University of Szeged
Albert Szent-Györgyi Medical School
Doctoral School of Theoretical Medicine

Plasminogen activator inhibitor type 1, as a novel faecal biomarker in Inflammatory Bowel Diseases

Ph.D. Thesis

Boldizsár Csaba Jójárt

Supervisor:
Dr. habil Klaudia Farkas Ph.D.

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List of publications

List of publications related to this thesis:

1. Jójárt Boldizsár; Resál Tamás; Kata Diána; Molnár Tünde; Bacsur Péter; Szabó Viktória; Varga Árpád; Szántó Kata Judit; Pallagi Petra; Földesi Imre; Tamás Molnár; József Maléth; Klaudia Farkas
   Plasminogen activator inhibitor 1 is a novel faecal biomarker for monitoring disease activity and therapeutic response in inflammatory bowel diseases
   JOURNAL OF CROHNS & COLITIS (2023) **D1/Q1 IF: 8 ***

List of publications not related to this thesis:

1. Péter Bacsur; Mariann Rutka*; András Asbóth; Tamás Resál; Kata Szántó; Boldizsár Jójárt; Anita Bálint; Eszter Ari; Walliyulahi Ajibola; Bálint Kintses et al.
   Effects of bowel cleansing on the composition of the gut microbiota in inflammatory bowel disease patients and healthy controls
   THERAPEUTIC ADVANCES IN GASTROENTEROLOGY (2023) **Q1 IF: 4.2 ***
2. Árpád Varga; Tamara Madácsy; Marietta Görög; Aletta Kiss; Petra Susánszki; Viktória Szabó; Boldizsar Jójárt; Krisztina Dudás; Gyula Jr. Farkas; Edit Szederkényi et al.
   Human pancreatic ductal organoids with controlled polarity provide a novel ex vivo tool to study epithelial cell physiology
   CELLULAR AND MOLECULAR LIFE SCIENCES (2023) **D1/Q1 IF: 8 ***
3. Szabó Viktória; Csákány-Papp Noémi; Görög Marietta; Madacsy Tamara; Varga Árpád; Kiss Aletta; Tél Balint; Jójárt Boldizsár; Crul Tim; Dudás Krisztina et al.
   Orai1 calcium channel inhibition prevents progression of chronic pancreatitis
   JCI INSIGHT (2023) **D1/Q1 IF: 8 ***
4. Kata Judit Szántó; Tamara Madácsy*; Diána Kata; Tamás Ferenci; Mariann Rutka; Anita Bálint; Renáta Bor; Anna Fábián; Ágnes Milassin; Boldizsár Jójárt et al.
   Advances in the optimization of therapeutic drug monitoring using serum, tissue and faecal anti-tumour necrosis factor concentration in patients with inflammatory bowel disease treated with TNF-α antagonists
   EXPERT OPINION ON BIOLOGICAL THERAPY (2021) **Q1 IF: 5.589**

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Introduction

Crohn's Disease (CD) and Ulcerative Colitis (UC) are the two most common forms of Inflammatory Bowel Disease (IBD), chronic, tissue-damaging conditions that develop as a result of uncontrolled activation of immune effector cells in the intestinal mucosa. The incidence of IBD is steadily increasing worldwide, driven by the global rise in the number of CD and UC patients. Symptoms and complications can lead to a significant reduction in the life quality of patients and to social and occupational difficulties. However, the long-term efficacy of currently available anti-inflammatory therapies is still far from optimal. With effective treatment, clinical remission can be achieved, but subclinical inflammation may persist within the intestinal mucosa, contributing to the risk of "symptomatic" relapse. A disease monitoring strategy based on objective markers of inflammation and timely escalation of therapy leads to better clinical and endoscopic outcomes than purely symptom-oriented treatment. In the long term, this approach would reduce the disease burden for patients and the costs to the healthcare system. Therefore, an important area of research is the discovery and development of biomarkers that can be useful diagnostic tools for clinicians, reducing the risk of complications of the disease.

Serum C-reactive protein (CRP) and faecal calprotectin (FC) are the most widely studied inflammatory biomarkers for disease monitoring. Although CRP is commonly used, it lacks sensitivity and specificity for intestinal inflammation. Recently, faecal biomarkers have emerged as a non-invasive, rapid, simple, and cost-effective diagnostic tool for the detection of intestinal inflammation. Most studies have been performed with FC, which is now considered the gold standard among faecal markers, but significant limitations prevent its use in daily practice.

