1 # The impact of inflammation on the
2 # expression of drug transporters and
3 # metabolic enzymes in colonic tissue from
4 # ulcerative colitis patients

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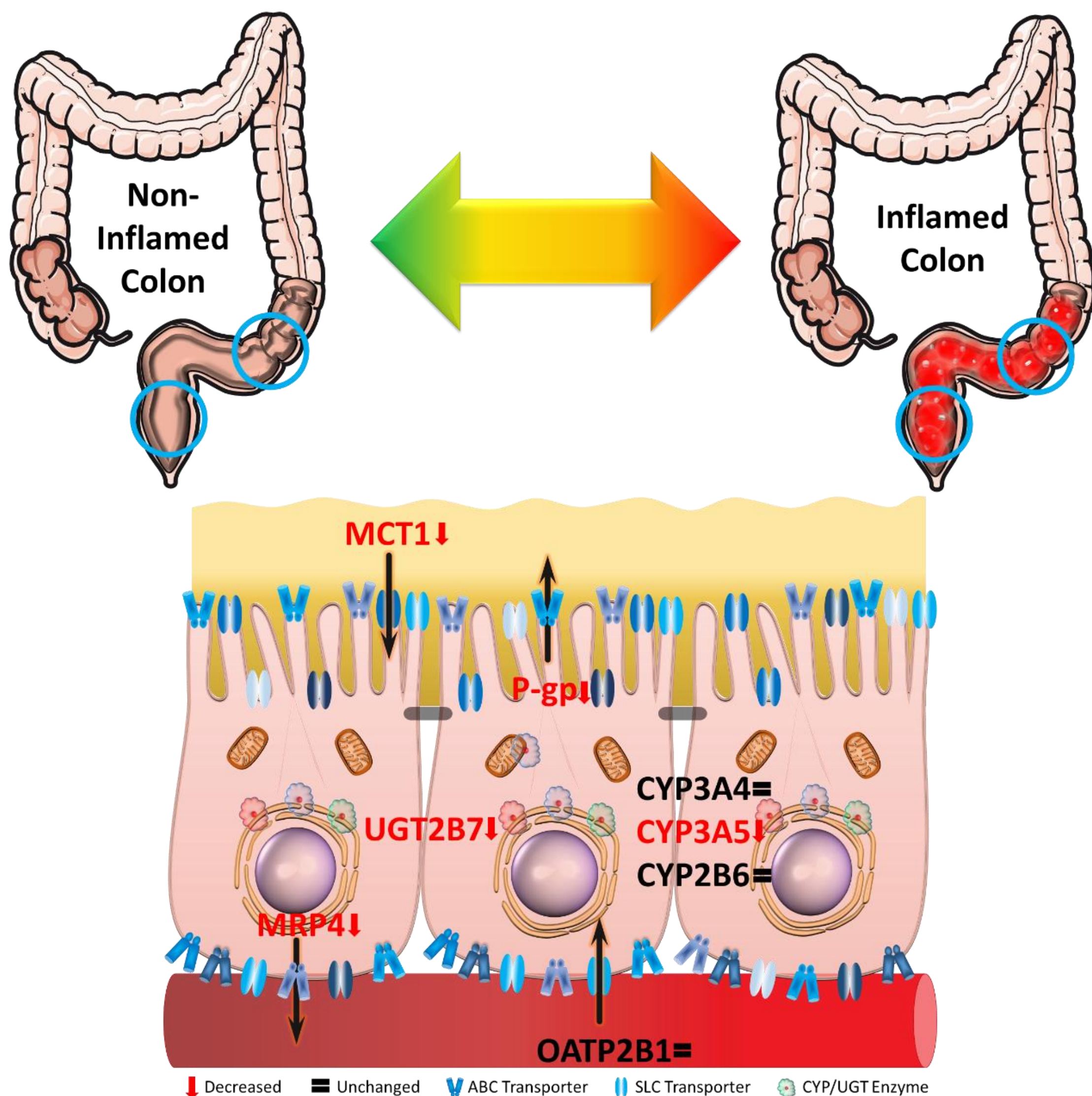

14 # Abstract

15 The intestinal tract forms an important barrier against xenobiotics while allowing nutrients to pass. In
16 ulcerative colitis (UC), a chronic inflammatory bowel disease, this barrier function is impaired leading
17 to an abnormal immune response and inflammation of the colonic mucosa. Transporter proteins and
18 metabolic enzymes are an integral part of the protective barrier in the gut and play an important role
19 in the disposition of nutrients toxins and oral drugs. In this study, the protein expression of 13
20 transporters and 13 enzymes was determined in the sigmoid and rectum of UC patients in endoscopic
21 remission and during active inflammation. In inflamed conditions (endoscopic Mayo sub-score 1, 2 or
22 3), a significant decrease ($q<0.05$) was observed in the median expression of the transporters P-gp
23 (0.046 vs. 0.529 fmol/µg protein), MRP4 (0.003 vs. 0.023 fmol/µg protein) and MCT1 (0.287 vs. 1.090
24 fmol/µg protein), and the enzymes CYP3A5 (0.031 vs. 0.046 fmol/µg protein) and UGT2B7 (0.083 vs
25 0.176 fmol/µg protein). Moreover, during severe inflammation, the decrease was even more
26 pronounced. Expression levels of other proteins were not altered during inflammation (e.g., OATP2B1,
27 CYP3A4, CYP2B6 and UGT2B15). The results suggest a decreased transport and metabolism of
28 xenobiotics in the colon of UC patients during active inflammation potentially altering local drug
29 concentrations and thus treatment outcome.

30

1. Introduction

The gastrointestinal epithelium forms a crucial barrier against environmental toxins, microbiota and xenobiotics including oral drugs. Vital components of this barrier are drug transporter proteins (DTPs) and drug metabolic enzymes (DMEs)[1]. Transporter proteins, categorized in the ATP binding cassette (ABC) and solute carrier (SLC) families, allow nutrients to enter the mucosa while excreting potentially harmful agents. Phase I and II metabolic enzymes within the cells transform nutrients and potential toxins into inactive forms and/or easier excretable substances. As these intestinal proteins affect xenobiotics, they may also have a pronounced effect on the bioavailability of orally administered drugs[2,3]. For this reason, a lot of effort has been made to explore the protein expression of DTPs and DMEs in the small intestine, the main site of drug absorption owing to its massive surface area[4–8]. Interest has expanded to the colon due to the rise of colon-targeted drug formulations to treat and prevent local colonic diseases such as inflammatory bowel diseases (IBD) and colorectal cancers[9]. Moreover, colon targeting formulations are being explored to enhance the absorption of peptides and proteins by circumventing the harsh environment of the stomach and the small intestine. Overall, the expression of DTPs and DMEs is markedly lower in the colon compared to the small intestine[5,10]. Despite their relatively limited expression, DTPs and DMEs may affect the colonic tissue concentrations of drugs treating, for instance, IBD[11–14]. Knowing the abundance of DTPs and DMEs in the colon is important to predict the efficacy of locally acting drugs, and recent advances in protein analysis allow to determine these low abundant proteins.

