

**NOVEL PATHOPHYSIOLOGICAL FACTORS AND
DIAGNOSTIC MARKER IN ORGANIC AND FUNCTIONAL
BOWEL DISORDERS**

Ph.D. thesis

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BOWEL DISORDERS**

Ph. D. Thesis

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Y, Piche T, Theodorou V, Wittmann T, Bueno L. Luminal cysteine-proteases degrade colonic tight junction structure and are responsible for abdominal pain in constipation-predominant IBS. *Am J Gastroenterol*. 2013 Aug;108(8):1322-31.

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TABLE OF CONTENTS

1.	INTRODUCTION	1
1.1.	Animal studies	1
1.2.	Human studies	2
2.	AIMS	4
3.	MATERIALS AND METHODS	5
3.1.	Ethical Considerations	5
3.2	Animal studies	6
3.2.1.	Animals	6
3.2.2	In vivo permeability analysis	6
3.2.3	Ussing chamber analysis	6
3.2.4	Follow-up of animal growth	7
3.2.5	Gastrointestinal transit	7
3.2.6	Gut microbiota composition analysis	7
3.2.7	Open field test	8
3.2.8	Colorectal distension (CRD)	8
3.2.9	C-Fos analysis	9
3.2.10	Statistical analysis of animal data	9
3.3	Human studies	9
3.3.1.	Patient selection for the observations of faecal MMP-9 levels in ulcerative colitis/IBS-D patients/healthy controls comparison (UC- study)	9
3.3.2	Patient selection for the faecal MMP-9 measurements in colon cancer (CRC study)	10
3.3.3	Sample storage	10
3.3.4	Validation assay of faecal MMP-9 measurement	11
3.3.5	Measurement of faecal MMP-9	11
3.3.6	Measurement of faecal calprotectin	12
3.3.7	Statistical analysis in the human studies	12
4.	RESULTS	13
4.1.	Animal studies	13
4.1.1.	In vivo and in vitro permeability measurements	13
4.1.2.	Body weight gain and intestinal transit	13

4.1.3. Microbiota analysis	13
4.1.4. Open field test	15
4.1.5. Colorectal distension	15
4.1.6. C-Fos analysis	15
4.2. Human studies	18
4.2.1. Validation assay of faecal MMP-9 measurement	18
4.2.2. Faecal MMP-9 levels in control, IBS-D and UC patients' samples	19
4.2.3. Correlation between faecal MMP-9 levels with disease activity in UC	20
4.2.4. Correlation of faecal MMP-9 concentration with faecal calprotectin levels in UC	21
4.2.5. Faecal MMP-9 levels in groups of non-adenoma/adenoma/CRC patients	22
4.2.6. Faecal MMP-9 levels according to CRC localisation and stage	25
5. DISCUSSION	27
5.1. Animal studies	27
5.2. Human studies	29
6. NEW RESULTS ESTABLISHED IN THE THESIS	34
6.1. Animal studies	34
6.2. Human studies	34
7. SUMMARY	35
8. ABBREVIATIONS	38
9. ACKNOWLEDGEMENTS	40
10. REFERENCES	41
ANNEXES	46

1. INTRODUCTION

1.1. Animal studies

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by continuous or remittent abdominal pain, bloating and altered bowel habits without recognized underlying organic aetiology [1]. Nowadays, 100 years after the first description of functional gastrointestinal diseases, 5-20% of patients seeking a gastroenterologist are diagnosed with IBS, causing considerably high medical and indirect costs [2]. Despite of its epidemiological significance, the pathophysiology of IBS is still not completely understood. The current classification of IBS is based on the Rome criteria [1] that only stratify patients according to their bowel habits as diarrhoea (IBS-D), or constipation (IBS-C) predominant, with mixed symptoms (IBS-M), and patients, who meet diagnostic criteria for IBS but their bowel habits cannot be accurately categorized in any of the above subtypes are labelled as unclassified IBS (IBS-U) group. The known elements of the pathomechanism are perturbation in the brain-gut axis causing altered pain sensation and modulation, misbalance in intestinal microbiota, moderate gut immune activation referred to as "low-grade inflammation", intestinal motor dysfunctions and elevated gut permeability [3]. In addition, a higher level of anxiety is often observed in these patients [4]. According to the high prevalence of the disease, a better understanding of the pathophysiology would help to focus on appropriate therapies.

In the present thesis, we focus on the role of the elevated gut permeability in the genesis of IBS symptoms. Intestinal barrier impairment has been associated with a variety of human diseases, e.g. inflammatory bowel disease (IBD), celiac disease, and IBS [5, 6]. Stressful life events, infection and dysbiosis can induce elevated gut permeability, which is associated with visceral hypersensitivity [7, 8], and pain severity in patients [9]. Animal models have shown that stress-induced elevation of paracellular permeability is linked to the decrease of the tight junction proteins expression (e.g. occludin, claudins). This is as a key factor in actinomyosin-driven epithelial cytoskeleton contraction, in which the phosphorylation of the myosin light chain by myosin light chain kinase (MLCK) plays the major role [10, 11].

Every subtype of IBS is represented by visceral hypersensitivity. Several factors were found playing a role in this phenomenon: modulation of the central nervous response to the intestinal derived stimuli, hyperexcitability of the afferent innervation, or sensitization of the sensory endings in the gut wall can contribute to the pain response [12]. End-organ hypersensitivity may be a result of the low-grade immune activation in the intestine. Moreover, mast cells and their mediators, such as histamine, trypsin, and tryptase have been documented in higher levels in colonic and jejunal biopsy supernatants from IBS patients, and increased mast cell infiltration has also been found in IBS gut mucosa. Similar results were found using animal models, and mast cell stabilizers have proven efficacy in preventing visceral hyperalgesia in animals [13, 14]. Transgenic mice with specific intestinal expression of a constitutively active form of MLCK (*CAMLCKTg*) have congenitally elevated gut permeability. Besides this increased gut permeability, these animals present a moderate intestinal immune activation in basal state without any sign of disease [15], which suggests that it could represent an ideal model to study IBS.

Nowadays it is well known, that alteration of the intestinal microbiota is present in IBS patients [16, 17], however the role of dysbiosis in the pathophysiology is unclear. Infective diarrhoea can provoke long-term IBS symptoms, and intestinal microbiota is permanently perturbed after the elimination of the pathogen (postinfectious IBS) [18]. On the other hand, modification of the intestinal microbiota (e.g. rifaximin or probiotic treatment) in IBS patients has only short term effect on patient's symptoms. [19]. The temporary efficacy of these treatments could suggest an endogenous trigger maintaining the "perturbed" bacterial composition in the intestine. *CAMLCKTg* animal model could help to clarify the potential relationship between the intestinal permeability changes and the composition of the intestinal microbiota.

1.2. Human studies

Ulcerative colitis (UC) is a chronic, relapsing inflammatory bowel disease characterized by continuous colonic mucosal ulceration and diarrhoea during relapses. The differentiation of UC from functional disorders such as IBS-D is clinically challenging, as

symptoms may include altered bowel habits and abdominal pain in both diseases, moreover activity signs can mix with anxiety-driven symptoms as well in UC. Several attempts have been made to establish non-invasive markers that can clearly distinguish IBD from functional disorders and reveal strong correlation not only with clinical activity, but also with the endoscopic picture of the mucosa in UC. Among biochemical laboratory markers serum C-reactive protein (CRP) is the most studied [20]; however, while the activity of Crohn's disease (CD) is accompanied by a strong CRP response in most patients, relapses in UC are characterized by only a modest or absent CRP increase [21] depending mostly on the extension of the involved area. The sensitivity of CRP in the detection of UC is only 50%–70% [22–24]. The pathogenesis of UC includes the complex dysregulation of mucosal immune cells [25] accompanied by the invasion of neutrophils, which leads to the formation of crypt abscesses and to the dysfunction of the colonic epithelial barrier [26]. During transmigration, neutrophils reach the colonic lumen and can be detected in the stool [27], similarly to their secreted mediators, such as polymorphonuclear elastase, calprotectin, or cathepsin G [28, 29]. Therefore, faecal biomarkers that reflect neutrophil activation seem promising in UC, and faecal calprotectin has been identified as a marker to discriminate IBD patients from IBS patients or controls [29, 30], and its faecal level correlates with disease activity [31, 32]. Matrix metalloproteases (MMPs) 1, 2, 3, and 9 are also released from neutrophils in IBD, and they are significantly elevated in colonic biopsies from UC patients compared to controls, with a higher increase in the ulcerated than in nonulcerated regions [33]. Among these MMPs, MMP-9 was the most abundant in UC biopsies [33]. Additionally, elevated MMP-2 and MMP-9 levels were detected in the urine of paediatric patients suffering from either UC or CD, and these MMPs were suggested as non-invasive biomarkers in the diagnosis of IBD [34]. Nevertheless, no study has so far focused on faecal MMP levels in UC compared with IBS-D. Furthermore, the correlation of MMPs with disease severity, clinical, and endoscopic activity has never been evaluated.

Colorectal cancer (CRC) represents a major cause of morbidity and mortality in Western societies, and the incidence in developing countries is also rapidly growing [35–37]. The high frequency of the disease and the fact that the prognosis well correlates with the stage at diagnosis makes CRC screening extremely important. Colonoscopy is the gold standard for CRC screening by its high sensitivity and specificity but has also disadvantages, such as risk of complications, high costs and lower acceptance by patients as a screening method.

Therefore, non-invasive methods are required to identify patients at high risk of CRC where colonoscopy needs to be performed. CRC screening recommendations vary between countries but mostly consist of an annual or biannual faecal occult blood test (FOBT) combined with colonoscopy in case of positivity or a colonoscopy every 10 years, in the population aged >50 years [38]. Two basic types of FOBT exist, based on the imperceptible intermittent leakage of blood from CRC and high-risk adenomas in stool [38]. The guaiac test (gFOBT) detects the peroxidase activity in the haemoglobin heme subunit, therefore it is nonspecific for human haemoglobin and theoretically requires dietary restrictions a few days before performing the test, but large studies have found no significant advantage of the dietary modifications, therefore lately they are not recommended by many authors [39]. A minimum of two faecal samples are necessary from different stools to obtain an appropriate sensitivity. Sensitivity for CRC ranges from 6.2% to 83.3%, depending on the test used, while specificity is >80% [38]. The immunochemical faecal occult blood test (FIT) is based on specific monoclonal or polyclonal antibodies against human haemoglobin, thus it does not require dietary alterations. Contrary to gFOBT, the analysis by FIT can be automated and its threshold value is adjustable by the user. According to the literature, the sensitivity of FIT is extremely variable, from 5.4% to nearly 98%, depending on the test used [38]. Specificity can range from 77% to 99%. The European Guidelines for quality assurance of CRC recommends the use of FIT rather than gFOBT [40]. However, reliable tests with a less variable sensitivity and specificity are still lacking. MMPs are a family of Zn^{2+} containing endopeptidases, secreted by various cell types, such as tumour cells, mesenchymal cells, fibroblasts and inflammatory cells (monocytes, lymphocytes and neutrophils). Increased expression of MMP-9 has been detected in tissue samples from rectal carcinoma and colon cancer, where it correlates with poor prognosis [41-43]. However, faecal MMP-9 levels in CRC patients have not been investigated.

2. AIMS

In animal experiments in *CAMLCKTg* mice our aims were:

- 1) to verify if the transgene has an effect on the gut permeability, animals' growth and intestinal transit;

- 2) to explore the effect of elevated intestinal permeability on the gut microbiota composition;
- 3) to study the moderately elevated gut permeability on behaviour and visceral sensitivity;
- 4) to investigate if the intestinal permeability elevation has an effect in the brain activity.

In human studies our aims were:

- 1) to validate if the faecal matrix has an influence on MMP-9 determination and whether the kit is able to determine MMP-9 from human faecal extracts;
- 2) to compare faecal MMP-9 levels in UC patients to control subjects and patients with a functional gastrointestinal disorder characterized by diarrhoea (IBS-D);
- 3) to test the correlation between UC disease activity and faecal levels of MMP-9;
- 4) to correlate faecal MMP-9 levels with a known faecal marker of UC activity, calprotectin;
- 5) To compare faecal MMP-9 levels in patients with normal colonoscopic results, colonic polyps and colon cancer.

3. MATERIALS AND METHODS

3.1. Ethical Considerations

Experimental procedures on animals were reviewed and authorized by the Ethical Committee CEEA-86, under the number APAFiS#4145. (INRA TOXALIM, Toulouse, France)

The human study protocols were approved by the Ethical Committee of the University of Szeged under the number 170/2011. All subjects provided written and informed consent to participate.

3.2 Animal studies

3.2.1. Animals

CAMLCKTg mice (Tg(Vil-FLAG-CAMLCK)#Jrt) were maintained as male heterozygotes on C57BL/6J background. These were mated with wild type (WT) C57BL/6J females to produce WT and *CAMLCKTg* littermates. At weaning, female mice were separated and housed at constant temperature ($22\pm 1^{\circ}\text{C}$) with a 12 hour light/dark cycle. Food (Teklad 2018, Envigo) and water were available ad libitum. All experiments were performed at 8 weeks of age.

3.2.2 In vivo permeability analysis

Mice were fasted for 4 hours before gavage with 150 μL of 100mg/mL FITC-4kDa dextran in tap water. Blood (200 μL) was collected after 4h and plasma fluorescence determined using Tecan Infinite 200 microplate reader (Tecan Group Ltd., Switzerland).

3.2.3 Ussing chamber analysis

Animals were sacrificed and jejunal sections were collected. Jejunal sections were mounted in Ussing chambers (Physiologic Instruments, USA) filled with Krebs buffer and continuously oxygenated (95% O_2 , 5% CO_2). After 1 hour of equilibration, Fluorescein (1mg/mL) was added in the apical chamber and fluorescence intensity of the basolateral chamber was measured after 1 hour using Tecan Infinite 200 microplate reader (Tecan Group Ltd., Switzerland).

3.2.4 Follow-up of animal growth

CAMLCKTg and WT mice were weighed weekly for 12 weeks. (Cubis, Sartorius, Germany)

3.2.5 Gastrointestinal transit

Animals received 70µL of 100mg/ml TRITC-70kDa dextran in tap water by gavage and were sacrificed 1 h later. Stomach, small and large intestine were cut in 11 equal parts. Luminal contents of each segment were centrifuged and fluorescence determined using Tecan Infinite 200 microplate reader (Tecan Group Ltd., Switzerland). Transit was calculated as the geometric center of the values for each mouse.

3.2.6 Gut microbiota composition analysis

Gut microbiota were analysed in two cohorts (15 WT and 16 *CAMLCKTg*) from 8 different WT dams. At sacrifice, colonic contents were stored at -80°C. DNA was extracted using the ZR faecal DNA MiniPrep kit (Zymo Research, USA) and adjusted to 1 ng/µL. Changes in relative abundance of 24 microbial 16S rRNA gene targets were obtained by qRTPCR using an adapted Gut Low- Density Array platform [44-46]. A universal bacterial primer set was included as the reference gene. The qRTPCR was performed in duplicate on a ViiA7 (Applied Biosystems, USA). Fluorescence data was imported into LinRegPCR to perform baseline corrections, calculate mean PCR efficiency per amplicon group and calculate initial quantities. Among the 24 targeted amplicon groups, 6 were not detected in any faecal samples and were removed from the analysis (*B. vulgatus*, *Alistipes spp.*, *Parabacteroidetes distasonis*, *Roseburia spp.*, *E. coli* and *A. muciniphila*). Normalized NO-values were log10-transformed and processed by MixOmics (v6.1.1) with RStudio (v1.0.44) to build a partial least-squares discriminant analysis (PLS-DA). This multivariate supervised

approach projects samples (X) onto a low-dimensional space of latent variables to maximize separation between groups according (Y=genotype). Leave-one-out cross-validation was used to select the optimal number of latent variables for PLS-DA models.

3.2.7 Open field test

Mice explored a 50x50 cm arena (illumination 300lux) for 10 min. Exploration was automatically assessed using a video tracking system (Bioseb, France). The percentage of distance travelled and time spent and in the center area (20x20 cm) and total distance travelled in the entire arena were assessed.

3.2.8 Colorectal distension (CRD)

Two 0,08 mm diameter electrodes were implanted in the abdominal external oblique muscle and a third in the abdominal skin. On postoperative days 3-6, CRD was performed using a balloon catheter (Fogarty 4F catheter, 1,1cm length, and tip 3,5 cm from the anus) in 10 sec periods with increasing volumes from 0,02 mL to 0,10 mL, with 5 min rest between distensions. Abdominal electromyography activity was registered after the amplification (10000x) and analysed (Powerlab Chart 5, AD Instruments, Australia). Basal EMG activity was subtracted from EMG activity registered during distension. Some mice were treated with ML-7 (2 mg/kg i.p.) or naloxone sulfate (2 mg/kg i.p.) 1 h before CRD. For others, water avoidance stress was induced on a floating platform (3x3 cm) in the middle of a water-filled tank (40x40 cm) for 1h daily over four days. Recovery (30 min) preceded CRD.

3.2.9 C-Fos analysis

Vibratome sections (40 μm) were stained using polyclonal rabbit anti-C-Fos (Santa Cruz Biotechnology, USA) and secondary HRP-conjugated goat anti-rabbit antisera (Jackson Immuno Research, UK). NDPI images (x20) were obtained (Nanozoomer, Hamamatsu Photonics, Japan) and converted into TIFF format using ImageJ (NDPI tools plugin, USA). Regions of interest (ROI) were manually circumscribed using ROI tools and C-Fos-immunoreactive cells quantified automatically using the particle analysis function (size: 5-20 μm^2 ; circularity: 0.5-1). For each animal, 3-6 sections of each brain area were assessed by a blinded observer.

3.2.10 Statistical analysis of animal data

Statistical significance was determined by two-tailed t-test, two-tailed Mann-Whitney U test, or 2-way ANOVA and set at $p < 0.05$. For microbial analyses, univariate analysis was realized in parallel to compare each amplicon separately using unpaired t-test followed by the Benjamini-Hochberg adjustment of p -values for multiple comparisons. For statistical analysis Graph Pad Prism 4.0 (GraphPad, San Diego, CA) was used.

3.3 Human studies

3.3.1. Patient selection for the observations of faecal MMP-9 levels in ulcerative colitis/IBS-D patients/healthy controls comparison (UC- study)

Overall, 94 patients were enrolled in the study. Healthy controls ($n = 24$; male/female: 9/15; mean age: 49.3 years), UC ($n = 47$; male/female: 19/28; mean age: 48.0 years), and IBS-D ($n = 23$; male/female: 4/19; mean age: 49.4 years) patients were recruited to provide faecal

samples at the First Department of Medicine, Szeged, Hungary. IBS-D patients fulfilled the Rome III criteria. Organic gastrointestinal disorders were excluded by detailed blood analyses and stool cultures, serologic assays for celiac disease, lactose-hydrogen breath test, and colonoscopy. UC patients were evaluated clinically and endoscopically. The activity of the disease was determined by clinical (partial and total Mayo score) and endoscopic (Mayo endoscopic subscore) scores. Based on the total Mayo score, 12 patients were determined as inactive, 17 as mildly active, 9 as moderately active, and 9 as severely active. At the time of the endoscopy, blood samples were taken from all patients for the determination of platelet and leukocyte count, CRP, erythrocyte sedimentation rate, serum iron level, and haemoglobin level.