Taking these into consideration, we believe it is highly important to develop new alternative biomarkers to overcome limitations, accurately monitor disease progression, and predict therapeutic response. In a recent publication, Kaiko et al. have identified a novel protein - Plasminogen activator inhibitor type 1 (PAI-1) - that plays an important role in the regulation of key inflammatory modulators during mucosal damage in IBD. They also highlighted that the expression of the mucosal Serpin E1 gene (the gene encoding PAI-1) was elevated in patients with active, more severe IBD who did not respond to anti-TNF therapy. In another study, Wang et al. showed that PAI-1 induces neutrophil-mediated chemokine expression by activating the NF-κB pathway in a mouse model of colitis. These studies highlighted a possible role for PAI-

1 in the pathogenesis of IBD, but the protein levels of PAI-1 in different biological samples and its correlation with disease activity or response to therapy remain unknown.

Aims

Therefore, our study aimed at a comprehensive analysis of the expression profile of PAI-1 in serum, tissue, and stool samples from IBD patients. This will allow us to investigate its selectivity, its association with disease activity, and its potential to predict therapeutic response in IBD.

Materials and methods

Patient population and disease activity measurements

Consecutive patients diagnosed with CD and UC who underwent colonoscopy between 2019 and 2023 at the Department of Medicine, University of Szeged, were enrolled in the study. All IBD patients were eligible for the study except those under 18 years of age, pregnant women, and those, who did not consent to take part in the study. The inactive IBD group consisted of patients showing no sign of activity at the index colonoscopy. For patients in the active IBD group, new therapeutic agents were introduced, or the current therapy was modified according to the step-up or accelerated step-up strategy, and/or combination therapy was used to increase the therapeutic effectiveness. Throughout the 12-month follow-up period, patients were strictly controlled at regular visits performed every 2 months. Demographic data, information of disease activity, and therapeutic response were documented at every visit. In CD patients, the clinical disease activity was determined using the Crohn's Disease Activity Index (CDAI), in patients with UC, the clinical disease activity was measured using the partial Mayo score (pMayo). Colonoscopy was performed at inclusion and week 52, except for the case of uncertain disease exacerbation requiring unscheduled endoscopy to clarify symptoms. The control group consisted of non-IBD subjects who underwent colonoscopy.

Sample collection

Biopsies were obtained from the inflamed and the non-inflamed parts of the colon of IBD patients and the healthy sigmoid colon of controls. The biopsies were discharged from the forceps into the transport media. The tissue samples were placed immediately in ice-cold Hank's Balanced Salt Solution (HBSS) and transferred to the laboratory. Serum and faecal specimens were collected within one to three days before endoscopy. Serum samples were obtained to determine the routine inflammatory parameters (CRP, total blood count) at every appointment. CRP was considered to be elevated above 5 mg/L. Blood specimens were

centrifuged, and the serum was snap-frozen and stored at -80°C until use. Faecal samples were also frozen until further investigations.

Human colonic organoid culture

Human colonic organoid cultures (OCs) were generated using 3-4 colon biopsy samples. First, the colonic crypts were isolated as previously described with some modifications (Farkas et al. 2011). The isolated crypts were resuspended in Matrigel, and 2 domes per well were placed into a 24-well plate. After the polymerization of Matrigel 1 ml feeding media, completed with 10 μM Rho kinase inhibitor was added per well. The media was changed every other day. OCs were passaged after 7 days of culturing using TrypLE Express Enzyme, completed with 10 μM Rho kinase inhibitor.

Protein isolation from tissue and faecal samples

For total protein isolation, colonic biopsy samples were minced into small pieces with a razor blade and were transferred into RIPA lysis buffer completed with protease inhibitor. The biopsy samples were homogenized with a sonicator. After that, the samples were centrifuged and the supernatant was transferred into a new tube, snap-frozen, and stored at -80°C until use. For total protein isolation from stool samples, 100 mg stool was vortexed in 300 μl RIPA lysis buffer with protease inhibitor. After that, the samples were centrifuged and the supernatant was transferred to a new, sterile tube, and the centrifuge step was repeated. The samples were stored at -80°C until use.

