Automated analysis of proliferation index in PCNA and BrdU stained jejunum in toxicological studies

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for the Colleagues of the ISTP, 26-28 OCTOBER 2018

Automated analysis of BrdU and PCNA in jejunum

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Problem

- Count proliferating and non-proliferating cells in small intestine crypts and calculate proliferation index
- 9000 crypts

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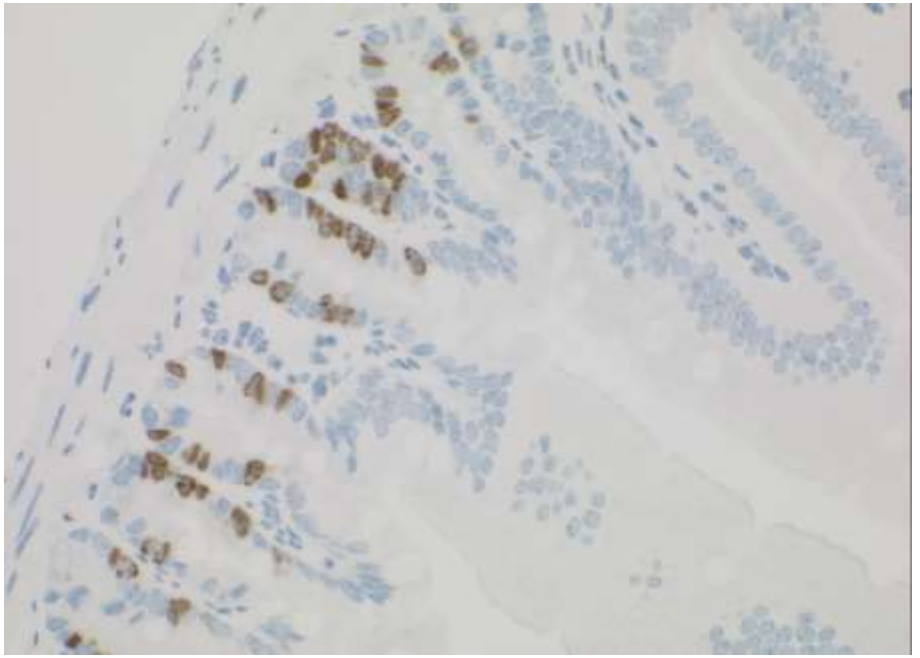
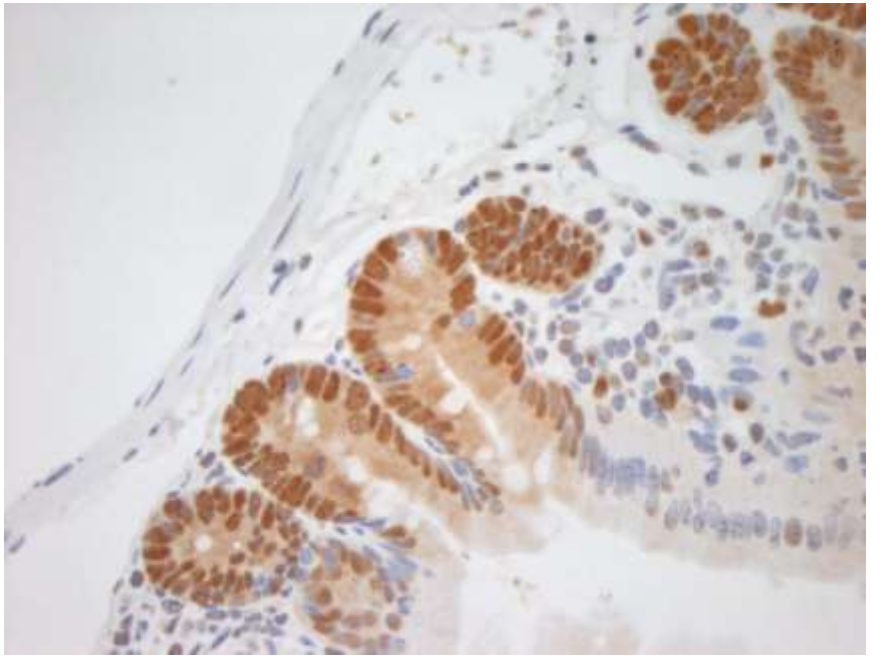
BrdU



PCNA



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Software Cellenger® from Definiens AG

entirely new, object oriented approach to image analysis

ability to extract and analyse structures at a new level of detail, using concepts such as embedding, distances, or the detailed description of the composition of substructures

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What we did

1. Benchmark study to compare manual counting with automated image analysis (Cellenger® software)
2. 13-week oral toxicity study in mice with a Xenobiotic under GLP and time pressure

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1.) Benchmark study

Jejunum stained for PCNA from a previous study

Images of 16 to 30 selected crypts from control and high dose group were used for the counting.