Dysregulation of the colonic barrier function has been associated with various disease states[1]. A popular hypothesis in IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is an abnormal inflammatory response triggered by environmental factors and the microbiome in genetically predisposed individuals[15,16]. Increased permeability and reduced detoxification may lay at the basis of this immune reaction and could be caused by a decreased expression of DTPs and DMEs[17–21]. Furthermore, recent studies implicate the microbiome, which is impaired in UC patients, to have a modulating role in the expression of DTPs[20,22,23].

UC is characterized by inflammation of the colon typically extending from the rectum and sigmoid to the more proximal colon[16]. In periods of remission, the inflammation in UC can temporarily subside; however, inflammation often reoccurs during so-called flare-ups. While the colonic barrier function and microbiome remain altered even in periods of remission, these changes become more apparent during active inflammation[18,19,24,25]. Inflammation in UC may range from mild friability with a decrease in vascular patterns and erythema to erosions, ulcers, and spontaneous bleeds[26]. Considering the possible link between inflammation and the expression of DTPs and DMEs, these different stages of remission and inflammation may cause variations in transport and metabolism of locally acting drugs,

65 potentially leading to suboptimal drug performance and/or unwanted side effects in UC patients[27,28].

66

67 The present study aimed to explore the effects of UC on the protein expression of both DTPs and DMEs
68 in colonic tissue during remission and different stages of inflammation. To this end, DTP and DME
69 protein expression was investigated in colonic tissue from the sigmoid and rectum of UC patients in
70 endoscopic remission (endoscopic Mayo sub-score 0) and varying stages of inflammation (endoscopic
71 Mayo sub-score 1, 2 and 3), utilizing a targeted LC-MS/MS approach. Data on the protein levels of DTPs
72 and DMEs in these patients may improve the prediction of local drug levels and guide dose regimens
73 in patients with different stages of inflammation. In addition, it could give new insights into the
74 potential role of DTPs and DMEs in the pathogenesis of IBDs. Furthermore, the expression levels may
75 improve physiologically based pharmacokinetic (PBPK) models that can steer the future development
76 of colon-targeted drug formulations[29].

77
2. Materials and methods

78
2.1 UC tissue biopsies

79 The clinical study was approved by the Institutional Review Board (Ethics Committee Research UZ/KU
80 Leuven) for research on IBD (VLECC registry B322201213950/S53684). Tissue biopsies were taken from
81 previously diagnosed UC patients (n=17) that gave written informed consent preceding the
82 colonoscopy. Patient demographics are summarized in Table 1 and detailed in Supplementary Table
83 S1. Colonoscopies were performed during check-ups on disease progression and treatment outcomes
84 at the University Hospitals Leuven, Belgium. Two to four biopsies were taken per region (i.e., sigmoid
85 and rectum) and immediately snap-frozen in liquid nitrogen and stored at -80 °C pending analysis.
86 Inflammation status was determined Using the Mayo score[30] during the colonoscopy by an endoscopist
87 with extensive experience in IBD endoscopy. In case of active inflammation, biopsies were taken from
88 the inflamed mucosa. The degree of inflammation varied from non-inflamed or macroscopically
89 normal tissue (Mayo 0), signs of mild inflammation with erythema and slightly decreased vascular
90 patterns (Mayo 1, mild disease), more pronounced erythema and decrease or absence of vascular
91 patterns and erosions (Mayo 2, moderate disease), and severe ulceration of the mucosa with
92 spontaneous bleeding (Mayo 3, severe disease). In some patients, inflammation scores differed
93 between regions (more frequent inflammation in the rectum compared to the sigmoid), implying that
94 for some patients, both non-inflamed (endoscopic remission, Mayo 0) and inflamed (Mayo 1,2 or 3)
95 tissue samples were analyzed. Of the 17 included patients, 9 patients had at least one inflammation-
96 free region, while 12 patients had active inflammation in at least one region.

97 *Table 1. Basic patient demographics*

Characteristics	Ulcerative Colitis			
Number of Patients	17			
Male/Female	4/13			
Median age (range)	40 (32 – 68)			
Mayo score	Mayo 0	Mayo 1	Mayo 2	Mayo 3
Number of Patients*	9	3	6	4
Individual samples analyzed	67**			

98 * Both rectal and sigmoid expression data were averaged, unless, both segments had a different Mayo score, in which case the segments
99 were averaged separately.
100 **A more detailed description of the samples can be found in Supplementary Table S1.

101 ## 2.2 Sample preparation

102 ### 2.2.1 Tissue lysis

103 Individual biopsies were placed in 0.5 mL homogenizing tubes filled with 1.4 mm ceramic beads and
104 100 µL lysis buffer (50 mM dithiothreitol (DTT), 2 w/v% sodium dodecyl sulfate (SDS) and 0.1 v/v%
105 protease inhibitor cocktail in 100 mM Tris buffer pH 7.8). The biopsies were homogenized (Precellys
106 24 tissue homogenizer, Bertin Instruments) in two subsequent 5-second cycles at 6500 rpm following
107 incubation at 95 °C for 5 minutes. The lysate was centrifuged for 5 minutes at 16000 g and the
108 supernatant was collected.

109 ### 2.2.2 Total protein and peptide concentrations

110 Total protein concentration was determined using the tryptophan fluorescence assay described by
111 Wiśniewski et al. (2015)[31]. In short, 2 µL of lysate was added to 200 µL 8M urea in 100 mM Tris pH 8.5.
112 Fluorescence (excitation 295 nm, emission 350 nm) was measured and compared to a calibration curve
113 of different tryptophan concentrations. For digested peptides, the sample was not diluted before
114 measurement. Total protein and peptide concentrations were calculated assuming the average
115 tryptophan content to be 1.17 %[31].

116 ### 2.2.3 Proteomic sample preparation before LC-MS/MS

117 Tissue lysates were processed according to the filter-aided sample preparation (FASP) protocol by
118 Wiśniewski et al.[32,33] with simultaneous digestion by endoproteinase Lys-C (Pierce™ MS grade,
119 ThermoFisher) and trypsin (Sequencing Grade Modified, Promega). In short, a volume of lysate
120 containing 120 µg total protein was mixed with 200 µL 1 mM DTT and 8 M urea in 100 mM Tris buffer
121 pH 8.5 in 30 KDa centrifugation filter units and centrifuged for 15 minutes at 10000 g. After discarding
122 the eluate, the filters were washed again with 200 µL of the same buffer and centrifuged for 15 minutes

123 at 10000 g. Following centrifugation, 100 µL 50 mM iodoacetamide in 50 mM Tris pH 8.5 with 8M urea
124 was added to the filter, protected from light and incubated at room temperature for 20 minutes. After
125 initial centrifugation for 10 minutes at 10000 g, filters were washed twice with 100 µL 8 M urea in 50
126 mM Tris pH 8.5 and trice with 100 µL 50 mM Tris pH 8.5 (DB). Proteins were digested in 60 µL DB
127 containing a mixture of endoproteinase LysC and trypsin in a 1:4 ratio. An enzyme-to-protein ratio of
128 1 to 40 was used for the digestion. Samples were digested by incubating for 16 hours in a humid
129 chamber at 37 °C. The peptides were collected by centrifugation for 10 minutes at 10000 g followed
130 by two consecutive elution steps with 100 µL 2.5 % formic acid (FA). Samples were evaporated using a
131 vacuum concentrator (GeneVac EZ-2 plus) before diluting to a final peptide concentration of 0.5 µg/µL
132 containing 5 nM stable isotope labeled (SIL) peptides as internal standard (IS).

