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Differential Expression of Mucosal Trefoil Factors and Mucins in Pediatric Inflammatory Bowel Diseases

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In the intestinal mucosa trefoil factors (TFF) and mucins (Muc) - primarily produced by goblet cells - are thought to play a major role in providing barrier function during infection and inflammation. To investigate their role in pediatric Crohn's disease (CD) and ulcerative colitis (UC) we obtained mucosal biopsies of children with CD, UC and healthy controls and analyzed genetic expression. Levels of TFF2 mRNA were lower in inflamed mucosal samples (terminal ileum (TI) and duodenum) of children with CD, but higher in non-inflamed mucosal samples when compared to healthy controls ($p < 0.05$). Similarly, TFF2 levels in the TI were significantly lower in inflamed UC tissue. Adjustment for goblet cell density revealed slightly less marked, yet significantly different gene expression in IBD and controls. Furthermore, TI expression of TFF2 and Muc2 was inversely correlated with interleukin-8 expression in CD ($p = 0.027$). In Summary, our data demonstrate significant changes in Muc and TFF mRNA expression in pediatric patients with IBD suggesting a role in mucosal healing. Further studies are needed to elucidate a potential use as biomarkers for disease progression.

Inflammatory bowel diseases (IBD), mainly comprised of the two entities Crohn's disease (CD) and ulcerative colitis (UC) are a world-wide health-care problem with increasing incidence[1,2]. While CD and UC have distinct clinical features, both disorders are characterized by relapsing inflammation in the gastrointestinal tract. Even though the etiology remains largely unknown, the underlying pathophysiological mechanisms are thought to involve genetic susceptibility, environmental factors, gut microbial composition and altered immune response patterns.

The trefoil factor family (TFF) is a group of peptides abundantly secreted onto the surface of the gastrointestinal tract by goblet cells. These 7–12 kDa small, protease-resistant proteins play an important role in maintaining epithelial integrity of the gastrointestinal tract through regulation of restitution and regeneration of the intestinal epithelium[3]. While the complexity of their biological functions and the molecular mechanisms involved remain to be fully understood, TFF proteins have been shown to play key roles following mucosal injury through inhibition of apoptosis and anti-inflammatory signaling[4]. Similar to TFF's, hepatocyte growth factor (HGF) and mucins are also involved in healing processes following intestinal epithelial damage. HGF modulates intestinal epithelial cell proliferation and migration, thus accelerating intestinal mucosal repair processes[5]. Mucins are epithelial glycoproteins important for the protection of mucosal integrity through preservation of the epithelial barrier function[6].

Currently only little information is available on the potential roles and regulations of mucins and HGF in the intestinal mucosa of adult IBD patients and there is a complete lack of data in pediatric IBD. As TFFs, mucins and HGF play important roles in mucosal protection, regeneration and restitution processes following inflammatory damage, we hypothesized that their expression may be altered in pediatric patients with IBD when compared to healthy children.

Results

Patient characteristics and IL8 mRNA levels are shown in Table 1. PCDAI and SES-CD differed significantly between the inflamed and the non-inflamed CD patient group with p-values of 0.04 and 0.02, respectively.

Table 1 | Clinical characteristics of the study population [median ± standard deviation (sd)]

	total	controls	crohn's disease		ulcerative colitis	
			inflamed	non-inflamed	inflamed	non-inflamed
number (n)	21	5	5	5	3	3
gender						
male	13	2	4	3	2	2
female	8	3	1	2	1	1
age (years)	12.6 ± 3.7	10.4 ± 6.1	13.6 ± 2.2	12.4 ± 3.6	13.7 ± 2.1	13.7 ± 2.1
PCDAI			55.5 ± 7.9	37 ± 14.3		
SES-CD#			20.4 ± 14.4	5.6 ± 4.1		
MAYO endoscopic index*					1,7 ± 0,6	0,3 ± 0,6
CU disease extent (None/Proctitis/left sided colitis/extensive colitis)					0/0/0/3	2/0/1/0
CRP (mg/dl)			2,6 ± 0,3	<0,5	5,6 ± 2,2	<0,5
IL8 mRNA copy numbers			145 ± 82.8	22 ± 17.3	-	-
duodenum	-	46 ± 128.3	12365 ± 56679	1698 ± 1458	659 ± 552.7	83 ± 56.4
terminal ileum	-	102 ± 99.5	13869 ± 26640	1815 ± 6297	1273 ± 3625	56 ± 32.5
ascending colon	-	51 ± 121.5				

#SES-CD = Simple Endoscopic Score;
*MAYO endoscopic index: 0 = Normal or inactive disease, 1 = Mild disease (erythema, decreased vascular pattern, mild friability), 2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions), 3 = Severe disease (spontaneous bleeding, ulceration).

