Chapter 7

5-Aminosalicylic acid inhibits colitis-associated but not sporadic colorectal neoplasia in a novel conditional Apc mouse model

Pim J. Koelink\textsuperscript{1}, Els C. Robanus-Maandag\textsuperscript{2}, Peter Devilee\textsuperscript{2},
Daniel W. Hommes\textsuperscript{1}, Cornelis B.H.W. Lamers\textsuperscript{1},
Hein W. Verspaget\textsuperscript{1}

Department of Gastroenterology-Hepatology\textsuperscript{1},
Department of Human Genetics\textsuperscript{2},
Leiden University Medical Centre,
Leiden, The Netherlands.

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Abstract

Genetic predisposition, life-style habits and inflammatory bowel diseases (IBD)-related colitis are a main risk factor for colorectal cancer (CRC). 5-aminosalicylic acid (5-ASA, mesalazine) is a mainstay therapy in IBD and believed to reduce the risk for developing CRC. We aimed to determine the ability of 5-ASA enemas to inhibit the development of sporadic and colitis-related neoplasia in mice. FabplCre:Apc\textsuperscript{15lox/+} mice, which spontaneously develop sporadic colorectal tumours, were treated at 5 weeks of age with 5-ASA or placebo enemas for 3 weeks and examined for colorectal tumourigenesis at 8 weeks of age. Colitis-related tumour development was investigated in these mice by administration of dextran sodium sulphate (DSS), inducing intestinal inflammation and accelerating colorectal tumourigenesis, combined with treatment of 5-ASA or placebo enemas during and/or after colitis induction. 5-ASA significantly reduced colitis-accelerated neoplasia development by 50\%, from 19.4 ± 2.7 to 9.4 ± 2.4 (mean tumour numbers ± SEM, P=0.02), in the distal part of the large intestine covered by the enema. 5-ASA was only effective when given during and/or after the intestinal inflammatory period. 5-ASA did not reduce, however, sporadic neoplasia development in the FabplCre:Apc\textsuperscript{15lox/+} mice. 5-ASA tended to reduce proliferation of epithelial cells in the colitis-associated colorectal tumours but not in the sporadic colorectal tumours. In conclusion, 5-ASA medication inhibits the development of colitis-associated tumours in FabplCre:Apc\textsuperscript{15lox/+} mice when administered during and/or after the induction of inflammation. 5-ASA does not reduce, however, sporadic tumour development in this mouse model.
**Introduction**

Inflammation and carcinogenesis are pathological consequences of injury and repair at a cellular and molecular level. Several studies suggest inflammation as a risk factor for the development of cancer, including CRC\(^1\). Individuals suffering from either of the two main chronic forms of IBD, ulcerative colitis (UC) and Crohn's disease (CD), are at increased risk of developing CRC\(^2,3\). 5-ASA is a non-steroid anti-inflammatory drug, widely used in the maintenance of remission and treatment of mild inflammatory exacerbations in IBD. Most epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC, although some studies failed to show this, as described in a recent meta-analysis by Velayos et al.\(^4\). To study prospectively whether 5-ASA has chemopreventive actions in patients is hardly feasible, due to duration of the study, high patient numbers needed, and ethical considerations. Therefore, for this kind of research animal models are used which mimic the human disease, although most models lack similarities.

The \(Apc^{\text{Min/+}}\) mouse model carries a germline mutation in one allele of the adenomatous polyposis coli (\(Apc\)) tumour suppressor gene. \(Apc^{\text{Min/+}}\) mice develop multiple intestinal neoplasia (\(min\)), mainly in the small intestine, due to loss of function of the intact \(Apc^+\) allele\(^5\). \(Apc\) is a crucial negative regulator of the canonical Wnt pathway, forming a complex with axins and glycogen synthase 3\(\beta\) kinase (GSK3\(\beta\)), targeting \(\beta\)-catenin for degradation. Loss of functional \(Apc\) prevents formation of the complex, resulting in accumulation of \(\beta\)-catenin. Upon translocation to the nucleus and association with the TCF-4 transcription factor, \(\beta\)-catenin induces transcriptional activation of many genes involved in cell adhesion, migration and proliferation\(^6\). The \(Apc^{\text{Min/+}}\) mouse model mimics the familial adenomatous polyposis (FAP) syndrome in humans, an inherited form of CRC, caused by a germline \(APC\) mutation and characterized by development of 100s-1000s adenomas in the large intestine. Most CRCs, however, are sporadic but they share early \(APC\) mutations in 80% of the cases.