133 2.3 Targeted proteomic analysis with LC-MS/MS

134 The peptide mixtures resulting from the sample preparation were analyzed by means of a targeted LC-
135 MS/MS-based proteomics approach to quantify the protein abundance of selected DTPs and DMEs
136 (Table 2). To ensure the clinical relevance of the investigated DTPs and DMEs, the selection included
137 the most abundant proteins in the small and large intestine, based on available protein and gene
138 expression data from organ donors or healthy individuals[4,5,34]. In addition, all selected DTPs and DMEs
139 have various drug substrates, inducers and inhibitors, indicating their potential to mediate drug-drug
140 interactions[35–37].

141 *Table 2. Overview of the studied proteins with corresponding genes.*

Transporters		Enzymes	
Protein	*Gene*	*Protein*	*Gene*
ASBT	*SLC10A2*	CYP1A2	*CYP1A2*
BCRP	*ABCG2*	CYP2B6	*CYP2B6*
MCT1	*SLC16A1*	CYP2C19	*CYP2C19*
MRP2	*ABCC2*	CYP2C8	*CYP2C8*
MRP3	*ABCC3*	CYP2C9	*CYP2C9*
MRP4	*ABCC4*	CYP2D6	*CYP2D6*
MRP6	*ABCC6*	CYP2E1	*CYP2E1*
OAT2	*SLC22A7*	CYP3A4	*CYP3A4*
OATP2B1	*SLCO2B1*	CYP3A5	*CYP3A5*
OCT1	*SLC22A1*	UGT1A1	*UGT1A1*
OCT3	*SLC22A3*	UGT1A3	*UGT1A3*
PEPT1	*SLC15A1*	UGT2B15	*UGT2B15*
P-gp	*ABCB1*	UGT2B7	*UGT2B7*

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143

144 Peptides were separated using a Waters ACQUITY H-Class UPLC system equipped with an ACQUITY
145 UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm). A separate method was used for the transporters and
146 enzymes, respectively. The mobile phase composition used in both methods consisted of a mixture of
147 water containing 0.1% formic acid (FA) as mobile phase A and acetonitrile containing 0.1% FA as mobile
148 phase B, following the gradients specified in Table 3. The flow rate was set at 0.55 mL/min.

149 *Table 3. LC gradient for transporters (left) and enzymes (right), (A) water, 0.1% FA and (B) acetonitrile, 0.1% FA*

Transporters			**Enzymes**		
Time (min)	*%A*	*%B*	*Time (min)*	*%A*	*%B*
0	98	2	0	98	2
2	98	2	2	98	2
3	92	8	4	90	10
7.5	82	18	11	83	17
16	73	27	15	65	35
17	10	90	16	10	90
17.8	10	90	16.8	10	90
18	98	2	17	98	2
19	98	2	19	98	2

150

151 Protein concentrations were determined by quantifying the surrogate peptides unique for the protein
152 (one per protein shown in Table 2 and further specified in Supplementary Table S2) using a QTRAP
153 6500 (AB Sciex) in positive ion mode with scheduled multiple reaction monitoring (MRM) of 78
154 transitions per run (three transitions per peptide). Data acquisition was performed with a target scan
155 time of 0.75 and 0.60 seconds and an MRM detection window of 45 and 60 seconds for transporters
156 and enzymes, respectively. Calibration curves (0.05 – 25 nM) and quality control samples (QC; 0.1, 1,
157 5 nM) were made from standard peptide mixtures to allow for protein quantification. Similar to the
158 samples, standards were spiked with SIL peptides as IS to a final concentration of 5 nM. Data were
159 processed using MultiQuant (Version 3.0.5373.0, AB Sciex). Protein concentrations were calculated by
160 the peak area ratio of the IS and sample peptide; peak areas represented the sum of the three peptide
161 transitions. The lower limit of detection (LOD) and quantification (LOQ) were determined by a signal-
162 to-noise (S/R) ratio ≥ 3 and ≥ 10, respectively, and are specified in Supplementary Table S2.

163 2.4 Data representation and statistics

164 Statistical analysis was performed with Graphpad Prism 9. Expression of the proteins under
165 investigation did not differ significantly between sigmoid and rectal tissue specimens (data not shown);
166 therefore, individual data points represent the average protein abundance per µg total protein of both
167 the rectal and sigmoid biopsies per patient. When the Mayo score differed between the sigmoid or
168 rectum of the same patient, the values were averaged for each segment separately (Table S1). For this

169 reason, a total of 22 (i.e., 9 non-inflamed and 13 inflamed) data points are represented from 17
170 patients (i.e., 5 patients with different inflammation between rectum and sigmoid). Expression levels
171 under the LOD or LOQ were allocated a value ($\frac{LOD}{\sqrt{2}}$ or $\frac{LOQ}{\sqrt{2}}$, respectively) to allow for nonparametric
172 statistical testing (Mann–Whitney U test and Kruskal-Wallis test). To account for multiple testing for
173 the different proteins, the Benjamini – Hochberg correction was applied; a False Discovery Rate (FDR)
174 corrected P value < 0.05 (q value) was considered statistically significant. Statistical testing was
175 performed between the non-inflamed (remission) and inflamed tissue specimens; the further division
176 in inflammation severity was not tested due to the limited number of patients in each subgroup.

177 3. Results

178 3.1. Drug transporters

179 3.1.1. ABC transporters

180 The ABC transporter proteins MRP4 and P-gp were quantifiable in the rectum and/or sigmoid tissue
181 biopsies of 7 (MRP4) and 16 (P-gp) out of the 17 included UC patients. The protein expression of MRP4
182 could only be quantified in non-inflamed tissue biopsies, with a median value of 0.023 fmol/µg protein
183 (range 0.003 – 0.099 fmol/µg protein). In the inflamed tissue biopsies, MRP4 expression was almost
184 completely abolished with no detectable amounts in any of the biopsies except for one (Figure 1A).
185 The inflammation-related decrease in MRP4 expression was statistically significant (q = 0.0011), but
186 independent of the inflammation intensity, as a complete lack of expression was already observed in
187 patients with Mayo score 1 (Figure 2A).
188 Although the apical efflux transporter P-gp could be detected in both inflamed and non-inflamed
189 tissue, its expression decreased significantly in tissue with active inflammation (non-inflamed: 0.529
190 (0.051 – 0.872) fmol/µg protein versus inflamed: 0.046 (0.003 – 0.363) fmol/µg protein; q = 0.0086)
191 (Figure 1B). Moreover, the decreased P-gp protein expression became more pronounced with
192 increasing severity of inflammation (Figure 2B).
193 Three other ABC transporter proteins (MRP3, MRP6 and BCRP) were only detected in 8, 10, and 4 out
194 of the 71 tissue biopsies, respectively. MRP2 was not detected in any of the samples.