3.3.2 Patient selection for the faecal MMP-9 measurements in colon cancer (CRC study)

Overall, 109 patients (male/female: 58/51, age: 29–91 years, mean: 66.0 years) referred to the First Department of Medicine, Szeged, Hungary were enrolled in the study, who all underwent colonoscopy. No patients had a history of UC or a history of other cancers. One patient, who was later identified as a rectal adenocarcinoma patient, had liver metastases. All colonoscopies were completed until reaching the terminal ileum. During colonoscopy, biopsies were taken from all suspect lesions, and histology was performed. Based on the colonoscopic and histological results, patients were allocated to five groups: negative, diverticulosis, hyperplastic polyp, adenoma, and CRC. Adenomas were considered at increased risk of malignisation when one of the followings was fulfilled: polyp size ≥ 10 mm; villous adenoma or tubulovillous adenoma with at least 20% villous component; high grade dysplasia; or multiple adenomas.

3.3.3 Sample storage

Faecal samples were collected from all patients 2–4 days before the colonoscopy and were frozen to -20°C within 1 hour after defecation.

3.3.4 Validation assay of faecal MMP-9 measurement

Our group was the first to use the commercial Human Quantitative Enzyme-Linked Immunosorbent Assay Kit from R&D Systems (Abingdon, UK, Cat. No. DMP900) for the measurement of MMP-9 levels in faeces. Therefore, we have performed a validation assay to test whether the faecal matrix itself has an influence on MMP-9 determination and whether the kit from R&D Systems is able to determine MMP-9 from human faecal extracts. Active human MMP-9 full-length protein (Abcam LTD, Cambridge, UK; Cat. No.: ab157344; LOT No: GR218764-1) was added to the extraction medium. Control faecal samples were weighed and 1ml extraction medium with or without added MMP-9 was given to 0.25 mg sample (the sample to extraction medium ratio was 1:4). The samples were immediately homogenised for 2×30 s and incubated for 10 min at room temperature. Subsequently, all homogenates were centrifuged at 1500 g for 10 min followed by a second centrifugation of the supernatants from the first centrifugation step at 10 000 g for 10 min. The final supernatants were processed for linearity (parallelism) test. The extract containing added MMP-9 was serially diluted 1+1 to get 50, 25, 12,5 and 6,25% of the original MMP-9 content with the extract of the same faeces containing no added MMP-9. Then all samples, including 100% of added MMP-9 extract and the native control extract without added MMP-9, were subjected to MMP-9 measurement by the above-mentioned ELISA kit for human MMP-9. The kit is able to detect the human 92 kDa Pro-MMP-9 and 82 kDa active form of MMP-9 as stated by the manufacturer. The added MMP-9 protein obtained for this evaluation was the 92 kDa Pro-MMP-9.

3.3.5 Measurement of faecal MMP-9

Samples were stored on -20 °C for a maximum of 3 months and then thawed at 4 °C for 1 day. In all, 1,0 g of each faecal sample was diluted, mixed and homogenised in 4 ml of ice-cold Tris-buffer (0,15 M NaCl+20mM Tris-HCl, pH:8,3). After centrifugation (10 min, 4500 r.p.m., 4 °C), pellets were discarded and supernatants were recentrifuged (10 min, 10 000 g; 4 °C). The final supernatants were filtered by 0,8-mm pore-sized syringe filters, and the aliquots

were stored at -20 °C until analysed. MMP-9 was measured by the quantitative enzyme linked immunosorbent assay described above.

3.3.6 Measurement of faecal calprotectin

In the second part of this study, faecal calprotectin was measured in a subset of UC patients (n=24, male/ female: 10/14, mean age: 45,7 years). For calprotectin measurements, faecal specimens were thawed at 48 °C and calprotectin level was determined by a quantitative lateral flow assay (Quantum Blue, Bühlmann Laboratories, Schönenbuch, Switzerland) according to the manufacturer's instructions.

3.3.7 Statistical analysis in the human studies

The results are presented as mean \pm standard deviation. Due to the non-normal distribution of the data, a Kruskal–Wallis test was used to compare faecal MMP-9 levels of the three groups (control, IBS-D, and UC) four different groups of UC related to disease activity, and control, hyperplastic polyp, adenoma and CRC. Correlation between MMP-9 levels and disease activity scores or blood test results were determined by Spearman correlation. Correlation between MMP-9 and calprotectin were also tested by Spearman correlation. The cut-off levels, specificity, and sensitivity were calculated using receiver operating characteristic (ROC) analysis in both data groups to determine the predictive power of the MMP-9 level. In UC patients, active disease has been determined as total Mayo score >3 and Mayo endoscopic sub-score >0 . The cut-off levels were calculated in case the area under the ROC curve (AUC) value was above 0,9 in UC study and 40,8 in the cancer study. The cut-off level was determined by the maximum value of Youden's index (sensitivity+specificity-1). MMP-9 levels of high- and low-risk adenoma subgroups were analysed by the Mann–Whitney test. The different subgroups according to tumour localisation or stage were compared with the Kruskal–Wallis test. Differences were considered statistically significant

when $P < 0.05$. Statistical analyses were performed by the R (version 3.1.2) statistical software (<https://www.r-project.org/>).

4. RESULTS

4.1. Animal studies

4.1.1. In vivo and in vitro permeability measurements

To define the impact of modestly-increased intestinal permeability, we studied transgenic mice with intestinal epithelial-specific constitutively-active myosin light chain kinase expression. This MLCK-dependent tight junction regulation increased intestinal permeability (Figure 1 A and B). (In vitro permeability measurement: Mean \pm SD: WT jejunum: $0,8165 \pm 0,4476$ nmol/h/cm²; *CAMLCKTg* jejunum: $1,528 \pm 0,5516$ nmol/h/cm²; $p = 0,0411$, In vivo measurement: Mean \pm SD: WT: $11,83 \pm 4,343$, *CAMLCKTg*: $15,59 \pm 5,622$, $p = 0,0245$).

4.1.2. Body weight gain and intestinal transit

Postnatal growth (Figure 1 C), reproduction, intestinal transit (Figure 1 D) are unaffected in CAMLCK transgenic (*CAMLCKTg*) mice. (Transit results, geometric centers Mean \pm SD: WT: $8,248 \pm 0,6189$ μ g/ml, *CAMLCKTg*: $8,595 \pm 0,5105$ μ g/ml, $p = 0,1988$).

4.1.3. Microbiota analysis

We initially analysed the gut microbiome of 31 WT and *CAMLCKTg* pups born to 8 WT dams. The microbiomes segregated by pup genotype but not dam (Figure 1 E) and included increased *Clostridium* and decreased Bacteroidetes, *Enterococcus spp*, and

Prevotella in *CAMLCKTg* mice (Figure 1 F). Increased intestinal permeability can therefore cause dysbiosis-like microbiome shifts.

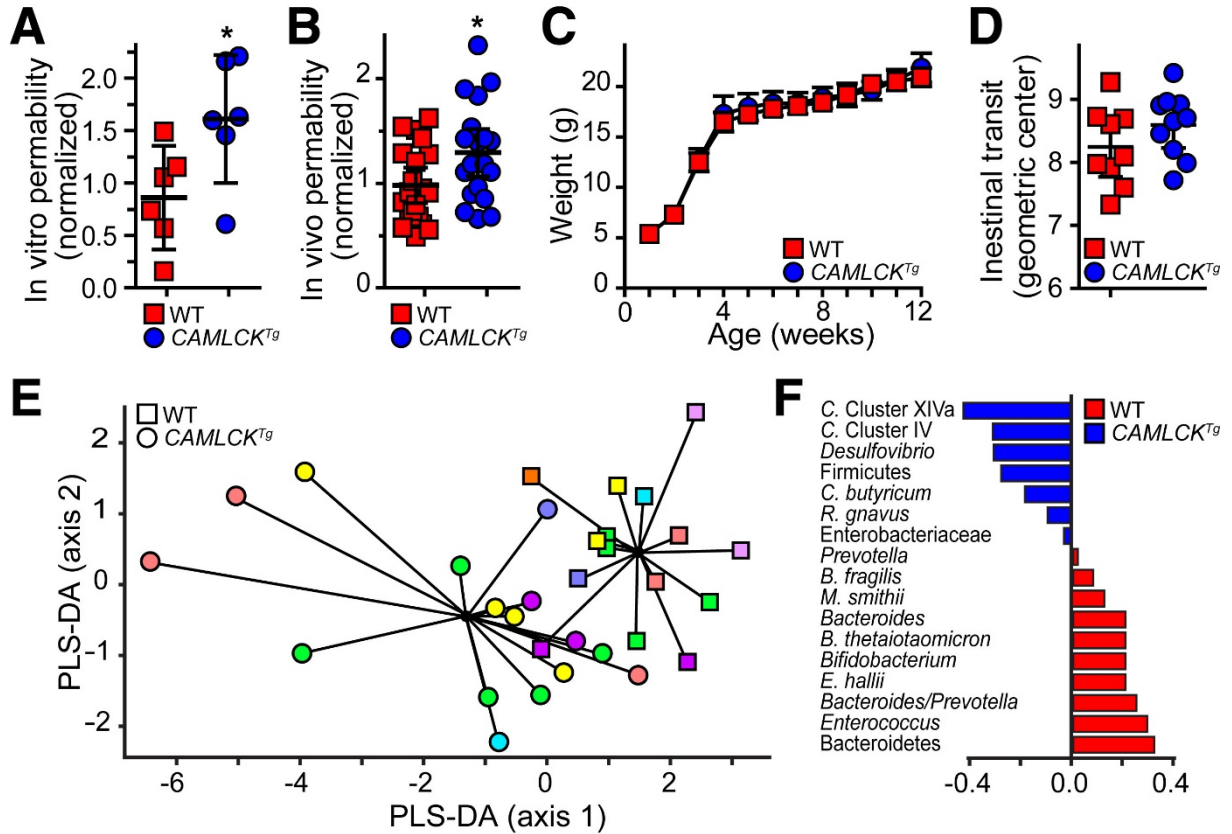


Figure 1: Trans-jejunal fluorescein flux was increased in *CAMLCKTg* (blue circles) relative to WT (red squares) littermates. mean \pm SD; *, $p < 0.05$, Mann-Whitney U test.

B. In vivo analysis using FITC-4kDa dextran demonstrated increased permeability of *CAMLCKTg* (blue circles, $n=19$) relative to WT (red squares, $n=20$) littermates. mean \pm SD; *, $p < 0.05$, t-test.

C. Weight gain was similar in WT (red squares, $n=6$) and *CAMLCKTg* (blue circles, $n=6$) littermates. mean \pm SD.

D. Intestinal transit was similar in WT (red squares, $n=10$) and *CAMLCKTg* (blue circles, $n=9$) littermates. mean \pm SD.

E. Partial least squares discriminant analysis (PLS-DA) score plot based on the relative abundances of 18 microbial taxa in gut contents of *CAMLCKTg* (circles, $n=16$) and WT (squares, $n=15$) born to 8 different dams (each colour represents one dam).

F. Relative abundances of microbial communities in *CAMLCKTg* (blue) and WT (red) mice. Diagrams indicate regions analysed.

4.1.4. Open field test

Both the percentage of distance travelled in the center and the fraction of time spent in the center of the open field were reduced in *CAMLCKTg* mice (Figure 2C); this did not reflect reduced locomotor activity, as total distance travelled in the entire area was similar in *CAMLCKTg* and WT mice (Figure 2C). (Time in center: (mean \pm SEM) WT: 10,18 \pm 1,219% , *CAMLCKTg*: 5,785 \pm 1,312%, $p=0,0278$, distance in center: WT: 12,22 \pm 0,7487%, *CAMLCKTg*: 7,860 \pm 0,7869%, $p=0,0011$, total distance travelled: WT: 3602 \pm 302,8 m, *CAMLCKTg*: 4260 \pm 419,8 m, $p=0,2157$)

4.1.5. Colorectal distension

CAMLCKTg mice displayed striking visceral analgesia to colorectal distension relative to WT littermates (Figure 2D). Sensitivity was restored by enzymatic MLCK inhibition, water avoidance stress, or naloxone-mediated opioid receptor antagonism (Figure 2D).

4.1.6. C-Fos analysis

C-Fos immunolabeling, an indicator of neuronal activity, was significantly greater in the paraventricular nucleus of the thalamus, the paraventricular nucleus of the hypothalamus, and the hippocampus, but not the medial prefrontal cortex, nucleus accumbens, or amygdala, of *CAMLCKTg*, relative to WT, mice (Figures. 3, 4). (Mean \pm SEM, paraventricular nucleus of the thalamus: WT: 102,4 \pm 27,49 cells/mm², *CAMLCKTg*: 204,1 \pm 29,23 cells/mm² $p= 0,0297$, paraventricular nucleus of the hypothalamus: WT: 41,27 \pm 11,57 cells/mm², *CAMLCKTg*: 114,2 \pm 28,41 cells/mm², $p= 0,0387$, hippocampus: WT: 24,50 \pm 2,397 cells/mm², *CAMLCKTg*: 46,78 \pm 8,083 cells/mm², $p=0,0296$, medial prefrontal cortex: 47,52 \pm 12,48 cells/mm², *CAMLCKTg*: 53,33 \pm 13,41 cells/mm², $p= 0,7577$, nucleus accumbens: WT: 55,36 \pm 12,30

cells/mm², CAMLCKTg: 70,82± 12,93 cells/mm², $p = 0,4068$, amygdala: WT: 65,98± 24,16 cells/mm², CAMLCKTg: 91,98± 23,87 cells/mm², $p = 0,4616$)

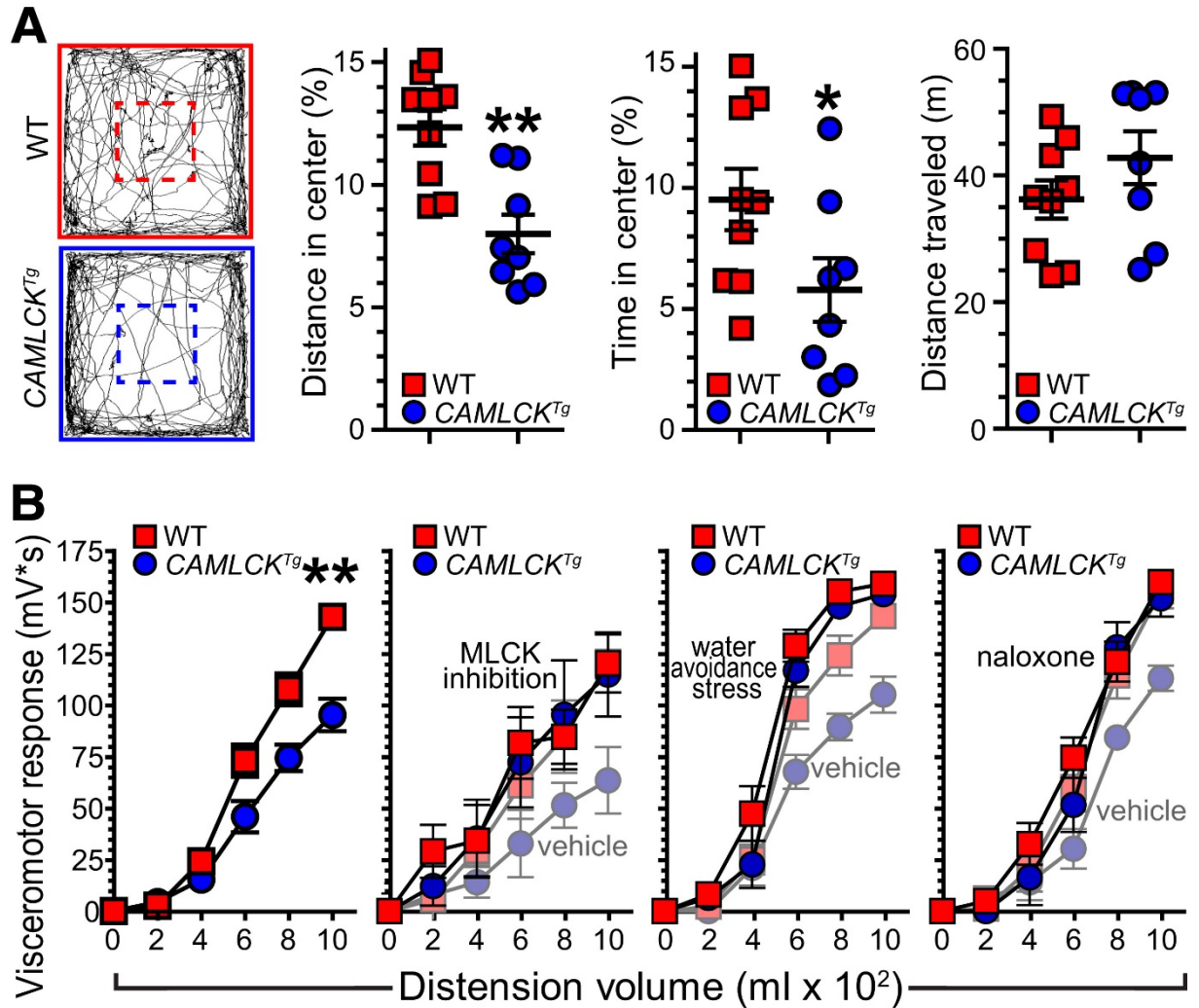


Figure 2: Increased intestinal permeability modifies behaviour and visceral sensitivity. A. Video tracking paths of representative WT and *CAMLCKTg* mice in the open field test. Percent distance travelled in the center (dashed lines), percent time in the center, and overall distance travelled in the entire field are shown. *CAMLCKTg* (blue circles, $n=8$) and WT (red squares, $n=9$) littermates were tested. mean±SEM. *, $p<0.05$; **, $p<0.01$, Mann-Whitney U test. **B.** Stepwise colorectal distension-induced visceromotor responses in *CAMLCKTg* (blue circles, $n=7$) were reduced relative to WT (red squares, $n=7$) littermates. Genotype-specific differences were eliminated by MLCK inhibition, water avoidance stress, or naloxone treatment. $n = 5-9$ per condition; for each treatment (vehicle control *CAMLCKTg* and WT mice from the same experiment are shown with pale symbols in the last three graphs). mean±SEM; **, $p<0.01$, 2-way ANOVA.