Reports on the effects of 5-ASA on the development of intestinal polyps in \(Apc^{\text{Min/+}}\) mice have been conflicting. McGregor et al.\(^7\) showed that 5-ASA reduced the number of polyps, whereas Ritland et al.\(^8\) found no reduction in polyp numbers. The \(Apc^{\text{Min/+}}\) mouse model is limited for studying the chemopreventive effect of 5-ASA on the development of CRC, because these mice develop only a small fraction of lesions in the colon compared to the small intestine, and intestinal tumourigenesis is most likely already initiated in the first 2 weeks of life in these mice.
Recently, we generated a novel tissue-specific, sporadic CRC mouse model, FabplCre:Apc\textsuperscript{15lox/+}. Compared to Apc\textsuperscript{Min/+} mice, FabplCre:Apc\textsuperscript{15lox/+} mice develop relatively few (<50) intestinal tumours, at a more advanced age, almost exclusively located in the distal part of the large intestine, similar to the human disease counterpart (Robanus-Maandag \textit{et al.}, manuscript in preparation).

A widely used and thoroughly described colitis model is the dextran sodium sulphate (DSS) model. DSS dissolved in the drinking water of rodents, induces intestinal inflammation, including crypt damage and crypt loss. When DSS is applied in a low concentration (2% w/v) for a few days, followed by a period of normal drinking water, the inflammatory period is followed by a healing and repair period, resembling exacerbation and remission stages in human UC\textsuperscript{9, 10}. DSS-induced colitis in the Apc\textsuperscript{Min/+} mouse model was shown to accelerate the development of colorectal tumours, and to be useful as a colitis-associated intestinal cancer model\textsuperscript{11, 12}.

In the present study we determined the effects of local 5-ASA treatment on the development of colorectal tumours in: 1) FabplCre:Apc\textsuperscript{15lox/+} mice, which develop distally located tumours, to mimic human sporadic CRC development, and 2) FabplCre:Apc\textsuperscript{15lox/+} mice exposed to DSS, to study colitis-associated development of neoplasia. 5-ASA was found to effectively reduce the development of colitis-associated tumours, whereas it was ineffective in inhibiting sporadic tumour development.

\textbf{Material\&Methods}

\textit{Mice}

All animal studies were approved by the ethical committee for animal studies of the Leiden University Medical Center and complied with national laws relating to the conduct of animal experiments. FabplCre:Apc\textsuperscript{15lox/+} C57Bl/6 mice were generated by mating Fabpl\textsuperscript{Cre} C57Bl/6 mice (line Fabpl\textsuperscript{4x at ~}132\textsuperscript{Cre} \textsuperscript{13}, kind gift of J. Gordon) with Apc\textsuperscript{15lox/15lox} C57Bl/6 mice and genotyped by PCR (Robanus-Maandag \textit{et al.}, manuscript in preparation). FabplCre:Apc\textsuperscript{15lox/+} mice were weaned at 4 weeks of age and housed individually under standard housing conditions with drinking water and food available \textit{ad libitum}.