195 3.1.2 SLC transporters

196 The expression of the SLC transporter MCT1 significantly decreased in inflamed tissue (non-inflamed:
197 1.090 (0.507 – 2.490) fmol/µg protein versus inflamed: 0.287 (0.005 – 1.737) fmol/µg protein; q =
198 0.0043) (Figure 1C). As for P-gp, the decrease became slightly more pronounced with an increasing
199 Mayo score (Figure 2C).
200 Inflammation did not significantly affect the protein expression of OATP2B1 in sigmoid and rectum

201 biopsies, even though a small numeric decrease in median expression was observed (non-inflamed:
202 0.196 (0.106 – 0.401) fmol/µg protein versus inflamed: 0.166 (0.081 – 0.254) fmol/µg protein; q = 0.16)
203 (Figure 1D and 2D). In tissues with severe inflammation (Mayo score 3), the decrease in expression
204 seemed more pronounced (Figure 2D). The other five analyzed SLC transporter proteins (PEPT1,
205 OCT1, OCT3, ASBT and OAT2) were not detected in any of the rectal or sigmoid tissue biopsies,
206 irrespective of the inflammation state.

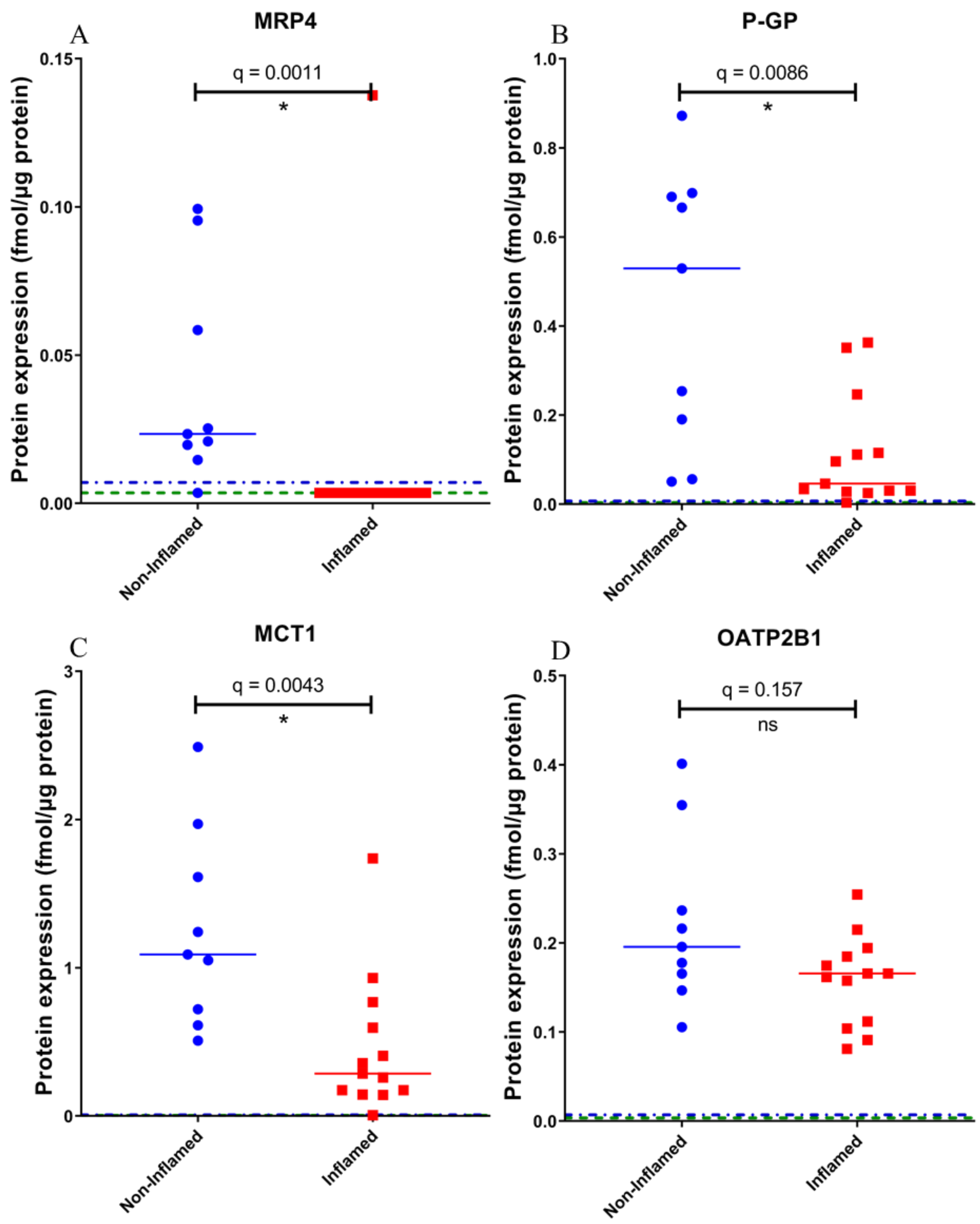

Figure 1. Transporter protein expression in non-inflamed and inflamed tissue biopsies from UC patients. (A) MRP4, (B) P-gp, (C) MCT1 and (D) OATP2B1. Individual data points represent the average of rectal and sigmoid biopsies per patient; the full lines represent the median over all patients. Expression levels below the LOD or LOQ were allocated an arbitrary value (LOD/√2 (dotted green line ---) or LOQ/√2 (dotted blue line ·-·), respectively) to allow statistical testing.

