Laboratory Animal Science

Full paper

Reduced differentiation of intestinal epithelial cells in wasting marmoset syndrome

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Running head: REDUCED CELL DIFFERENTIATION OF MARMOSET
Abstract

Wasting marmoset syndrome (WMS) is a serious disease in captive common marmoset (Callithrix jacchus) colonies. Because of the high mortality rates, elucidation of the underlying mechanisms is essential. In this study, we compared the histopathology, the number of each epithelial cell in the jejunum and colon, and the expression patterns of some molecular markers between healthy and WMS-affected marmosets. Atrophy of villi in the jejunum and mononuclear cell infiltration in the lamina propria were observed in the intestinal tract of WMS-affected marmosets. Although the numbers of transient amplifying cells and tuft cells were increased, the number of goblet cells was obviously decreased in the jejunum and colon of WMS-affected marmosets compared to healthy marmosets. In addition, the number of enterocytes in the jejunum was decreased in WMS animals. There was no apparent difference in the numbers of stem cells, enteroendocrine cells, or Paneth cells. The expression of β-catenin and Tcf7l2 was increased in WMS, and the co-existence of β-catenin and Tcf7l2/Cyclin D1 was observed around the crypts in WMS-affected marmosets. These findings suggest that cell proliferation continues, but cell differentiation is halted in the intestinal tract due to the enhanced β-catenin/Tcf7l2/Cyclin D1 signaling pathway in WMS, which results in malfunction of the villus and mucosa.

Keywords: common marmoset, differentiation, intestinal epithelial cell, wasting marmoset syndrome
INTRODUCTION

The common marmoset (*Callithrix jacchus*) is a small new world primate native to Brazil that is used increasingly as an alternative primate model in biomedical research areas including preclinical tests, reproduction, neurobiology, immunology, endocrine signaling, obesity, aging, and fetal or postnatal development [1, 16, 25]. A transgenic marmoset line has been established with germline transmission [22] and a genome-edited marmoset model has also been reported [23], attracting attention to the potential of this animal model.

However, a serious problem in rearing marmosets in a captive environment is the so-called wasting marmoset syndrome (WMS). WMS is considered a unique disease in this species and the main symptoms include weight loss, decreased muscle mass, and chronic diarrhea. Although a number of studies have been conducted on the nutritional and infectious factors, the pathogenesis of this life-threatening disease remains unclear. Previous studies [14, 28] reported that chronic enteritis was observed commonly in marmosets with WMS. We reported that serum matrix metalloproteinase 9 (MMP9) levels are elevated in WMS-affected marmosets [32], and we developed a new treatment for WMS using tranexamic acid [33]. However, our clinical experience indicates that it is difficult to cure WMS-affected marmosets with serum levels of albumin <2.0 g/dl. To elucidate the underlying mechanisms for the hypoalbuminemia, we measured the levels of fecal α1-proteinase inhibitor (also known as α1-antitrypsin) and identified an intestinal protein loss, a known reason for hypoalbuminemia, in WMS animals [19].

In this study, to address WMS in this valuable animal model, we investigated the pathogenesis of WMS as a basis for the development of a more powerful treatment for WMS-affected marmosets.
MATERIALS AND METHODS

Animals

All common marmosets used in this study were born and reared at the RIKEN Center for Brain Science (Saitama, Japan) and maintained on a 12 hr light-dark cycle at 27°C and 50% humidity. The information of marmosets used in this study is shown in Table 1. Marmosets were allowed ad libitum access to water and food pellets (CMS-1M; CLEA Japan Inc., Tokyo, Japan) with added vitamin C and D, calcium, and acidophilus. In this study, four WMS-affected marmosets at the humane endpoint (more than 20% decrease in body weight, no visible indications of recovery) were sacrificed and the organs were dissected. The intestinal tracts of healthy marmosets were dissected from four individuals which were sacrificed because of other than WMS (e.g., end of an experiment). This study was approved by the Animal Experiments Committee of RIKEN (Saitama, Japan, approval number; W2019-2-011 (4)), and was conducted in accordance with the Institutional Guidelines for Experiments using Animals.