\textit{Colitis induction}

Colonic inflammation was induced in 5 weeks old mice, with a median body weight of 17.5 gram (range 13.0-22.0), by administration of 2% (w/v) DSS (MP Biomedicals, Aurora, Ohio, USA; MW=36,000–50,000) in tap water to the mice \textit{ad libitum}. The solution
was refreshed every other day, and the consumption was monitored daily by weighing the drinking bottle. In order to induce inflammation followed by a recovery period, the 2% DSS solution was given for 5 days followed by 16 days of tap water (Figure 1A). Mice were sacrificed at the end of the experiment at 8 weeks of age, or before the end of the experiment if they exhibited >20% weight loss, rectal bleeding, anaemia, and lethargy. These latter mice were excluded from the results of the neoplasia scoring.

5-ASA medication
Salofalk 4gr 5-ASA/60 gr enemas (Tramedico BV, Weesp, The Netherlands) were given daily by rectal injection of approximately 200 µl of enema solution in animals that were anesthetized with isoflurane/O₂. The control animals were treated with 0.9% NaCl “placebo enema”. In the first experiment the effect of 5-ASA was tested on DSS-induced colitis-associated neoplasia development in FabplCre;Apc^{15lox/+} mice at 5 weeks of age, starting 2 days before DSS administration, in order to be effective before the colitis-induction in the prevention protocol and to adjust the animals to the enema treatment protocol, up to the end of the 2-week-recovery period (DSS/5-ASA and DSS/Placebo groups, experiment 1, Figure 1A). Similarly, FabplCre;Apc^{15lox/+} mice were treated at 5 weeks of age with either 5-ASA or placebo enemas for 3 weeks without DSS administration, to determine the effect of 5-ASA on sporadic colorectal tumour development. (5-ASA and Placebo groups, experiment 1, Figure 1A). In the second experiment the FabplCre;Apc^{15lox/+} mice were treated simultaneously with DSS and 5-ASA or placebo medication during the DSS-induced colitis (DSS+5-ASA and DSS+Placebo groups), or with 5-ASA only in the subsequent two week recovery period (5-ASA-post-DSS group, experiment 2, Figure 1B).

Assessment of colitis
The presence of blood in faeces, stool consistency, and general appearance were recorded daily for each animal. Together, these factors constitute the disease activity index (DAI, range 0-6, Table 1), as adapted from Cooper et al. 9. The daily recorded body weight was not included in the DAI.
**Figure 1: Experimental protocols.**

*FabplCre;Ap<sup>15lox/+</sup> mice were treated with placebo or 5-ASA enemas from 5 to 8 weeks of age without DSS administration (Placebo and 5-ASA panel, respectively), or in combination with 2% DSS administration for 5 days at 5 weeks of age (DSS/Placebo and DSS/5-ASA panel, respectively) (A). FabplCre;Ap<sup>15lox/+</sup> mice were treated with placebo or 5-ASA enemas for 7 days at 5 weeks of age during 2% DSS administration for 5 days (DSS+Placebo and DSS+5-ASA panel, respectively) or with 5-ASA enemas after the DSS administration, from 6 to 8 weeks of age (5-ASA-post-DSS panel) (B).*

**Histopathology**

The large intestine (from caecum to anus) of sacrificed mice was isolated and the length was measured in a relaxed position without stretching. The large intestine was opened longitudinally, cleaned in phosphate-buffered saline (PBS), spread onto filter paper (luminal side up), fixed in buffered 4% formalin (pH 7.2) for 24 hours at RT, and stored in 70% ethanol at 4°C. The neoplastic lesions were macroscopically counted under a dissection microscope (with an up to 40x magnification) in a blinded fashion. The intestinal tissues were embedded in paraffin (Swiss roll technique), sectioned at 5 µm and stained with hematoxylin and eosin (H&E), according to standard procedures. The sections were graded,
in a blinded fashion, for the residual inflammatory infiltrate, with a range from 0-4 as to the amount and depth of infiltration: 0 = no infiltrate, 1 = focal infiltrate crypt base, 2 = infiltrate in lamina propria, 3 = infiltrate throughout the lamina propria and mucosal thickening, and 4 = infiltrate extending into the submucosa. In addition, the lymphoid activity based on the number of lymph follicles was scored from 0 = none to 4 = more than three follicles. In combination a score of 0 to 8 could be obtained, a modification from the DSS-colitis microscopic activity index (MAI) as reported before\textsuperscript{14}.