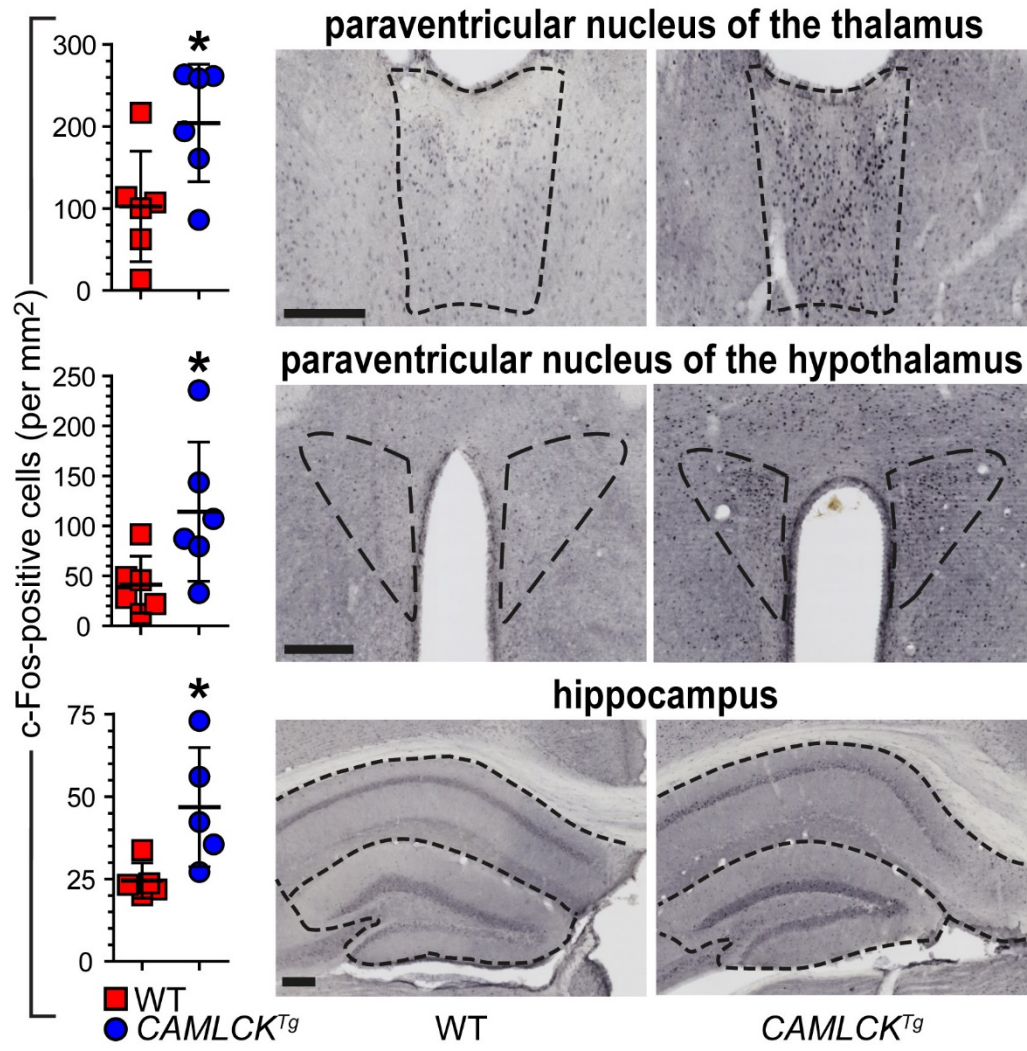


Figure 3: Increased intestinal permeability induces increased C-Fos immunolabelling in selected brain regions. *CAMLCK^{Tg}* (blue circles, n=5-6) and WT (red squares, n=5-6) littermates. Representative images of C-Fos immunolabeled brains from *CAMLCK^{Tg}* and WT mice. Bars = 200 µm; mean±SEM; *, $p < 0.05$, t-test.

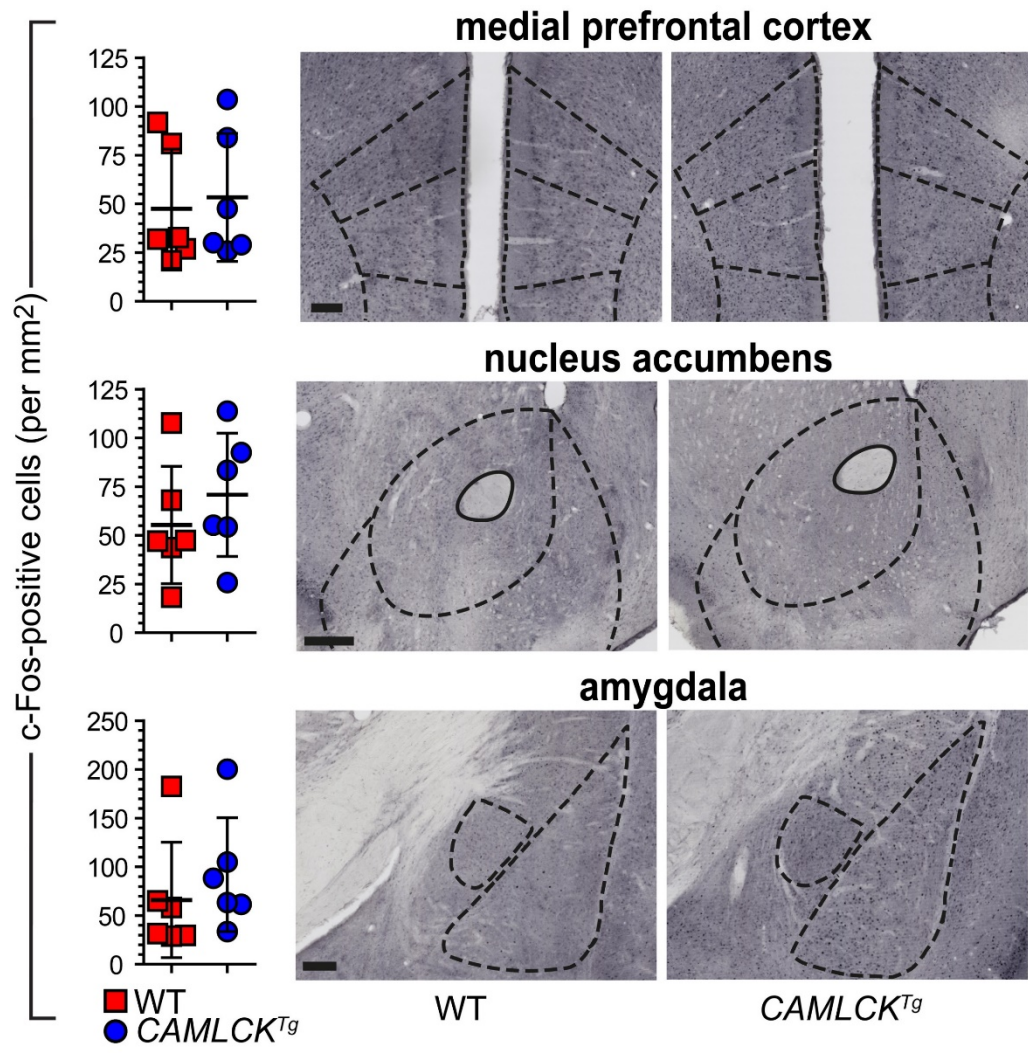


Figure 4: CAMLCKTg (blue circles, n=5-6) and WT (red squares, n=5-6) littermates. Representative images of C-Fos immunolabeled brains from CAMLCKTg and WT mice.

4.2. Human studies

4.2.1. Validation assay of faecal MMP-9 measurement

The concentrations measured in ng/mL are shown in Figure 5. The figure clearly shows a linear concentration change corresponding to dilution. It indicates that (i) the faecal

matrix itself has no influence on MMP-9 determination; and (ii) the kit from R&D Systems is able to determine MMP-9 from human faecal extracts.

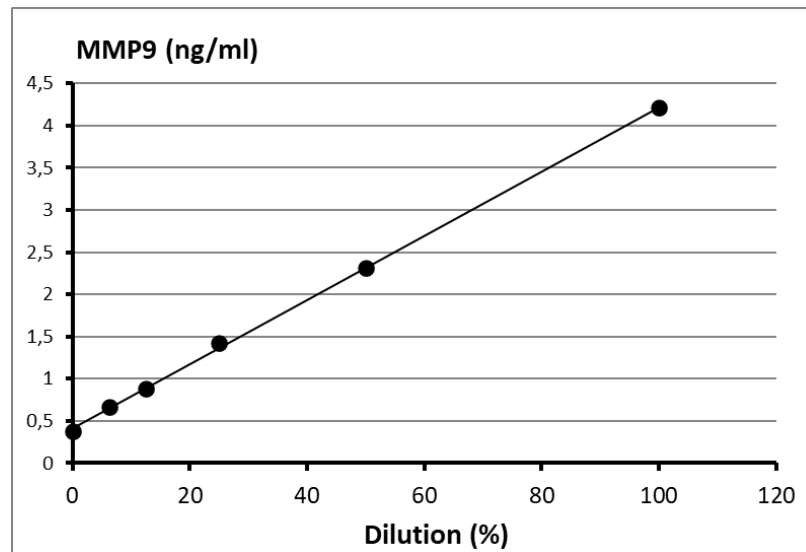
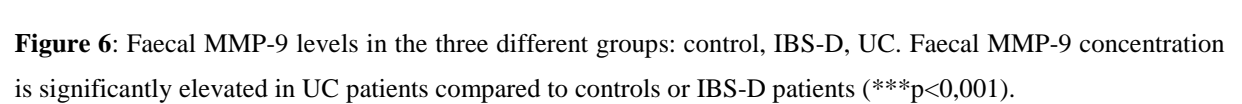


Figure 5: MMP-9 concentration levels at different dilutions in the validation assay.

4.2.2. Faecal MMP-9 levels in control, IBS-D and UC patients' samples

There were no significant differences between the three groups (control, IBS-D, and UC) regarding age ($P=0,916$). Faecal MMP-9 contents were significantly different between the three groups (Figure 6, $P < 0,001$). Indeed, specimens originating from healthy subjects had very low MMP-9 levels; nearly half of them were found to be below the detection limit of the assay (mean: $0,0208 \pm 0,045$ ng/mL). Similarly, IBS-D patients had low faecal MMP-9 levels (IBS-D: $0,0017 \pm 0,008$ ng/mL). However, in the UC group the average MMP-9 level was significantly higher (UC: $15,65 \pm 20,14$ ng/mL). To distinguish UC patients from healthy controls or IBS-D patients, the cut-off level was determined at 0,245 ng/mL ($AUC=0,939$). By means of this cut-off, sensitivity was 85.1% and specificity 99,99% (Figure 6)



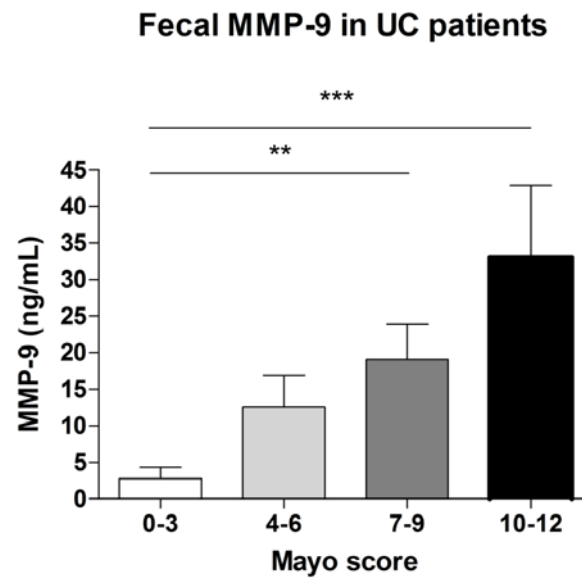


Figure 7: Faecal MMP-9 levels in UC patients depending on disease activity. Faecal MMP-9 concentration is significantly elevated in UC patients with moderate (** $p < 0,01$) and severe (** $p < 0,001$) activity compared to those in remission.

Blood test result	CRP	Erythrocyte sedimentation rate	Serum ferrous level	White blood cell count	Haemoglobin	platelet
MMP-9	R=0,456 P=0,002	R=0,228 P=0,137	R=0,454 P=0,003	R=0,404 P=0,005	R=0,151 P=0,317	R=0,296 P=0,046

Table 1. Correlation of faecal MMP-9 levels with blood test results. Significance is accepted at $p < 0,05$.

4.2.4. Correlation of faecal MMP-9 concentration with faecal calprotectin levels in UC

In a subset of 24 UC patients, faecal calprotectin levels were also determined. Significant correlation was found between the faecal MMP-9 and calprotectin levels ($R=0,495$; $P=0,014$). In this subset of patients, the correlation of endoscopic and overall Mayo scores was calculated with both MMP-9 and calprotectin. In this smaller cohort of patients, faecal MMP-9 levels showed a slightly stronger correlation with the endoscopic component of

the Mayo score than calprotectin ($R=0,587$; $P=0,003$, $AUC=0,895$ for calprotectin and $R=0,653$; $P=0,002$, $AUC=0,939$ for MMP-9), and a higher correlation with the overall Mayo score than calprotectin ($R=0,587$; $P=0,016$, $AUC=0,895$ for calprotectin and $R=0,718$; $P=0,002$, $AUC=0,905$ for MMP-9).

4.2.5. Faecal MMP-9 levels in groups of non-adenoma/adenoma/CRC patients

Seventeen patients (male/female: 8/9; mean age: 59,7 years) were found negative on total colonoscopy and 17 patients had no other finding from non-inflamed diverticulosis (male/female: 3/14; mean age: 71,8 years). There was no significant difference between these two groups in terms of faecal MMP-9 levels ($P=0,49$), therefore these 34 patients were used as polyp-free/non-malignant controls (mean age: 65,7 years). Out of the other patients, 15 had hyperplastic polyps (male/female: 9/6; mean age: 62,3 years), 32 had adenomas (male/female: 21/11; mean age: 67,0) and 28 had CRC (all adenocarcinomas; male/female: 17/11; mean age: 67,2 years) according to the endoscopic and histological examinations. There were no statistically significant differences between the four groups (controls, hyperplastic polyps, adenomas, CRC) regarding the age. Faecal MMP-9 levels were significantly different between the four groups (Figure 8, $P<0,0001$). Faecal samples from control patients had very low MMP-9 levels (mean: $0,118\pm0,057$ ng/mL). Patients with hyperplastic polyps also presented with low faecal MMP-9 levels (mean: $0,112\pm0,073$ ng/mL). Adenoma patients had slightly, but not significantly elevated faecal MMP-9 levels (mean: $0,656\pm0,396$ ng/mL). Nevertheless, patients with CRC had a significantly increased mean of faecal MMP-9 levels (mean: $7,028\pm3,722$ ng/mL). To distinguish CRC patients from controls, the cut-off level was determined at 0,23 ng/mL ($AUC=0,913$; 95% CI: 0,833–0,994; $P<0,001$). This cut-off level was associated with a sensitivity of 89,3% and a specificity of 91,2% (Figure 9). Patients with hyperplastic polyps ($AUC=0,526$; 95% CI: 0,340–0,711; $P=0,779$) were not distinguishable from control individuals by ROC analysis. Among adenomas, 22 out of 32 were at increased risk of malignancy, with a mean faecal MMP-9 level significantly elevated compared with low-risk adenomas (mean of high-risk adenomas: $0,936\pm0,570$ vs mean of low-risk adenomas: $0,040\pm0,017$; $P<0,05$; Figure 10). Low-risk adenomas could not be distinguished

from controls by faecal MMP-9 levels (AUC=0,456; 95% CI: 0,236–0,676; P=0,706) When ROC analysis was performed with an aim to select high-risk adenomas and CRC from controls, the cut-off level was determined at 0.085 ng/ml. In this case, sensitivity was 76% and specificity was 85,3% (AUC=0,806; 95% CI: 0,710–0,902; P<0,001). With this lower cut-off level, 59% of high-risk adenomas were identified. By ROC analysis of high-risk adenomas vs controls, the cut-off level would be similarly 0.085 ng/ml⁻¹ (AUC=0,670; 95% CI: 0,507–0.833; P=0,033).

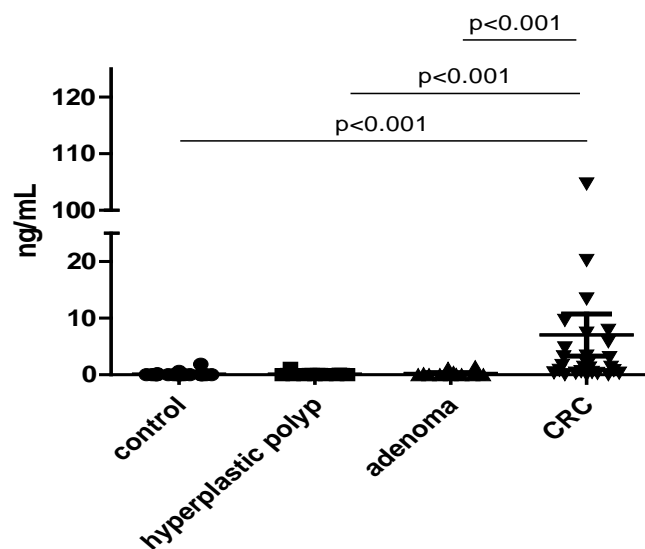


Figure 8: Faecal MMP-9 levels of the different groups (based on the colonoscopy and histology). Abbreviation: CRC=colorectal carcinoma

