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

Ph.D. thesis

Orsolya Inczefi M.D.

First Department of Medicine
University of Szeged
Szeged
2020

LIST OF FULL PAPERS THE THESIS BASED UPON

I. **Inczefi** O, Bacquié V, Olier-Pierre M, Rincel M, Ringot-Destrez B, Ellero-Simatos S, Eutamène H, Bétoulières C, Thomas J, Laine J, Gros L, Lévêque M, Leonard R, Harkat C, Robbe-Masselot C, Roka R, Mercier-Bonin M, Theodorou V, Darnaudéry M, Turner JR, Ferrier L.: Targeted intestinal tight junction hyperpermeability alters the microbiome, behavior, and visceromotor responses. Cell Mol Gastroenterol Hepatol. 2020 Mar 5 [Epub ahead of print]

D1

II. Annaházi A, Molnár T, Farkas K, Rosztóczy A, Izbéki F, Gecse K, **Inczefi O**, Nagy F, Földesi I, Szűcs M, Dabek M, Ferrier L, Theodorou V, Bueno L, Wittmann T, Róka R.: Fecal MMP-9: a new noninvasive differential diagnostic and activity marker in ulcerative colitis Inflamm Bowel Dis. 2013 Feb;19(2):316-20.

IF: 5.475, D1

III. Annaházi A, Ábrahám S, Farkas K, Rosztóczy A,**Inczefi O**, Földesi I, Szűcs M, Rutka M, Theodorou V, Eutamene H, Bueno L, Lázár G, Wittmann T, Molnár T, Róka R.: A pilot study on faecal MMP-9: a new noninvasive diagnostic marker of colorectal cancer Br J Cancer. 2016 Mar 29;114(7):787-92.

IF: 6.176, D1

Sum of impact factors (IF) for base references implicated in the thesis: **11,651**

LIST OF PUBLICATIONS NOT RELATED TO THE THESIS

I. Bálint L, Tiszai A, Kozák G, Dóczi I, Szekeres V, **Inczefi O**, Ollé G, Helle K, Róka R, Rosztóczy A. Epidemiologic characteristics of Helicobacter pylori infection in southeast Hungary World J Gastroenterol. 2019 Nov 14;25(42):6365-6372.

IF^{2018}: 3,411, Q1

II. Annaházi A, Ferrier L, Bézirard V, Lévêque M, Eutamène H, Ait-Belgnaoui A, Coëffier M, Ducrotté P, Róka R, **Inczefi O,** Gecse K, Rosztóczy A, Molnár T, Ringel-Kulka T, Ringel 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.

IF: 9.213, D1

Sum of IF for base references: 24,275

1. INTRODUCTION

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. 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. 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 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. In addition, a higher level of anxiety is often observed in these patients. According to the high prevalence of the disease, 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. Stressful life events, infection and dysbiosis can induce elevated gut permeability, which is associated with visceral hypersensitivity, and pain severity in patients. 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. 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, which suggests that it could represent an ideal model to study IBS.

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; 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 depending mostly on the extension of the involved area. The sensitivity of CRP in the detection of UC is only 50%–70%.. The pathogenesis of UC includes the complex dysregulation of mucosal immune cells accompanied by the invasion of neutrophils, which leads to the formation of crypt abscesses and to the dysfunction of the colonic epithelial barrier. During transmigration, neutrophils reach the colonic lumen and can be detected in the stool, similarly to their secreted mediators, such as polymorphonuclear elastase, calprotectin, or cathepsin G. 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, and its faecal level correlates with disease activity. 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. Among these MMPs, MMP-9 was the most abundant in UC biopsies. 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. 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. 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. Two basic types of FOBT exist, based on the imperceptible intermittent leakage of blood from CRC and high-risk adenomas in stool. 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. 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. According to the literature, the sensitivity of FIT is extremely variable, from 5.4% to nearly 98%, depending on the test used. Specificity can range from 77% to 99%.. 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. 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

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.

4. 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.

NEW RESULTS ESTABLISHED IN THE THESIS AND CONCLUSIONS

4.1. 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.

Based on our experimental studies *CAMLCKTg* mouse could be a promising model of IBS, further investigations on this transgenic animal could provide novel informations concerning the pathophysiology of the disease.

5.2 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.

Based on our clinical observations 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.

6. ACKNOWLEDGEMENTS

I express my gratitude to **Dr. Richard Róka**, my tutor, without his support this work could not be done. I would like to express my gratitude to **Prof. Vassilia Theodorou** and **Dr. Laurent Ferrier** my tutors at INRA, who guided and supported my experimental work in Toulouse.I would like to express my deepest appreciation to **Prof. János Lonovics**, **Prof. Tibor Wittmann**, **Prof. György Ábrahám**, **Prof. Csaba Lengyel** the former and present heads of the First Dept. of Medicine for providing the conditions of experimental and clinical research in the department.

I am grateful to my collegue, **Dr. Anita Annaházi** for her enthusiastic help in both clinical and experimental studies.

I would like to thank my colleagues and co-authors **Valerie Bacquié, Dr. Maïwenn Olier-Pierre, Dr. Marion Rincel, Dr. Belinda Ringot-Destrez, Sandrine Ellero-Simatos, Prof. Hélène Eutamène, Colette Bétoulières, Julie Thomas, Justin Lainé, Louise Gros, Mathilde Lévêque, Dr. Renaud Leonard, Cherryl Harkat, Dr. Catherine Robbe-Masselot, Dr. Muriel Mercier-Bonin, Prof. Muriel Darnaudéry, Prof. Tamás Molnár, Dr. Klaudia Farkas, Dr. András Rosztóczy, Prof. Ferenc Izbéki, Dr. Krisztina Gecse, Prof. Ferenc Nagy, Dr. Imre Földesi, Mónika Szűcs, Dr. Marta Dabek, Prof. Lionel Bueno, Dr. Szabolcs Ábrahám, Dr. Mariann Rutka, Klára Vadászi** and **Prof. György Lázár** for their help.

Last but not least I would like to express my deepest gratitude to my family members who are always with me and support my work.