# Neuropeptide receptor expression in inflammatory bowel disease
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# Motilin Receptor Expression in Smooth Muscle, Myenteric Plexus and Mucosa of Human Inflamed and Noninflamed Intestine

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# Abstract

**Background:** Besides regulation of upper gastrointestinal motility, motilin seems to play a role in the inflammatory response. Motilin receptor expression in human intestine has not been studied thoroughly. This study aimed to describe the intestinal distribution of motilin receptors in inflammatory bowel disease (IBD) and control patients. **Methods:** Quantitative autoradiography, immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR) were used to detect motilin receptors in tissue of 25 IBD patients (13 Crohn's disease (CD), 12 ulcerative colitis (UC)) and 19 patients with a neoplasm (controls). **Results:** Median muscular motilin binding was 3 and 8 fmol/g tissue in colon and ileum, respectively. In the gastroduodenal region the median was higher (93 fmol/g). In UC colonic muscular motilin binding was significantly increased compared to controls (7 versus 3 fmol/g, $P \leq 0.05$). Expression in CD was similar to controls. Besides the binding found in the muscular compartment, motilin binding was also found in the mucosa, which was even higher than in the muscle (3 versus 11 and 8 versus 27 fmol/g for colon and ileum ($P \leq 0.06$), respectively). RT-PCR and immunohistochemistry confirmed mucosal motilin receptor expression. The mucosal motilin receptors were located in the epithelial cells. In the muscular compartment receptors were strongly present in the myenteric plexus and weakly in the smooth muscle cells. In IBD tissue the expression pattern was not different. **Conclusions:** The motilin receptor is expressed in human colonic and ileal smooth muscle. Further, motilin receptor expression was also shown in the mucosa. Muscular binding in UC patients is increased but no different expression pattern was found.

# Introduction

Motilin, a 22 amino-acid biological active peptide, is released by endocrine cells of the upper gastrointestinal mucosa. The 1- to 2-hour interval increases in plasma motilin levels are synchronized with phase III myoelectric contraction of the stomach and intestine [1] and it is now well accepted that motilin is involved in initiating the interdigestive motor complex. Studies with intravenous infusion of motilin in humans showed that motilin induces increased antrum contraction frequency [2,3]. Motilin receptor location studies, to obtain more information about possible functions of motilin are mainly performed in animals. These studies showed some interspecies differences. In the rabbit, the most frequently studied animal, receptors for motilin are found in the antrum, small and large intestine with exception of the cecum. Most receptors were found in colon followed by antrum from where an aborally decreasing gradient was seen over the small intestine [4-7]. Studies in antrum and colon to test if the motilin receptor expression is mostly located on nerve or muscle cells are contradictory [4,5,8-10]. In the small intestine receptors are only found in the smooth muscle fraction. In cat and guinea pig, in contrast with the results in rabbit, no receptors are found in the colon but antrum and small intestine do express binding sites [11-13] and motilin receptors are clearly present in the myenteric plexus of guinea pig ileum [14]. Only a few articles described studies on motilin binding sites in humans. The highest frequency was found in nerve fractions of antrum, but also binding sites in duodenum and colon were detected while there were no receptors demonstrated in ileum and jejunum [15-17]. Due to the species differences it is important that more studies are performed on motilin receptor expression in human intestine. A few years ago an orphan human G-protein receptor (GPR38-A) was identified as a motilin receptor [18] and more techniques became available to detect this motilin receptor. GPR38-A mRNA was detected in enteric neurons of human ileum and colon [18].

Many gastrointestinal hormones also act as a neuropeptide and the presence of the motilin receptor in nerve fractions and in enteric neurons indicates that this could also be the case for motilin. From other neuropeptides in the gastrointestinal tract it is known that they have a function in the regulation of the inflammatory process. For example substance P has been proven to be an attractant for

macrophages and its receptor expression is increased in the inflamed intestine [19]. In rabbit there have been some preliminary studies to the role of motilin in the inflammatory process. It was shown that in a TNBS colitis model the motilin receptor expression in smooth muscle was decreased and that there was less contractility in response to motilin administration [20,21]. They also showed that interleukin-11 could reverse these effects. The aims of our study were to describe the distribution of the motilin receptor in the human lower gastrointestinal tract (ileum and colon), and to see if in tissue of patients with inflammatory bowel disease (IBD) motilin receptor expression is changed as compared with normal controls. Here 3 different techniques where used i.e. quantitative autoradiography, immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR).