Similarly, the MAYO clinical disease index differed significantly between inflamed and non-inflamed UC patients ($p = 0.03$, Table 1). In patients with acutely inflamed CD we observed significantly lower mRNA levels of TFF2, TFF3 and MUC2 in the mucosa of the terminal ileum (TI) when compared to healthy controls. These genes were shown to have significantly higher mRNA levels in non-inflamed IBD tissue when compared to healthy controls (Fig. 1). Furthermore, mRNA expression levels of TFF1 and Muc1 and HGF were significantly higher in CD during clinical remission (Fig. 1). Interestingly, in duodenal mucosa of CD patients TFF2 levels were lower in active disease and higher in clinical remission, whereas MUC1 levels were significantly higher regardless of disease activity state when compared to healthy controls (Fig. 1). When we compared CD with UC patients we found higher mRNA levels for TFF2, MUC1, MUC2 and HGF in the non-inflamed TI, whereas in the ascending colon only HGF mRNA levels differed significantly (Fig. 1). Since mucins and trefoil factors are primarily produced by goblet cells (GC), we determined GC densities and analyzed GC adjusted mRNA levels. While this revealed lower numbers of GCs and less marked gene expression changes in inflamed tissues, differences in genetic expression still remained clear (Fig. 2). This is also reflected by immunohistochemistry showing lower GC density as well as different intensities of protein expression, which correlate well with the observed mRNA levels (Fig. 3). Subsequently, we investigated the potential correlation between expression of TFFs and mucins and inflammatory status as measured by expression of IL8 and the PCDAI. Interestingly, these analyses revealed TFF2 and MUC2 in the TI to inversely correlate with the expression of interleukin-8 in CD ($r^2 = 0.53$ ($p = 0.03$) and 0.61 ($p = 0.017$), respectively). Moreover TFF2 and MUC2 in the TI were correlated to PCDAI. The best fitting model was found to be a plateau followed by a one phase exponential decay with increasing PCDAI ($r^2 = 0.60$ ($p = 0.03$) and 0.56 ($p = 0.0005$), respectively).

Discussion

This is the first study to analyze TFFs and their role of GI healing processes in pediatric IBD. It is also the only study to sub-divide CD and UC specimens into groups of acutely inflamed and non-inflamed tissues which helps to gain valuable insight into the dynamics of genetic expression in the course of IBD.

Comparing GI genetic expression of TFF peptides in healthy children and IBD patients, we found the most distinct degree of dysregulation in the terminal ileum (TFF 1 and 2) and duodenum (TFF2) rather than in the colon. The consistently high levels of TFFs seen in IBD patients with clinical remission is supported by both *in vitro* and *in vivo* findings promoting their role in mucosal healing processes and by clinical data suggesting TFF up-regulation both in GI tissue biopsies and sera of patients with IBD[7–9]. Moreover, our finding of low small intestinal mRNA levels of TFF2 in patients with active CD is consistent with data from an experimental rat model in which TFF2-deficient rats showed increased susceptibility to GI mucosal injury from environmental stimuli[10].

In addition, we observed strong inverse correlations of MUC2 and TFF2, respectively, with the degree of inflammation (IL-8). This may qualify TFF2 and MUC2 as biomarkers for mucosal healing and regeneration processes. We also observed stunningly parallel expression profiles for TFF2 and MUC2 which can most probably be explained by co-localization of TFF2 and MUC2 in goblet cell vesicles[11]. As a reduced mucosal goblet cell density is a known phenomenon in IBD, we normalized mRNA expression levels to it. On the one hand, we found that differences in gene expression patterns can be partly explained by a decline in goblet cells. On the other hand, the overall differential regulation still remained significant after normalization which indicates that functional differences underlie the observed expression patterns besides the decreased absolute amount of goblet cells. This raises