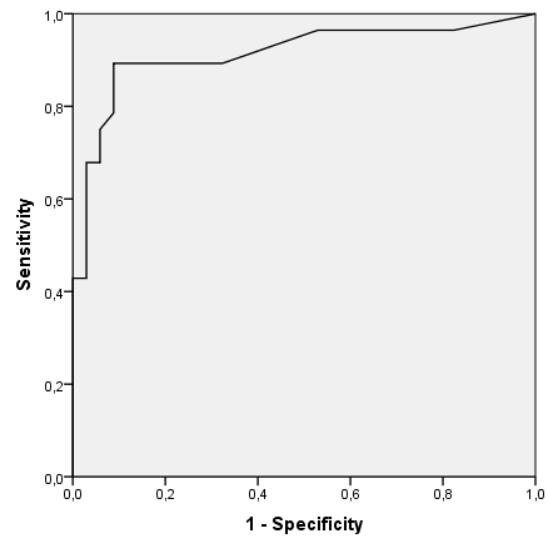


Figure 9: ROC (receiver operating characteristic) curve of CRC vs. controls

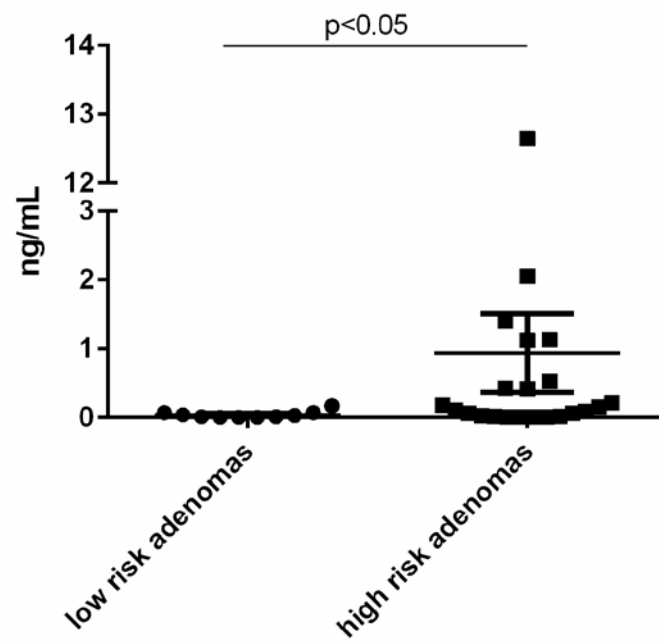
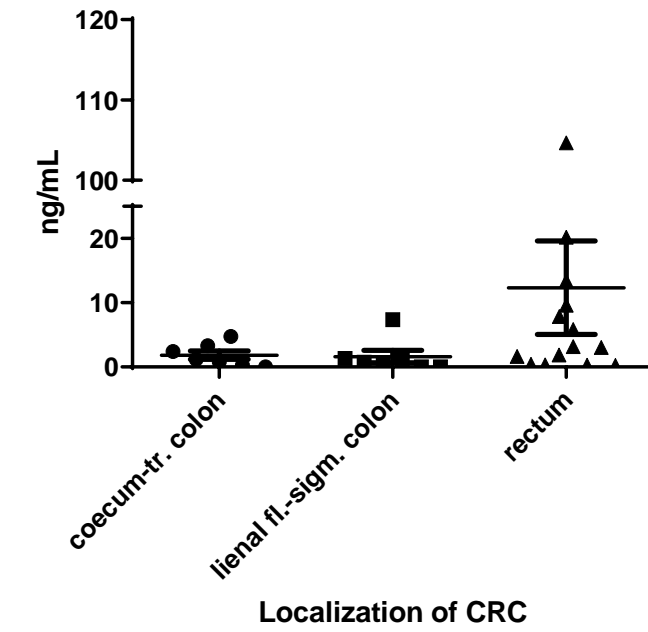


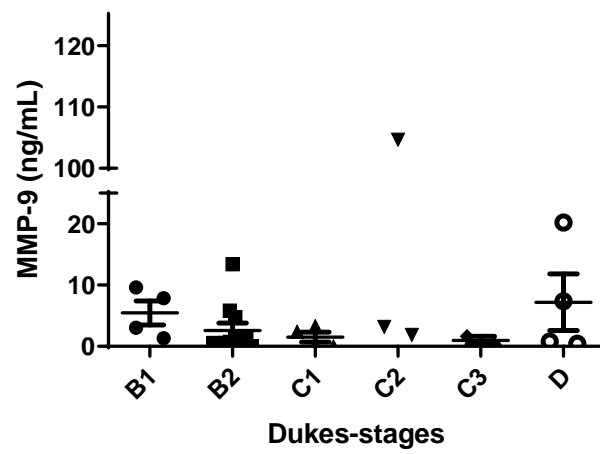
Figure 10. Faecal MMP-9 levels in low-risk and high-risk adenomas.

4.2.6. Faecal MMP-9 levels according to CRC localisation and stage

The number of patients regarding the site of the CRC was as follows: rectum: 14, sigmoid colon: 6, lineal flexure: 1, transverse colon: 2, hepatic flexure: 2, ascending colon: 1, and caecum: 2 patients. If faecal MMP-9 levels are analysed according to the site of the CRC, no significant difference was observed ($p=0,146$), although a trend was seen towards higher levels in case of a rectal tumour (mean: $12,33\pm 7,274$ ng/ml) compared with left-sided colon (lineal flexure–sigmoid colon; mean: 1.619 ± 0.978 ng/ml) and transverse-right-sided colon (from caecum to transverse colon; mean: 1.830 ± 0.656 ng/mL; Figure 11A). According to Dukes' classification, no significant difference could be seen between the groups (Figure 11B; $p=0,236$).



A



B

Figure 11: Faecal MMP-9 levels in CRC patients. Values according to (A) localisation and (B) Dukes' stages of CRC.

5. DISCUSSION

5.1. Animal studies

Our results showed that mice exhibiting a permanent and discrete elevation of intestinal permeability are characterized by modifications of colonic microbiota composition. Moreover, *CAMLCKTG* animals exhibit a naloxone-sensitive visceral hyposensitivity to colorectal distension, associated with an anxiety-prone phenotype.

In the present work we have used a transgenic mouse that expresses a constitutively active myosin light chain kinase. MLCK is expressed throughout the gut epithelium and controls the cytoskeleton contraction through MLC phosphorylation upon TNF stimulation [47]. *CAMLCKTg* animals exhibit discrete increased permeability, without alterations of tight junction organization, associated with low-grade mucosal inflammation evoked by increased expression of TNF α , IFN γ , IL-10 and TGF β [15]. However, transgenic mice grew normally and did not present any modification of the gastrointestinal transit. We have confirmed that *CAMLCKTg* mice display an increased permeability *in vivo*, which seems limited to the small intestine as shown by Ussing chambers experiments. This observation is consistent with the higher expression of the transgene denoted in the jejunum compared with the colon [15].

Microbiota analysis has been performed in colonic samples from WT and *CAMLCKTg* animals using Gut low density Array (GuLDA) developed by Bergström [44], allowing the determination of abundance of targeted species using 16S rRNA. It is important to note that we performed this analysis on littermates, *i.e.* on animals from the same dam and bred together until weaning. We have observed an increased abundance of Firmicutes, especially *Clostridium* clusters XIVa and IV, which encompass bacterial species able to adhere and degrade intestinal mucus [48]. Among them, *Ruminococcus gnavus* has been shown to degrade mucins through a specific bacterial adhesion molecule that also present a sialydase activity [49]. In addition, higher relative levels of *Desulfovibrio spp*, a strain known to bind mucins, especially in a context of inflammation [50], have also been denoted in transgenic animals. Of interest, *Akkermansia muciniphila* was not detected in both genotypes, while we have found these bacteria in older mice (data not shown). On the other hand, transgenic mice

microbiota is characterized by a lower abundance of *Bacteroidetes*. This leads to an increase in the Firmicutes/Bacteroides ratio, which has also been observed in IBS patients [51], despite the clinical relevance of this parameter remains a matter of debate.

Based on our observations *CAMLCKTg*, animals display a phenotype close to what could be observed in IBS patients [52], in terms of epithelial barrier dysfunction, mucosal immune alterations and dysbiosis. The diagnosis of IBS relies on a symptom-based approach that identifies, according to Rome IV criteria, four subtypes: constipation, diarrhoea, mixed symptoms, and unsubtyped [1]. Altered intestinal barrier function is frequently observed whatever the IBS subgroup considered. Of interest, increased permeability has been characterized in biopsies harvested from patients suffering from IBS [53]. Luminal contents of these patients are also likely involved in triggering epithelial barrier impairment since faecal supernatants promote increased permeability associated with occludin disruption [54]. In addition, alterations of the barrier function do not appear to be limited to the colon, but extend to the small bowel [55], suggesting the commitment of the whole gut in this phenomenon. Increased intestinal permeability has been suspected to be a prerequisite for the enhanced visceral pain experienced by patients. In humans, the severity of IBS symptoms in IBS-D patients is positively correlated to intestinal permeability measured by the lactulose/mannitol ratio [9]. In rats submitted to partial restraint stress, a valuable model to induce visceral hypersensitivity to colorectal distension associated with impaired intestinal barrier function, normalization of increased permeability by probiotics [56] or an MLCK inhibitor [7] resulted in visceral normosensitivity. Therefore, we hypothesized that *CAMLCKTg* mice, showing increased permeability, would also present visceral hypersensitivity to colorectal distension. Unexpectedly, we observed a marked, naloxone-sensitive hyposensitivity to colorectal distension for moderate to high volumes of distension applied. Visceral sensitivity can be negatively or positively modulated by descending pathways. Indeed, Wilder-Smith *et al.* have shown an abnormal activation of endogenous pain inhibitory pathways in IBS patients [57]. In rats, Larauche *et al.* have established that animals acutely exposed to water avoidance stress present analgesia to colorectal distension in females but not in males [58]. This analgesia was sensitive to naloxone. Conversely, one day after stress, female rats exhibited a visceral hyperalgesia. The authors hypothesize that the initial hypoalgesia could be linked with the engagement of antinociceptive pathways in response to anxiety or fearfulness associated with

stress exposure. Therefore, we determined the levels of anxiety of *CAMLCKTg* and WT animals, and we observed that transgenic animals present an anxiety-prone phenotype evidenced by the open field. This suggests that the increased level of anxiety of transgenic animals compared with their WT littermates may determine a lower sensitivity to colorectal distension. Our works on neuronal activity in non-stressed animals show, that the permeability elevation due to the transgene induced a cerebral activity pattern, with altered activation in regions of the brain usually associated with visceral sensation and the affective response to pain. Literature data show, paraventricular thalamus as responsible for wakefulness [59], and in a latest post-traumatic stress response study similar regions alteration was observed (nucleus paraventricularis of the thalamus, medial prefrontal cortex, hippocampus) [60]. In *CAMLCKTg* transgenic mice, the modification of the intestinal permeability provoked an anxious behaviour through the central nervous system.

5.2. Human studies

To the best of our knowledge our study is the first to report elevated faecal MMP-9 levels in patients with active UC compared to functional (IBS-D) patients or controls. In UC patients faecal MMP-9 levels are excellent predictors of disease activity and show a significant correlation with the clinical and endoscopic scores. Faecal MMP-9 levels correlate significantly with faecal calprotectin, a well-known marker of disease activity in UC. MMPs are a family of Zn²⁺-containing endopeptidases capable of degradation of the extracellular matrix. They are secreted by various cell types, such as fibroblasts, mesenchymal cells, tumour cells, and several inflammatory cells like monocytes, lymphocytes, and neutrophils. MMP-9 is a gelatinase that represents $\approx 30\%$ of protein content of neutrophil granules [61]. In a murine experimental model, it has been shown that epithelial cells and granulocytes are the main sources of MMP-9 in colitic tissue, and exogenous MMP-9 impairs epithelial wound healing and cell attachment in vitro [62]. Urinal MMP-2 and MMP-9 levels have been suggested to discriminate children with IBD from controls; nevertheless, urine samples were found positive for MMP-2 in 25%, for MMP-9 in 20% of control individuals, leading to false positivity in the diagnosis [34]. Further, as MMPs play important roles in cancer development

and metastasis formation[63], urinary MMPs were found elevated in various cancer types including prostate, bladder, breast, brain, and hematologic malignancies [64-67]. These findings point out that urinary MMPs are not specific for IBD. We hypothesize that faecal MMP level may be more characteristic of gastrointestinal pathologies than of other organ-specific or systemic disorders, although its specificity to UC compared to other colon diseases needs to be verified. In colonic biopsies, MMP-9 levels were significantly increased in UC patients, and less significantly increased in CD patients compared to controls [33]. Based on colonic biopsies, a recent study demonstrated that in contrast to UC, MMP-9 is not involved in the inflammatory process of lymphocytic colitis and collagenous colitis [68]. By means of a cut-off level of faecal MMP-9 at 0,245 ng/mL, none of our controls and IBS-D patients tested positive. Nevertheless, faecal MMP-9 negativity does not rule out IBD, as seven UC patients, all in remission, were also below the cut-off level. It is important to note that none of them had colonic ulcerations on endoscopy (Mayo endoscopic subscore ≤ 1). Therefore, our data suggest that low faecal MMP-9 level is a negative predictor of mucosal lesions. Faecal MMP-9 positivity suggests the need of further evaluation of inflammatory diseases or other gastrointestinal pathologies. Interestingly, in an earlier study on CD patients, MMP-9 plasma levels were found elevated compared to controls, but no correlation could be observed with the Crohn's Disease Activity Index (CDAI) [69]. Similarly, it has been demonstrated that other disease activity markers, such as calprotectin [70], lactoferrin, or CRP, and even endoscopic appearance correlate poorly with CDAI [71]. In our study, however, faecal MMP-9 concentration showed a significant correlation with the clinical and endoscopic scores in UC patients. Additionally, faecal MMP-9 level significantly correlated with CRP, a laboratory value referring to inflammation, widely used as a disease activity marker in the blood. In the last part of our study we compared faecal MMP-9 to an established faecal marker in UC, calprotectin, and a significant correlation was found between them. In some centres, faecal calprotectin is already used as a routine test to discriminate IBS from IBD[72]. These data suggest that faecal MMP-9 can be a novel marker to help in the differential diagnosis of patients with diarrhoea and abdominal pain. Further, it could be a new complementary non-invasive method to assess mucosal inflammation and lesions in UC patients.

To the best of our knowledge, we report for the first time that MMP-9 levels are elevated in faecal specimens from CRC patients compared with patients without polypoid

lesions. Furthermore, faecal MMP-9 can be used to distinguish CRC patients from patients with no polypoid lesions, with a high sensitivity and specificity. Additionally, with a lower cut-off level, faecal MMP-9 is able to identify nearly 60% of patients with a high-risk adenoma. MMP-9 is a gelatinase capable of the degradation of the extracellular matrix, which is believed to have a role in the progression and metastasis formation of many tumours, including colon cancer [43]. Apart from colon cancer cells, tumour-infiltrating neutrophils are also an important source of MMP-9, as a major angiogenesis-inducing factor [73, 74]. Serum MMP-9 was suggested as an early non-invasive biomarker for breast cancer [75] and a prognostic marker for lung cancer [76]. Urinary MMPs were found elevated not only in bladder, renal and prostate cancers but also in anatomically distant tumours, including breast, brain, and haematological malignancies [64-67]. Recently, significant efforts have been made to explore the potential role of MMP-9 as a biomarker in CRC. In the serum, it has been suggested as a biomarker of CRC in symptomatic patients [77], and its levels correlated with the Dukes' stage [78]. Furthermore, it has been recently shown that serum levels of neutrophil gelatinase-associated lipocalin in complex with MMP-9 are elevated in CRC patients, but it was found unsuitable as a diagnostic marker, as its discriminative power was very poor [79]. We have found the discriminative power of faecal MMP-9 levels in CRC patients sufficient, which can be explained by the fact that faecal MMP-9 could be more informative for gastrointestinal malignancies than urine or serum. Our method does not require a preceding diet, and one faecal sample gave a sufficient sensitivity for discrimination. Its analysis is automated, and the cut of concentration is adjustable. In terms of these characteristics, faecal MMP-9 measurement, similarly to FIT, is more convenient for clinical use than gFOBT. Furthermore, faecal MMP-9 measurement provides a higher sensitivity and specificity for CRC than gFOBT and many types of FIT. In the past decade, numerous faecal molecular markers have been described, which are based on the detection of genetic mutations and epigenetic alterations, linked to the carcinogenetic process [80]. Genetic markers are claimed to be more sensitive than FOBTs [80]. However, one disadvantage of these markers is that they are not present in every colorectal carcinoma. To overcome this problem, genetic markers are often combined in a panel, but this may lead to a decrease in specificity [80]. Furthermore, an important limitation of these methods is their high costs. Current evidence is not sufficient to accept the routine use of faecal genetic markers, and the European Guidelines for quality assurance have stated that they are currently unsuitable for CRC screening [40].

Similarly to molecular tests, our method also has the advantage over FOBTs in that it detects a marker originating directly from cancer, which, contrary to bleeding, is supposed to be permanent. However, the costs of faecal MMP-9 measurement are far beyond those of molecular tests, as they are <10 USD per sample, which is comparable to the costs of FOBT. This method is markedly cheaper than faecal DNA tests, which cost around 350 USD per sample [81]. The process of transformation from normal epithelium to cancer takes 10–15 years in most cases, leaving a possibility to remove precancerous lesions before they turn to definitive cancer [38]. Therefore, a reliable marker detecting high-risk adenomas is of high importance. The sensitivity of FIT for advanced adenomas is only around 20–33% [82, 83]. DNA tests are characterised by a much higher sensitivity, for example, K-ras mutations are detected in the faeces of around 50% of individuals with adenomas >1 cm [84], p53 mutations in up to 64% of severely dysplastic polyps [85] and 4–26% of adenomas [84]. Methods based on the detection of epigenetic changes appear to have a wide range of sensitivity, for example, the methylation of SFRP2 can be detected in the faeces of 52,4% of individuals with adenomas [86], while the methylation of methylguanine DNA methyltransferase can be found in 36% of the faeces of adenoma patients but that of the human mut I homolog-1 in only 11% [87]. Faecal tumour M2 pyruvate kinase, a promising marker in CRC screening, showed a sensitivity of 22% for advanced adenomas [88]. Serum MMP-9 levels were elevated not only in cancer but also in colorectal adenoma patients compared with healthy subjects [78]. Similarly, MMP-9 expression was found increased not only in tissue samples from CRC but also in those from highly dysplastic adenomatous polyps compared with hyperplastic polyps or adenomas with low dysplasia [89]. Furthermore, MMP-9 protein expression has been elevated in adenomas with high grade dysplasia compared with other adenomas or normal colonic tissue [90]. Our results have shown that faecal MMP-9 levels from high-risk adenoma patients are significantly elevated compared with patients with low-risk adenomas. However, with a lower cut-off level, our marker identified 59% of high-risk adenoma patients as positive. This result is definitely higher than the usual sensitivity of FOBTs and comparable to that of molecular tests but without the high costs of the latter. We did not find any significant difference between the faecal MMP-9 levels of the groups with different tumour location. Nevertheless, a tendency could be seen towards the highest faecal MMP-9 levels in rectal adenocarcinoma patients. This is probably not related to the nature of rectal tumours but could be explained with a less time for degradation of the marker in the intestinal lumen,

similarly to that seen by FIT [82]. Our study has some limitations. As our department is a specific colorectal centre, our patients were a selected population not representing the general population. Patients were on a low-fibre diet at the time of faecal sample collection, as a preparation for the upcoming colonoscopy. Data on the time of the day at faecal sample production was not collected in our study. Although the percentage of cancer and adenoma found on colonoscopy was relatively high, the number of patients in the different subgroups (i.e., tumour localisation, Dukes' stage) may be insufficient for the detection of minor differences. Moreover, MMP-9 is also elevated in gut inflammation, which may limit its use in case of, for example, IBD. We hypothesise that, in a more general population, elevated faecal MMP-9 levels in some cases could be related to inflammation or other cancers in the gastrointestinal tract. However, even in this case, the method would be a good predictor of the presence of a non-functional gastrointestinal disorder, based on our previous results showing that faecal MMP-9 measurement can well discriminate IBS-D patients and healthy subjects from UC patients. A further limitation is that, owing to the different cut off levels calculated on the same sample groups, the performance of the method may be overestimated. A further study with a larger and more general patient population could help us to clarify whether diet, time of the day at sampling, tumours of the upper GI tract and so on can affect faecal MMP-9 levels and could also serve to verify the performance of our method. A comparison with FIT on the same samples could be useful. Moreover, a larger study on a general cohort could also provide more information if this method is valuable for CRC screening. Nonetheless, our study was suitable to identify a new marker, faecal MMP-9, capable of discrimination of CRC from polyp-free controls. Faecal MMP-9 is characterised by many advantages of FIT and also of the molecular markers, without high costs. Further studies on a larger and more general population are needed to confirm whether this promising method is suitable for CRC screening as a complementary assay.