The following counting methods were compared:

- **manual counting by four different people (manual 1-4)**
- **semi-automatic counting with the analySIS® system software**
- **automatic counting with the Definiens Cellenger ® software**

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Benchmark study

For the manual counting, the crypt area was interactively defined and measured with the analySIS software, in images from these crypts. Negative and PCNA positive cell nuclei were counted.

For the semi-automatic counting, results from a previous study with the analySIS system software were taken (same samples).

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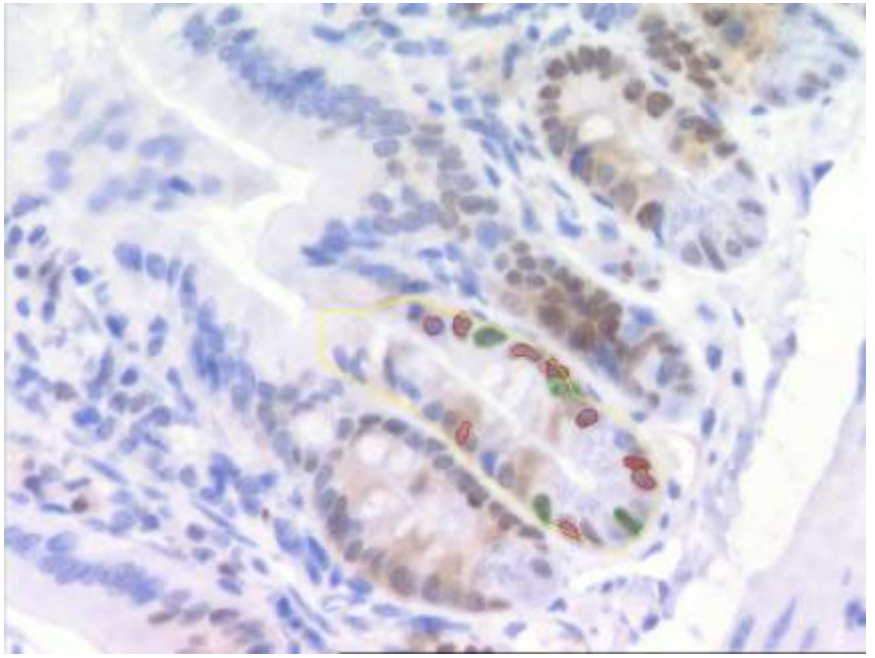
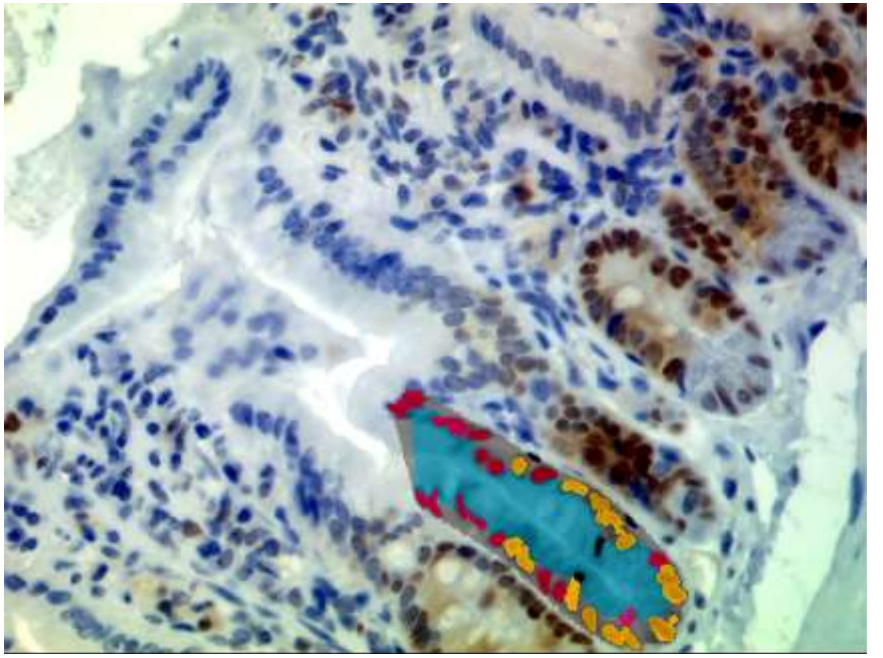
Image comparison