Observation of the cytokine expression in serum, and colonic biopsy

To define the cytokine expression pattern of the serum and colonic mucosa, the Proteome Profiler Human Cytokine Array Kit was used according to the manufacturer's protocol. Briefly, 200 μl sera were used without dilution in all serum cytokine determination measurements. Whereas for the mucosal cytokine expression investigation, 500 μg total protein was applied at all membranes after total protein isolation. The total protein concentrations of biopsy samples were determined by Bradford assay. Blots were imaged with a ChemiDoc MP.

Determination of PAI-1 in serum, colonic biopsy, organoids, and faecal samples

To determine the PAI-1 concentration in the serum, mucosa, and fecal samples, a commercially available ELISA kit was used according to the manufacturer's protocol. Multiskan FC plate reader was used for the ELISA and Protein Bradford measurements. Serum specimens were collected and stored as described above. For serum ELISA measurements, samples were diluted to 20-100x with the Sample Diluent NS buffer of the ELISA kit. For the tissue and organoid samples, the total protein concentrations were defined by the Protein

Bradford method and protein levels were normalized to the total protein concentration. For the measurement of PAI-1 in tissue and faecal samples, the ab269373 ELISA kit was validated.

Gene expression analyses of colonic biopsy samples and organoid cultures

Gene expression analyses were performed as previously described (Molnár et al. 2020). Briefly, total RNA was isolated from homogenized biopsies with NucleoSpin RNA Plus Kit, whereas for homogenized organoids NucleoZOL was used according to the instructions of the manufacturer. iScript cDNA Synthesis Kit was used for the reverse transcription. The primers were designed in the NCBI Primer-BLAST. Amplicons were detected by a Lightcycler 96 using SYBR.

Immunofluorescence staining

Immunostainings were performed as previously described (Szabó et al. 2023). Briefly, colon biopsies were fixed in 4% PFA-PBS and 30% sucrose was used for cryoprotection. Fixed biopsy samples and organoids were embedded into Cryomatrix, frozen at -20°C, and 7 µm thick sections were cut. Organoids were fixed after sectioning in 4% PFA-PBS. Biopsies were permeabilized with 0.02% Triton-X100-TBS. For organoids, the permeabilization was performed using citrate-Tween 20 solutions and boiled in a rice cooker by indirect way. Unspecific antibody binding was blocked with 10% BSA-TBS containing 1% goat serum. Mouse monoclonal anti-PAI-1 primary antibody was diluted in 1:200 in the blocking buffer. For the secondary antibody, Alexa Fluor Plus 488 conjugated goat anti-mouse secondary antibody was applied, and the nuclei were labeled with DAPI. Sections were placed into Fluoromount, and images were obtained by Zeiss LSM 880 confocal microscope with a 40x oil immersion objective.

PAI-1 stability in faecal samples

The stability of the PAI-1 was defined in stool samples. 100 mg faeces were separated into sterile tubes and incubated at room temperature and 4°C for up to 7 days. Total protein isolation was performed every day. The isolation protocol and the PAI-1 level determination were the same as described above.

Data and statistical analysis

Statistical analysis was performed with GraphPad Prism software. Between two groups, unpaired Mann-Whitney or paired Wilcoxon tests were performed. Kruskal-Wallis test with Dunn's multiple comparisons test was utilized for three or more groups. The significance was accepted at $p<0.05$. For the correlation analysis, non-parametric Spearman's correlation was utilized. MedCalc statistical software was used for the receiver operator characteristic (ROC) analysis.

Ethical Considerations

Ethical approval for the study was obtained from the Regional and Institutional Human Medical Biological Research Ethics Committee, University of Szeged (247/2018-SZTE; 4412). The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki), and written informed consent was obtained from the enrolled patients.

Results

Patient population

The study population consisted of 132 IBD patients (56 CD and 76 UC) and 40 non-IBD patients (10 without any abnormalities, 7 with diverticulosis, 14 with colon polyps, and 9 with colorectal cancer). The mean age was 39 years for both CD (22-72) and UC (18-72). Patients in clinical remission accounted for 37.5% of CD and 28.9% of UC, while endoscopic remission was confirmed in 30.4% of CD and 25% of UC patients. New therapy was introduced in 21.4% of CD and 21.1% of UC patients. Switch and dose escalation of biological therapy was carried out in 16.1% and 14.3% of patients with CD and in 17.1% and 9.2% of patients with UC.