Table 1: Table to determine the disease activity index.

<table>
<thead>
<tr>
<th>Score</th>
<th>Stool</th>
<th>Blood loss</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>none</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Loose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Diarrhea</td>
<td>Blood loss</td>
<td>Lethargic</td>
</tr>
</tbody>
</table>

The disease activity index (DAI) is defined as the total score for all three parameters (stool consistency, blood loss, and appearance), range 0-6.

Immunohistochemistry

Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked in 0.3% H\textsubscript{2}O\textsubscript{2} (Merck, Darmstadt, Germany) in methanol for 30 minutes at room temperature (RT) and rehydrated in a graded ethanol series to PBS. For antigen retrieval the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes in a microwave oven. The slides were cooled down to RT, rinsed with PBS (3x), and blocked with 5% goat serum (Dakocytomation, Glostrup, Denmark) in 1% bovine serum albumin (BSA) in PBS for 20 minutes at RT. Sections were incubated with the primary antibody in 1% BSA/PBS: Ki67 (1:1000, Monosan San Bio BV, Uden, The Netherlands) 1 hour at RT, active caspase-3 (1:200, Cell Signaling Technology Inc., Danvers, MA, USA), or β-catenin (1:3200, BD Transduction Laboratories, Lexington, KY, USA) overnight at 4°C. The slides were rinsed with PBS (3x) and incubated with biotinylated secondary antibodies (1:200, Dakocytomation) in 1% BSA/PBS for 45 minutes at RT. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin-biotin complex/HRP (Dakocytomation) for 45 minutes at RT, followed by 0.015% H\textsubscript{2}O\textsubscript{2}/0.05% DAB (Sigma, Schneldorf, Germany)/0.01 M Tris-HCL pH 7.6 for 10 minutes at RT. Sections were counterstained with Mayers’ haematoxylin (Merck), dehydrated, and mounted in entallan (Merck). Negative controls without primary antibodies were included. The Ki67 index was determined in 5 representative areas of neoplastic tissue and in 5 normal appearing
crypts at a 400x magnification, and expressed as the percentage of Ki67-positive epithelial
cells. Photomicrographs were taken with a Nikon Elipse E800 microscope equipped with a
Nikon DXM 200 digital camera.

Statistical analysis

Statistical analysis of the changes in bodyweight, DAI, MAI, length of the large
intestine, the number of neoplastic lesions and the (immuno)histology scores were performed
using the Student t-test. Differences were considered significant when \( P \leq 0.05 \).

Results

Increased disease activity index and loss of body weight due to DSS

The typical colitis symptoms, recorded as the DAI, increased dramatically in
\( \text{Fabp1Cre;Apc}^{15\text{lox/}+} \) mice during and shortly after the five-day-DSS period in both DSS/5-
ASA and DSS/Placebo groups (Figure 2A). In addition, both DSS-treated groups displayed a
gradual loss of body weight at the end and shortly after the DSS period in contrast to the two
non-DSS-treated groups (Figure 2B). The DSS-treated animals recovered from this severe
colitis during the next two weeks, as indicated by a decrease in DAI and gain of body weight.
In general, 5-ASA treatment by enema tended to a faster/better recovery of the DSS-treated
mice (Figure 2A/B). This difference could not be attributed to a difference in consumption of
DSS-supplemented drinking water (Figure 2C).