analySIS



Cellenger



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Result crypt area (Benchmark study)

quantity	group	n	method	mean	std. dev.	min	median	max	quotient D./m.
area	control	13	**manual 1**	**3.04**	0.88	1.77	2.88	5.09	
			Definiens	**3.11**	1.25	1.72	2.88	5.68	1.025
	treated	15	**manual 1**	**5.80**	1.26	3.46	5.61	8.97	
			Definiens	**6.03**	1.50	3.56	6.13	9.58	1.039
total nuclei/crypt	control	13	**manual 1**	**32.00**	4.76	25	31	41	
			Definiens	**33.69**	9.42	18	35	50	1.053
	treated	15	**manual 1**	**48.87**	10.51	36	48	77	
			Definiens	**52.47**	10.74	38	52	80	1.074

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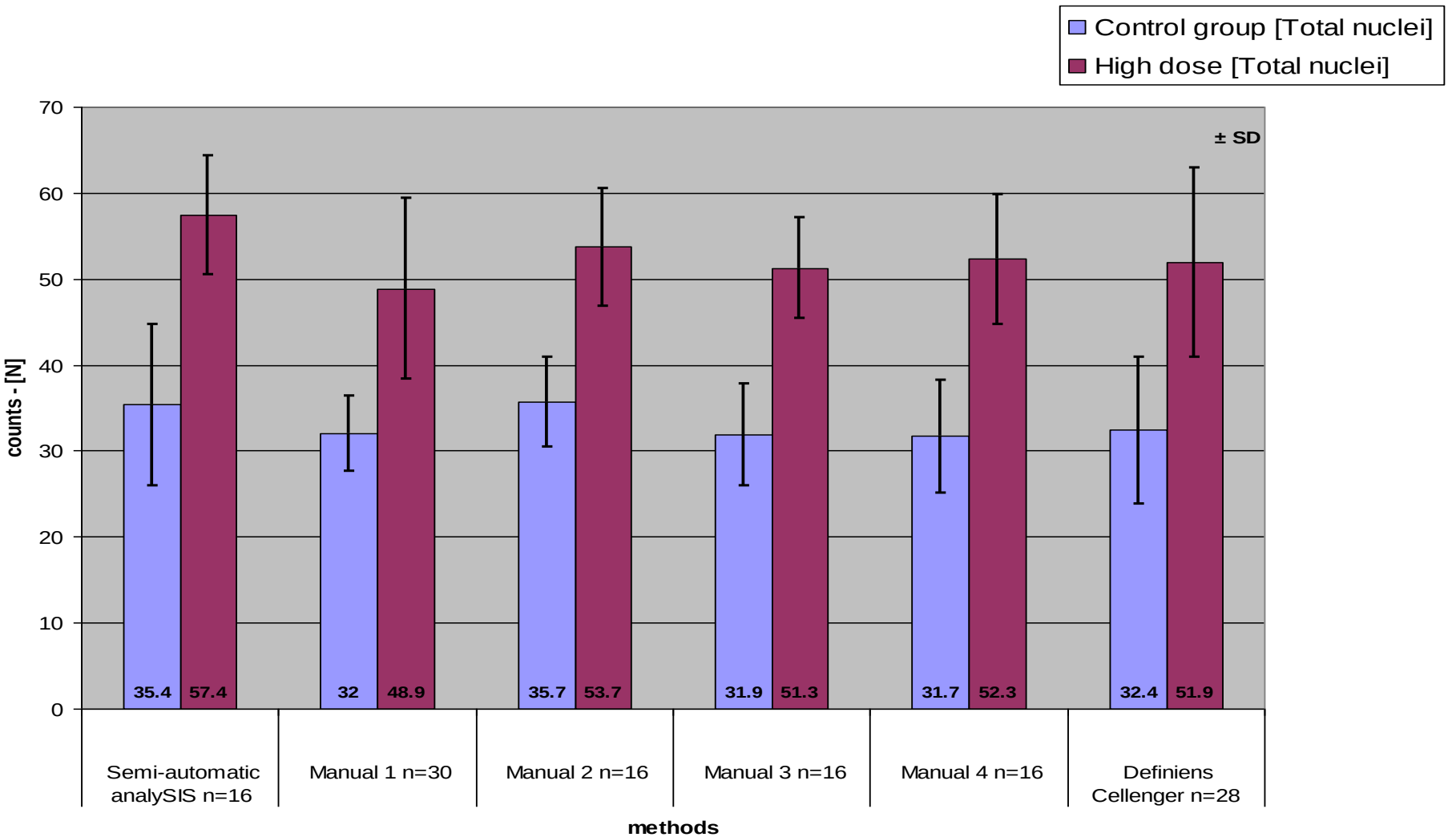
Result total nuclei (Benchmark study)



