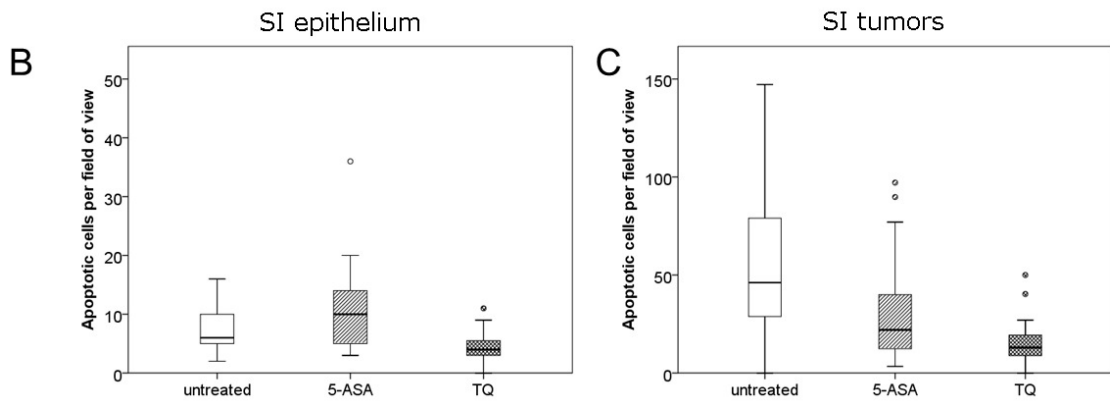
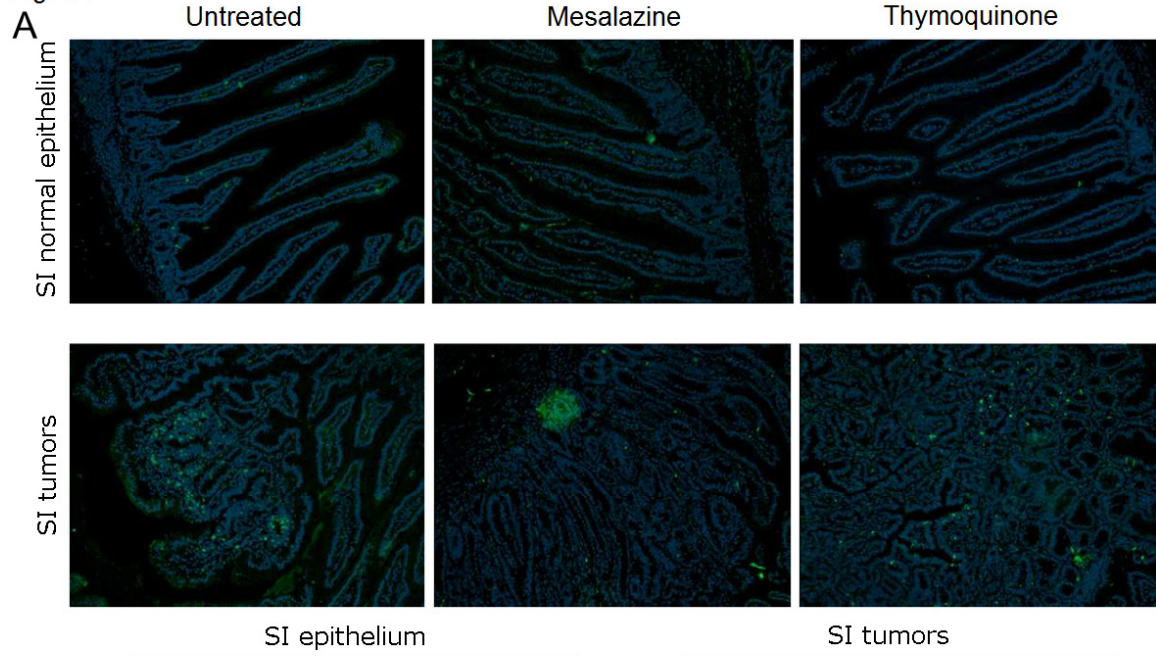
**Supplementary Figure S3: Tumour development over time.** 7 mice of the euthanasia control were terminated to assess tumour development after 20, 24, 34 and 39 weeks. (A) Tumour incidence increased over time and after 39 weeks all mice examined showed at least one tumour. (B) Tumour multiplicity increased in mean from 0.29 to 2 tumours per mouse between 20 and 39 treatment weeks.

Fig. S3



**Supplementary Figure S4: Induction of apoptosis.** (A) TUNEL-assay was performed on Msh2-deficient small intestinal (SI) epithelium (upper row) and tumour in untreated (left), mesalazine (middle) and thymoquinone treated mice (right). The number of apoptotic cells was quantified per field of view (ACpF). No significant effect on apoptosis was detectable in either treatment group both in SI epithelium (B) and tumours (C).

Fig. S4



**Supplementary Figure S5: Induction of proliferation.** (A) IHC was performed for the nuclear proliferation marker KI-67. While treatment with mesalazine and thymoquinone significantly increased percentage of KI-67-positive cells per crypt in the small intestine, neither treatment affected counts of KI-67-positive cells in tumours. Boxplots display percentages of KI-67-positive crypt cells in SI epithelium for each treatment group (B) and KI-67-positive cells in tumours pooled for the respective substance (C).

Fig. S5