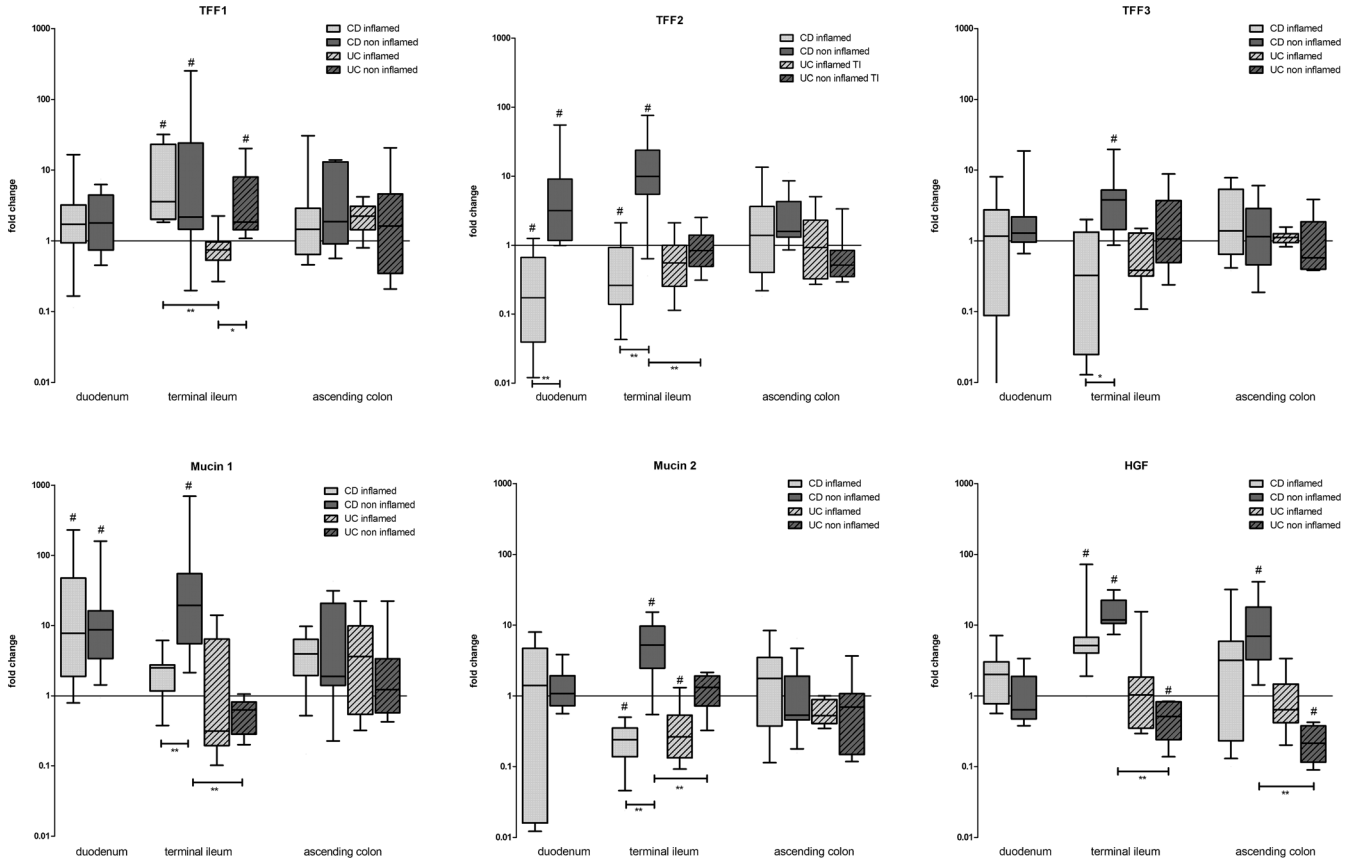

Figure 1 | Gene expression of TFFs and mucins in the intestinal mucosa. Expression levels were normalized against the median of GAPDH, b-actin and RPL19. Data is expressed as median with interquartile range, minimum and maximum. * and # indicate p-values < 0.05 and ** indicates p-values < 0.01, respectively.

the suspicion that the altered expression pattern is rather the consequence of ongoing inflammatory cell damage rather than the cause of the inflammation itself. Overall, the complexity and heterogeneity of both trefoil peptides and mucin glycoproteins make it difficult to decipher their various roles in pathophysiological processes as cause for or consequence of IBD. Further studies are needed to identify their immunological functions and possible interacting partners.