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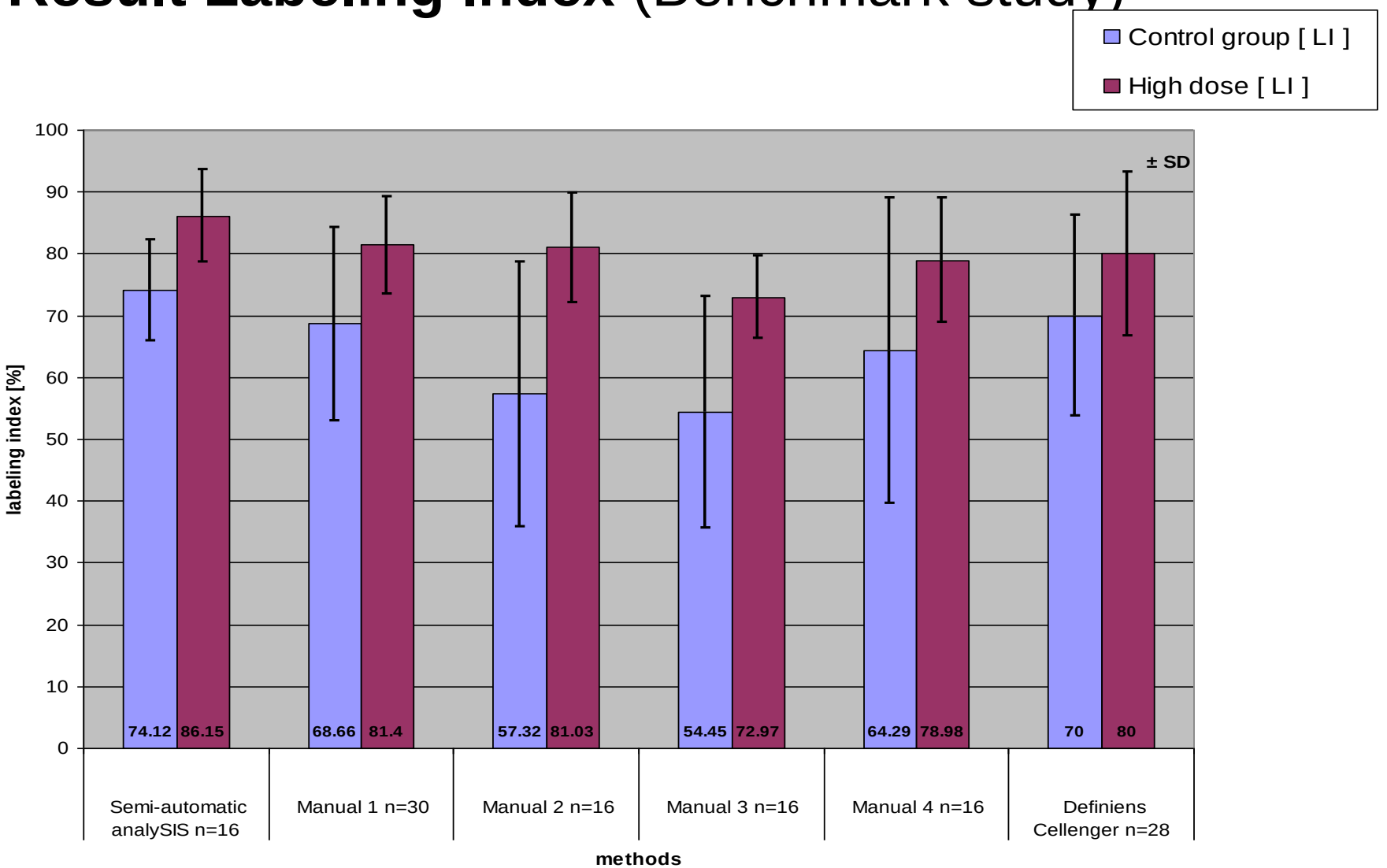
Result Labeling Index (Benchmark study)



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Conclusion (Benchmark study)

Counting with the Definiens Cellenger® software gives results very similar to the manual and the semi-automatic analySIS method.

Automated image analysis with Cellenger® allows repeatable and standardized counting of cell nuclei and crypt area. This in 10 instead of 40 days.