DSS-induced colitis accelerates large intestinal tumourigenesis

Due to the DSS-induced colitis, the length of the large intestine of the DSS/Placebo
group was significantly decreased compared to that of non-treated mice on average from 9.3
to 7.5 cm (\( P < 0.0001 \), Figure 3A/B). The DSS-induced colitis strongly accelerated intestinal
tumourigenesis as indicated by the increased average number of large intestinal tumours from
5.2 tumours in non-treated mice to 28.8 tumours in DSS/Placebo mice (\( P < 0.0001 \), Figure
3C).
5-ASA inhibits colitis-associated CRC

5-ASA suppresses colitis-accelerated large intestinal tumourigenesis

5-ASA treatment tended to increase the length of the large intestine of Fabp1Cre;Apc\(^{16lox/+}\) mice treated with DSS (on average from 7.5 cm to 7.9 cm, P=0.17, Figure 3B). More importantly, however, 5-ASA treatment significantly reduced the average
Figure 3: 5-ASA suppresses colitis-accelerated large intestinal tumourigenesis.

(A,B) DSS reduced the length of the large intestine. Individual measurements are indicated by closed circles and mean values are indicated by horizontal lines. (C) DSS increased the number of tumours. 5-ASA suppressed DSS-induced, colitis-accelerated tumourigenesis, but not sporadic tumourigenesis of the large intestine. 5-ASA exerted its effect during the recovery period. Individual tumour numbers are shown for males (closed circles) and females (open boxes). Mean tumour numbers are indicated by horizontal lines. Significant P-values ≤0.05 are shown in bold. Full-colour of A on page 186.
number of colitis-accelerated large intestinal tumours by 37.4%, from 28.8 tumours in the DSS/Placebo group to 18.0 tumours in the DSS/5-ASA group (P=0.03, Figure 3C). In contrast, 5-ASA treatment was unable to reduce the average number of sporadic tumours in the large intestine (5.2 and 4.8 tumours in the 5-ASA group and Placebo group, respectively, P=0.93, Figure 3C).

5-ASA exerts tumour-inhibiting effect during recovery period

To determine whether 5-ASA exerted its preventive effect due to anti-inflammatory actions on the DSS-induced colitis or due to effects during the recovery period, mice were treated with 5-ASA or placebo enemas during the DSS administration period and 2 days thereafter (DSS+5-ASA group and DSS+Placebo group), and with 5-ASA enemas only during the two weeks of subsequent colitis recovery (5-ASA-post-DSS group, experiment 2, Figure 1B). The DAI and the body weight changes of these DSS-treated mice showed similar curves as those of the corresponding DSS-treated animals of experiment 1 (not shown). Importantly, 5-ASA treatment during the DSS administration was not able to reduce the average number of tumours (29.5 and 29.2 tumours in the DSS+Placebo group and DSS+5-ASA group, respectively, P=0.96, Figure 3C), whereas 5-ASA treatment during the recovery period tended to reduce the average tumour numbers (20.8 and 28.8 tumours in the 5-ASA-post-DSS and DSS/Placebo groups respectively, P=0.07, and 29.2 tumours in the DSS+5-ASA group, P=0.19 vs 5-ASA-post-DSS).

5-ASA exerts tumour-inhibiting effect in the distal large intestine

We examined the tumour-inhibiting effect of the 5-ASA treatment in more detail. First, the area covered by the 5-ASA enemas was determined in the DSS/5-ASA, 5-ASA-post-DSS, and DSS+5-ASA groups. Mice were sacrificed shortly after rectal installation of the 5-ASA enemas on the last day of the experiment and the distance covered by the 5-ASA enemas was measured. The 5-ASA enemas reached to approximately 3 cm from the anus (Figure 4A), which was defined as the treatment area. 5-ASA significantly reduced the number of tumours in the treatment area by 51.3% in the DSS/5-ASA group as compared to the DSS/Placebo group (on average 9.4 and 19.4 tumours, respectively, P=0.02, Figure 4B). The reduction concerned the development of both small (<2mm) and larger (≥2mm) sized tumours (both, P=0.02), leaving 15.3% of the tumours ≥2 mm in the DSS/5-ASA group compared with 23.9% of the tumours ≥2 mm in the DSS/Placebo group. The large intestine
proximal to the 5-ASA-treated area clearly showed no reduction in total tumour number, or in size of tumours (Figure 4C), and served as internal control, nicely confirming the tumour-inhibiting effect of the 5-ASA enema.