Cytokine expression in serum and colon mucosa discriminates IBD patients from controls

First, we used a semi-quantitative method to select the most prominent cytokines, which will be assessed in detail. For this analysis serum and biopsy samples were collected from controls and IBD patients and the cytokine expression patterns were compared. CCL-5, CD40 Ligand, C5/C5a, CXCL-12, ICAM-1, MIF, and PAI-1 were detected in all the serum samples.

Next, we investigated the cytokine expression of biopsies captured from the inflamed and non-inflamed part of the colon mucosa and compared them to control biopsies. The expression of CXCL-12, ICAM-1, MIF, IL-1RA, and IL-16 were detected in both IBD and control samples. The PAI-1, IL-8, IL-1β, CXCL-10, CXCL-1, and MIP-1α/β showed remarkably higher number of detections in the inflamed versus non-inflamed colons of the same patients. The PAI-1 pattern of IBD patients in different biological samples is unknown, therefore we did further experiments.

Serum concentration of PAI-1 is elevated in IBD patients and decreases in response to effective therapy

In the study by Kaiko et al. PAI-1 has been highlighted as a potential link between the epithelium and inflammation in the pathogenesis of IBD. We demonstrated that the level of PAI-1 is significantly elevated in the serum samples of IBD patients versus the control subjects

(24.62 ng/mL vs 30.48 ng/mL, p=0.0409). PAI-1 levels did not differentiate between UC and CD (27.77 vs 27.44 ng/mL, p=0.99). On the other hand, PAI-1 levels were significantly higher in IBD patients with endoscopic or clinical activity compared to inactive and control patients (endoscopically active vs inactive: 27.77 vs 21.67 ng/mL, p=0.0018, endoscopically active vs control: 27.77 vs 24.62 ng/mL, p=0.0239; clinically active vs inactive: 27.44 vs 21.7 ng/mL, p=0.0069, clinically active vs control: 27.44 vs 24.62 ng/mL p=0.0273). Of note, CRP was significantly increased in endoscopically or clinically active patients, but in UC patients, it was significantly lower compared to CD patients.

The increased serum PAI-1 level correlated with the endoscopic activity (r=0.3265, p=0.0008). Furthermore, ROC curves demonstrated that serum PAI-1 showed a moderate power to distinguish between active and inactive IBD patients (area under the curve (AUC) = 0.71; specificity: 48%; sensitivity: 87%; cut-off: 19.99 ng/mL) and between controls and active IBD patients (AUC = 0.69; specificity: 48%; sensitivity: 87%; cut-off: 27.96 ng/mL).

The PAI-1 level decreased significantly after the induction of therapy in responders, and this was not the case in non-responders (responders: 30.96 ng/mL vs 21.14 ng/mL, p<0.0147; non-responders: 26.49 ng/mL vs 26.87 ng/mL, p=0.93). Additionally, after the treatment, the serum PAI-1 concentration was significantly lower in responders than in non-responders. However, CRP did not show a significant difference between the responder and non-responder patients. Furthermore, ROC curves demonstrated that CRP showed a moderate power to distinguish between active and inactive IBD patients (AUC = 0.74; specificity: 96%; sensitivity: 50%; cut-off: 9.3 mg/mL) and between CD and UC patients (AUC = 0.82; specificity: 78%; sensitivity: 74%; cut-off: 15.3 mg/mL).

Serpin E1 gene expression in the colonic biopsies was significantly elevated in IBD patients and decreased in responders

We determined the relative gene expression fold changes (Fc) of Serpin E1 (the gene encoding PAI-1) in colonic biopsy samples from non-IBD control subjects, therapy-naive, active IBD patients, and responders and non-responders. Compared to the control samples, mucosal Serpin E1 expression was significantly higher in therapy-naive, active IBD patients (2.585 vs 18.17, p=0.006) but showed no significant difference between CD and UC patients. In addition, Serpin E1 gene expression was significantly lower in endoscopically inactive patients compared to active subjects (0.96 vs 14.32; p<0.0001) and in responders compared to the untreated patients (0.9326 vs 18.17, p<0.0001), or to non-responders (0.9326 vs 8.487; p=0.002).