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2.) 13-week oral toxicity study in mice

Animals and dosage form

20 male mice (Crl:CD-1(ICR)BR) per group

Oral application in feed

- **Control**
- **7 dose groups (5, 15, 60, 150, 200, 300, and 600 mg/kg/day)**

180 animals total

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Method (Immunohistochemistry)

- **Jejunum from male mice of control and treated groups**
- **fixed in 10% buffered formalin**
- **5-15 specimens from jejunum put in one paraffin block**
- **1-2 µm-thick sections**
- **Immunohistochemistry with antibodies against PCNA and BrdU in Ventana immunostainer**

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Counting

- **4-12 cross sections of jejunum (on 1 slide) per animal examined**
- **Images of 25 selected crypts per animal (total 9000 images)**
- **Positive and negative nuclei**
- **Crypt area**
- **performed with the Cellenger® analysis system and a light microscope (magnification x400).**
- **Data of 20 crypts per animal used for calculation**

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Counting

- Only crypts sectioned longitudinally with both base and surface present were selected.
- Automatically counted crypts were visually checked, and manually accepted or rejected, or if necessary edited.
- The counting of 20 crypts corresponds approximately to 650 and 1000 nuclei per animal for the control groups and the high dose group, respectively.

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Evaluation

- resulting data were transferred to the Excel system
- PCNA and BrdU labeling indices (LI) were calculated as percentage of positive/total number of nuclei per crypt
- Crypt area was calculated in μm^2
- results from the treated groups were tested against control groups using analysis of variance (area) and a generalized linear model (LI)

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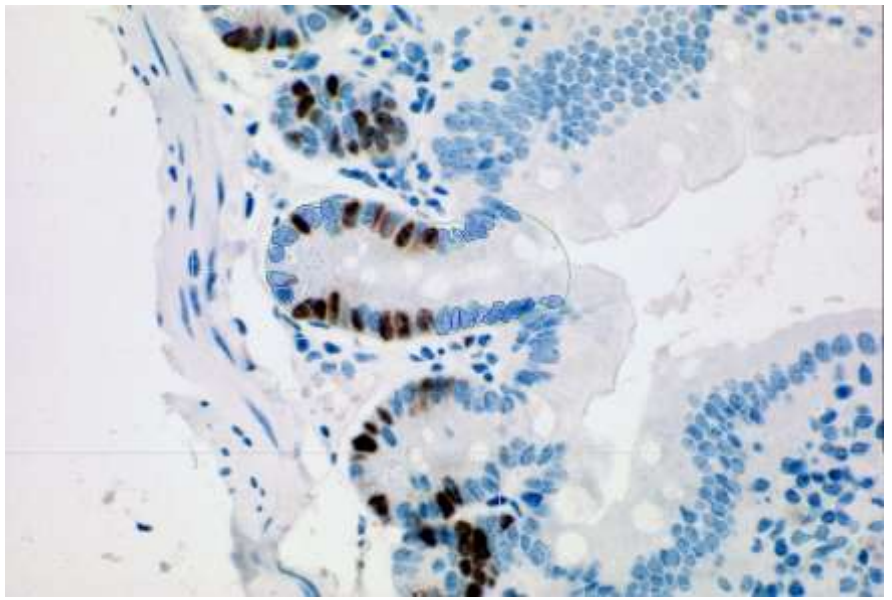
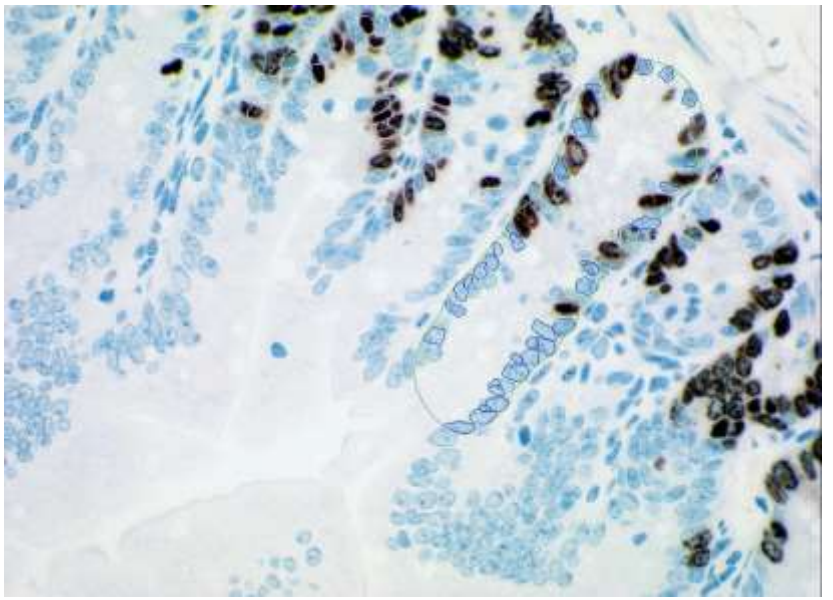
Results BrdU, ***from control (left) and high dose animal (right)***

binary pictures with counting area (ROI, green), positive nuclei (red) negative nuclei (blue)