Sample preparation and staining

Marmosets were anesthetized with 15 mg/kg of Ketamine and 2-3% of Isoflurane and perfused with saline followed by G-Fix (Genostaff Co., Ltd., Tokyo, Japan). As the main symptoms of WMS are chronic diarrhea and malnutrition followed by loss of body weight and muscle mass, we focused on the jejunum and colon where absorption of nutrients and water, respectively, is done. The jejunum and colon were longitudinally dissected after the perfusion, fixed in the G-Fix, embedded in paraffin on CT-Pro20 (Genostaff Co., Ltd.) using G-Nox (Genostaff Co., Ltd.) as a less toxic organic solvent than xylene. The tissue
blocks were sectioned at 6 µm and stained with hematoxylin and eosin (HE), Masson trichrome, and alcian blue stain (pH 2.5) by routine techniques.

**In situ hybridization**

*In situ* hybridization (ISH) was performed similarly to Tsukasaki et al. [27] as below using the ISH Reagent Kit (Genostaff Co., Ltd.) according to the manufacturer’s instructions. Tissue sections were de-paraffinized with G-Nox, and rehydrated through an ethanol series and phosphate-buffered saline (PBS). The sections were fixed with 10% neutral buffered formalin (NBF, 10% formalin in PBS) for 30 min at 37°C and washed in distilled water, placed in 0.2% HCl for 10 min at 37°C and washed in PBS, treated with 4 µg/ml Proteinase K (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in PBS for 10 min at 37°C and washed in PBS, then placed within a coplin jar containing 1 × G-Wash (Genostaff Co., Ltd.), equal to 1 × saline-sodium citrate (SSC) buffer. Hybridization was performed with probes (250 ng/ml) in G-Hybo-L (Genostaff Co., Ltd.) for 16 hr at 60°C. The probes for Leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5), Chromogranin A (Chga), WD repeat domain 43 (Wdr43), Cyclooxygenase 1 (Cox1), Cadherin 1 (Cdh1), Transcription factor 7 like 2 (Tcf7l2), and Cyclin D1 (Ccd1) were purchased from Genostaff Co., Ltd. (Table 2). After hybridization, the sections were washed 3 times with 50% formamide in 0.5 × G-Wash for 30 min at 50°C, and 5 times in tris-buffered saline (TBS) with Tween20 (TBST, 0.1% Tween20 in TBS) at room temperature (RT). After treatment with 1 × G-Block (Genostaff Co., Ltd.) for 15 min at RT, the sections were incubated with anti-DIG AP conjugate (Roche, Basel, Switzerland) diluted 1:2000 with G-Block (diluted 1/50) in TBST for 1 hr at RT. The sections were washed twice in TBST and then incubated in 100 mM NaCl, 50 mM MgCl2, 0.1% Tween20, 100 mM Tris-HCl, pH9.5. Coloring reactions
were performed with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) Solution (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and then washed in PBS. The sections were counterstained with Kernechtrot Stain Solution, and mounted with G-Mount (Genostaff Co., Ltd.).

**Immunohistochemistry (IHC)**

IHC was performed similarly to Matsumoto *et al.* [17] as below. Tissue sections were de-paraffinized with xylene, and rehydrated through an ethanol series and PBS. Antigen retrieval was performed by microwave treatment with Citrate Buffer, pH6.0 (Genostaff Co., Ltd.). Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by incubation with G-Block and Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA). The sections were incubated with Anti-Beta Catenin Rabbit Monoclonal Antibody (Cell signaling Technology, Beverly, MA, USA) at 4°C overnight. They were incubated with Biotin-Conjugated Anti-Rabbit Ig (Agilent, Santa Clara, CA, USA), for 30 min at RT, followed by the addition of Peroxidase conjugated Streptavidin (Nichirei Bioscience Inc., Tokyo, Japan) for 5 min. Peroxidase activity was visualized by diaminobenzidine. The sections were counterstained with Mayer’s Hematoxylin (Muto Pure Chemical Co., Ltd., Tokyo, Japan), and mounted with Malinol. We compared the signal intensity of β-catenin using the free software “ImageJ (version 2.0.0)” in 10 different 200 µm squares from a single section. The combined values from two independent sections were compared.