# Material and methods

## Tissue samples

Colonic or ileal tissue samples were collected at the Leiden University Medical Centre, the Netherlands. Twenty-five IBD patients (13 Crohn's disease (CD), 12 ulcerative colitis (UC); mean age 39), were included in our study and tissue was taken from both inflamed as noninflamed areas (table 1). Eighty-two percent of the IBD patients used anti-inflammatory drugs; the type of drugs is specified in table 2. As controls, macroscopically normal intestine was taken at least 10 cm from the affected site of 19 patients (mean age 58) with colonic neoplasm's. None of the control patients used anti-inflammatory drugs. Mucosa was snap frozen in isopentane on dry ice; full thickness tissue samples were embedded in Tissue-Tek® O.C.T. compound and frozen on dry ice. A further 2 antrum samples and 3 duodenal samples were collected as positive controls. Tissue was stored at -80°C until use.

**Table 1. Patients Characteristics**

| Group | n | age in years | sex M | F | tissue location ileum | colon | inflam-mation yes | no |
|---|---|---|---|---|---|---|---|---|
| **Controls** | 19 | 34-74 (mean 58) | 10 | 9 | 8 | 12 | - | 20 |
| **Crohn's disease** | 13 | 18-73 (mean 37) | 3 | 10 | 9 | 8 | 10 | 7 |
| **Ulcerative colitis** | 12 | 19-72 (mean 39) | 4 | 8 | - | 14 | 9 | 5 |

**Table 2.  Type of Anti-inflammatory Drugs Used by the Patients**

| Group | Controls | Crohn's disease | Ulcerative colitis |
|---|---|---|---|
| **none** | 19 | 1 | 1 |
| **Corticosteroids** | 0 | 2 | 2 |
| **Corticosteroids + 5- aminosalicylicacid** | 0 | 6 | 5 |
| **Corticosteroids + 5- aminosalicylicacid + immunosuppressors** | 0 | 2 | 2 |
| **Corticosteroids + immunosuppressors** | 0 | 0 | 1 |
| **5-aminosalicylic acid** | 0 | 1 | 0 |
| **Immunosuppressors** | 0 | 0 | 1 |

## MPO-assay

The extent of neutrophil infiltration was quantified by measuring myeloperoxidase activity (MPO) to confirm the macroscopic classification of inflammation and to grade the inflammation. Tissue was homogenized and 25 µl of the homogenate was used in an assay described by Krawisz *et al.* [22] to detect MPO activity. The reaction kinetics was followed for 30 minutes and a sample of human polymorphonuclear neutrophils was used for standardization. MPO activity is expressed in arbitrary units.

## Peptide labelling

Motilin (Bachem AG, Switzerland) was iodinated using the chloramine T oxidation method. Iodinated peptide was separated from unincorporated iodide by chromatography on a Sephadex-G50 column with Tris-HCL elution buffer (50 mM,

pH 8.0) [23]. The specific activity of $^{125}$I-motilin was estimated to be ≈2000 Ci/mmol.

## Storage phosphor autoradiography

Cryostat tissue sections (14 µm) were cut at -20°C mounted on gelatin-coated glass slides and stored overnight at -80°C. Binding of $^{125}$I-motilin to tissue sections was carried out by a modification of Sakai et al's protocol [5], which was optimized in our laboratory. In brief, slides were air dried for 30 min and preincubated in 50 mM Tris-HCl (pH 8.0) containing 0.05% PMSF and 0.4% BSA for 20 min. For total binding, slides were incubated with 50 mM Tris-HCl, 10 mM MgCl$_2$, 0.05% PMSF, 0.4% BSA and 600 pM $^{125}$I-motilin at pH 8.0 for 180 min. Alternate serial sections were incubated with addition of 1µM nonradioactive motilin to determine nonspecific binding. After incubation sections were washed five times for 5 min with 50 mM Tris-HCl pH 8.0 containing 0.05% PMSF and 0.4% BSA at 4°C. Washed slides were rapidly dried with a stream of cold air. Slides were placed in a storage phosphor cassette for 40 hours at room temperature. The latent image stored in the storage phosphor screen was visualized by laser scanning of the screen in PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). The data of the digitized image were processed with ImageQuant® software (Molecular Dynamics), the nonspecific binding image was subtracted from the total binding image to create the specific binding image. Slides with 10 µl drops of different concentrations of radiolabeled ligand were used for standardization. Binding is expressed as fmol/g tissue. Serial sections were stained with hematoxylin/eosin to distinguish between the smooth muscle and mucosa.