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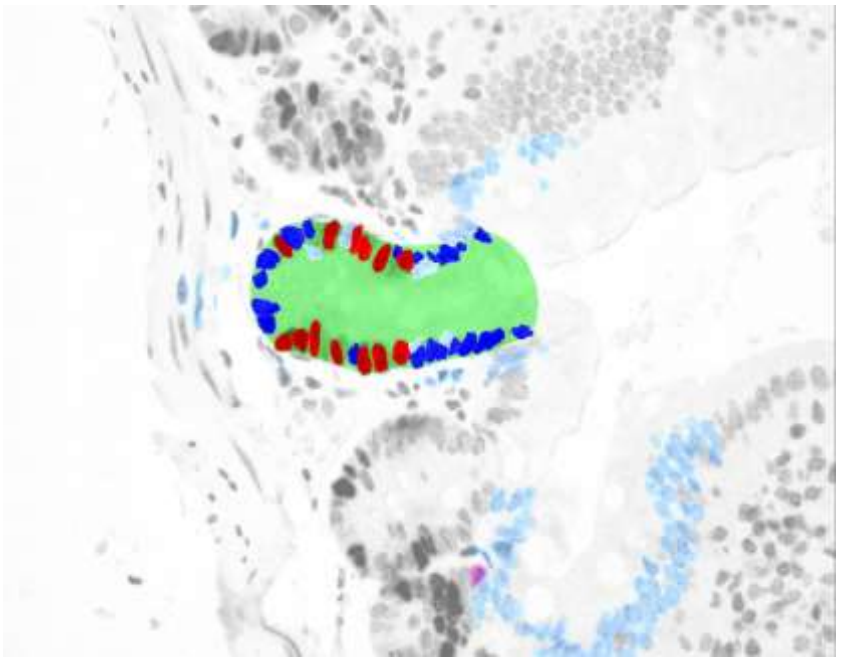