6. NEW RESULTS ESTABLISHED IN THE THESIS

6.1. Animal studies

1. *CAMLCKTg* results discrete permeability elevation in the small intestine in mice. This modification does not change the body weight and intestinal transit, but causes dysbiosis-like microbiota composition, increased *Clostridium* and decreased Bacteroidetes, *Enterococcus spp*, and *Prevotella* compared to WT littermates.
2. Visceral sensitivity responses in *CAMLCKTg* mice are reduced relative to WT littermates. Genotype-specific differences are eliminated by MLCK inhibition, water avoidance stress, or naloxone treatment.
3. *CAMLCKTg* mice show behaviour modifications compared to WT littermates, intestinal permeability elevation causes anxiety-like phenotype.
4. Neuronal activity is significantly greater in the paraventricular nucleus of the thalamus, the paraventricular nucleus of the hypothalamus, and the hippocampus, but not the medial prefrontal cortex, nucleus accumbens, or amygdala in *CAMLCKTg*, relative to WT mice.

6.2. Human studies

1. Standard ELISA kit is able to detect MMP-9 from faecal samples.
2. Elevated faecal MMP-9 levels are present in patients with active UC compared to functional (IBS-D) patients or controls.
3. In UC patients faecal MMP-9 levels are excellent predictors of disease activity and show a significant correlation with the clinical and endoscopic scores.
4. Faecal MMP-9 levels correlate significantly with faecal calprotectin in UC.
5. Faecal MMP-9 can be used to distinguish CRC patients from patients with no polypoid lesions, with a high sensitivity and specificity.
6. With a lower cut-off level, faecal MMP-9 is able to identify nearly 60% of patients with a high-risk adenoma.

7. SUMMARY

Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by continuous or remittent abdominal pain, bloating and altered bowel habits without recognized underlying organic aetiology. 5-20% of patients seeking a gastroenterologist are diagnosed with IBS, causing considerably high medical and indirect costs. Despite its epidemiological significance, the pathophysiology of IBS is still not completely understood. Animal models, which reveal the pathophysiological background, could provide better treatments options for patients suffering from this disease. Impaired intestinal barrier function is one of the potential key steps of the development of IBS symptoms, therefore transgenic mice with constantly elevated intestinal permeability could be ideal animal model of the disease.

Clinically it is a great challenge to distinguish between functional and organic gastrointestinal diseases. Symptoms of ulcerative colitis (UC) in remission are often similar to those of IBS. Furthermore, colorectal cancer (CRC) is one of the leading malignancies worldwide, therefore cheap non-invasive screening methods are of great importance. Matrix metalloprotease 9 (MMP-9) is elevated in colonic biopsies, urine, and blood plasma of UC patients and its level is elevated in colonic tumour biopsies. So far, faecal MMP-9 levels have never been measured.

Aims

The aims of the animal experiments in *CAMLCKTg* transgenic mice were: 1) to verify if the transgene has an effect on the gut permeability, animals' growth and intestinal transit, 2) to explore the effect of elevated intestinal permeability on the gut microbiota composition, 3) to study the moderately elevated gut permeability on behaviour and visceral sensitivity, 4) to investigate if the intestinal permeability elevation has an effect in the brain activity. In human studies our aims were: 1) to verify if the Standard ELISA Kit is able to detect MMP-9 from faecal samples 2) to compare faecal MMP-9 levels in UC patients to control subjects and a functional gastrointestinal disorder characterized by diarrhoea (IBS-D); 3) to test the

correlation between UC disease activity and faecal levels of MMP-9; 4) to correlate faecal MMP-9 levels with a known faecal marker of UC activity, calprotectin. 5) to assess the faecal MMP-9 levels in patients undergoing total colonoscopy according to endoscopic and histological diagnosis.

Methods

Animal studies: To examine the permeability elevation on IBS-like symptoms, we used transgenic mice that specifically express Constitutively Active Myosin Light Chain Kinase (*CAMLCKTg*) in gut epithelium. Faecal microbiota composition, relative bacterial abundances were analysed using qPCR technique. Visceral sensitivity to colorectal distension (CRD) was determined in groups of wild type (WT) and *CAMLCKTg* female mice. Some groups were previously submitted to water avoidance stress, others treated with naloxone. Specificity of MLCK-driven modifications of visceral sensitivity was addressed using ML-7 as a MLCK inhibitor. Behavioural modifications were evaluated using the open-field test. Neuronal activity was examined using c-Fos immunostaining. **Human investigations:** Faecal MMP-9 and calprotectin levels were measured by enzyme-linked immunosorbent assay and lateral flow assay, respectively. To measure the efficacy of MMP-9 to detect UC or CRC, first we verified if the standard ELISA Kit is able to measure MMP9 from faecal samples. For the UC-study UC, IBS-D patients, and control subjects provided faecal samples for MMP-9 analysis. In UC patients, disease severity was evaluated by the Mayo score. In the CRC study 104 patients provided faecal samples for MMP-9 analysis. A total colonoscopy was performed; suspicious lesions were evaluated by histology. Based on the colonoscopic and histological results, patients were allocated to five groups: negative, diverticulosis, hyperplastic polyp, adenoma, and CRC.

Key results

Animal studies: *CAMLCKTg* animals have an altered faecal microbiota composition, similarly as observed in IBS. At the basal state, *CAMLCKTg* mice showed a visceral hyposensitivity compared with WT, which was abolished with ML7 as well as naloxone pre-treatment. Upon stress stimulation, transgenic mice displayed a strong hypersensitivity to

CRD. Submitted to the open-field, *CAMLCKTg* mice showed an anxiety-prone phenotype. *CAMLCKTg* mice have altered activity in certain brain regions compared to the wild type mice, what can be responsible to the behaviour modification. Human investigations: Level of MMP-9 was undetectable or very low in the faeces of all healthy controls and IBS-D patients. In UC patients, faecal MMP-9 levels significantly correlated with the overall Mayo score, endoscopic score, and the serum C-reactive protein level. Additionally, in UC patients faecal MMP-9 levels showed a significant correlation with a known disease activity marker, faecal calprotectin. In the CRC study faecal MMP-9 was significantly increased in CRC compared with all other groups. Faecal MMP-9 was suitable to distinguish CRC patients from controls.

Conclusions & Inferences

Expression of *CAMLCKTg* within the mouse gut epithelium triggers increased permeability, which is associated with visceral hyposensitivity to CRD and anxiety. ML7 pre-treatment abolished visceral hyposensitivity showing that visceral hyposensitivity is linked to MLCK. Moreover, inhibition induced by naloxone suggests the involvement of endogenous opioid pathways. Transgenic mice were also hyperresponsive to water avoidance stress. Dysbiosis is observed in the group of animals having CAMLCK transgene induced intestinal permeability elevation with an increased activity pattern in certain brain regions.

Faecal MMP-9 is a useful tool in the differential diagnosis of diarrheic disorders and in the non-invasive evaluation of disease activity and mucosal healing in UC. Faecal MMP-9 may be a promising new non-invasive marker in CRC.

8. ABBREVIATIONS

AUC: area under the curve

CAMLCKTg: transgenic with constitutively active myosin light chain kinase

CD: Crohn's disease

CDAI: Crohn's Disease Activity Index

CRC: colorectal cancer

CRD: colorectal distension

CRP: C-reactive protein

ELISA: enzyme-linked immunosorbent assay

FIT: immunochemical faecal occult blood test

FITC: fluorescein isothiocyanate

FOBT: faecal occult blood test

gFOBT: guaiac faecal occult blood test

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome

IBS-C: constipation predominant irritable bowel syndrome

IBS-M :mixed type irritable bowel syndrome

IBS-U: unclassified irritable bowel syndrome

IL : interleukin

EMG : electromyograph

MLCK : myosin light chain kinase

MMP: matrix metalloprotease

ROC: receiver operating characteristic

ROI: region of interest

SD: standard deviation

SFRP2: secreted frizzled-related protein 2

TGF β : transforming growth factor beta

TNF: tumour necrosis factor

TRITC: tetramethyl-rhodamine

UC: ulcerative colitis

WT: wild type

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ANNEXES



Targeted intestinal tight junction hyperpermeability alters the microbiome, behavior, and visceromotor responses

Orsolya Inczeffi, Valérie Bacquié, Maiwenn Olier-Pierre, Marion Rincel, Belinda Ringot-Destrez, Sandrine Ellero-Simatos, Hélène Eutamène, Colette Bétoulières, Julie Thomas, Justin Laine, Louise Gros, Mathilde Lévêque, Renaud Leonard, Cherryl Harkat, Catherine Robbe-Masselot, Richard Roka, Muriel Mercier-Bonin, Vassilia Theodorou, Muriel Darnaudéry, Jerrold R. Turner, Laurent Ferrier

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Targeted intestinal tight junction hyperpermeability alters the microbiome, behavior, and visceromotor responses

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RUNNING HEADER: Diverse responses to intestinal hyperpermeability

CONFLICTS OF INTEREST: JRT is a cofounder of Thelium Therapeutics. LF is currently an employee of Nestlé Research (Société des Produits Nestlé S.A.).

CONTRIBUTIONS: Conceptualization: MOP, MMB, MD, VT, JRT, LF; Experimentation: OI, VB, MOP, BRD, MR, SES, CB, ML, JT, JL, LG, RL, CH; Data analysis: OI, VB, MOP, HE, CRM, MR, RR, MMB, MD, JRT, LF; Manuscript preparation and revision: MD, JRT, LF.

KEYWORDS: myosin light chain kinase, MLCK, tight junction, microbiome, stress

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1 Markedly increased intestinal permeability occurs in inflammatory bowel disease (IBD), graft-
2 versus-host disease (GVHD), celiac disease, and multiple organ dysfunction. In these
3 diseases, effectors of increased permeability include immune signaling,¹ microbiome,² and
4 corticosteroids³ that, in part, signal through epithelial myosin light chain kinase (MLCK). More
5 modest permeability increases occur in other disorders, including irritable bowel syndrome
6 (IBS), autism spectrum disorder (ASD), depression, and stress-related disorders. Data directly
7 linking barrier loss to disease phenotypes, however, are lacking.

8 To define the impact of modestly-increased intestinal permeability, we studied transgenic mice
9 with intestinal epithelial-specific constitutively-active myosin light chain kinase (CAMLCK)
10 expression. This MLCK-dependent tight junction regulation increased intestinal permeability
11 (Fig. S1A,B).¹ Nevertheless, postnatal growth (Fig. S1C), reproduction, intestinal transit (Fig.
12 S1D), and intestinal histology, epithelial proliferation (a sensitive indicator of epithelial damage),
13 and epithelial turnover are unaffected in CAMLCK transgenic (*CAMLCK^{Tg}*) mice.¹ In contrast,
14 mucosal tumor necrosis factor- α , interferon- γ , IL-10, and IL-13 transcripts as well as numbers of
15 lamina propria neutrophils, CD4⁺ T cells, and IgA⁺ plasma cells are modestly increased by
16 CAMLCK expression.^{1,2} Subclinical inflammation is, therefore, present and, by microbiome-
17 dependent, IL-17-mediated processes, affords partial protection from acute pathogen invasion.²
18 Immune activation is nevertheless unlikely to amplify CAMLCK-driven permeability increases,
19 as barrier function and ZO-1 anchoring are both acutely normalized by enzymatic MLCK
20 inhibition.^{1,4}

21 We initially analyzed the gut microbiome of 31 WT and *CAMLCK^{Tg}* pups born to 8 WT dams.
22 The microbiomes segregated by pup genotype but not dam (Fig. S1E) and included increased
23 *Clostridium* and decreased Bacteroidetes, *Enterococcus spp*, and *Prevotella* in *CAMLCK^{Tg}* mice
24 (Fig. S1F). Increased intestinal permeability can therefore cause dysbiosis-like microbiome
25 shifts. Interestingly, maternal separation, which increases intestinal permeability, causes similar
26 alterations and can be partially corrected by MLCK inhibitor-induced barrier restoration.⁵

27 Microbiome alterations overlapping with the above have been reported in IBS and ASD. We
28 therefore asked if *CAMLCK^{Tg}* mice displayed anxiety-like behavior, as occurs in those disorders,
29 using the open-field test (Fig. 1C). Both the percentage of distance traveled in the center and
30 the fraction of time spent in the center of the open field were reduced in *CAMLCK^{Tg}* mice (Fig.
31 1C); this did not reflect reduced locomotor activity, as total distance traveled in the entire area
32 was similar in *CAMLCK^{Tg}* and WT mice (Fig. 1C). These data are consistent with increased
33 anxiety-like behavior in *CAMLCK^{Tg}* mice. Although the results cannot differentiate between

34 direct effects of increased permeability and those requiring intermediate mediators, these data
35 demonstrate that intestinal permeability increases can influence behavior.

36 Stress and increased permeability have been associated with enhanced visceral sensitivity in
37 humans and rodents. Surprisingly, *CAMLCK^{Tg}* mice displayed striking visceral analgesia to
38 colorectal distension relative to WT littermates (Fig. 1D). Sensitivity was restored by enzymatic
39 MLCK inhibition, water avoidance stress, or naloxone-mediated opioid receptor antagonism
40 (Fig. 1D). Although this effect of increased permeability on visceral sensitivity was unexpected,
41 it is remarkably similar to the naloxone-reversible visceral analgesia reported in chronically-
42 stressed female rats⁶ and naloxone-sensitive inhibition of nociceptive neurons by supernatants
43 of colitic human and murine tissues.⁷

44 Studies of female IBS patients have linked increased permeability to altered functional and
45 structural brain connectivity.⁸ Thus, although responses to colorectal distension can be
46 mediated by spinal reflexes and sensory, limbic, and paralimbic regions of the brain,⁹ we asked
47 if neuronal activation was modified by CAMLCK-induced permeability increases. C-Fos
48 immunolabeling, an indicator of neuronal activity, was significantly greater in the paraventricular
49 nucleus of the thalamus, the paraventricular nucleus of the hypothalamus, and the
50 hippocampus, but not the medial prefrontal cortex, nucleus accumbens, or amygdala, of
51 *CAMLCK^{Tg}*, relative to WT, mice (Figs. 2, S2). Increased intestinal permeability may therefore
52 increase basal neuronal activity in areas of the brain that regulate responses to visceral pain or
53 stress⁹ but not those associated with conscious visceral sensation.

54 These results demonstrate that increased intestinal permeability can impact i) gut microbiome
55 composition; ii) behavior; iii) visceral pain responses; and iv) neuronal activation within the
56 brain. Critically, these changes are all results, rather than causes, of intestinal barrier loss, as
57 the latter was induced by targeted CAMLCK expression.

58 The sites of neuronal activation in *CAMLCK^{Tg}* mice support the hypothesis that increased
59 intestinal permeability can activate the hypothalamic-pituitary-adrenal axis.¹⁰ Conversely,
60 hypothalamic-pituitary-adrenal axis activation by exogenous stress can induce intestinal
61 permeability increases.³ Thus, as has been proposed in IBD and GVHD, a self-amplifying cycle
62 may ultimately direct the diverse phenotypes induced by MLCK-dependent, intestinal
63 permeability increases. Further study is needed to define the complex relationships between
64 intestinal permeability, stress, behavioral alterations, visceromotor responses, microbiome
65 composition, and other abnormalities.

66 These data are the first to assess behavior in a model where a targeted increase in intestinal
67 tight junction permeability is the only direct perturbation. The results demonstrate,
68 unequivocally, that modest tight junction permeability increases induced via a physiologically-
69 and pathophysiologically-relevant mechanism are sufficient to trigger local and systemic
70 microbial, behavioral, and neurosensory changes. This provides new perspective with which to
71 understand previously hypothesized cause-effect relationships that have been proposed on the
72 basis of correlative data.

73

Figure legends:**Figure 1: Increased intestinal permeability modifies behavior and visceral sensitivity. A.**

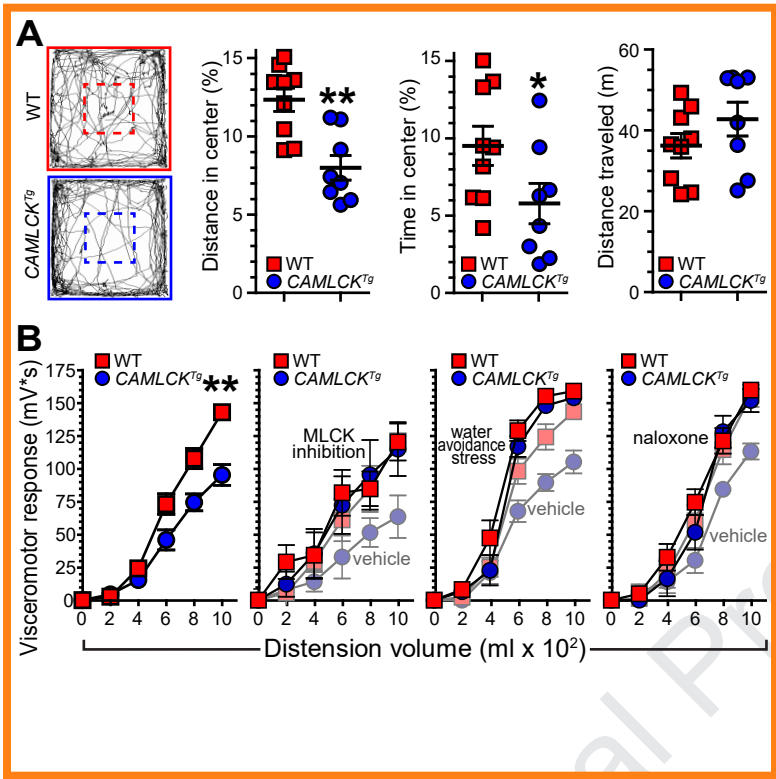
Videotracking paths of representative WT and *CAMLCK^{Tg}* mice in the open field test. Percent distance traveled in the center (dashed lines), percent time in the center, and overall distance traveled in the entire field are shown. *CAMLCK^{Tg}* (blue circles, n=8) and WT (red squares, n=9) littermates were tested. mean±SEM. *, $p<0.05$; **, $p<0.01$, Mann-Whitney U test. **B.** Stepwise colorectal distension-induced visceromotor responses in *CAMLCK^{Tg}* (blue circles, n=7) were reduced relative to WT (red squares, n=7) littermates. Genotype-specific differences were eliminated by MLCK inhibition, water avoidance stress, or naloxone treatment. n = 5-9 per condition; for each treatment (vehicle control *CAMLCK^{Tg}* and WT mice from the same experiment are shown with pale symbols in the last three graphs). mean±SEM; **, $p<0.01$, 2-way ANOVA.