## Immunohistochemistry

Immunolocalization of motilin receptors was assessed by an indirect peroxidase-labeled antibody method [24] with a polyclonal antibody directed against motilin receptor GPR38-A in a subset of 8 control patients and 19 IBD (11 CD, 8 UC) patients. In brief, cryostat tissue sections (7 µm) were cut at -20°C and mounted on poly-L-lysine coated glass slides. After 50 min of air drying tissue was fixed in acetone (-20°C) for 10 min and then air dried for another 30 min. Next the tissue

was rinsed in Tris buffered saline (TBS, 0.05 M, pH 7.5) and treated for 1 min with 0.25% periodic acid in TBS to block endogenous peroxidase. After 2 wash steps with TBS the slides were incubated for 20 minutes with 1.5% normal rabbit serum (NRS) to block nonspecific binding. Excess serum was drained off and sections were incubated for 2 hours at room temperature with goat antihuman GPR38-A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), appropriately diluted (1:640) in TBS containing 1.5% NRS. The sections were rinsed thoroughly in TBS, and subsequently incubated with biotinylated rabbit anti-goat Ig (Dako, Glostrup, Denmark; 1:200 in TBS) and peroxidase-labeled streptavidin (Dako; 1:100 in TBS) for 30 minutes each. Sections were stained by incubation in 0.1 M acetate buffer (pH 5.2) containing 0.03% 3-amino-9-ethylcarbazole and 0.03% $H_2O_2$ for 10 minutes, resulting in a red staining. Finally, sections were lightly counterstained in Mayer's hematoxylin and mounted in Aquamount™ (BDH, Germany). To asses the specificity of the staining, control experiments where performed on serial sections in which the primary antibody was replaced by TBS or preabsorbed with the blocking peptide.

## RT-PCR

The expression of GPR38 mRNA was determined with a semiquantitative RT-PCR; β-actin mRNA expression was used for standardization of the amount of used cDNA. Total RNA from 8 control and 17 IBD (9 CD, 8 UC) mucosal tissue samples was isolated by phenol chloroform extraction of guanidinium isothiocynate lysates [25]. RNA of TE671 cells was used as positive control. With M-MLV reverse transcriptase (Invitrogen, La Jolla, CA, USA) and a random primer mix (Hoffmann-La Roche, Switzerland) cDNA was synthesized from 2 μg RNA. The obtained cDNA was serially diluted from 1:4 to 1:1024. The diluted cDNA served as a template for the PCR using REDTaq™ DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and primers for GPR38 (forward, CACGTTGGCAGAATCATTTAC; reverse, TCCCATCGTCTTCACGTTAG) or β-actin (forward, GGGTCAGAAG-GATTCCTATG; reverse, GGTCTCAAACATGATCTGGG) (Sigma-Genosys, UK). Following amplification programs were used 1 minute 95℃; 1 minute 53℃; 1 minute 72℃ 36 cycles and 30 seconds 94℃; 45 seconds 56℃; 1 minute 72℃ 30

cycles for GPR38 and β-actin respectively. PCR fragments were loaded on a 1.5% agarose gel and after electrophoresis DNA was visualized with ethidium bromide under UV light and a digital picture was made. Intensity of the bands was measured with Scion (Washington D.C., USA) imaging software and plotted against starting amount of cDNA on log scale. Ratios of the integrated optical density per µg cDNA between samples and positive control were calculated.

## Statistics

Data is expressed as median values with confidence intervals. For statistical assessment of differences between groups, Wilcoxon signed ranks test and a Mann-Whitney U-test were used for paired and unpaired data respectively. Values of $P < 0.05$ were considered significant.