**ISH-IHC double stain**

ISH was performed similarly to Tsukasaki *et al.* [27] as below with the ISH Reagent Kit according to the manufacturer’s instructions. Tissue sections were de-paraffinized
with G-Nox, and rehydrated through an ethanol series and PBS. The sections were fixed
with 10% NBF for 30 min at 37°C and washed in distilled water, placed in 0.2% HCl for
10 min at 37°C and washed in PBS, treated with 4 µg/mL Proteinase K in PBS for 10 min
at 37°C and washed in PBS, then placed within a coplin jar containing 1x G-Wash.
Hybridization was performed with probes (250 ng/mL) in G-Hybo-L (Genostaff Co., Ltd.)
for 16 hr at 60°C. After the hybridization, the sections were washed 3 times with 50%
formamide in 0.5 × G-Wash for 30 min at 50°C, and 5 times in TBST (0.1% Tween20
in TBS) at RT. After treatment with 1 × G-Block for 15 min at RT, the sections were
incubated with Anti-DIG AP Conjugate diluted 1:2000 with G-Block (diluted 1/50) in
TBST for 1 hr at RT. The sections were washed twice in TBST and then incubated in 100
mM NaCl, 50 mM MgCl₂, 0.1% Tween20, 100 mM Tris-HCl, pH9.5. Coloring reactions
were performed with NBT/BCIP Solution and then washed in PBS.

IHC was performed similarly to Matsumoto et al. [13] as a second staining after the ISH.
The sections were treated 0.3% hydrogen peroxide in PBS for 30 min, followed by
incubation with G-Block and Avidin/Biotin Blocking Kit. They were incubated with Anti-
beta Catenin Rabbit Monoclonal Antibody at 4°C overnight. They were incubated with
Biotin-Conjugated Anti-Rabbit Ig, for 30 min at RT, followed by the addition of
Peroxidase Conjugated Streptavidin for 5 min. Peroxidase activity was visualized by
diaminobenzidine. The sections were counterstained with Kernechtrot stain solution, and
mounted with G-Mount.

**Statistical analysis**

Two-tailed Mann-Whitney U-tests were used to compare the healthy and WMS-affected
groups (GraphPad Prism ver. 7, GraphPad Software Inc., La Jolla, CA, USA). Results
were considered significant at 5% or less probability of error.
RESULTS

Histopathology

HE stain of the jejunum and colon showed atrophy or disappearance of villi and an increase in distorted and irregular crypts in the jejunum and mononuclear cell infiltration in the lamina propria of the jejunum and the colon in WMS-affected marmosets (Fig. 1). Villus height in the jejunum of WMS-affected marmosets was significantly lower compared with healthy individuals.

Comparison of the numbers of each epithelial cell type

We compared the numbers of each cell type in the intestinal epithelial tissue between healthy and WMS-affected marmosets (Fig. 2). We counted stem cells, transient amplifying (TA) cells, paneth cells, tuft cells, enterocytes, goblet cells, and enteroendocrine cells per crypt/villus (in jejunum) or per intestinal gland (in colon) from a single section. The combined values from two independent sections were compared.

Lgr5 is used as a marker for stem cell because of the expression pattern revealed by the knock-in mice [3]. We used Chga as a marker for enteroendocrine cells, since it is expressed in the large dense-core secretory vesicles [10]. Wdr43 coordinates hyperactive transcription [6], thus, it is considered a marker for TA cells. Cox1 is implicated in inflammation, and is used as a marker for tuft cells [5]. Since Chd1 is used as a marker for differentiation [9], Cdh1-positive cells in villi were considered enterocytes, the major
differentiated cells in villi. Goblet cells are stained light blue by alcian blue [29], while Paneth cells are stained dark red in Masson’s trichrome staining [11]. The numbers of TA cells and tuft cells were significantly greater in WMS-affected marmosets than those in healthy marmosets in the jejunum and colon. On the other hand, goblet cells were significantly decreased in the jejunum and colon of WMS-affected marmosets. The number of enterocytes in the jejunum of WMS-affected marmosets was also significantly decreased compared with that of healthy marmosets. There was no apparent difference in numbers of stem cells, enteroendocrine cells, or Paneth cells between healthy and WMS-affected marmosets.

Comparison of expression patterns of the molecular markers

The β-catenin expression in the jejunum was significantly stronger in WMS-affected marmosets compared with that of healthy marmosets (Fig. 3). In particular, an accumulation of cytoplasmic β-catenin was observed in the cells surrounding crypts. The numbers of Tcf7l2-positive cells were significantly greater in the jejunum and colon of WMS-affected marmosets and the numbers of Tcf7l2 and β-catenin double-positive cells were also significantly higher in WMS-affected marmosets (Fig. 3). The numbers of Ccnd1-positive cells were significantly greater in WMS-affected marmosets than healthy marmosets in the jejunum and colon (Fig. 3). Moreover, expression of Ccnd1 in the cells with accumulated β-catenin was significantly higher in the jejunum and colon of WMS-affected marmosets than in healthy individuals (Fig. 3).