We observed significantly higher levels of TFF1 in the TI in CD when compared to healthy controls and patients with active UC. This TFF1 neo-expression in IBD was also observed before in both adult patients with severe CD and in an experimental dog model[12,13] and is probably a compensatory repair mechanism to counteract the decreasing mucosal barrier function due to inflammatory tissue damage. This potentially qualifies TFF1 as a marker to discriminate between CD and UC in ambiguous cases of acute IBD with TI involvement. Finally, we observed high levels of HGF in CD when compared to UC and healthy controls which is in accordance with the concept that CD is a process affecting the entire intestinal wall whereas UC only affects the mucosa.

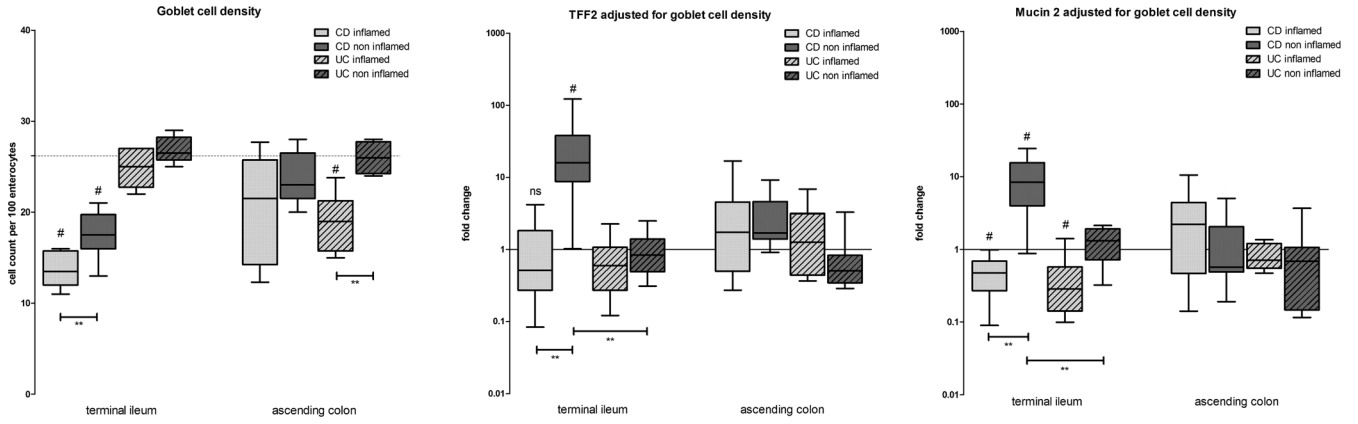

Figure 2 | Gene expression adjusted for goblet cell density. Left: Goblet cells (GC) density was analysed in relation to total numbers of enterocytes counted in PAS stained tissue sections. The dashed line represents the mean goblet cell number in healthy controls. Middle and right: Expression levels were adjusted for GC density and normalized against the median of GAPDH, b-actin and RPL19. Data is expressed as median with interquartile range, minimum and maximum. * and # indicate p-values < 0.05 and ** indicates p-values < 0.01, respectively.