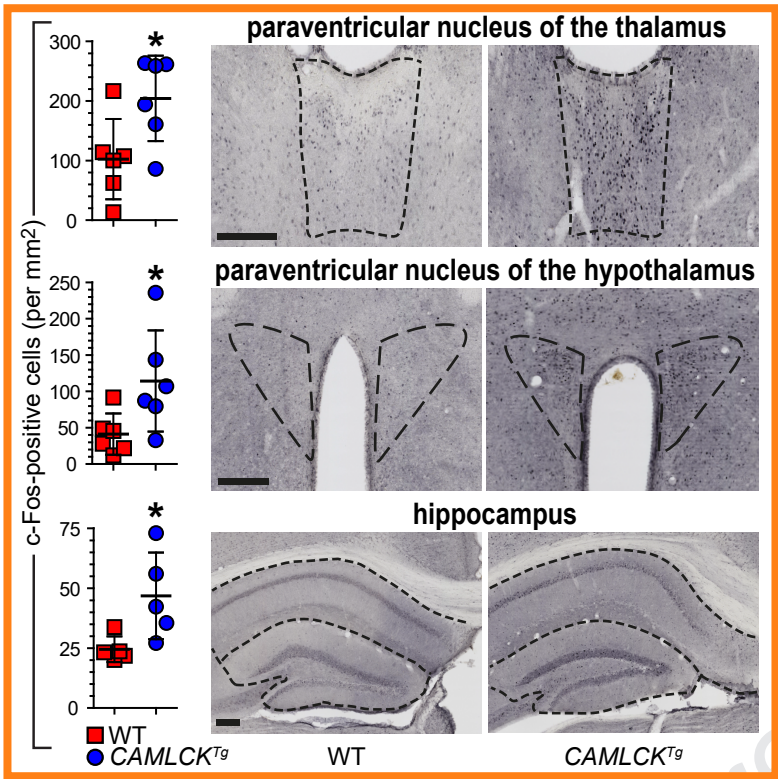
Figure 2: Increased intestinal permeability induces increased C-Fos immunolabelling in selected brain regions. *CAMLCK^{Tg}* (blue circles, n=5-6) and WT (red squares, n=5-6)

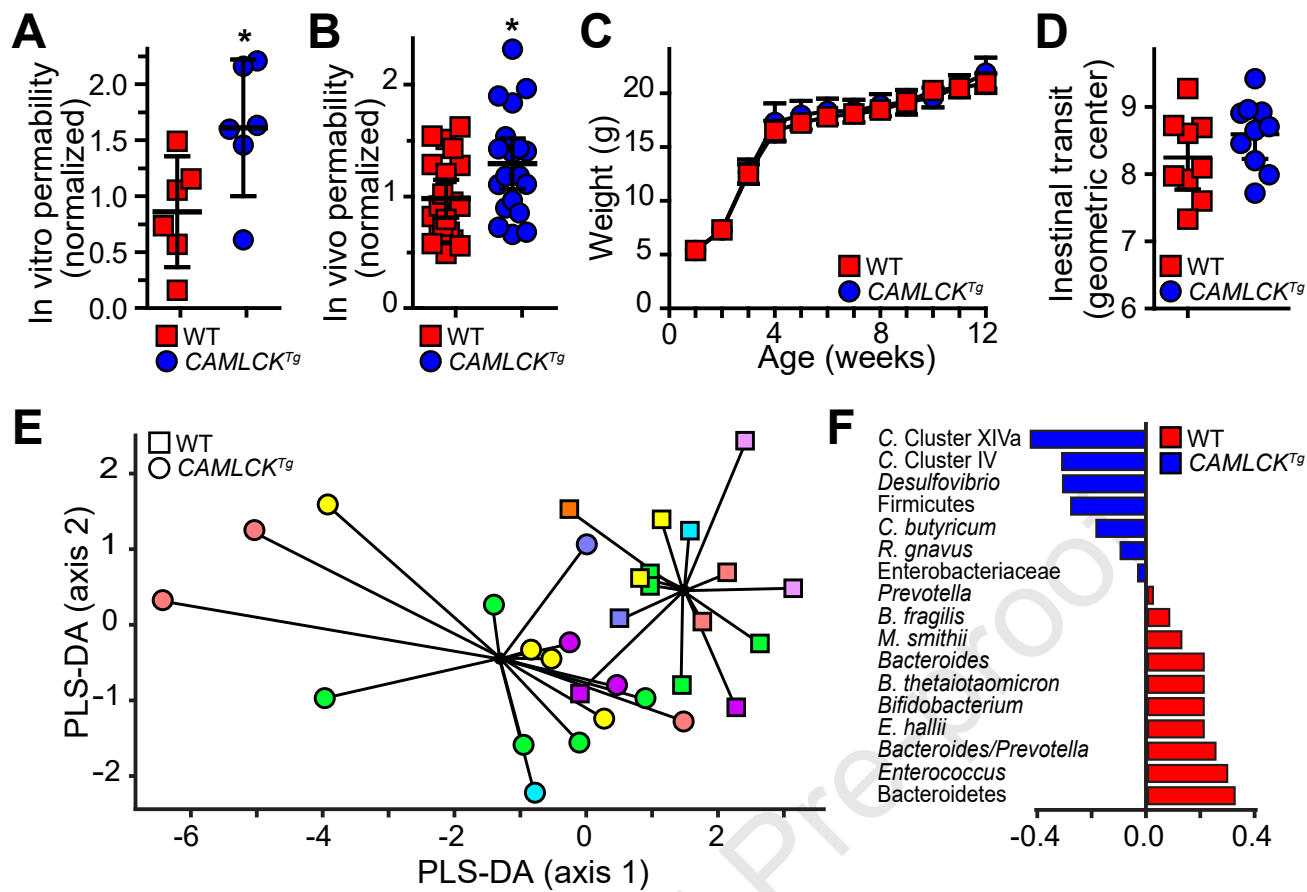
littermates. Representative images of C-Fos immunolabeled brains from *CAMLCK^{Tg}* and WT mice. Bars = 200 µm; mean±SEM; *, $p<0.05$, t-test.

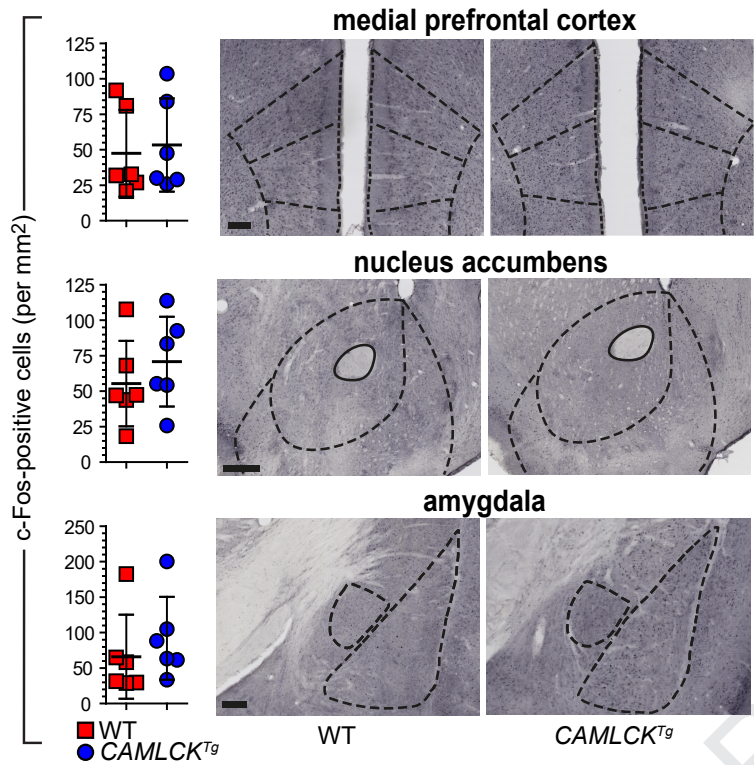
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Supplemental Figure 1:

A. Trans-jejunal fluorescein flux was increased in *CAMLCK^{Tg}* (blue circles) relative to WT (red squares) littermates. mean±SD; *, $p<0.05$, Mann-Whitney U test.

B. In vivo analysis using FITC-4kDa dextran demonstrated increased permeability of *CAMLCK^{Tg}* (blue circles, n=19) relative to WT (red squares, n=20) littermates. mean±SD; *, $p<0.05$, t-test.

C. Weight gain was similar in WT (red squares, n=6) and *CAMLCK^{Tg}* (blue circles, n=6) littermates. mean±SD.

D. Intestinal transit was similar in WT (red squares, n=10) and *CAMLCK^{Tg}* (blue circles, n=9) littermates. mean±SD.

E. Partial least squares discriminant analysis (PLS-DA) score plot based on the relative abundances of 18 microbial taxa in gut contents of *CAMLCK^{Tg}* (circles, n=16) and WT (squares, n=15) born to 8 different dams (each color represents one dam).

F. Relative abundances of microbial communities in *CAMLCK^{Tg}* (blue) and WT (red) mice. Diagrams indicate regions analyzed.

Supplemental Figure 2:

CAMLCK^{Tg} (blue circles, n=5-6) and WT (red squares, n=5-6) littermates. Representative images of C-Fos immunolabeled brains from *CAMLCK^{Tg}* and WT mice. Bars = 200µm; mean±SEM; *, $p<0.05$, t-test.

Supplemental Methods

Animals

CAMLCK^{Tg} mice¹⁻⁴ (Tg(Vil-FLAG-CAMLCK)#Jrt) were maintained as male heterozygotes on C57BL/6J background. These were mated with WT C57BL/6J females to produce WT and *CAMLCK^{Tg}* littermates. At weaning, female mice were separated and housed at constant temperature (22±1°C) with a 12 hour light/dark cycle. Food (Teklad 2018, Envigo) and water were available ad libitum. All experiments were performed at 8 weeks of age. Procedures were approved by the Ethical Committee CEEA-86, under the number APAFiS#4145.

Gut microbiota composition analysis

Gut microbiota were analyzed in two cohorts (15 WT and 16 *CAMLCK^{Tg}*) from 8 different WT dams. At sacrifice, colonic contents were stored at -80°C. DNA was extracted using the ZR fecal DNA MiniPrep kit (Zymo Research) and adjusted to 1 ng/μL. Changes in relative abundance of 24 microbial 16S rRNA gene targets were obtained by qRT-PCR using an adapted Gut Low-Density Array platform.⁵⁻⁷ A universal bacterial primer set was included as the reference gene. qRT-PCR was performed in duplicate on a ViiA7 (Applied Biosystems).

Fluorescence data was imported into LinRegPCR to perform baseline corrections, calculate mean PCR efficiency per amplicon group, and calculate initial quantities. Among the 24 targeted amplicon groups, 6 were not detected in any fecal samples and were removed from the analysis (*B. vulgatus*, *Alistipes* spp., *Parabacteroides distasonis*, *Roseburia* spp., *E. coli* and *A. muciniphila*). Normalized N₀-values were log₁₀-transformed and processed by MixOmics (v6.1.1) with RStudio (v1.0.44) to build a partial least-squares discriminant analysis (PLS-DA). This multivariate supervised approach projects samples (X) onto a low-dimensional space of latent variables to maximize separation between groups according (Y=genotype). Leave-one-out cross-validation was used to select the optimal number of latent variables for PLS-DA models.

Open field test

Mice explored a 50x50cm arena (illumination 300lux) for 10min. Exploration was automatically assessed using a video tracking system (Bioseb). The percentage of distance traveled and time spent in the center area (20x20cm) and total distance traveled in the entire arena were assessed.

Colorectal distension (CRD)

Two 0.08mm diameter electrodes were implanted in the abdominal external oblique muscle and a third in the abdominal skin. On postoperative days 3-6, CRD was performed using a balloon catheter (Fogarty 4F catheter, 1.1cm length, tip 3.5cm from the anus)⁸ in 10 sec periods with increasing volumes from 0.02 mL to 0.10 mL, with 5min rest between distensions. Abdominal electromyography activity was registered after the amplification (10000x) and analyzed (Powerlab Chart 5). Basal EMG activity was subtracted from EMG activity registered during distension. Some mice were treated with ML-7 (2 mg/kg i.p.) or naloxone sulfate (2 mg/kg i.p.) 1h before CRD. For others, water avoidance stress was induced on a floating platform (3cmx3cm) in the middle of a water-filled tank (40cmx40cm) for 1h daily over four days. Recovery (30min) preceded CRD.

Gastrointestinal transit

Animals received 70µL of 100mg/ml TRITC-70kDa dextran in tap water by gavage and were sacrificed 1 h later.⁹ Stomach, small and large intestine were cut in 11 equal parts. Luminal contents of each segment were centrifuged and fluorescence determined. Transit was calculated as the geometric center of the values for each mouse.

Ussing chamber analysis

Jejunal sections were mounted in Ussing chambers (Physiologic Instruments) filled with Krebs buffer and continuously oxygenated (95% O₂, 5% CO₂). After 1 hour of equilibration, Fluorescein (1mg/mL) was added in the apical chamber and fluorescence intensity of the basolateral chamber was measured after 1 hour.

In vivo permeability analysis

Mice were fasted for 4 hours before gavage with 150µL of 100mg/mL FITC-4kDa dextran in tap water. Blood (200µL) was collected after 4h and plasma fluorescence determined.

C-Fos analysis

Vibratome sections (40µm) were stained using polyclonal rabbit anti-C-Fos (Santa Cruz) and secondary HRP-conjugated goat anti-rabbit antisera (Jackson ImmunoResearch). NDPI images (x20) were obtained (Nanozoomer, Hamamatsu Photonics) and converted into TIFF format using ImageJ (NDPI tools plugin). Regions of interest (ROI) were manually circumscribed using ROI tools and C-Fos-immunoreactive cells quantified automatically using the particle analysis function (size: 5-20 µm²; circularity: 0.5-1). For each animal, 3-6 sections of each brain area were assessed by a blinded observer.

Statistical analysis

Statistical significance was determined by two-tailed t-test, two-tailed Mann-Whitney U test, or 2-way ANOVA and set at $p < 0.05$. For microbial analyses, univariate analysis was realized in parallel to compare each amplicon separately using unpaired t-test followed by the Benjamini-Hochberg adjustment of p -values for multiple comparisons.

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Fecal MMP-9: A New Noninvasive Differential Diagnostic and Activity Marker in Ulcerative Colitis

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Background: Ulcerative colitis (UC) is characterized by frequent relapses, with the presence of colorectal inflammation and mucosal lesions. Matrix-metalloprotease 9 (MMP-9) is elevated in colonic biopsies, urine, and blood plasma of UC patients. MMP-9 has been suggested as a predictor of UC in the urine of children; however, 20% of the controls tested positive. So far, fecal MMP-9 levels have never been measured. Our aims were: 1) to compare fecal MMP-9 levels in UC patients to control subjects and a functional gastrointestinal disorder characterized by diarrhea (IBS-D); 2) to test the correlation between UC disease activity and fecal levels of MMP-9; and 3) to correlate fecal MMP-9 levels with a known fecal marker of UC activity, calprotectin.

Methods: UC ($n = 47$), IBS-D ($n = 23$) patients, and control subjects ($n = 24$) provided fecal samples for MMP-9 analysis. In UC patients, disease severity was evaluated by the Mayo score. Fecal MMP-9 and calprotectin levels were measured by enzyme-linked immunosorbent assay and lateral flow assay, respectively.

Results: MMP-9 was undetectable or ≤ 0.22 ng/mL in the feces of all controls and IBS-D patients. In UC patients, fecal MMP-9 levels significantly correlated with the overall Mayo score ($P < 0.001$), the endoscopic score ($P < 0.001$), and the serum C-reactive protein levels ($P = 0.002$). Additionally, in UC patients fecal MMP-9 levels showed a significant correlation with a known disease activity marker, fecal calprotectin ($P = 0.014$).

Conclusions: These results highlight fecal MMP-9 as a useful tool in the differential diagnosis of diarrheic disorders and in the noninvasive evaluation of disease activity and mucosal healing in UC.

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Key Words: ulcerative colitis, MMP-9, disease activity score, calprotectin

Ulcerative colitis (UC) is a chronic, relapsing inflammatory bowel disease (IBD) characterized by continuous colonic mucosal ulceration and diarrhea during relapses. The differentiation of UC from functional disorders such as diarrhea predominant irritable bowel syndrome (IBS-D) is clinically challenging, as symptoms may include altered bowel habits and abdominal pain in both diseases and activity signs can mix with anxiety-driven symptoms as well in UC. Several

attempts have been made to establish noninvasive markers that can clearly distinguish IBD from functional disorders and reveal strong correlation not only with clinical activity, but also with the endoscopic picture of the mucosa in UC. Among biochemical laboratory markers serum C-reactive protein (CRP) is the most studied¹; however, while the activity of Crohn's disease (CD) is accompanied by a strong CRP response in most patients, relapses in UC are characterized by only a modest or absent CRP increase,² depending mostly on the extension of the involved area; the sensitivity of CRP in the detection of UC is only 50%–70%.^{3–5}

The pathogenesis of UC includes the complex dysregulation of mucosal immune cells⁶ accompanied by the invasion of neutrophils, which leads to the formation of crypt abscesses and to the dysfunction of the colonic epithelial barrier.⁷ During transmigration, neutrophils reach the colonic lumen and can be detected in the stool,⁸ similar to their secreted mediators, such as polymorphonuclear elastase, calprotectin, or Cat-G.^{9,10} Therefore, fecal biomarkers that reflect neutrophil activation seem promising in UC, and fecal calprotectin has been identified as a marker to discriminate IBD patients from irritable bowel syndrome (IBS)

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patients or controls,^{10,11} and its fecal level correlates with disease activity.^{12,13}

Matrix metalloproteases (MMPs) 1, 2, 3, and 9 are also released from neutrophils in IBD, and they are significantly elevated in colonic biopsies from UC patients compared to controls, with a higher increase in the ulcerated than in non-ulcerated regions.¹⁴ Among these MMPs, MMP-9 was the most abundant in UC biopsies.¹⁴ Additionally, elevated MMP-2 and MMP-9 levels were detected in the urine of pediatric patients suffering from either UC or CD, and these MMPs were suggested as noninvasive biomarkers in the diagnosis of IBD.¹⁵ Nevertheless, no study has so far focused on fecal MMP levels in UC compared with IBS-D. Furthermore, the correlation of MMPs with disease severity, clinical, and endoscopic activity has never been evaluated.

Therefore, in our study we aimed to compare fecal MMP-9 levels in UC patients with those of IBS-D patients and controls. As a second step, we correlated fecal MMP-9 levels of UC patients with disease severity based on clinical and colonoscopic scores. In the last part of our study, we compared the diagnostic value of MMP-9 and fecal calprotectin levels in a separate group of UC patients.

MATERIAL AND METHODS

Patient Selection and Sample Collection

Overall, 94 patients were enrolled in the study. Healthy controls ($n = 24$; male/female: 9/15; mean age: 49.3 years), UC ($n = 47$; male/female: 19/28; mean age: 48.0 years), and IBS-D ($n = 23$; male/female: 4/19; mean age: 49.4 years) patients were recruited to provide fecal samples at the First Department of Medicine, Szeged, Hungary.