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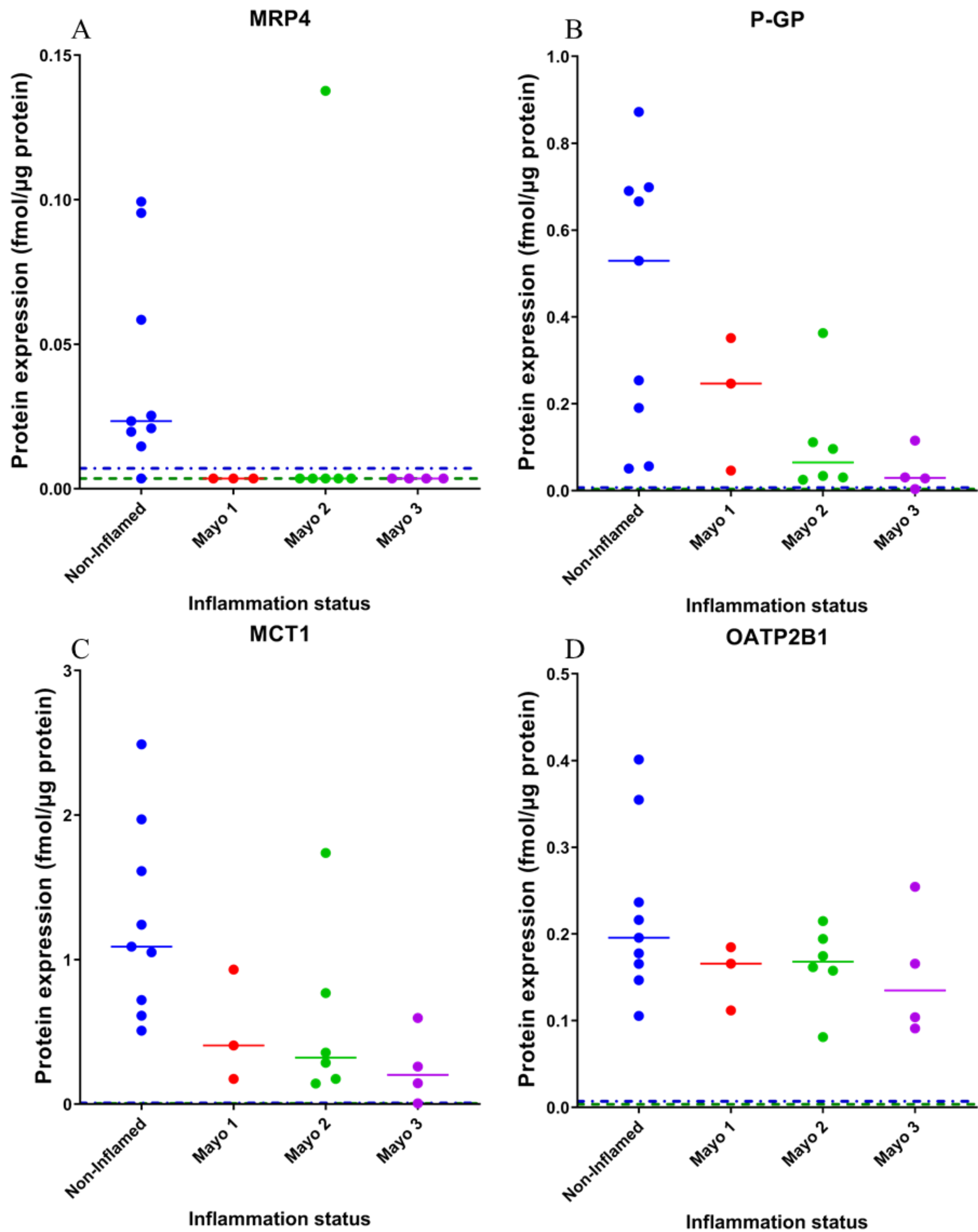

Figure 2. Transporter protein expression in tissue biopsies from UC patients with different severity of inflammation, as expressed by the endoscopic Mayo sub-score (Mayo 1: mild inflammation, Mayo 2: moderate inflammation, Mayo 3: severe inflammation). (A) MRP4, (B) P-gp, (C) MCT1 and (D) OATP2B1. Individual data points represent the average of rectal and sigmoid biopsies per patient; the full lines represent the median over all patients. Expression levels below the LOD or LOQ were allocated an arbitrary value (LOD/√2 (dotted green line ---) or LOQ/√2 (dotted blue line ·-·), respectively) to allow statistical testing.

209

210 ## 3.1 Metabolic enzymes

211 ### 3.2.1. Phase I metabolic enzymes

212 Three phase I metabolic enzymes (CYP3A4, CYP3A5 and CYP2B6) were quantifiable in the rectum
213 and/or sigmoid tissue of all included UC patients. The protein expression of CYP3A4 did not differ
214 between inflamed and non-inflamed tissue (non-inflamed: 0.091 (0.053 – 0.152) fmol/µg protein
215 versus inflamed: 0.081 (0.055 – 0.217) fmol/µg protein; q = 0.65) (Figure 3A). Within the inflamed
216 tissue specimens, similar expression levels were seen for all Mayo scores (Figure 4A).
217 In contrast, CYP3A5, belonging to the same subfamily with similar substrate selectivity as CYP3A4, had
218 a significantly lower protein expression in inflamed tissue (non-inflamed: 0.046 (0.030 – 0.053) fmol/µg
219 protein versus inflamed: 0.031 (0.016 – 0.048) fmol/µg protein; q = 0.0019) (Figure 3B). Moreover, this
220 decrease appeared slightly more pronounced with increasing severity of inflammation (Figure 4B).
221
222 Another phase I enzyme, CYP2B6, did not show a significant difference in the expression in inflamed
223 compared to non-inflamed tissue (non-inflamed: 0.194 (0.144 – 0.330) fmol/µg protein versus
224 inflamed: 0.16 (0.057 – 0.290) fmol/µg protein; q = 0.072) (figure 3C). Although not statistically
225 significant, a slight decrease was observed in the inflamed tissue specimens, which seemed
226 independent of the inflammation severity (figure 4C).

227 Three enzymes (CYP2C8, CYP2C9, and CYP1A2) were only sporadically detected in the samples (in 16,
228 4 and 14 out of 71 tissue biopsies, respectively). One enzyme (CYP2C19) was detected in almost all
229 samples but could not be quantified due to a high LOQ for this protein. The high LOQ could be
230 attributed to the absence of 2 out of 3 selected transitions for the surrogate peptide in the sub 1nM
231 range. The last phase I metabolic enzyme (CYP2E1) could not be detected in any of the rectal and
232 sigmoid biopsies.

233 ### 3.2.2 Phase II metabolic enzymes

234 In addition to the phase I enzymes, two phase II enzymes (UGT2B7 and UGT2B15) could be quantified
235 in all UC patients. The expression levels of UGT2B7 were significantly lower in inflamed tissue
236 compared to non-inflamed tissue (non-inflamed: 0.176 (0.101 – 0.311) fmol/µg protein versus
237 inflamed 0.083 (0.003 – 0.166) fmol/µg protein; q = 0.0004) (Figure 3D). Although this drop was already
238 apparent in tissue samples with mild to moderate inflammation (Mayo 1 and 2), a slightly more
239 pronounced effect was visible in severely inflamed tissue specimens (Mayo 3) (Figure 4D).

240 The expression of UGT2B15 was slightly lower in inflamed compared to non-inflamed biopsies.
241 However, this decrease was not significant (non-inflamed: 0.041 (0.033 – 0.062) fmol/µg protein versus
242 inflamed: 0.036 (0.030 – 0.053) fmol/µg protein; q = 0.088) (Figure 3E). No trend was observed

243 between the inflammation intensity and the decrease in UGT2B15 expression (Figure 4E).
244 Finally, UGT1A1 could only be detected in 8 out of 71 tissue biopsies, and UGT1A3 was not detected
245 in any of the samples.