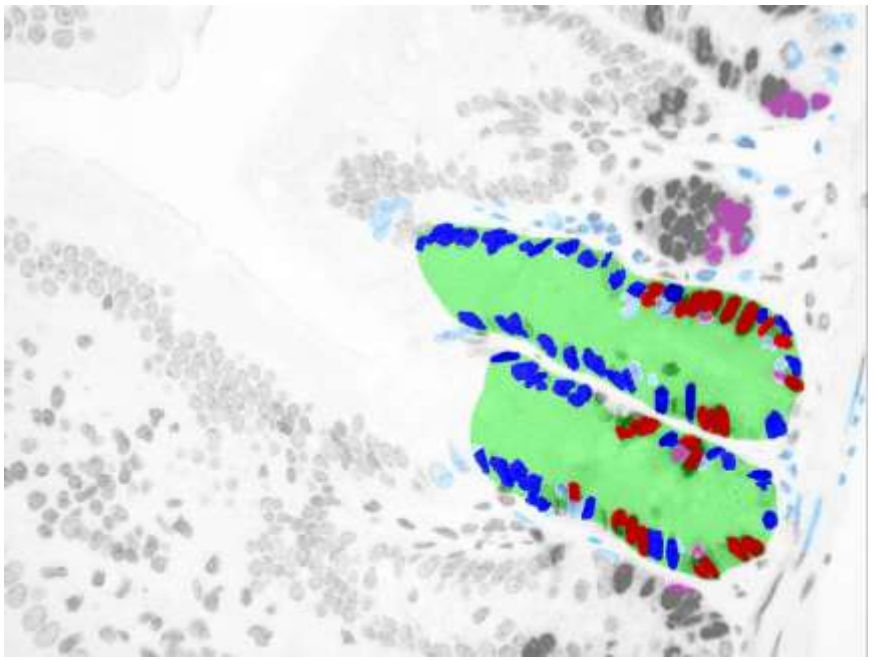

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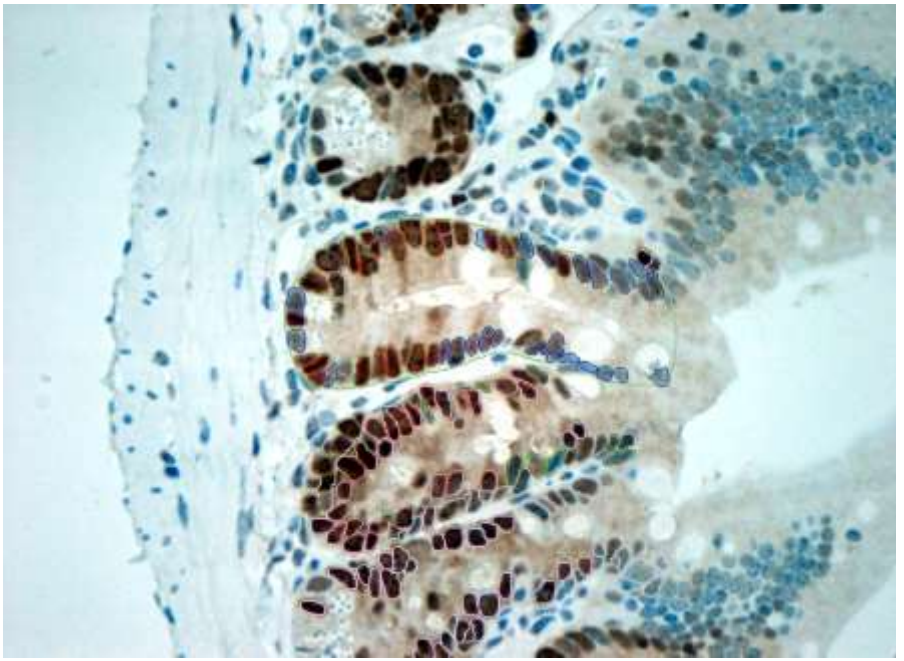

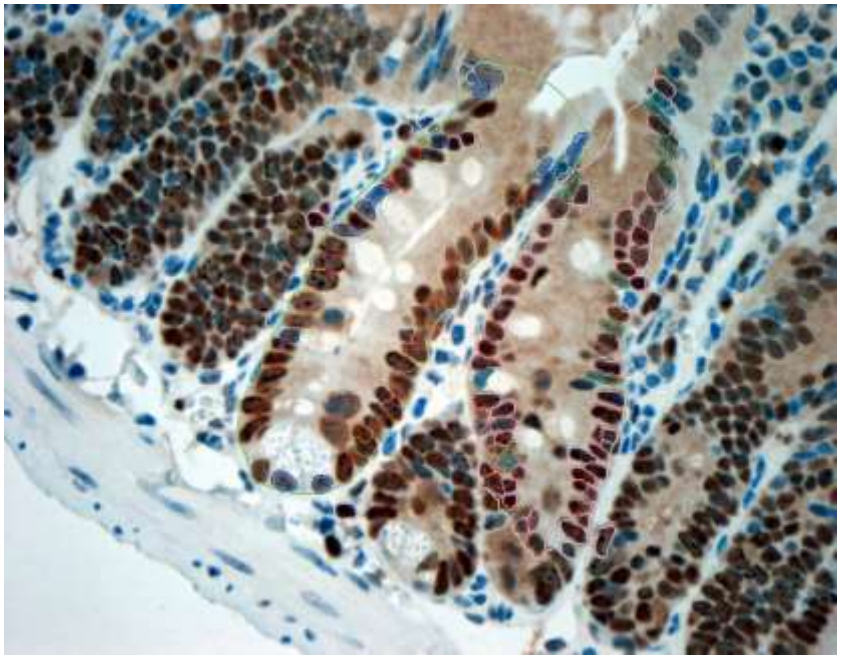

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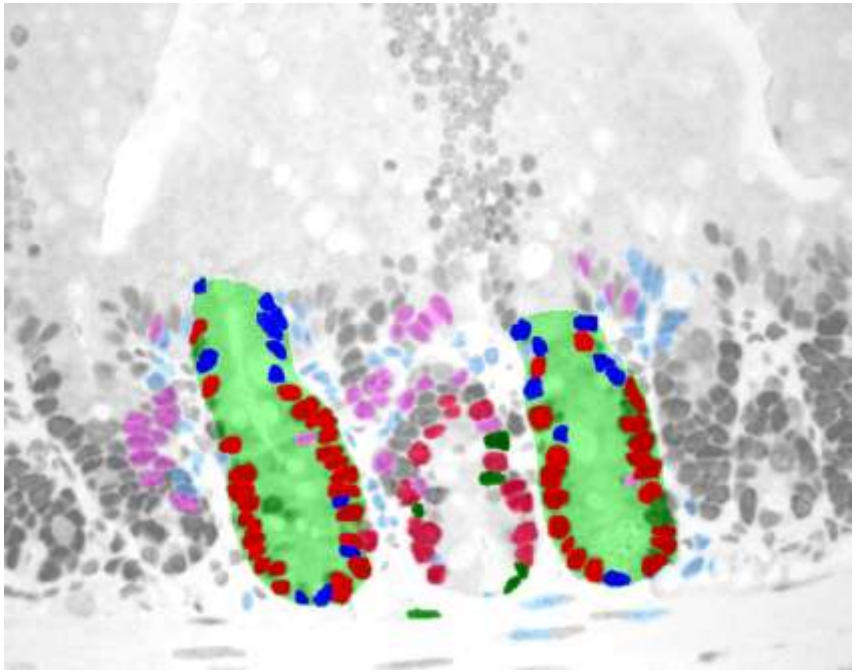