We also determined the relative expression of four well-known inflammatory genes, TNF-α, IL-1β, IL-6, and TGF-β, from the same samples. In these experiments, IL-1β and TGF-β showed elevated expression in the untreated IBD biopsies, whereas TNF-α and IL-6 were not significantly elevated. Among the four other genes, only TGF-β and IL-1β were upregulated in the endoscopically active IBD patients and decreased in responder subjects.

Colonic mucosal expression of PAI-1 is higher in IBD and decreases significantly in response to the therapy

Immunofluorescence staining was performed in biopsy samples to determine the protein expression and localization of PAI-1 in the colonic mucosa. Following the elevated gene expression, the number of PAI-1 positive cells was significantly higher in biopsy samples obtained from the inflamed part of the colon (IBD vs control: 18.52% vs 5.718%, p=0.0014; IBD vs inactive IBD: 18.52% vs 6.651%, p=0.0126), whereas the non-inflamed part showed no difference when compared to control samples (5.716% vs 6.641%, p>0.9999). Notably, the majority of the PAI-1-positive cells were observed primarily in the epithelial cell layer.

Next, we isolated the total protein from the biopsy samples and determined the PAI-1 concentration in the colonic tissue, which was significantly higher in the IBD samples (0.00 vs 55.96 pg/mg, p=0.001). However, no significant difference was detected between CD and UC patients (29.24 vs 40.55 pg/mg, p=0.7903). Moreover, PAI-1 concentration was significantly higher in patients with endoscopic (active vs inactive: 40.83 vs 0.23 pg/mg, p<0.0001; active vs control: 40.83 vs 0.00, p<0.0001) or clinical activity (active vs inactive: 40.55 vs 2.770 pg/mg, p=0.0015; active vs control: 40.55 vs 0.00 pg/mg, p<0.0001) compared to the controls or inactive patients. Of note, the mucosal PAI-1 concentration was not significantly different in inactive patients and controls.

The increased mucosal PAI-1 level showed a correlation for the endoscopic activity (r=0.578, p<0.0001). ROC curves demonstrated that mucosal PAI-1 showed a relatively high power to distinguish between active and inactive IBD patients (AUC=0.81; specificity: 83%; sensitivity: 72%; cut-off: 18.16 pg/mg) and between controls and active IBD patients (AUC=0.88; specificity: 100%; sensitivity: 72%; cut-off: 15.49 pg/mg). Finally, when patients were categorized into responder and non-responder groups based on their response to the selected anti-inflammatory therapy, we found that the initial PAI-1 level was significantly higher in the responder patients compared to the non-responders (62.77 vs 34.15 pg/mg, p=0.03). Notably, mucosal PAI-1 concentration decreased significantly in responders, and this is not the case in non-responders (responders: 62.77 vs 0.23 pg/mg, p=0.0001, non-responders:

34.15 vs 24.58 pg/mg, p=0.58). These results suggest that the tissue concentration of PAI-1 correlates with the disease activity and decreases in response to therapy.

PAI-1 expression is higher in IBD patient-derived colonic organoids compared to controls

To further investigate whether epithelial cells could be the primary sources of PAI-1 in the colonic tissue, we established colonic organoid cultures (OC) from biopsy samples obtained from control subjects and IBD patients with active disease. Adult stem cell-based organoids consist of epithelial cells that display apical-to-basal polarity and maintain features of the original tissue in 3D cell cultures. In our experiments, OCs were used for experiments between the first and second passage to avoid changes in the inflammatory phenotype. The immunostaining revealed that the expression of PAI-1 was higher in IBD organoids. On the other hand, analysis of Serpin E1 revealed that the relative gene expression Fc was higher in the inflamed IBD organoids, however, the difference was not significant (2.034 vs 1.003 ng/g, p=0.3095). Moreover, the PAI-1 concentration in the organoids (35.29 vs 0.00 ng/g, p=0.0286) and in the media (31.28 vs 28.50 ng/g, p=0.0423) were significantly higher in the IBD organoids compared to the control.