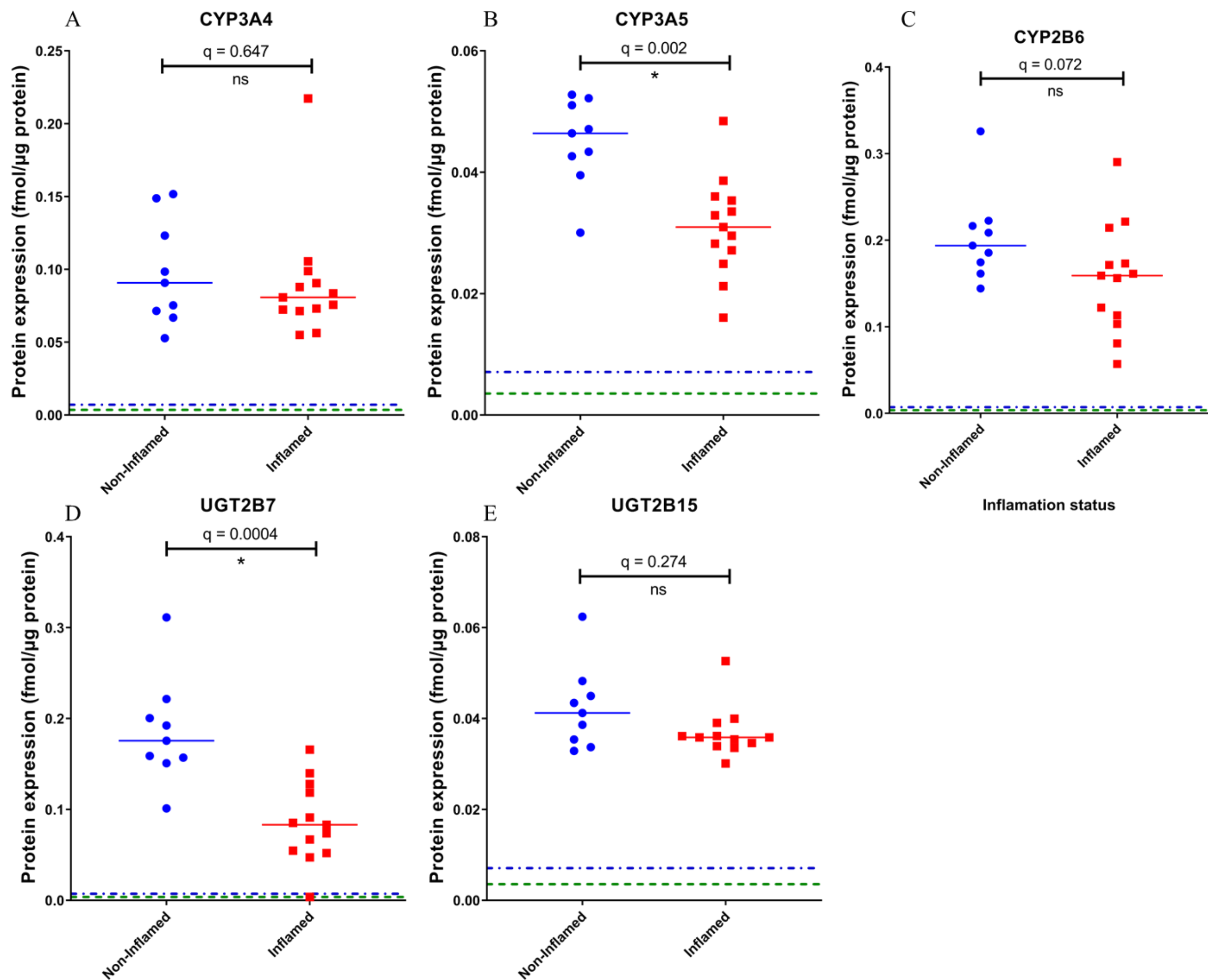

Figure 3. Metabolic enzyme expression in non-inflamed and inflamed tissue biopsies from UC patients. (A) CYP3A4, (B) CYP3A5, (C) CYP2B6, (D) UGT2B7 and (E) UGT2B15. Individual data points represent the average of rectal and sigmoid biopsies per patient; the full lines represent the median over all patients. Expression levels below the LOD or LOQ were allocated an arbitrary value (LOD/√2 (dotted green line ---) or LOQ/√2 (dotted blue line ·-·), respectively) to allow statistical testing.

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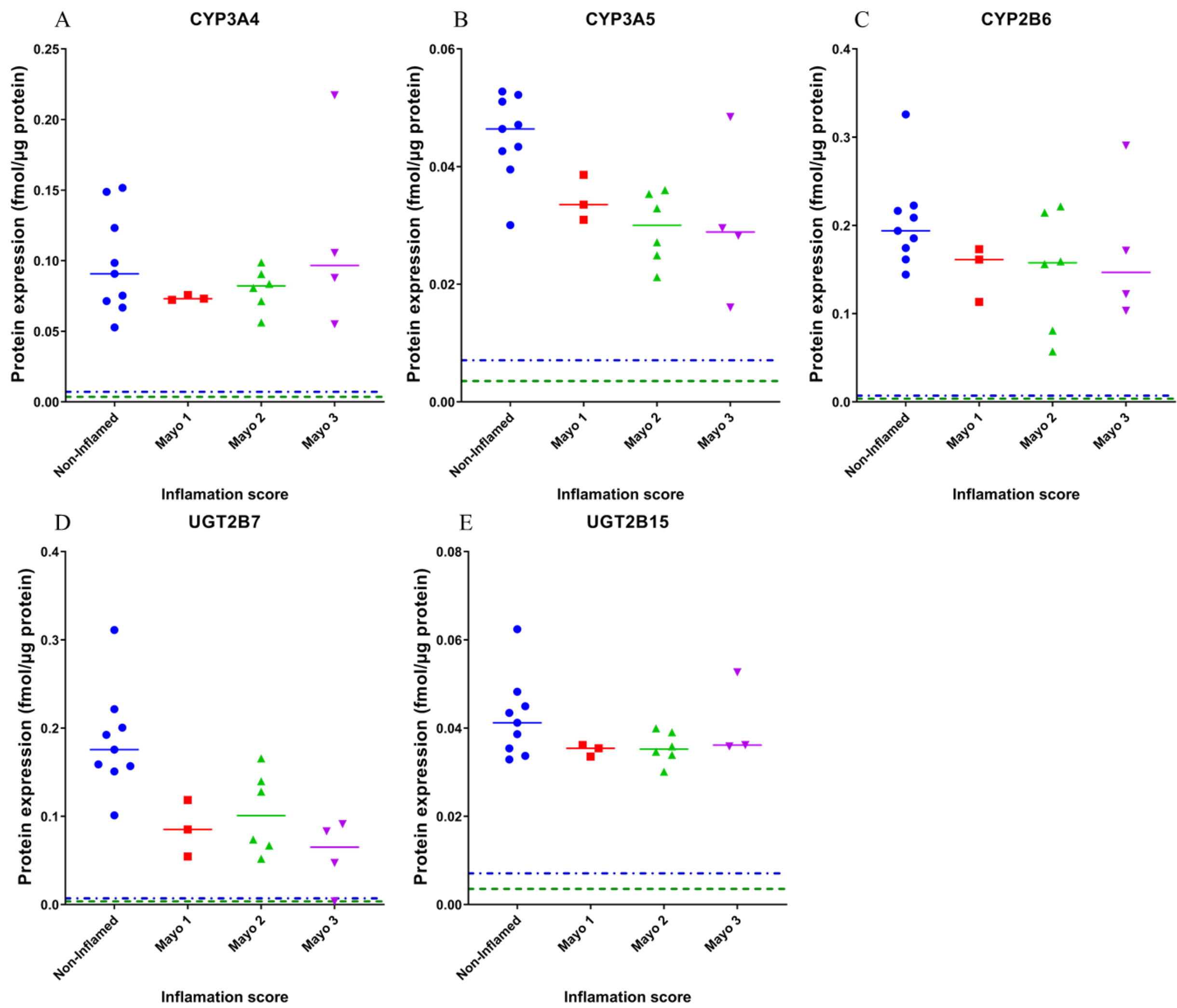

Figure 4. Metabolic enzyme expression in tissue biopsies from UC patients with different severity of inflammation, as expressed by the endoscopic Mayo sub-score (Mayo 1: mild inflammation, Mayo 2: moderate inflammation, Mayo 3: severe inflammation). (A) CYP3A4, (B) CYP3A5, (C) CYP2B6, (D) UGT2B7 and (E) UGT2B15. Individual data points represent the average of rectal and sigmoid biopsies per patient; the full lines represent the median over all patients. Expression levels below the LOD or LOQ were allocated an arbitrary value (LOD/√2 (dotted green line ---) or LOQ/√2 (dotted blue line ·-·), respectively) to allow statistical testing.