# Results

## Motilin receptors in normal human gastrointestinal tract

### Quantification of motilin receptor expression

With autoradiography the median motilin binding found in the colonic and ileal smooth muscle was 3 and 8 fmol/g tissue, respectively (table 3). This is lower ($P < 0.05$) than the amount of binding sites present in the human gastroduodenal region (93 fmol/g tissue; range 90-167) which was used as positive control. Besides binding in the muscular compartment, specific motilin binding was found in intestinal mucosa (table 3). In colon and ileum the motilin receptor expression in the mucosa was higher than in the smooth muscle (colon: 11 vs. 3 fmol/g, p≤0.06; ileum: 27 vs. 8  fmol/g, P≤0.05) (table 3), while in the gastroduodenal region mucosal binding tended to be lower than the binding found in smooth muscle (44 vs. 93 fmol/g). Figure 1 shows representative pictures of the location and intensity of motilin binding in normal human colon and ileum.

**Table 3. Quantity of $^{125}$I-motilin Binding (fmol/g tissue) in Control Human Intestine as Measured by Autoradiography**

| | mucosa | | | muscle | | |
|---|---|---|---|---|---|---|
| | median | CI | n | median | CI | n |
| **Gastroduodenal region** | 44 | 3-100 | 3 | 93 | 8-225 | 3 |
| **Ileum** | 27 | 9-48 | 8 | 8* ╞ | 4-14 | 8 |
| **Colon** | 11 | 4-36 | 11 | 3* | 2-7 | 12 |

* P<0.05 versus antrum and duodenum, ╞ P<0.05 vs. paired mucosa, CI = 95% confidence interval, n = number of tested tissue samples
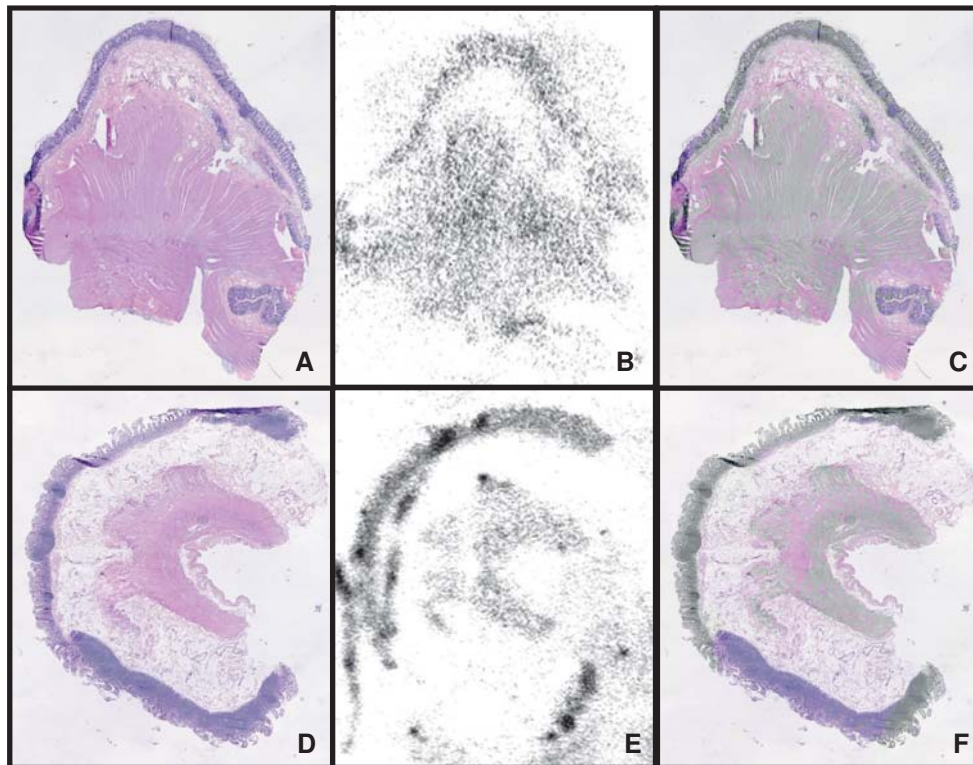


**Figure 1** $^{125}$I-motilin Binding to Control Colon (A-C) and Ileum (D-F).

A, D: The eosin/haematoxylin staining. B, E: Specific binding of $^{125}$I-motilin on the serial sections, the intensity of the greyscale equals the amount of binding sites. C, F: The precise location of motilin-binding is shown by merging the binding image with the eosin/haematoxylin staining, which gives a qualitative result.