PAI-1 as a potential faecal biomarker in IBD

The widely accepted tight disease monitoring strategy in IBD requires rapid, cheap, and easily accessible biomarkers, allowing patient self-testing. Faecal disease markers are suitable for these goals, therefore, in the next step, we analyzed the faecal concentration of PAI-1 in IBD patients. First, we assessed the stability of PAI-1 in the faeces at room temperature and 4°C, for up to 7 days, as this is a crucial parameter for a faecal biomarker. Our results suggest that PAI-1 remains stable for 7 days at 4ºC in faecal samples collected from IBD patients. In addition, we showed that PAI-1 level decreased in the first 24 hours at room temperature but remained stable until 7 days. Notably, in these experiments, we didn't use any stabilizer or enzyme inhibitor agent, which presumably could further improve the stability of PAI-1.

In the next step, we compared the concentration of PAI-1 in faecal samples collected from controls and IBD patients. Our results demonstrated that the level of PAI-1 was significantly higher in the faecal samples of IBD patients in general (0.00 vs 0.84 ng/g, p<0.0001). Similarly, to the serum and tissue PAI-1 levels, we could not find any significant difference between UC and CD patients (0.77 vs 1.15 ng/g, p=0.73). Importantly, the faecal PAI-1 concentration correlated with the endoscopic and clinical disease activity, and it was significantly higher in patients with endoscopically or clinically active disease compared to inactive patients and controls (endoscopically active IBD vs inactive IBD: 1.535 vs 0.215 ng/g, p<0,0001, endoscopically active IBD vs control: 1.535 vs 0.02 ng/g, p<0.0001; clinically active

IBD vs inactive IBD: 1.45 vs 0.01 ng/g, p<0,0001, clinically active IBD vs control: 1.45 vs 0.02 ng/g, p<0.0001).

The increased faecal PAI-1 level correlated with the endoscopic activity (r=0.5501, p<0.0001), whereas ROC curves demonstrated that faecal PAI-1 showed a relatively high power to distinguish between active and inactive IBD patients (AUC = 0.82; specificity: 80%; sensitivity: 74%; cut-off: 0.6 ng/g) and between controls and active IBD patients (AUC = 0.83; specificity: 62%; sensitivity: 88%; cut-off: 0.2 ng/g).

In the same patient cohort, CRP was significantly increased in the clinically or endoscopically active patients as well, however, no correlation was detected between CRP and faecal PAI-1 level. Finally, the faecal PAI-1 concentration was significantly lower in responders vs. non-responders after the treatment (0.33 vs 1.1 pg/g, p=0.047). Importantly, CRP did not show a significant difference between the responder and non-responder patients. These results suggest that faecal PAI-1 could be utilized as a novel biomarker in the clinical follow-up of IBD patients.

PAI-1 as a potential faecal marker for differential diagnosis of colorectal diseases

The controversial selectivity is one of the most significant limitations of the current gold standard FC, which could also be elevated in other organic gastrointestinal (GI) diseases. To assess the selectivity of faecal PAI-1, we collected samples from negative controls, IBD patients, and patients who had organic lesions detectable at colonoscopy except for IBD and were allocated to 6 groups: negative, active IBD, inactive IBD, adenoma, colorectal cancer, and diverticulosis (without inflammation). We found that faecal PAI-1 was elevated only in active IBD. In contrast, in other GI diseases, such as adenoma, colorectal cancer, or diverticulosis, it did not show a significant elevation compared to the control patients.

To gain information about the dependency on disease localization, we compared the faecal concentration of PAI-1 in patients with ileal, colonic, or ileocolonic localizations. In this cohort, the faecal PAI-1 concentration was significantly higher in patients with colonic and ileocolonic localization. Although patients with ileal localization displayed elevated faecal PAI-1 concentration, the difference was not significant compared to the controls. Notably, there were no significant differences among the faecal PAI-1 concentrations in the ileal, colonic, or combined localizations. Due to the low n numbers, these observations require further validation.

Discussion

PAI-1 emerged recently as a critical link between the epithelium and inflammation, which showed elevated mucosal gene expression in patients with IBD who did not respond to

anti-TNF biologic therapy. The present thesis demonstrated that the serum, mucosal, and faecal PAI-1 concentration is selectively elevated in IBD patients showing clinical and endoscopic activity but not in other organic gastrointestinal diseases and decreased significantly upon successful therapy in responders.