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4. Discussion

The available data on the protein expression of DTPs and DMEs in the colon of UC patients are limited. Most information is based on either mRNA data with limited correlation to actual protein expression, or semiquantitative Western Blot protein expression data[19,20,24,25,38]. Moreover, most of these studies focus on a limited number of DTPs or DMEs comparing healthy subjects with UC patients in remission or with active inflammation. Considering that the abundance of DTPs and DMEs in the colon is relatively low in comparison to the liver and small intestine, the adequate assessment of their protein expression is challenging. However, recent advances in LC-MS/MS-based proteomics now allow quantifying low abundant proteins, such as DTPs and DMEs in the colon. In this respect, a recent study by Erdmann et al.[25] investigated the protein expression of several DTPs using LC-MS/MS-based proteomics; for DMEs, only the mRNA expression was assessed. In that study, specimens of inflamed and non-inflamed tissue were collected from the same individual and compared. However, previous studies indicated that endoscopically healthy tissue in proximity to the inflammation site may also show structural abnormalities and altered P-gp expression[16,39]. The present study explored the impact of the inflammatory state on the abundance of 13 DTPs and 13 DMEs in the sigmoid and rectum of UC patients. To this end, the protein expression was compared between healthy tissue taken from UC patients in regions with endoscopic remission and inflamed tissue taken in regions with active inflammation. Moreover, the effect of the inflammation severity (evaluated with the endoscopic Mayo sub-score) on the expression of DTPs and DMEs was explored. Obviously, colonic drug disposition might be affected by other DTPs and DMEs, not assessed in the present study. Here, we focused on a selection of the most abundant DTPs and DMEs in the colon with known potential for drug-drug interactions.

Of the 13 DTPs analyzed, 4 were sufficiently abundant in the colon of UC patients to evaluate the impact of inflammation on their expression (Figure 1). No effect of inflammation was seen on the expression of OATP2B1, which is in line with previously published proteomics data [25]. In contrast, the protein expression of ABCB1 (P-gp), ABCC4 (MRP4) and SLC16A1 (MCT1) decreased significantly during active inflammation when compared to non-inflamed tissue from UC patients in remission. This agrees with previous reports on mRNA and protein expression data in IBD patients(19,20,24,34). In contrast, Erdmann et al.[25] found a higher protein expression of P-gp, MRP4 and MCT1 in inflamed tissue when comparing non-inflamed and inflamed tissue within the same UC patients. However, these results conflicted with a recent study by Foley et al.[20] who reported significantly lower P-gp expression in inflamed versus healthy tissue biopsies within the same UC patients using Western blot. This illustrates the ambiguous data that may be obtained with intra-patient comparisons between healthy and inflamed tissue.

288 For the other DTPs analyzed, the impact of inflammation could not be evaluated in the present study
289 due to their expression being below the LOQ in the majority of the collected tissue biopsies. For OAT2
290 and MRP6, this result is in line with literature (i.e., no reports have shown colonic expression) and
291 confirms their absence or extremely low abundance in the human colon. In contrast, ABCC3 (MRP3)
292 has been reported to be expressed in the colon[5,25,40], but could not be detected in the present study.
293 This discrepancy is likely due to the selected surrogate peptide (HIFDHVIGPEGVLAGK) which
294 unfortunately showed poor results in the MS resulting in a high LOD. Reanalysis was impossible due to
295 the limited tissue available. Similar to P-gp, the efflux transporter ABCG2 (BCRP) has previously been
296 reported to decrease in UC patients with active inflammation, although mostly based on mRNA data
297 and to a lesser extent protein expression data (via Western blot)[24,25]. Although BCRP is found in the
298 colon, the levels are relatively low and for the majority of samples in the current study, it remained
299 under the limit of detection. Finally, PEPT1, OCT1, OCT3 and ASBT have sporadically been reported to
300 be expressed in colon tissue[5] but remained under the LOD in the present study. In this respect, it must
301 be stressed that in some studies a membrane extraction was performed to increase the concentration
302 of membrane bound proteins[25,34,41]. In the present study, however, whole tissue homogenates were
303 used for the quantification, as the enrichment of membrane bound proteins is often incomplete and
304 may favor certain proteins over others, potentially leading to biased results[41]. Moreover, non-
305 membrane bound enzymes will be lost during membrane enrichment leading to an underprediction of
306 these proteins.
307
308 In addition to the transporter expression, the expression of enzymes in the human colon is even more
309 unclear, let alone during inflammation. Available data on DMEs in the colon are all based on gene
310 expression rather than protein expression or focus on a single enzyme. Fritz et al.[4] reported gene
311 expression data along the entire human intestinal tract for some major phase I and II enzymes,
312 indicating a markedly decreasing expression from the small intestine to the colon. In a publication by
313 Drozdzik et al.[34], colonic CYP expression remained under the limit of quantification in deceased organ
314 donors, except for CYP3A4 which was low compared to the small intestine. More recently, data on
315 polymorphisms of several CYP families and their effect on drug metabolism in IBD were published[21,27].
316 Erdmann et al.[25] reported mRNA data in their above-mentioned UC study, but the membrane
317 extraction protocol did not allow analysis on the protein level. In the present study, two of the
318 investigated enzymes, i.e., CYP3A5 and UGT2B7 were significantly reduced during inflammation (figure
319 3). Furthermore, CYP2B6 and UGT2B15 showed a marginal decrease which did not reach statistical
320 significance. Interestingly, CYP3A4, which is closely related to CYP3A5, did not decrease during
321 inflammation, not even in the severely inflamed (Mayo 3) sigmoid or colon (figure 4). The remaining
322 enzymes (i.e. CYP2C8, CYP2C9, CYP2E1, UGT1A1 and UGT1A3) were either not detected or only in a

323 few patients. The limited data on protein expression of enzymes in the colon does not allow for a good
324 comparison. However, studies on gene expression have shown low levels of CYP2C9 and UGT1A, which
325 are sporadically detected on protein level in the current study[4].