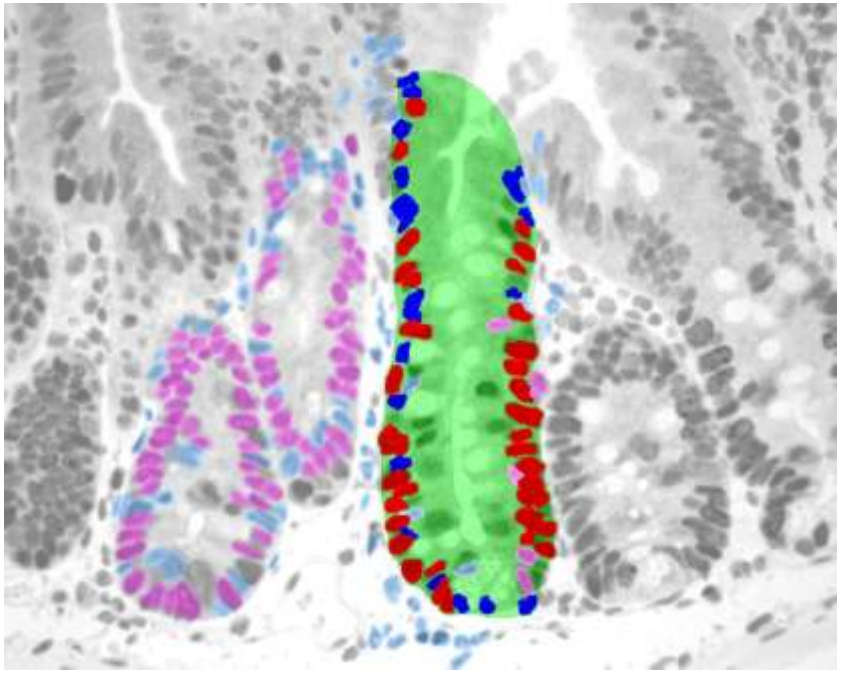

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Results BrdU

group	no. of animals	area [µm^2]		labeling index %	
		geometric mean	CV	mean	std. dev.
1 (C1)	20	3031.80	0.17	23.92	2.26
2 (C2)	20	2922.85	0.16	23.27	2.80
6 (150 mg/kg)	20	2958.46	0.13	23.24	3.13
7 (200 mg/kg)	20	2971.97	0.16	23.04	3.52
8 (300 mg/kg)	20	3148.59	0.11	27.27***	3.32
9 (600 mg/kg)	20	4106.33***	0.12	29.01***	5.26

*** $p < 0.0001$

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Results (PCNA)

group	no. of animals	area [µm^2]		labeling index %	
		geometric mean	CV	mean	std. dev.
1 (C1)	20	3186.45	0.15	61.34	5.62
2 (C2)	20	3184.35	0.12	57.93	5.10
6 (150 mg/kg)	20	3058.48	0.15	60.39	5.71
7 (200 mg/kg)	20	3095.39	0.14	60.12	5.72
8 (300 mg/kg)	20	3396.94	0.13	59.89	6.64
9 (600 mg/kg)	20	4638.41***	0.15	62.81*	7.74

* $p < 0.05$, *** $p < 0.0001$

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Conclusion from the 13-week study

The present investigation of cell proliferation determined by PCNA and BrdU revealed a statistically significant difference in cell proliferation and crypt area between the control and the high dose group

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General conclusion

Evaluated 9000 images

Time saved:

Instead of 16 weeks for manual counting,

only 4 weeks for Automated analysis

Tremendous impact for the mentioned project in terms of approval of the drug

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Until now 5 different applications with the Cellenger software from Definiens AG within Novartis, Basel

- **Intestine crypts (proliferation)**
- **Nerves (g-ratio)**
- **Brain (proliferation)**
- **Lymphiod tissue (B- and T-cells)**
- **Angiogenesis (growing vessles)**

Acknowledgements

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