DISCUSSION
In the present study, we observed an atrophy or disappearance of villi in the jejunum and increases in aberrant crypts and mononuclear cell infiltration in the lamina propria in the jejunum and colon of WMS-affected marmosets. This observation is consistent with previous reports [14, 15, 28]. As suggested by Ludlage et al. [15], most of the infiltrated cells in the lamina propria were thought to be T cells because our preliminary IHC experiment using a CD3 antibody often exhibited positive signals on these cells (data not shown). Further experiments are needed to elucidate the immunological mechanisms involved in WMS. Previously, we reported intestinal protein loss in WMS-affected marmoset [19]. In human, primary intestinal lymphangiectasia usually causes protein loss, whereas secondary intestinal lymphangiectasia after intestinal bowel disease is observed in protein loss in dogs [7]. In the present study, lymphangiectasia was not observed in WMS-affected marmoset, which suggests that other mechanisms might be involved in the protein loss in WMS.

To understand these phenomena that occurred in the intestinal tract of WMS-affected marmosets, we compared the numbers of each epithelial cell type between healthy and WMS-affected marmosets. As a result, a greater number of TA cells and tuft cells were observed in the jejunum and colon of WMS-affected marmosets compared to healthy marmosets. However, goblet cell numbers were decreased in the jejunum and colon of WMS-affected marmosets. The number of enterocytes in jejunum of WMS-affected marmosets was also decreased compared with that of healthy marmosets. There was no detectable difference in numbers of stem cells, enteroendocrine cells, or Paneth cells between healthy and WMS-affected marmosets. The symptoms of WMS resemble human Crohn’s disease with respect to chronic enteritis, chronic diarrhea and weight loss [26]. There are also histological similarities such as aberrant (distorted, non-parallel, and
irregular) crypt structure, shortening, widening, and blunting villi, and lymphangiectasia [8]. However, increased goblet cells have been reported in Crohn’s disease [8, 18], whereas goblet cell numbers were decreased in WMS. In addition, SAMP1/YitFcsJ mice are known as a spontaneous model of Crohn’s disease [20], and they have similar histological features to WMS such as decreased surface area of the villi, increased depth of intestinal crypts, and increased number of TA cells [12]. However, the number of Paneth cells is decreased in SAMP1/YitFcsJ mice, whereas there was no detectable change in the number of Paneth cell in WMS. Taken together, the underlying mechanisms for WMS might differ from Crohn’s disease.

In the mammalian intestinal epithelium, stem cells at the bottom of the crypt give rise to a transient population of undifferentiated cells that vigorously proliferate while migrating toward the lumen of the intestine. This process occurs continuously, and replacement of the tip of the villus occurs over 4 to 5 days [21]. However, an increase of branching crypts and atrophy of villi in the jejunum were observed in this study. Thus, regeneration of villi in the jejunum of WMS-affected marmosets does not occur. As shown in Fig. 2, there was no difference in the number of stem cells between healthy and WMS-affected marmosets. Previous studies reported that β -catenin and TCF (transcription factor) mediate cell positioning in the intestinal epithelium [4] and that β -catenin/TCF-4 (also known as TCF7L2) complex imposes a crypt progenitor phenotype [30]. These findings suggest that β -catenin/TCF signaling is essential for controlling the proliferative/undifferentiated state of intestinal epithelial cells. According to Van de Wetering et al., downregulation of β -catenin/TCF7L2 activity results in cell cycle arrest and the start of differentiation [30]. Thus, β -catenin/Tcf7l2 signaling may be upregulated, which suppresses differentiation of epithelial cells in WMS-affected marmoset, resulting in extended crypts and the atrophy of villi.
To confirm this hypothesis, we compared the expression levels of β-catenin and Tcf7l2 between healthy and WMS-affected marmosets. As a result, increased β-catenin expression was observed in WMS-affected marmosets, especially around the crypts, and β-catenin was present both in the cell membrane and the cytoplasm. β-catenin can translocate through the nuclear pores from the cytoplasm [31], thus, nuclear accumulation of β-catenin may have occurred in WMS-affected marmosets, resulting in the formation of β-catenin-TCF complex in the nucleus. In fact, in cells with accumulated nuclear β-catenin, the expression of Tcf7l2 was also observed, as shown in Fig. 3. In addition, it has been reported that Cyclin D1 expression is activated by β-catenin/TCF and that it has a direct effect on cell proliferation [24]. Cyclin D1 is known as a critical target of proliferative signals in G1 phase of the cell cycle [2]. We observed the expression of Cyclin D1, with increased expression in the cells around the crypts in WMS-affected marmosets. In addition, the co-existence of Cyclin D1 and β-catenin in the same cells around crypts was also observed.