**Localization of motilin receptor GPR38-A**

The result of motilin binding in the mucosa was confirmed by immunohistochemistry with an antibody against the GPR38-A receptor. In addition to the manufacturer's assurance of specificity of the antibody for the human GPR28-A receptor, control experiments in which the antibody was preabsorbed with the blocking peptide confirmed the specificity of the antibody. The staining in smooth muscle and myenteric plexus were completely blocked and the pronounced staining in the mucosa was reduced by half. Control sections in which the primary antibody was replaced by TBS where all negative. It was shown that the motilin receptor was expressed in the epithelial cells of the mucosa and there was a difference between colon and ileum in the fact that in the ileum the top of the villi were negative for the motilin receptor and the crypts stained positive while in colon all epithelial cells stained positive (figure 2). In mucosa of antrum also all epithelial cells stained positive while in duodenum a comparable gradient was seen as in ileum. The smooth muscle of all parts of the intestine stained weakly positive, with staining in the antrum being strongest and in ileum weakest. Within the smooth muscle the expression of the motilin receptor was clearly positive in the myenteric plexus in all parts of the gastrointestinal tract and this staining was stronger than that in the surrounding muscle cells (figure 3).
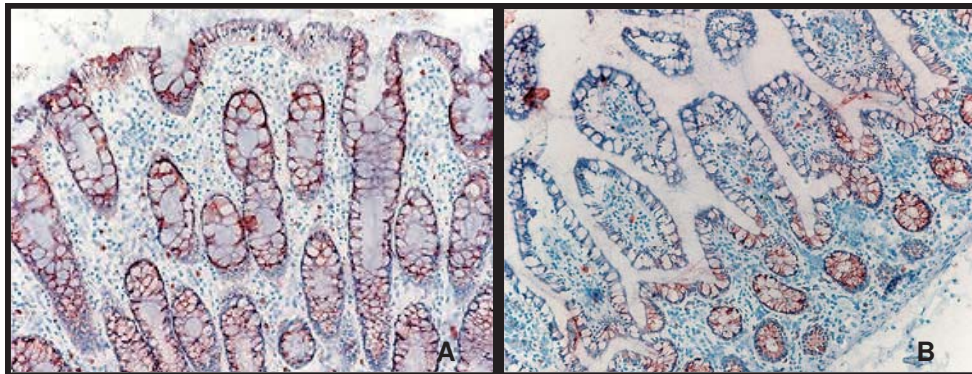


**Figure 2. Immunohistochemical Staining (brown/red) for GPR38-A in Control Colonic (A) and Ileal (B) Mucosa.** Note that the epithelial expression in ileum is only present in the crypts.
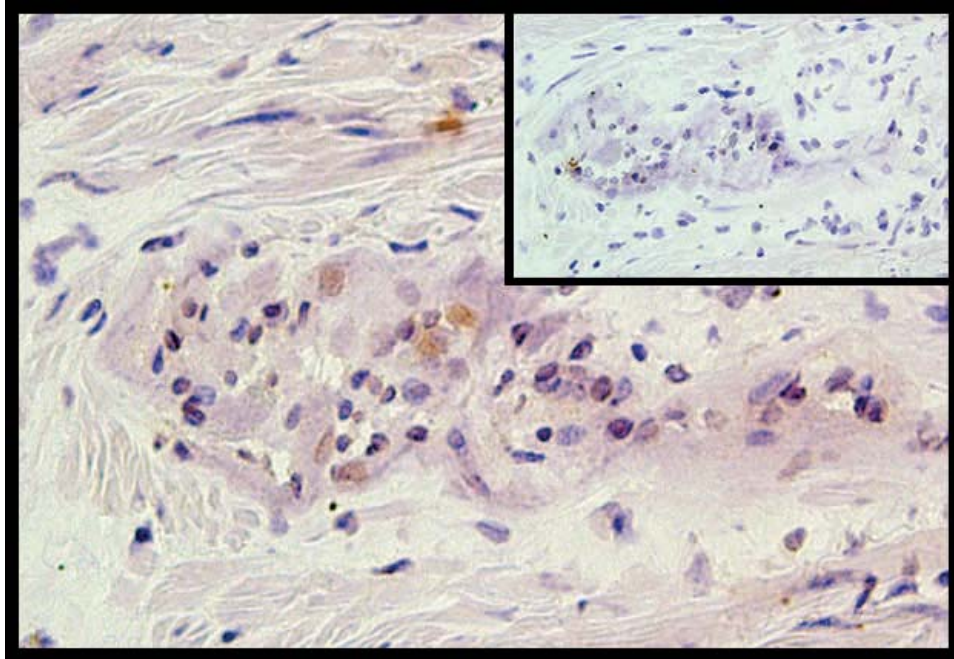
**Figure 3. Immunohistochemical Staining (brown/red) for GPR38-A in Control Colonic Myenteric Plexus.** The corresponding control where the primary antibody was preabsorbed with the blocking peptide is inserted.