IBS-D patients fulfilled the Rome III criteria. Organic gastrointestinal (GI) disorders were excluded by detailed blood analyses and stool cultures, serologic assays for celiac disease, lactose-hydrogen breath test, and colonoscopy.

UC patients were evaluated clinically and endoscopically. The activity of the disease was determined by clinical (partial and total Mayo score) and endoscopic (Mayo endoscopic subscore) scores. Based on the total Mayo score, 12 patients were determined as inactive, 17 as mildly active, 9 as moderately active, and 9 as severely active. At the time of the endoscopy, blood samples were taken from all patients for the determination of platelet (PLT) and leukocyte count (WBC), CRP, erythrocyte sedimentation rate (ESR), serum iron level (Fe), and hemoglobin level (Hgb). Fecal samples were collected from all patients 2–4 days before the colonoscopy and were frozen to -20°C within 1 hour after defecation.

Measurement of Fecal MMP-9

Samples were thawed at 4°C . One g of each fecal sample was diluted, mixed, and homogenized in 4 mL of ice-cold Tris-buffer (0.15 M NaCl + 20 mM Tris-HCl, pH 8.3). After

centrifugation (10 min, 4500 rpm, 4°C), pellets were discarded and supernatants were recentrifuged (10 min, 10,000g; 4°C). The final supernatants were filtered by $0.8\ \mu\text{m}$ pore-sized syringe filters and the aliquots were stored at -20°C until analyzed. MMP-9 was measured by a quantitative enzyme-linked immunosorbent assay (Quantikine, R&D Systems, Abingdon, UK).

Measurement of Fecal Calprotectin

In the second part of this study, fecal calprotectin was measured in a subset of UC patients ($n = 24$, male/female: 10/14, mean age: 45.7 years). For calprotectin measurements, fecal specimens were thawed at 4°C and calprotectin level was determined by a quantitative lateral flow assay (Quantum Blue, Bühlmann Laboratories, Schönenbuch, Switzerland) according to the manufacturer's instructions.

Statistical Analysis

The results are presented as mean \pm standard deviation. Due to the nonnormal distribution of the data, a Kruskal–Wallis test was used to compare fecal MMP-9 levels of the three groups (control, IBS-D, and UC) and four different groups of UC related to disease activity. Correlation between MMP-9 levels and disease activity scores or blood test results were determined by Spearman correlation. Correlation between MMP-9 and calprotectin were also tested by Spearman correlation. The cutoff levels, specificity, and sensitivity were calculated using receiver operating characteristic (ROC) analysis. In UC patients, active disease has been determined as total Mayo score >3 and Mayo endoscopic subscore >0 . The cutoff level was calculated in case the area under the ROC curve (AUC) value was above 0.9. The cutoff level was determined by the maximum value of Youden's index (sensitivity + specificity - 1).

Ethical Considerations

The study protocol was approved by the Ethical Committee of the University of Szeged. All subjects provided written and informed consent to participate.

RESULTS

Fecal MMP-9 Levels Between Groups

There were no significant differences between the three groups (control, IBS-D, and UC) regarding age ($P = 0.916$). Fecal MMP-9 contents were significantly different between the three groups (Fig. 1, $P < 0.001$). Indeed, specimens originating from healthy subjects had very low MMP-9 levels; nearly half of them were found to be below the detection limit of the assay (mean: $0.0208 \pm 0.045\ \text{ng/mL}$). Similarly, IBS-D patients had low fecal MMP-9 levels (IBS-D: $0.0017 \pm 0.008\ \text{ng/mL}$). However, in the UC group the average MMP-9 level was significantly higher (UC: $15.65 \pm 20.14\ \text{ng/mL}$). To distinguish UC patients from healthy controls or IBS-D patients, the cutoff level was determined at $0.245\ \text{ng/mL}$ (AUC = 0.939). By means of this cutoff, sensitivity was 85.1% and specificity 99.99% (Fig. 1).

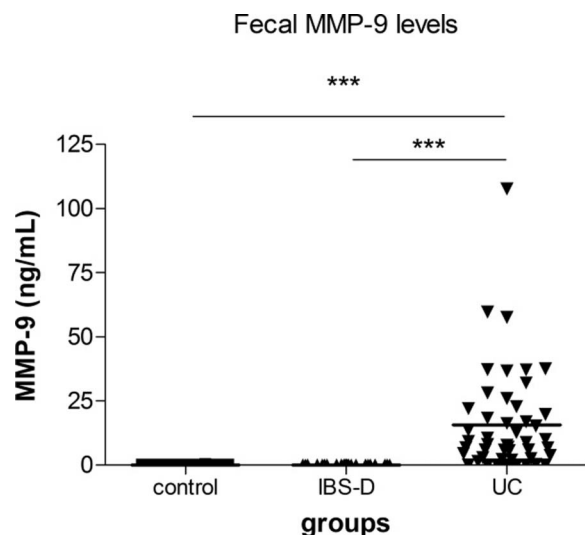


FIGURE 1. Fecal MMP-9 levels in the three different groups: control, IBS-D, UC. Fecal MMP-9 concentration is significantly elevated in UC patients compared to controls or IBS-D patients ($***P < 0.001$).

Correlation of Fecal MMP-9 Levels with Disease Activity in UC

MMP-9 activity significantly correlated with the overall Mayo score ($R = 0.616$; $P < 0.001$; $AUC = 0.905$). Fecal MMP-9 levels were significantly lower in case of inactive disease compared to moderately or highly active (Fig. 2). Further, within the components of the Mayo score a strong correlation was observed with the endoscopic evaluation of the colorectal mucosa ($R = 0.653$; $P < 0.001$, $AUC = 0.900$). To separate active UC patients from those in remission, the cutoff level of MMP-9 was determined at 2.38 ng/ mL. This cutoff resulted in a sensitivity of 97.1% and a specificity of 83.3%. Correlation of fecal MMP-9 with laboratory findings is illustrated in Table 1.

Correlation of Fecal MMP-9 Concentration with Fecal Calprotectin Levels in UC

In a subset of 24 UC patients, fecal calprotectin levels were also determined. Significant correlation was found between the fecal MMP-9 and calprotectin levels ($R = 0.495$; $P = 0.014$). In this subset of patients the correlation of endoscopic and overall Mayo scores was calculated with both MMP-9 and calprotectin. In this smaller cohort of patients, fecal MMP-9 levels showed a slightly stronger correlation with the endoscopic component of the Mayo score than calprotectin ($R = 0.587$; $P = 0.003$, AUC = 0.895 for calprotectin and $R = 0.653$; $P = 0.002$, AUC = 0.939 for MMP-9), and a higher correlation with the overall Mayo score than calprotectin ($R = 0.587$; $P = 0.016$, AUC = 0.895 for calprotectin and $R = 0.718$; $P = 0.002$, AUC = 0.905 for MMP-9).

DISCUSSION

To the best of our knowledge this study is the first to report elevated fecal MMP-9 levels in patients with active UC compared

to functional (IBS-D) patients or controls. In UC patients fecal MMP-9 levels are excellent predictors of disease activity and show a significant correlation with the clinical and endoscopic scores. Fecal MMP-9 levels correlate significantly with fecal calprotectin, a well-known marker of disease activity in UC. MMPs are a family of Zn²⁺-containing endopeptidases capable of degradation of the extracellular matrix. They are secreted by various cell types, such as fibroblasts, mesenchymal cells, tumor cells, and several inflammatory cells like monocytes, lymphocytes, and neutrophils. MMP-9 is a gelatinase that represents ≈ 30% of protein content of neutrophil granules.¹⁶ In a murine experimental model, it has been shown that epithelial cells and granulocytes are the main sources of MMP-9 in colitic tissue, and exogenous MMP-9 impairs epithelial wound healing and cell attachment *in vitro*.¹⁷

Urinal MMP-2 and MMP-9 levels have been suggested to discriminate children with IBD from controls; nevertheless, urine samples were found positive for MMP-2 in 25%, for MMP-9 in 20% of control individuals, leading to false positivity in the diagnosis.¹⁵ Further, as MMPs play important roles in cancer development and metastasis formation,¹⁸ urinary MMPs were found elevated in various cancer types including prostate, bladder, breast, brain, and hematologic malignancies.^{19–22} These findings point out that urinary MMPs are not specific for IBD. We hypothesize that fecal MMP level may be more characteristic of GI pathologies than of other organ-specific or systemic disorders, although its specificity to UC compared to other colon diseases needs to be verified. In colonic biopsies, MMP-9 levels were significantly increased in UC patients, and less significantly

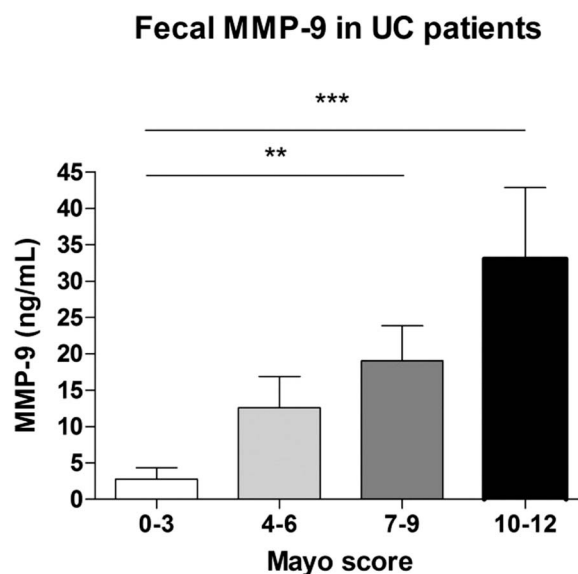


FIGURE 2. Fecal MMP-9 levels in UC patients depending on disease activity. Fecal MMP-9 concentration is significantly elevated in UC patients with moderate (** $P < 0.01$) and severe (***) ($P < 0.001$) activity compared to those in remission.

TABLE 1. Correlation of Fecal MMP-9 Levels with Blood Test Results

	Blood Test Results					
	CRP	ERS	Fe	WBC	Hgb	PLT
MMP-9	R = 0.456 P = 0.002	R = 0.228 P = 0.137	R = 0.454 P = 0.003	R = 0.404 P = 0.005	R = 0.151 P = 0.317	R = 0.296 P = 0.046

Significance is accepted at $P < 0.05$.

increased in CD patients compared to controls.¹⁴ Based on colonic biopsies, a recent study demonstrated that in contrast to UC, MMP-9 is not involved in the inflammatory process of lymphocytic colitis and collagenous colitis.²³ By means of a cutoff level of fecal MMP-9 at 0.245 ng/mL, none of our controls and IBS-D patients tested positive. Nevertheless, fecal MMP-9 negativity does not rule out IBD, as seven UC patients, all in remission, were also below the cutoff level. It is important to note that none of them had colonic ulcerations on endoscopy (Mayo endoscopic subscore ≤ 1). Therefore, our data suggest that low fecal MMP-9 level is a negative predictor of mucosal lesions. Fecal MMP-9 positivity suggests the need of further evaluation of inflammatory diseases or other GI pathologies.

Interestingly, in an earlier study on CD patients, MMP-9 plasma levels were found elevated compared to controls, but no correlation could be observed with the Clinical Disease Activity Index (CDAI).²⁴ Similarly, it has been demonstrated that other disease activity markers, such as calprotectin,²⁵ lactoferrin, or CRP, and even endoscopic appearance correlate poorly with CDAI.²⁶ In our study, however, fecal MMP-9 concentration showed a significant correlation with the clinical and endoscopic scores in UC patients. Additionally, fecal MMP-9 level significantly correlated with CRP, a laboratory value referring to inflammation, widely used as a disease activity marker in the blood.¹

In the last part of our study we compared fecal MMP-9 to an established fecal marker in UC, calprotectin, and a significant correlation was found between them. In some centers, fecal calprotectin is already used as a routine test to discriminate IBS from IBD.²⁷ These data suggest that fecal MMP-9 can be a novel marker to help in the differential diagnosis of patients with diarrhea and abdominal pain. Further, it could be a new complementary noninvasive method to assess mucosal inflammation and lesions in UC patients.

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The authors thank Valérie Bezirard and Mathilde Leveque for help in the measurements.

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Keywords: colorectal cancer; noninvasive diagnostic marker; matrix-metalloprotease-9

A pilot study on faecal MMP-9: a new noninvasive diagnostic marker of colorectal cancer

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Background: Colorectal cancer (CRC) is one of the leading malignancies worldwide, therefore cheap noninvasive screening methods are of great importance. Matrix-metalloproteinase-9 (MMP-9) has a role in the progression of CRC, and its level is elevated in tumour biopsies. Faecal MMP-9 levels are increased in active ulcerative colitis patients, but in CRC patients, they have never been measured. We aimed to assess the faecal MMP-9 levels in patients undergoing total colonoscopy according to endoscopic and histological diagnosis.

Methods: One hundred and nine patients provided faecal samples for MMP-9 analysis. A total colonoscopy was performed; suspicious lesions were evaluated by histology. Faecal MMP-9 levels were measured by ELISA.

Results: The number of patients allocated to different groups were: negative/diverticulosis: 34 (referred to as controls); hyperplastic polyps: 15; adenomas: 32 (22 at high risk); and CRC: 28. Faecal MMP-9 was significantly increased in CRC compared with all other groups ($P < 0.001$). Faecal MMP-9 was suitable to distinguish CRC patients from controls (sensitivity: 89.3%; specificity: 91.2%). By means of a lower cutoff level, faecal MMP-9 identified high-risk adenomas besides CRC (sensitivity: 76%; specificity: 85.3%). This lower cutoff level screened 59% of high-risk adenomas.

Conclusions: Faecal MMP-9 may be a promising new noninvasive marker in CRC.

Colorectal cancer (CRC) represents a major cause of morbidity and mortality in Western societies, and the incidence in developing countries is also rapidly growing (Sung *et al*, 2008; Siegel *et al*, 2012). In Europe, CRC is the second most frequent type of malignancy among males and females, with an estimated incidence of >400 000 new cases per year (Ferlay *et al*, 2013). The high frequency of the disease and the fact that the prognosis correlates well with the stage at diagnosis makes CRC screening extremely important. Colonoscopy is the gold standard for CRC screening by

its high sensitivity and specificity but has also disadvantages, such as risk of complications, high costs and lower acceptance by patients as a screening method. Therefore, noninvasive methods are required to identify patients at high risk of CRC where colonoscopy needs to be performed.

CRC screening recommendations vary between countries but mostly consist of an annual or biannual faecal occult blood test (FOBT) combined with colonoscopy in case of positivity or a colonoscopy every 10 years, in the population aged >50 years

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(Binefa *et al*, 2014). Two basic types of FOBT exist, based on the imperceptible intermittent leakage of blood from CRC and high-risk adenomas in stool (Binefa *et al*, 2014). The guaiac test (gFOBT) detects the peroxidase activity in the haemoglobin haeme subunit, therefore it is nonspecific for human haemoglobin and theoretically requires dietary restrictions a few days before performing the test, but large studies have found no significant advantage of the dietary modifications, therefore lately they are not recommended by many authors (Konrad, 2010). A minimum of two faecal samples are necessary from different stools to obtain an appropriate sensitivity. Sensitivity for CRC ranges from 6.2% to 83.3%, depending on the test used, while specificity is >80% (Binefa *et al*, 2014). The immunochemical faecal occult blood test (FIT) is based on specific monoclonal or polyclonal antibodies against human haemoglobin, thus it does not require dietary alterations. Contrary to gFOBT, the analysis by FIT can be automated and its threshold value is adjustable by the user. According to the literature, the sensitivity of FIT is extremely variable, from 5.4% to nearly 98%, depending on the test used (Binefa *et al*, 2014). Specificity can range from 77% to 99%. The European Guidelines for quality assurance of CRC recommends the use of FIT rather than gFOBT (Halloran *et al*, 2012). However, reliable tests with a less variable sensitivity and specificity are still lacking.

Matrix-metalloproteinases (MMPs) are a family of Zn^{2+} -containing endopeptidases, secreted by various cell types, such as tumour cells, mesenchymal cells, fibroblasts and inflammatory cells, such as monocytes, lymphocytes and neutrophils. Increased expression of MMP-9 has been detected in tissue samples from rectal carcinoma and colon cancer, where it correlates with poor prognosis (Unsal *et al*, 2008; Araujo *et al*, 2015; Yang *et al*, 2014). Recently, we have shown that faecal MMP-9 levels are elevated in active ulcerative colitis (UC) patients compared with patients with a functional gastrointestinal disorder (diarrhoea-predominant irritable bowel syndrome) or healthy controls (Annahazi *et al*, 2013). Faecal MMP-9 levels were found to be a predictor of disease activity in UC patients (Annahazi *et al*, 2013), and had a high specificity in the diagnosis of pouchitis (Farkas *et al*, 2014). However, faecal MMP-9 levels in CRC patients have not been investigated.

Based on this background, we aimed to measure faecal MMP-9 levels in patients undergoing colonoscopic examination in our hospital and to analyse the results according to the different colonoscopic and histological findings.

MATERIALS AND METHODS

Ethical considerations. The study protocol was approved by the Ethical Committee of the University of Szeged, Szeged, Hungary. All subjects provided written and informed consent to participate.

Patient selection and sample collection. Overall, 109 patients (male/female: 58/51, age: 29–91 years, mean: 66.0 years) referred to our specialist colorectal unit in the First Department of Medicine, Szeged, Hungary were enrolled in the study, who all underwent colonoscopy. No patients had a history of UC or a history of other cancers. One patient, who was later identified as a rectal adenocarcinoma patient, had liver metastases. Faecal samples were collected from all patients 2–4 days before the colonoscopy and were frozen to -20°C within 1 h after defaecation. All colonoscopies were completed until reaching the terminal ileum. During colonoscopy, biopsies were taken from all suspect lesions, and histology was performed. Based on the colonoscopic and histological results, patients were allocated to five groups: negative, diverticulosis, hyperplastic polyp, adenoma, and CRC. Adenomas were considered at increased risk of malignisation when one of the

followings was fulfilled: polyp size >10 mm; villous adenoma or tubulovillous adenoma with at least 20% villous component; high-grade dysplasia; or multiple adenoma (>3).

Validation assay. Our group was the first to use the commercial Human Quantitative Enzyme-Linked Immunosorbent Assay Kit from R&D Systems (Abingdon, UK, Cat. No. DMP900) for the measurement of MMP-9 levels in faeces. Therefore, we have performed a validation assay to test whether the faecal matrix itself has an influence on MMP-9 determination and whether the kit from R&D Systems is able to determine MMP-9 from human faecal extracts.