Our findings suggest that an enhanced β-catenin/Tcf7l2/Cyclin D1 signaling pathway in WMS-affected marmosets leads to the malfunction of villi because, although cell proliferation continues, cell differentiation is suppressed due to the enhanced signaling pathway. Therefore, the malfunction of villi in the jejunum seems to be one of the mechanisms underlying the decrease in body weight and muscle mass, hypoalbuminemia and diarrhea observed in WMS. Therapies to facilitate intestinal epithelial cell differentiation may be effective as a new treatment for WMS.

Liu et al. reported that a significant decrease in goblet cells and a significant increase in proliferation were observed in MMP9 transgenic mice (Tg-villin-MMP9), in which MMP9 is overexpressed in the intestinal epithelium [13], and we previously reported treatment effects of MMP9 inhibition for WMS [33]. Taken together, there is a possibility
that inhibition of MMP9 might increase the number of goblet cells and suppress overproliferation, resulting in recovery of villus function.

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FIGURE LEGENDS

Figure 1. Representative photomicrographs of a section of the jejunum and colon in marmosets. Hematoxylin and eosin (HE). HE stain showed atrophy or disappearance of villi and an increase in distorted and irregular crypts in the jejunum and mononuclear cell infiltration in the lamina propria in the jejunum and colon of wasting marmoset syndrome (WMS)-affected marmosets. The height of the villus in the jejunum of WMS-affected marmosets was significantly lower compared with healthy individuals. The square frames in the low-power fields indicate the area in each high-power field. Scale bar, 50 μm. The
bar in each graph represents the median value. *; P<0.05

Figure 2. Comparison of the numbers of each epithelial cell type in the intestinal tract between healthy and wasting marmoset syndrome (WMS)-affected marmosets. *; P<0.05

Figure 3. Comparison of the expression patterns of molecular markers in the intestinal tract between healthy and wasting marmoset syndrome (WMS)-affected marmosets. *; P<0.05
| Case No. | Age (years old) | Sex     | Remarks                          |
|---------|----------------|---------|----------------------------------|
| A       | 5              | Male    | Healthy, used for breeding       |
| B       | 5              | Male    | Healthy, used for breeding       |
| C       | 6              | Male    | Healthy, used for breeding       |
| D       | 5              | Male    | Healthy, used for breeding       |
| E       | 7              | Female  | WMS-affected, used for breeding  |
| F       | 3              | Male    | WMS-affected, used for breeding  |
| G       | 4              | Female  | WMS-affected, used for breeding  |
| H       | 5              | Female  | WMS-affected, used for breeding  |

WMS, Wasting marmoset syndrome
Table 2. Reagents used in this study.

| Reagent                  | Catalog number | Manufacture                                                                 | Use         |
|--------------------------|----------------|-----------------------------------------------------------------------------|-------------|
| ISH Reagent Kit          | SRK-02         | Genostaff Co., Ltd. (Tokyo, Japan)                                          | ISH         |
| G-Nox                    | GN04           | Genostaff Co., Ltd.                                                         | ISH         |
| Proteinase K             | 162-22751      | FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan)                      | ISH         |
| G-Wash                   | SHW-01         | Genostaff Co., Ltd.                                                         | ISH         |
| G-Hybo-L                 | RPD-02         | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Lgr5           | CMP-L-01       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Chga           | CMP-C-04       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Wdr43          | CMP-W-01       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Cox1           | CMP-C-05       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Chdh1          | CMP-C-03       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Tcf7l2         | CMP-T-02       | Genostaff Co., Ltd.                                                         | ISH         |
| probe for Ccdn1          | CMP-C-06       | Genostaff Co., Ltd.                                                         | ISH         |
| G-Block                  | GB-01          | Genostaff Co., Ltd.                                                         | ISH, IHC    |
| anti-DIG AP conjugate    | 11093274910    | Roche (Basel, Switzerland)                                                  | ISH         |
| NBT                      | N6876          | Sigma-Aldrich (St. Louis, MO, USA)                                          | ISH         |
| BCIP                     | B8503          | Sigma-Aldrich                                                               | ISH         |
| G-Mount                  | GM-01          | Genostaff Co., Ltd.                                                         | ISH         |
| Citrate Buffer, pH6.0    | ARSC6-01       | Genostaff Co., Ltd.                                                         | IHC         |
| Avidin/Biotin Blocking Kit | SP-2001    | Vector Laboratories (Burlingame, CA, USA)                                  | IHC         |
| Anti-Beta Catenin Rabbit Monoclonal Antibody | 8480 | Cell signaling Technology (Beverly, MA, USA)                                | IHC         |
| Biotin-Conjugated Anti-Rabbit Ig | E0432 | Agilent (Santa Clara, CA, USA)                                              | IHC         |
| Peroxidase conjugated Streptavidin | 426062 | Nichirei Bioscience Inc. (Tokyo, Japan)                                     | IHC         |
| Mayer’s Hematoxylin      | 30142          | Muto Pure Chemical Co., Ltd. (Tokyo, Japan)                                 | IHC         |
| Malinol                  | 20093          | Muto Pure Chemical Co., Ltd.                                                | IHC         |