### GPR38 mRNA expression

RT-PCR of RNA extracted from the mucosal intestinal tissue samples showed that mRNA of the motilin receptor GPR38 was in detectable levels present in the mucosa of antrum, duodenum, ileum, and colon (figure 4).

## Motilin receptor expression in IBD

There was a 20-year age difference between controls and IBD patients. However, we found no correlation between age and motilin receptor expression in the control group, indicating that age does not influence the motilin receptor expression. The MPO assay showed that the classification in macroscopically inflamed and non-inflamed areas correlated well with the degree of neutrophil infiltration (r = 0.42, P=0.009). MPO values in the inflamed IBD group were significantly higher than
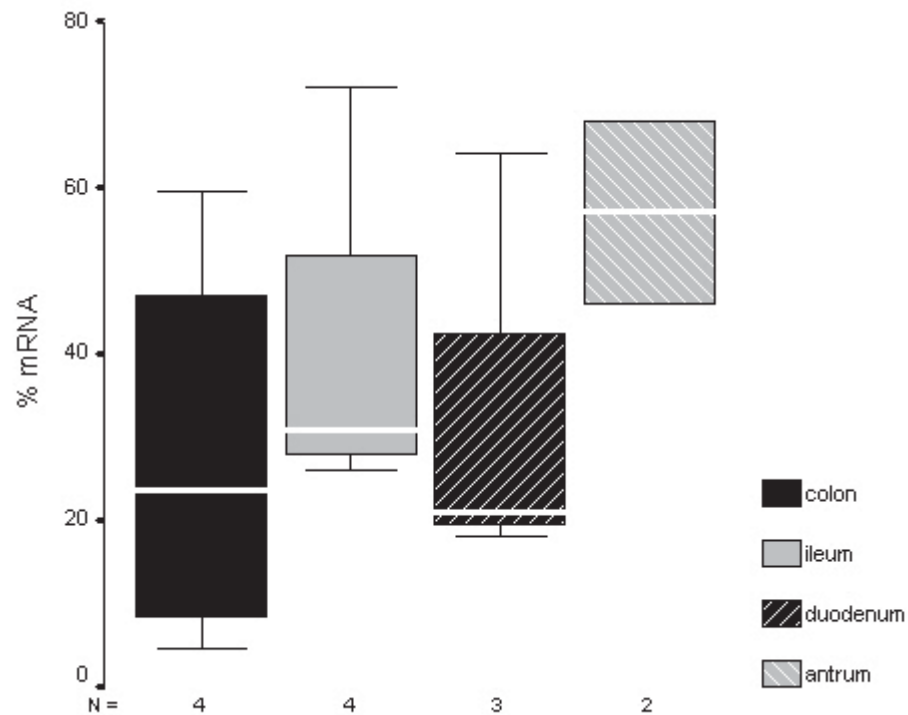
**Figure 4. GPR38 mRNA Expression in Control Human Intestinal Mucosa.** Data is expressed as a ratio of the amount of mRNA expression in mucosa and TE671 cell line. Data are corrected for β-actin expression. The white line represents the median value and the box 50% of the values.

those in the non-inflamed IBD group (10.0±1.1 versus 5.6±1.8 U/mg tissue; P<0.05). Neutrophil infiltration in control tissue was comparable to the non-inflamed IBD group (5.8±0.7 versus 5.6±1.8 U/mg tissue). In UC colonic muscular motilin binding was higher versus controls and lower versus adjacent mucosa (7 versus 3, 7 versus 19 fmol/g, P≤0.05). There was no correlation with the presence/intensity or absence of inflammation. There were no differences in the amount of motilin binding in CD patients compared to controls. In both, the motilin binding was lower in colonic and ileal smooth muscle than adjacent mucosa (colon: 4 versus 9, ileum: 9 versus 23 fmol/g, P≤0.02), with the binding in ileal smooth muscle being significantly higher (P≤0.01) than that in colonic smooth muscle (figure 5). In patients with IBD immunohistochemistry showed that there was no difference in the