326 In addition to the comparison between non-inflamed and inflamed sigmoid and rectal tissue biopsies,
327 the present study further divided the inflamed tissue biopsies according to their inflammation severity
328 using the Mayo score. Interestingly, the expression levels of P-gp and CYP3A5 and to a lesser extent
329 MCT1 and UGT2B7 show a more pronounced decrease with increasing inflammation severity (i.e.
330 Mayo 3 > Mayo 2 > Mayo 1) (figure 2 and 4). This indicates that during acute and heavy flare-ups a
331 potentially larger impact on drug transport and metabolism can be expected. This may be important
332 for the dosing of drugs and/or the decision to switch to parenteral modes of administration.
333 In addition, this subdivision clearly shows the possible impact of mild inflammation scored as Mayo 1,
334 which was often considered endoscopic healing in the past [42]. More recent works refer to Mayo 1 as
335 endoscopic improvement and Mayo 0 to be endoscopic remission, which appears more in line with the
336 effects on protein expression[26,43].
337
338 The results of the present study point to a specific mechanism that affects only certain drug
339 transporters and metabolic enzymes during inflammation, while others remain unaffected. The precise
340 mechanism is not fully understood as there are multiple theories on how inflammation mediates
341 protein expression. For instance, the nuclear Pregnane X receptor (PXR) has been associated with the
342 regulation of intestinal transporters proteins, although contradicting results have been published[18,19].
343 Inflammatory mediators such as IL-6 reduced P-gp through the mediation of PXR[44]. Other nuclear
344 hormone receptors have also been linked to protein regulation during inflammation. Another
345 mechanism is through the modulating function of the microbiome in UC patients. A recent study found
346 dysregulation of the gut microbiome associated with UC that led to decreased P-gp expression
347 potentially linked to changes in butyrate synthesis and bile salt metabolism[20]. It should further be
348 noted that all patients including those in remission were taking medication (mesalazine and less
349 frequently corticosteroids such as budesonide) that are capable of affecting the expression of drug
350 transporters and enzymes[12,13,45,46]. However, it is unlikely that these drugs are the primary cause for
351 the observed results, considering (i) the typical effect of drugs such as budesonide, i.e., inducing
352 protein expression by activation of PXR[12,13,45,46], and (ii) the overall uniformity of the results despite the
353 diverse medication schemes.

354 In the present study, we normalized the protein abundance for total protein content. As such,
355 infiltration of tissue by leukocytes during inflammation, which would increase total protein content,
356 might also contribute to a decrease in normalized protein abundance. However, this would result in

357 an overall decrease for all proteins, which was not observed. An alternative normalization approach is
358 to correct expression data for a stable marker protein such as villin-1. However, studies have suggested
359 the role of villin-1 as an anti-apoptotic protein that is regulated by mediators released during cell stress
360 (i.e., inflammation). For the purpose of the present study, villin-1 based normalization could therefore
361 potentially lead to faulty interpretation of the results[47] and normalization with total protein content
362 was more appropriate[24,25,48].

363

364 While inflammation may affect the expression of DTPs and DMEs, literature also suggests that a loss
365 in the protective function of the intestinal barrier by reduced expression of certain DTPs and DMEs
366 might be involved in the progress of inflammation. For instance, Mdr1 deficient mice (i.e., not
367 expressing P-gp) formed spontaneous colitis[49,50]. The effects were mitigated by antibiotics, indicating
368 that the loss of efflux of bacterial toxins by P-gp resulted in inflammation. The decrease in P-gp
369 observed in the present study could therefore exacerbate the inflammation[15,19]. Another example is
370 the decrease in butyrate uptake by the colonocytes due to the reduced MCT1 transporter expression.
371 Butyrate is an important energy source for the colonocytes and promotes the production of anti-
372 inflammatory cytokines, thereby protecting the cells against inflammation[38,51]. Lastly, MRP4 and P-gp
373 are involved in the signaling pathways for cell survival and apoptosis[52,53].

374 In addition to their role in the (patho-)physiology of the colonic tissue, DTPs and DMEs may also affect
375 the disposition of drugs in the colon. Overall, active transport and metabolism of drugs in the colon is
376 assumed to have a limited effect on the systemic disposition of orally administered drugs due to the
377 limited amount of drug remaining in the colon and the relatively low abundance of transporters and
378 enzymes compared to the small intestine and the liver[6,34]. When considering locally acting drugs and
379 colon targeting formulations, however, their disposition in colonic tissue can potentially be affected
380 by transporters and enzymes[9,11,54,55]. In this respect, a reduced P-g efflux during active inflammation
381 may lead to higher intracellular concentrations of multiple drugs used in UC patients, including
382 cyclosporin, budesonide, sulfasalazine and tacrolimus. Considering the commonly observed substrate
383 overlap between P-gp and CYP3A, the reduced expression of CYP3A5 might lead to an even more
384 pronounced effect. This may be especially true for tacrolimus which is extensively metabolized by
385 CYP3A5[27,28]. Budesonide, an often used corticosteroid in UC, is primarily metabolized by CYP3A
386 followed by glucuronidation. As inflammation affects both pathways, local budesonide concentrations
387 during inflammation may increase[12]. A similar effect may be observed for azathioprine and
388 mercaptopurine, both substrates for the basolateral efflux transporter MRP4, which showed a marked
389 decrease with even slight inflammation (Mayo 1)[56]. Although the protein expression data suggest
390 possibly increased local concentration of these drugs, *in vivo* data are needed to confirm these findings

as protein expression may not adequately predict activity. In this respect, it is worth noting that polymorphism of MRP4, resulting in a dramatic reduction in MRP4 function, caused increased intracellular concentrations of 6-thioguanine nucleotide, a metabolite of azathioprine and mercaptopurine, thereby affecting the patients' sensitivity to thiopurines[57].

In conclusion, the present study demonstrated a link between inflammation in UC patients and the downregulation of drug transporters and metabolic enzymes in the distal colon (i.e., P-gp, MRP4, MCT1, CYP3A5 and UGT2B7) by comparing UC patients in remission with patients with active inflammation. Our data further suggest a more pronounced effect with increasing severity of inflammation. As such, the outcome of this study warrants future research to assess the clinical relevance of these findings by elucidating possible UC-induced effects on drug transport and metabolism *in vivo*. In addition, the reduced protein levels during inflammation can be used as input data in PBPK models to explore the impact on local (colon) tissue concentrations of UC drugs. Furthermore, as some transporters and enzymes remained elusive in the current study, more advanced techniques (i.e., global proteomics) could be used to further explore the changes during inflammation.

Author contributions

Patrick Augustijns, **Joachim Brouwers**, **Tom de Waal**, **Per Artursson**, **Marc Ferrante**, **Séverine Vermeire** and **Tim Vanuytsel** conceptualized the study. **Patrick Augustijns**, **Joachim Brouwers**, **Per Artursson** and **Tim Vanuytsel** secured the funding. **Marc Ferrante** and **Séverine Vermeire** provided the colonic biopsies. The analysis, data curation and visualization were performed by **Tom de Waal** and **Niklas Handin**, supervised by **Joachim Brouwers**, **Per Artursson** and **Patrick Augustijns**. The manuscript was written by **Tom de Waal** and **Joachim Brouwers** and reviewed and edited by all authors. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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