Figure 4: 5-ASA affects the distal large intestine covered by the enema.
(A) The enema treatment area was determined in sacrificed animals shortly after enema treatment on the last day of the experiment. Individual measurements are indicated by closed circles and the mean value is indicated by the horizontal line. (B) 5-ASA reduces the number of both small (<2 mm) and larger (≥2 mm) tumours in the most distal 3 cm of the large intestine, defined as the 5-ASA treatment area, of the DSS/5-ASA and 5-ASA-post-DSS panels. (C) No reduction in total tumour number, or in size of tumours in the large intestine proximal to the 5-ASA-treated area. Bars (mean + SEM) indicate total tumour numbers (black), tumours <2 mm (grey), and tumours ≥2 mm (white) (B,C). Significant P-values ≤0.05 are shown in bold (B,C).
Post-DSS treatment with 5-ASA also showed a consistent tendency towards a decrease in the number and size of tumours in the 5-ASA-treated area (P=0.14-0.21, Figure 4B), leaving the more proximal large intestine unaffected (Figure 4C).

Figure 5: Histopathology of sporadic and IBD-associated tumours, treated with 5-ASA or otherwise. H&E stained sections of the distal large intestine of a non-treated, DSS/Placebo-treated, and DSS/5-ASA-treated FabplCre;Apc^{16110/10} mouse (original magnification 20 x). Neoplastic lesions (arrows) and infiltration of inflammatory cells (arrowhead) are indicated. The distal parts of the intestines are oriented at the right side of the picture. Full-colour image on page 187.
The effect of 5-ASA on histopathology, β-catenin expression, cellular proliferation, and apoptosis

Histopathology of the distal large intestines of the DSS groups also showed many neoplastic lesions and a more severe inflammatory infiltrate ($P=0.009$), compared with that of non-treated Fabpl$^{Cre; Apc^{15lox/+}}$ mice (Figure 5 and 6).

Histopathological evaluation of the DSS/5-ASA group revealed less inflammatory infiltration, although not statistically significant, compared to DSS/placebo (Figure 6, $P=0.19$), but not in the 5-ASA post-DSS group. The microscopic evaluations confirmed the reduced tumour numbers (Figure 5), but an exact histological quantification of the neoplastic lesions was not performed.

![Figure 6: Intestinal inflammatory infiltration of the different treatment groups.](image)

Intestinal inflammatory infiltration as scored by the MAI (mean + SEM), significant $P$-value $\leq 0.05$ shown in bold.

Immunohistochemistry showed nuclear accumulation of β-catenin in all tumours analyzed (illustrated in Figure 7A,D,G), indicating that the Wnt pathway was upregulated due to functional loss of the Apc$^+$ allele in all tumours. We did not detect any obvious differences in intensity or localization, i.e., nuclear versus membranous/cytoplasmic, of β-catenin between the different groups.

The extent of proliferation, as determined by immunohistochemistry for Ki67, of both neoplastic cells and normal intestinal epithelial cells was similar for the non-treated,
DSS/Placebo, and DSS/5-ASA groups, showing a higher percentage of proliferating neoplastic cells (all groups P<0.003) than that of normal epithelial cells (Figure 7B,E,H,K,J).