localization of the motilin receptor when compared with the control patients. Also RT-PCR showed no differences in GPR38 mRNA expression in mucosal tissue In samples of CD, UC or control patients (figure 6 and 7).
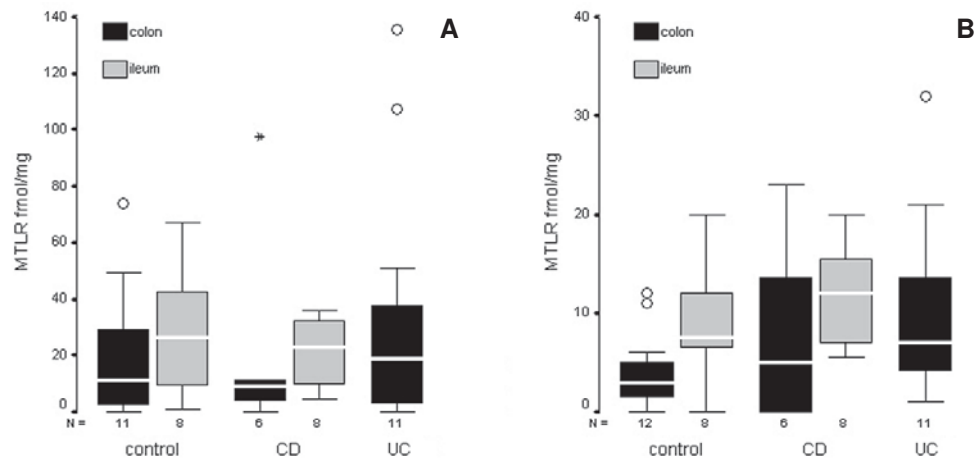


**Figure 5. Quantity of $^{125}$I-motilin Binding (fmol/g tissue) in Mucosa (A) and Smooth Muscle (B) of Colon and Ileum of IBD Patients and Controls as Measured by Autoradiography.** The white line represents the median value and the box 50% of the values.

**Figure 6. GPR38 mRNA Expression in Colonic and Ileal Mucosa of IBD Patients and Controls.** The first lane shows a marker, lane 2 until 7 colonic mucosal tissue and lane 8 till 11 ileal mucosa tissue from
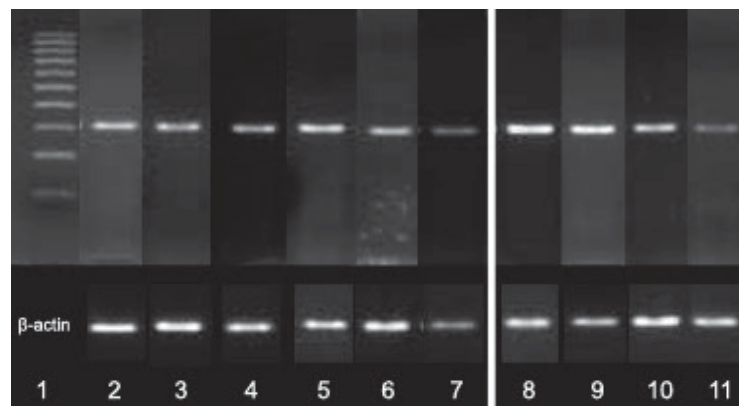