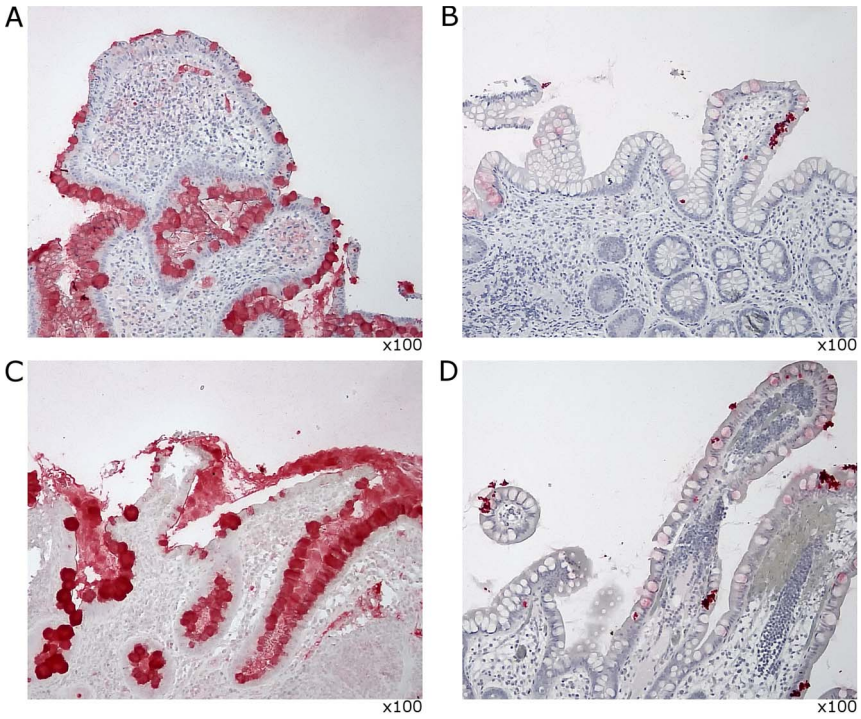

Figure 3 | Immunohistochemistry of terminal ileum specimens of patients with CD and UC. Expression of mucin 2 (dark red) is substantially reduced in the terminal ileum of patients with active Crohn's disease (A) when compared to patients in clinical remission (C). In ulcerative colitis, patients with active disease show lower expression of trefoil factor 1 (light brown) in the terminal ileum (B) when compared to UC patients in clinical remission (D). Microscopic magnification is ×100 in all slides.

Methods

Over a period of 18 months 21 pediatric subjects were recruited from three centers in Germany (Wuppertal) and the UK (London and Cambridge). Diagnosis of CD and UC was based on clinical, radiological, endoscopic, and histopathological findings in accordance with the Porto criteria and Montreal classification. Lower and upper gastrointestinal (GI) endoscopy was performed by experienced pediatric gastroenterologists and biopsies were collected from the mucosa of duodenum, terminal ileum and ascending colon. Patients with CD and UC were sub-divided into groups of patients with active inflammation and without active inflammation. This differentiation was based on histological findings and mRNA expression levels of the inflammatory cytokine interleukin-8 (IL-8). Patients without macroscopic or histopathologic abnormalities and with no evidence for underlying GI pathology served as controls.

Biopsy samples were immediately placed in RNAlater (Qiagen, Hilden, Germany) and stored at −80°C until processing. RNA was extracted using the RNeasy extraction kit (Qiagen) and RNA integrity was verified with agarose gel electrophoresis. Next, 500 ng of RNA was reverse-transcribed and DNAse-treated utilizing the QuantiFast kit (Qiagen). Real-time PCR (rt-PCR) was performed on a Rotor Gene 600 real-time rotary cycler (Corbett Life Science, Qiagen) using SYBR-green methodology. GAPDH, b-actin and RPL19 served as reference housekeeping genes. All samples were analysed in technical triplicates. Results are presented as boxplots with median, interquartile range, minimum and maximum.

Immunohistochemistry was performed on formalin/PFA-fixed, paraffin-embedded sections using commercially available antibodies for TFF1 (rabbit monoclonal antibody [EPR3972] to Estrogen Inducible Protein pS2/TFF1, *ab92377*, Abcam, Cambridge, United Kingdom) and Muc2 (rabbit monoclonal antibody [EPR6145] anti-MUC2, *ab134119*, Abcam, Cambridge, United Kingdom). Goblet cell density was counted in PAS stained tissue sections in relation to total numbers of enterocytes.

Calculation of statistical significance was performed with GraphPad Prism, version 5.0 (GraphPad Software, San Diego,CA, USA), using the Kruskal-Wallis test followed by Dunn's Test. Spearman's rank coefficient and Pearson regression analysis was used for correlation analyses. P-values < 0.05 were considered statistically significant. The study was performed under consideration of the declaration of Helsinki with amendments from Tokyo 1975, Edinburgh 2000 and Seoul 2008, in accordance with the responsible privacy authorities and carried out in accordance with the approved guidelines. Ethical approval for this study was obtained by the Witten/Herdecke University Ethics Committee and by all other participating hospitals. Written informed consents were obtained from children and/or legal guardians where appropriate.

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Author contributions

K.H. and V.B. have performed gene expression experiments and analyzed the data. K.H. wrote the manuscript. M.Z. and R.H. had helped recruiting patients and collecting tissue

specimens. S.W. and M.Z. critically reviewed the manuscript. S.V. and D.G. performed immunohistochemistry. A.J. and J.P. took part in the main study design, data analyses and supervision of the study.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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