Figure 7: Effect of 5-ASA on β-catenin expression, proliferation and apoptosis.
Immunohistochemical analysis of neoplastic lesions in the distal large intestine from non-treated (A-C), DSS/Placebo-treated (D-F), and DSS/5-ASA-treated (G-I) FabplCre;Apc<sup>1611</sup>/<sup>+</sup> mice and normal crypts from non-treated mice (J,K,L) for β-catenin (A,D,G,J), for Ki67 to determine the extent of proliferation (B,E,H,K), and for active caspase-3 to determine the extent of apoptosis (C,F,G,L). Original magnification 400 x. Full-colour image on page 188.
Quantification illustrated that 5-ASA tended to reduce the proliferation of neoplastic cells in the DSS/5-ASA group when compared with the DSS/Placebo group (on average from 55.6% to 52.7%, \( P=0.09 \), Figure 8), but 5-ASA did not reduce proliferation of normal epithelial cells in these two groups (\( P=0.47 \), Figure 8). The tendency to reduce proliferation of neoplastic cells by 5-ASA was not found in the groups without DSS treatment (\( P=0.73 \), Figure 8). Overall, proliferation of neoplastic and normal epithelial cells in the DSS-treated groups was very similar to that in the non-DSS-treated groups. Finally, we investigated the extent of apoptosis by immunohistochemical detection of active caspase-3, an apoptotic protease. Apoptotic cells were mainly found at the top of normal crypts (Figure 7L) and incidentally in the lamina propria, whereas a heterogeneous distribution of apoptotic cells was seen in tumour tissue (Figure 7C,F,I). Quantification of the number of caspase-3 positive apoptotic cells within the epithelial lining and in the (sub)mucosa, excluding crypt abscesses and extruded cells, in the distal 3 cm of the colons revealed that 5-ASA did not significantly affect overall apoptosis in the DSS/5-ASA group as compared to the DSS/Placebo group, with a mean ± SEM of 26.1 ± 6.8 versus 18.3 ± 1.7 of apoptotic cells per cm muscularis mucosa, respectively.

*Figure 8: Quantification of proliferation.*

Quantification of ki67 positive cells in the distal large intestines of different mouse panels. Percentages of normal (grey bars) and neoplastic cells (black bars) and \( P \)-values are shown (mean ± SEM).
Discussion

The use of the Fabp1Cre;Apc<sup>15lox/+</sup> mouse model in the present study provided a unique opportunity to evaluate the chemopreventive effect of 5-ASA on both sporadic and IBD-associated neoplasia. Our results show that 5-ASA is able to reduce IBD-associated tumour development by approximately 50%, but not that of sporadic tumours. 5-ASA medication is prescribed to IBD patients as 5-ASA pro-drugs, like sulphasalazine, specially coated (pH-released) tablets, or enemas to circumvent 5-ASA acetylation by cells in the upper digestive tract or 5-ASA oxidation. In animal studies 5-ASA is usually given orally as supplement of the drinking water or food. However, this administration seems not to be the most ideal treatment in animal models, since it does not really mimic 5-ASA medication in humans. Therefore, we have chosen to treat the mice by rectal installation of a 5-ASA enema solution that is also used to treat IBD patients, and allows a good distribution and high luminal concentration of the drug. Rectal administration of 5-ASA was not effective in suppressing the acute clinical signs of DSS-colitis, i.e., the DAI and body weight loss, during the induction phase. 5-ASA was nevertheless able to significantly reduce the number and size of colorectal tumours in that part of the intestine covered by the enemas, the tumourigenesis of which was accelerated due to the DSS-induced colitis, mimicking colitis-associated colorectal carcinogenesis. Our observation that the tumour development in the proximal part of the colon is not affected by the 5-ASA enemas also illustrate that 5-ASA does primarily act locally and not systemically. Interestingly, 5-ASA treatment during the DSS-induced inflammation period, mimicking an inflammatory exacerbation in UC, was not able to reduce the development of colorectal tumours indicating that 5-ASA does not exert its cancer-preventive effects due to anti-inflammatory actions. In contrast, it was the 5-ASA treatment during the subsequent recovery period, mimicking the remission stage in UC, which showed a reduction in the number of tumours, although borderline not significantly, but not in the intestinal inflammatory infiltrate indicating tumour preventive effects of 5-ASA during the recovery period. Two recent reports showed that 5-ASA is also able to reduce colitis-associated neoplasia by approximately 50% in the DSS/azoxymethane model. Clapper et al. described a suppressive effect of 5-ASA on the colitis-induced colorectal carcinogenesis when 5-ASA was administered before, during and after 3 DSS periods. Surprisingly, in this report only the lowest of 3 different dosages of 5-ASA supplemented to the drinking water, simultaneously with DSS during the DSS periods, was effective. Ikeda et al. found a reduction in large intestinal polyp numbers and size when 5-ASA-supplemented to the food was administered in the remission stage, after 2 cycles of DSS-induced colitis. In
both reports, however, it was not exactly clear which 5-ASA dosage the animals received, because the animals were not housed individually and the dosages are only based on the assumption that each animal has the same consumption of 5-ASA supplemented to the water and the food. However, due to the induced-colitis each individual animal alters its eating or drinking pattern, which might influence the dosage intake considerably. In Clapper et al. 20 the mean different dosages of 5-ASA display different effects, indicating that the exact dosage per animal is very important. Moreover, both reports lacked an internal control and did not investigate the effect on sporadic tumour development without colitis induction. In the present study we show that local administration of 5-ASA specifically represses colitis-associated neoplasia in the treatment area, when applied chronically during and after acute intestinal inflammation. This corresponds to human epidemiological studies showing a chemopreventive effect on colitis-associated CRC by chronic medication of 5-ASA 4. One of the mechanisms of action of 5-ASA could be to reduce the proliferation of neoplastic cells as seen in our colitis-associated CRC model, in line with the report from Ikeda et al. 21.