controls (2,3,8,9) Crohn's disease patients (4,5,10,11) and ulcerative colitis patients (6,7) respectively. The upper band is GPR38 mRNA (294 kb) and underneath the corresponding β-actin band is inserted.
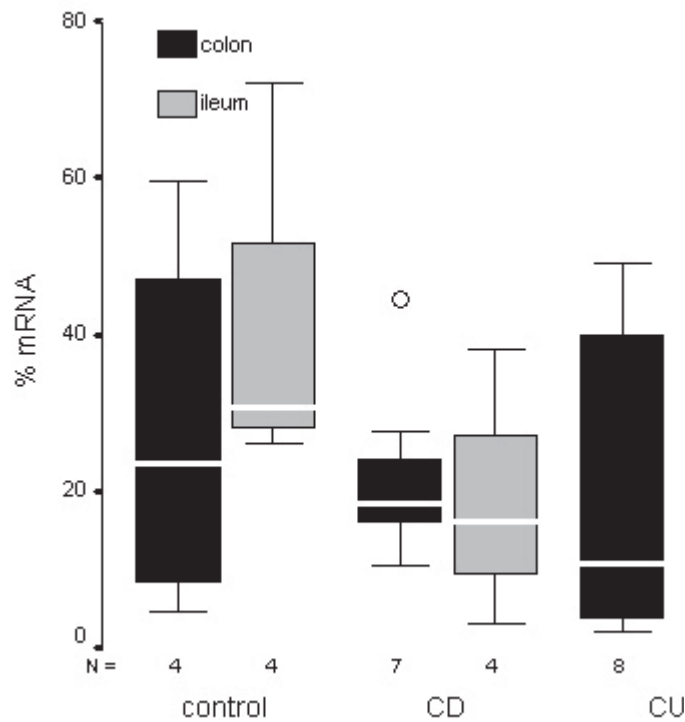
**Figure 7. GPR38 mRNA expression in colonic and ileal mucosa of IBD patients and controls.** Values are a ratio of the amount of mRNA expression in TE671 cell line. Data are corrected for β-actin expression. The white line represents the median value and the box 50% of the values.

# Discussion

Motilin is a peptide that is involved in the intestinal motility but the distribution of the receptor through which motilin exerts its effect is not well know for humans. This study describes the motilin receptor expression in humans as measured by 3 different techniques. The most commonly known function of motilin is the regulation of upper gastrointestinal motility, and indeed most binding sites for motilin are found in the smooth muscle compartment of antrum and duodenum. But this is not the only place were motilin receptors are expressed; we showed that in a lesser extent motilin binding sites are present in colonic and ileal muscular compartment. Earlier, Feighner *et al.* already showed mRNA expression in enteric neurons of colon and ileum [18]. We now show that beside mRNA also the receptor itself with autoradiography and immunohistochemistry. The presence of motilin receptors in colon and ileum smooth muscle and myenteric plexus indicates that motilin beside

its regulatory function in the upper gastrointestinal tract can also be involved in the regulation of lower gastrointestinal motility. Motilin agonists are now used therapeutically to accelerate gastric emptying but the finding of receptors in the lower gastrointestinal tract gives the possibility to study if these compounds are also useful in diseases as slow transit obstipation where the colonic motility is disturbed.

Besides its presence in the smooth muscle the motilin receptor was also expressed in the human intestinal mucosa. Most studies that examined the location of the motilin receptor in humans discarded the mucosa from the smooth muscle. We found that the amount of receptors in the mucosa varies along the gastrointestinal tract. Highest concentrations were seen in duodenum and antrum and lowest in colon. It is not known yet what the function of motilin receptors is in this intestinal compartment. In ileal and duodenal mucosa it was seen that only the crypts stained positive while the top of the villi were negative, while in colon and antrum all epithelial cells stained positive. It could be that motilin plays a role in epithelial secretion and in other epithelial functions. The fact that beside muscular motilin receptor expression the receptor was also in relatively high quantities present in the mucosa of the intestinal tract suggests that motilin receptors might have some other functions apart from its role in the regulation of motility.

Beside motilin receptor expression in normal human intestine we also studied the expression in the intestine of IBD patients. In recent years motilin has been more acknowledged as a neuropeptide and studies in the past have shown that the expression of some neuropeptide receptors are changed in IBD [26]. In experimental colitis studies in rabbits the contractility in response to motilin is decreased due to a decrease in motilin receptor density [20,27], but no studies were done in human intestine. Furthermore, location studies in normal intestine have already shown that there are pronounced interspecies differences. It is therefore important to obtain information about the motilin receptor expression and distribution in human inflamed intestine. This study shows a small increase of motilin receptors in the colonic smooth muscle of UC patients, but no major changes in the motilin receptor expression pattern were seen. There was no relation with the grade of inflammation. This result is in contrast with the above

mentioned study in rabbits, emphasizing the difference between rabbits and humans in case of neuropeptide receptor expression and the importance of such studies in humans.

Overall we can conclude that beside its role in the regulation of the upper gastrointestinal motility, motilin may have a role in the motility regulation of the lower intestinal tract since receptors for motilin are also expressed at that location. Besides the expression of motilin receptors in the smooth muscle, the receptors are also expressed in the mucosa as shown by 3 different techniques. Further studies have to be done to obtain more information about the precise role of the motilin receptors in the mucosa.

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