Active human MMP-9 full-length protein (Abcam LTD, Cambridge, UK; Cat. No.: ab157344; LOT No: GR218764-1) was added to the extraction medium. Control faecal samples were weighed and 1 ml extraction medium with or without added MMP-9 was given to 0.25 mg sample (the sample to extraction medium ratio was 1:4). The samples were immediately homogenised for 2×30 s and incubated for 10 min at room temperature. Subsequently, all homogenates were centrifuged at 1500 g for 10 min followed by a second centrifugation of the supernatants from the first centrifugation step at 10 000 g for 10 min. The final supernatants were processed for linearity (parallelism) test. The extract containing added MMP-9 was serially diluted 1 + 1 to get 50, 25, 12.5 and 6.25% of the original MMP-9 content with the extract of the same faeces containing no added MMP-9. Then all samples, including 100% of added MMP-9 extract and the native control extract without added MMP-9, were subjected to MMP-9 measurement by the above-mentioned ELISA kit for human MMP-9. The kit is able to detect the human 92 kDa Pro-MMP-9 and 82 kDa active form of MMP-9 as stated by the manufacturer. The added MMP-9 protein obtained for this evaluation was the 92 kDa Pro-MMP-9.

Measurement of faecal MMP-9. Samples were stored on -20°C for a maximum of 3 months and then thawed at 4°C for 1 day. In all, 1.0 g of each faecal sample was diluted, mixed and homogenised in 4 ml of ice-cold Tris-buffer (0.15 M NaCl + 20 mM Tris-HCl, pH:8.3). After centrifugation (10 min, 4500 r.p.m., 4°C), pellets were discarded and supernatants were recentrifuged (10 min, 10 000 g; 4°C). The final supernatants were filtered by 0.8- μm pore-sized syringe filters, and the aliquots were stored at -20°C until analysed. MMP-9 was measured by the quantitative enzyme-linked immunosorbent assay described above.

Statistical analysis. Continuous data were presented as mean \pm s.d. Kruskal–Wallis test was used to compare faecal MMP-9 levels of the different groups (control, hyperplastic polyp, adenoma and CRC). MMP-9 levels of high- and low-risk adenoma subgroups were analysed by the Mann–Whitney test. The different subgroups according to tumour localisation or stage were compared with the Kruskal–Wallis test. Differences were considered statistically significant when $P < 0.05$.

Receiver operating characteristic (ROC) curve analysis was performed to determine the predictive power of the MMP-9 level. Area under ROC curve (AUC), confidence interval for AUC, sensitivity and specificity were calculated. The optimal cutoff values were determined using Youden's index, if the AUC was >0.8 (Baker and Kramer, 2007). The cutoff level was determined by the maximum value of Youden's index (sensitivity + specificity – 1). Statistical analyses were performed by the R (version 3.1.2) statistical software (<https://www.r-project.org/>).

RESULTS

Validation assay. The concentrations measured in ng ml^{-1} are shown in Figure 1. The figure clearly shows a linear concentration

change corresponding to dilution. It indicates that (i) the faecal matrix itself has no influence on MMP-9 determination; and (ii) the kit from R&D Systems is able to determine MMP-9 from human faecal extracts.

Faecal MMP-9 levels between groups. Seventeen patients (male/female: 8/9; mean age: 59.7 years) were found negative on total colonoscopy and 17 patients had no other finding from noninflamed diverticulosis (male/female: 3/14; mean age: 71.8 years). There was no significant difference between these two groups in terms of faecal MMP-9 levels ($P=0.49$), therefore these 34 patients were used as polyp-free/non-malignant controls (mean age: 65.7 years). Out of the other patients, 15 had hyperplastic polyps (male/female: 9/6; mean age: 62.3 years), 32 had adenomas (male/female: 21/11; mean age: 67.0) and 28 had CRC (all adenocarcinomas; male/female: 17/11; mean age: 67.2 years) according to the endoscopic and histological examinations. There were no statistically significant differences between the four groups (controls, hyperplastic polyps, adenomas, CRC) regarding the age. Faecal MMP-9 levels were significantly different between the four groups (Figure 2, $P<0.0001$). Faecal samples from control patients had very low MMP-9 levels (mean: $0.118 \pm 0.057 \text{ ng ml}^{-1}$). Patients with hyperplastic polyps also presented with low faecal MMP-9 levels (mean: $0.112 \pm 0.073 \text{ ng ml}^{-1}$). Adenoma patients

had slightly, but not significantly elevated faecal MMP-9 levels (mean: $0.656 \pm 0.396 \text{ ng ml}^{-1}$). Nevertheless, patients with CRC had a significantly increased mean of faecal MMP-9 levels (mean: $7.028 \pm 3.722 \text{ ng ml}^{-1}$). To distinguish CRC patients from controls, the cutoff level was determined at 0.23 ng ml^{-1} (AUC = 0.913; 95% CI: 0.833–0.994; $P<0.001$). This cutoff level was associated with a sensitivity of 89.3% and a specificity of 91.2% (Figure 3). Patients with hyperplastic polyps (AUC = 0.526; 95% CI: 0.340–0.711; $P=0.779$) were not distinguishable from control individuals by ROC analysis. Among adenomas, 22 out of 32 were at increased risk of malignancy, with a mean faecal MMP-9 level significantly elevated compared with low-risk adenomas (mean of high-risk adenomas: 0.936 ± 0.570 vs mean of low-risk adenomas: 0.040 ± 0.017 ; $P<0.05$; Figure 4). Low-risk adenomas could not be distinguished from controls by faecal MMP-9 levels (AUC = 0.456; 95% CI: 0.236–0.676; $P=0.706$). When ROC analysis was performed with an aim to select high-risk adenomas and CRC from controls, the cutoff level was determined at 0.085 ng ml^{-1} . In this case, sensitivity was 76% and specificity was 85.3% (AUC = 0.806; 95% CI: 0.710–0.902; $P<0.001$). With this lower cutoff level, 59% of high-risk adenomas were identified. By ROC analysis of high-risk adenomas vs controls, the cutoff level would be similarly 0.085 ng ml^{-1} (AUC = 0.670; 95% CI: 0.507–0.833; $P=0.033$).

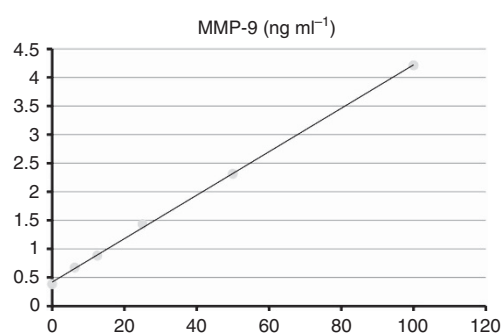


Figure 1. MMP-9 concentration levels at different dilutions in the validation assay.

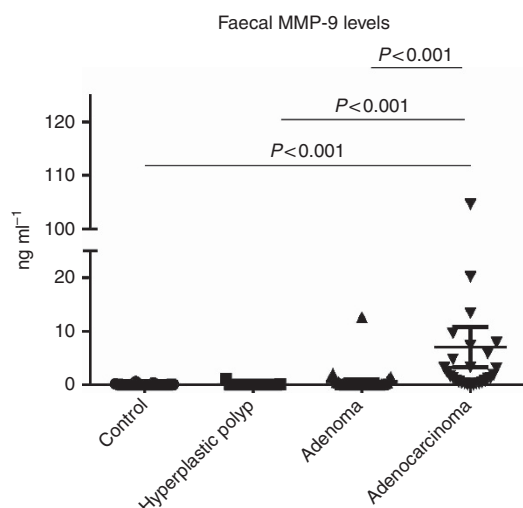


Figure 2. Faecal MMP-9 levels of the different groups (based on colonoscopy and histology). Abbreviation: CRC = colorectal carcinoma.

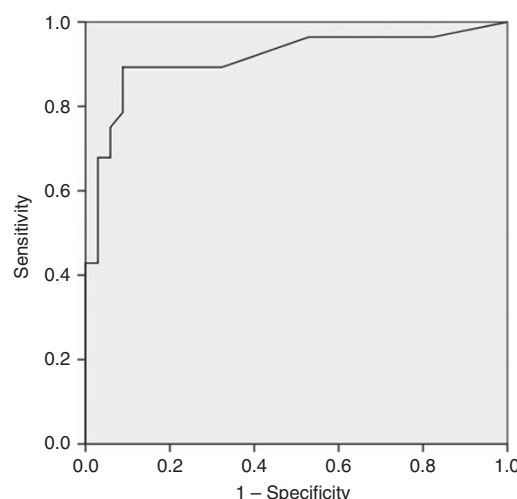


Figure 3. ROC curve of CRC vs controls.

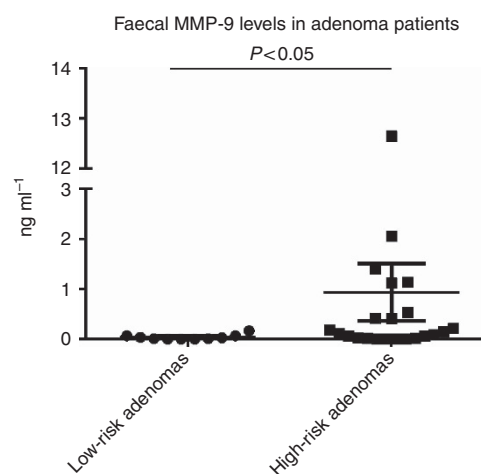


Figure 4. Faecal MMP-9 levels in adenomas.

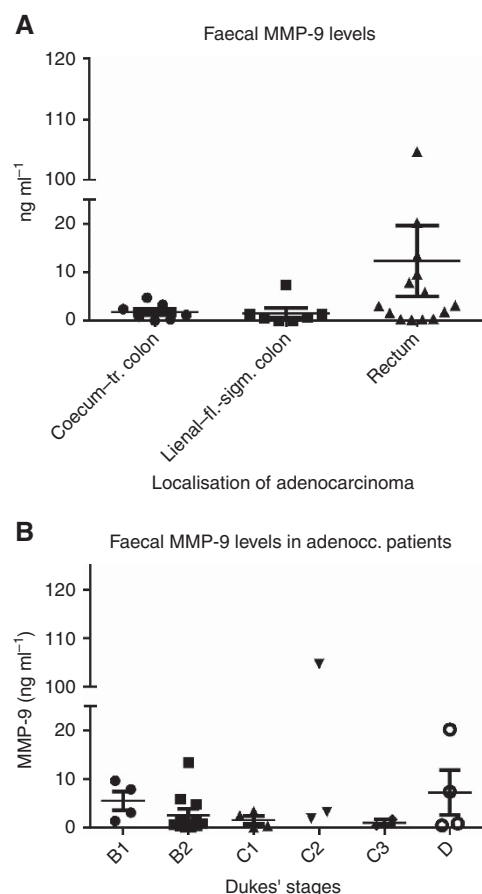


Figure 5. Faecal MMP-9 levels in CRC patients. Values according to (A) localisation and (B) Dukes' stages of CRC.

Faecal MMP-9 levels according to CRC localisation and stage.

The number of patients regarding the site of the CRC was as follows: rectum: 14, sigmoid colon: 6, lineal flexure: 1, transverse colon: 2, hepatic flexure: 2, ascending colon: 1, and coecum: 2 patients. If faecal MMP-9 levels are analysed according to the site of the CRC, no significant difference was observed ($P=0.146$), although a trend was seen towards higher levels in case of a rectal tumour (mean: 12.33 ± 7.274 ng ml⁻¹) compared with left-sided colon (lineal flexure–sigmoid colon; mean: 1.619 ± 0.978 ng ml⁻¹) and transverse-right-sided colon (from coecum to transverse colon; mean: 1.830 ± 0.656 ng ml⁻¹; Figure 5A). According to Dukes' classification, no significant difference could be seen between the groups (Figure 5B; $P=0.236$).

DISCUSSION

To the best of our knowledge, we report for the first time that MMP-9 levels are elevated in faecal specimens from CRC patients compared with patients without polypoid lesions. Furthermore, faecal MMP-9 can be used to distinguish CRC patients from patients with no polypoid lesions, with a high sensitivity and specificity. Additionally, with a lower cutoff level, faecal MMP-9 is able to identify nearly 60% of patients with a high-risk adenoma.

MMP-9 is a gelatinase capable of the degradation of the extracellular matrix, which is believed to have a role in the progression and metastasis formation of many tumours, including colon cancer (Yang *et al*, 2014). Apart from colon cancer cells, tumour-infiltrating neutrophils are also an important source of MMP-9, as a major angiogenesis-inducing factor (Hyuga *et al*,

1994; Deryugina *et al*, 2014). Serum MMP-9 was suggested as an early noninvasive biomarker for breast cancer (Provatopoulou *et al*, 2009) and a prognostic marker for lung cancer (Ylisirnio *et al*, 2000). Urinary MMPs were found elevated not only in bladder, renal and prostate cancers but also in anatomically distant tumours, including breast, brain, and hematological malignancies (Moses *et al*, 1998; Chan *et al*, 2004; Fernandez *et al*, 2005; Smith *et al*, 2008). Recently, significant efforts have been made to explore the potential role of MMP-9 as a biomarker in CRC. In the serum, it has been suggested as a biomarker of CRC in symptomatic patients (Hurst *et al*, 2007), and its levels correlated with the Dukes' stage (Mroczo *et al*, 2010). Furthermore, it has been recently shown that serum levels of neutrophil gelatinase-associated lipocalin in complex with MMP-9 are elevated in CRC patients, but it was found unsuitable as a diagnostic marker, as its discriminative power was very poor (Duvillard *et al*, 2014). We have found the discriminative power of faecal MMP-9 levels in CRC patients sufficient, which can be explained by the fact that faecal MMP-9 could be more informative for gastrointestinal malignancies than urine or serum. Our method does not require a preceding diet, and one faecal sample gave a sufficient sensitivity for discrimination. Its analysis is automated, and the cut of concentration is adjustable. In terms of these characteristics, faecal MMP-9 measurement, similarly to FIT, is more convenient for clinical use than gFOBT. Furthermore, faecal MMP-9 measurement provides a higher sensitivity and specificity for CRC than gFOBT and than many types of FIT.

In the past decade, numerous faecal molecular markers have been described, which are based on the detection of genetic mutations and epigenetic alterations, linked to the carcinogenic process (for a review, see Kanthan *et al*, 2012). Genetic markers are claimed to be more sensitive than FOBTs (Kanthan *et al*, 2012). However, one disadvantage of these markers is that they are not present in every colorectal carcinoma. To overcome this problem, genetic markers are often combined in a panel, but this may lead to a decrease in specificity (Kanthan *et al*, 2012). Furthermore, an important limitation of these methods is their high costs. Current evidence is not sufficient to accept the routine use of faecal genetic markers, and the European Guidelines for quality assurance have stated that they are currently unsuitable for CRC screening (Halloran *et al*, 2012). Similarly to molecular tests, our method also has the advantage over FOBTs in that it detects a marker originating directly from cancer, which, contrary to bleeding, is supposed to be permanent. However, the costs of faecal MMP-9 measurement are far beyond those of molecular tests, as they are <10 USD per sample, which is comparable to the costs of FOBT. This method is markedly cheaper than faecal DNA tests, which cost around 350 USD per sample (Lansdorp-Vogelaar *et al*, 2010).

The process of transformation from normal epithelium to cancer takes 10–15 years in most cases, leaving a possibility to remove precancerous lesions before they turn to definitive cancer (Binefa *et al*, 2014). Therefore, a reliable marker detecting high-risk adenomas is of high importance. The sensitivity of FIT for advanced adenomas is only around 20–33% (Morikawa *et al*, 2005; Haug *et al*, 2010). DNA tests are characterised by a much higher sensitivity, for example, K-ras mutations are detected in the faeces of around 50% of individuals with adenomas >1 cm (Mak *et al*, 2004), p53 mutations in up to 64% of severely dysplastic polyps (Tagore *et al*, 2004) and 4–26% of adenomas (Mak *et al*, 2004). Methods based on the detection of epigenetic changes appear to have a wide range of sensitivity, for example, the methylation of SFRP2 can be detected in the faeces of 52.4% of individuals with adenomas (Huang *et al*, 2007), while the methylation of methylguanine DNA methyltransferase can be found in 36% of the faeces of adenoma patients but that of the human mut I homolog-1 in only 11% (Baek *et al*, 2009). Faecal tumour M2 pyruvate kinase, a promising marker in CRC screening, showed a

sensitivity of 22% for advanced adenomas (Haug *et al*, 2008). Serum MMP-9 levels were elevated not only in cancer but also in colorectal adenoma patients compared with healthy subjects (Mroczko *et al*, 2010). Similarly, MMP-9 expression was found increased not only in tissue samples from CRC but also in those from highly dysplastic adenomatous polyps compared with hyperplastic polyps or adenomas with low dysplasia (Odabasi *et al*, 2014). Furthermore, MMP-9 protein expression has been elevated in adenomas with high grade dysplasia compared with other adenomas or normal colonic tissue (Herszenyi *et al*, 2008). Our results have shown that faecal MMP-9 levels from high-risk adenoma patients are significantly elevated compared with patients with low-risk adenomas. However, with a lower cutoff level, our marker identified 59% of high-risk adenoma patients as positive. This result is definitely higher than the usual sensitivity of FOBTs and comparable to that of molecular tests but without the high costs of the latter.

We did not find any significant difference between the faecal MMP-9 levels of the groups with different tumour location. Nevertheless, a tendency could be seen towards the highest faecal MMP-9 levels in rectal adenocarcinoma patients. This is probably not related to the nature of rectal tumours but could be explained with a less time for degradation of the marker in the intestinal lumen, similarly to that seen by FIT (Morikawa *et al*, 2005).

Our study has some limitations. As our department is a specific colorectal center, our patients were a selected population not representing the general population. Patients were on a low-fibre diet at the time of faecal sample collection, as a preparation for the upcoming colonoscopy. Data on the time of the day at faecal sample production was not collected in our study. Although the percentage of cancer and adenoma found on colonoscopy was relatively high, the number of patients in the different subgroups (i.e., tumour localisation, Dukes' stage) may be insufficient for the detection of minor differences. Moreover, MMP-9 is also elevated in gut inflammation, which may limit its use in case of, for example, IBD. We hypothesise that, in a more general population, elevated faecal MMP-9 levels in some cases could be related to inflammation or other cancers in the gastrointestinal tract. However, even in this case, the method would be a good predictor of the presence of a nonfunctional gastrointestinal disorder, based on our previous results showing that faecal MMP-9 measurement can well discriminate IBS-D patients and healthy subjects from UC patients. A further limitation is that, owing to the different cutoff levels calculated on the same sample groups, the performance of the method may be overestimated. A further study with a larger and more general patient population could help us to clarify whether diet, time of the day at sampling, tumours of the upper GI tract and so on can affect faecal MMP-9 levels and could also serve to verify the performance of our method. A comparison with FIT on the same samples could be useful. Moreover, a larger study on a general cohort could also provide more information if this method is valuable for CRC screening.

Nonetheless, our study was suitable to identify a new marker, faecal MMP-9, capable of discrimination of CRC from polyp-free controls. Faecal MMP-9 is characterised by many advantages of FIT and also of the molecular markers, without high costs. Further studies on a larger and more general population are needed to confirm whether this promising method is suitable for CRC screening as a complementary assay.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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