In contrast to several in vitro and in vivo studies on the effect of 5-ASA on CRC cells 24-27, we found a somewhat higher but no significant increase of apoptosis in the intestinal tissues due to 5-ASA administration. We used active caspase-3 immunohistochemistry to investigate apoptosis, which worked well but hampered the exact identification and counting of the percentage of apoptotic cells, because of additional staining of other (non-epithelial) apoptotic cells and cellular debris in the crypts and of extruded cells. Detection of the caspase-degraded product of cytokeratin 18 by M30 immunohistochemistry, widely used on human tissues and easy to quantify epithelial apoptosis, unfortunately did not work in our hands (not shown) since it is probably not specific for mouse. 5-ASA might also induce cell death via non-caspase mechanisms (Chapter 5).

5-ASA was not able to prevent the development of sporadic tumours in the Fabp1Cre;Apc15lox/lox/+ mice. Apparently 5-ASA can not prevent colorectal tumourigenesis which is solely driven by the Apc mutation, and thereby mimicking sporadic CRC development. Moreover, 5-ASA did not affect the proliferation of neoplastic cells in these sporadic CRCs, in line with the absence of a preventive effect. These observations correspond to the data from Ritland et al. 8, describing no effect on the development of adenomas in the ApcMin/+ mouse model. The only study performed in humans, addressing whether 5-ASA prevents sporadic CRC, examined the recurrence rate of sporadic colorectal adenomas in patients who underwent polypectomy 28. This study revealed that oral 5-ASA
was not able to reduce the adenoma recurrence rate, and was therefore unlikely to prevent sporadic CRC, in line with the results presented here.

As far as we know this is the first report that used a mouse model to study the effect of 5-ASA enema treatment on the development of both sporadic and colitis-associated colorectal neoplasia. One could argue that the used Fabpl\textit{Cre};\textit{Apc}^{15loxl/+} mouse model in combination with DSS-induced colitis is a too robust model, in which colorectal tumourigenesis is so strongly accelerated, that it does not really mimic colitis-associated CRC in human IBD patients, which takes decades to develop. One could also argue that due to the robustness of the model a small chemopreventive effect is unlikely to be found. The fact that we did find an effect of 5-ASA shows the contrary: the Fabpl\textit{Cre};\textit{Apc}^{15loxl/+} mouse in combination with DSS is a very useful model to investigate the chemopreventive effects of certain agents on colitis-associated carcinogenesis rather quickly and can also be used to study prevention of sporadic CRC.

In conclusion, in the present study we observed that chronically administered 5-ASA inhibited colitis-associated neoplasia in a mouse model, whereas it did not prevent the development of sporadic tumours, very much in line with the available data from human studies.

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