ISH, *In situ* hybridization
IHC, Immunohistochemistry
NBT, Nitroblue tetrazolium
BCIP, 5-Bromo-4-chloro-3-indolyl phosphate
Fig. 1

| Jejunum       | Healthy | WMS-affected |
|---------------|---------|--------------|
| Colon         | Healthy | WMS-affected |

- **Jejunum**
  - Healthy
  - WMS-affected

- **Colon**
  - Healthy
  - WMS-affected

Histograms showing:
- Height of villus (um) in Jejunum for Healthy and WMS-affected groups.
- Height of mucosa (um) in Colon for Healthy and WMS-affected groups.

* denotes a statistically significant difference.
Fig. 2

|                | Jejunum               | Colon                |
|----------------|-----------------------|----------------------|
|                | Healthy               | WMS-affected         |
| Lgr5 (Stem cell) | ![Lgr5 Healthy Jejunum](image) | ![Lgr5 WMS-affected Jejunum](image) |
| Chga (Entero-endocrine cell) | ![Chga Healthy Jejunum](image) | ![Chga WMS-affected Jejunum](image) |
| Wdr43 (TA cell) | ![Wdr43 Healthy Jejunum](image) | ![Wdr43 WMS-affected Jejunum](image) |
| Cox1 (Tuft cell) | ![Cox1 Healthy Jejunum](image) | ![Cox1 WMS-affected Jejunum](image) |
| Cdh1 (Enterocyte) | ![Cdh1 Healthy Jejunum](image) | ![Cdh1 WMS-affected Jejunum](image) |
| Alcian blue stain (Goblet cell) | ![Alcian Healthy Jejunum](image) | ![Alcian WMS-affected Jejunum](image) |
| Masson trichrome stain (Paneth cell) | ![Masson Healthy Jejunum](image) | ![Masson WMS-affected Jejunum](image) |

**Graphs:**
- Lgr5 (Stem cell) number per crypt
- Wdr43 (TA cell) number per crypt
- Cox1 (Tuft cell) number per villus/intestinal gland
- Cdh1 (Enterocyte) number per villi
- Goblet cell number per crypt
- Paneth cell number per crypt
### Fig. 3

|                | Jejunum          | Colon           |
|----------------|------------------|-----------------|
|                | Healthy          | WMS-affected    | Healthy          | WMS-affected    |
| **β-catenin**  | ![Image]         | ![Image]        | ![Image]         | ![Image]        |
| **β-catenin + Tcf7l2** | ![Image]         | ![Image]        | ![Image]         | ![Image]        |
| **Ccnd1**      | ![Image]         | ![Image]        | ![Image]         | ![Image]        |
| **β-catenin + Ccnd1** | ![Image]         | ![Image]        | ![Image]         | ![Image]        |

**Intensity per 200µm square**

- Healthy: ![Plot](image)
- WMS-affected: ![Plot](image)

**Number per crypt**

- Healthy: ![Plot](image)
- WMS-affected: ![Plot](image)

**Jejunum**

- **β-catenin**: ![Plot](image)
- **Tcf7l2**: ![Plot](image)
- **β-catenin + Ccnd1**: ![Plot](image)

**Colon**

- **β-catenin**: ![Plot](image)
- **Ccnd1**: ![Plot](image)
- **β-catenin + Ccnd1**: ![Plot](image)