Timely management and optimization of treatment strategies in relapses are essential in IBD. Optimal management of IBD relies on early diagnosis and intervention, treat-to-target strategies, and tight disease control. Despite the number of available medications, there are appreciable rates of primary non-response, loss of response, or adverse reactions thereby necessitating additional treatment options, whereas the assessment of relapse risk is challenging. A crucial feature of IBD is the unbalanced inflammatory response, where different circulating immunological cells receive and secrete transcellular signals that can have a major impact on the disease course. Therefore, comprehensive analysis of the pro-, and anti-inflammatory cytokine and chemokine composition may help to identify novel biomarkers. In our study, we used a semi-quantitative screening approach to compare the expression profile of cytokines in the serum and the inflamed and non-inflamed part of the colon mucosa captured from control subjects and IBD patients. This approach identified PAI-1, which was elevated in the inflamed mucosal samples of IBD patients, however in controls the expression was not detected. Moreover, PAI-1 also seemed to be able to discriminate between the inflamed and non-inflamed areas, therefore in the downstream analysis we focused on this protein.

PAI-1, encoded by the gene Serpin E1, is a member of the serine protease inhibitor (serpin) family, and its primary function is to inhibit the tPA and uPA, which has a major role in the fibrinolysis and the homeostasis of the extracellular matrix. The expression of PAI-1 is regulated by multiple growth factors, hormones, and inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, and TGF-β), whereas it can be secreted by different cell types (e.g., endothelial-, epithelial- and immune cells) and act as a pleiotropic cytokine. Kaiko et al. identified an immune-coagulation gene axis in IBD in several independent cohorts where elevated PAI-1 was central in controlling key inflammatory modulators and mucosal damage in colitis. Our results also confirmed the elevated mucosal Serpin E1 gene expression in active IBD patients. Additionally, we detected a significantly increased concentration of PAI-1 in the serum and mucosal samples of active IBD patients. Importantly, the serum and mucosal PAI-1 concentration and the Serpin E1 gene expression in the colonic tissue decreased significantly in patients who responded to the anti-inflammatory therapy. Notably, the PAI-1 positive cells were detected in the epithelium of the colonic crypts. A similar expression pattern of PAI-1 positive cells was described by Kaiko et al. To demonstrate the ability of epithelial cells to

express and secrete PAI-1, we utilized colonic organoids. OCs consist of primary epithelial cells, which can comprehensively mimic the biological features of the original cell types. In our study, the PAI-1 concentration in the organoids and the secreted PAI-1 concentration in the media was higher in the IBD organoids compared to the control confirming that the epithelial cells could be a major source of the expressed PAI-1 in the colon in IBD.

The costs of overall care for IBD have also increased in the last 5 years, and the annual mean healthcare costs are over 3-fold higher than for patients without IBD. Biological therapy-based individualized treatment that focuses more on preventing disease progression is expensive at the onset but may ultimately lead to decreased rates of surgeries and hospitalizations, potentially yielding lower long-term costs for treatment. To achieve these goals, reliable biomarkers that are easy and relatively cheap to measure are crucially needed. Serological laboratory parameters, total leucocyte count, CRP, and ESR offer indirect, objective, but nonspecific markers for IBD. In this study, both serum PAI-1 and CRP were elevated in active IBD patients. Additionally, serum PAI-1 concentration decreased significantly in therapy responder patients, whereas CRP remained elevated. Notably, a large number of studies have demonstrated relatively poor sensitivity and specificity of serum biomarkers in IBD diagnosis and patient monitoring.

Recent studies indicated that faecal biomarkers strongly correlate with mucosal inflammation in IBD. Among many potential faecal biomarkers, most studies have been performed with FC, which is today the gold standard among faecal markers. Several studies reported that the AUC of FC is between 0.8-0.9 in different patient populations, which increased above 0.9 when FC was combined with other markers, such as Oncostatin M. In our experiments, faecal PAI-1 concentration was significantly higher in active IBD patients compared to the controls (AUC = 0.83) and in active versus inactive IBD and control samples (AUC=0.82). These results suggest that faecal PAI-1 is comparable to FC. However, there are considerable limitations of FC in clinical settings. First, several studies demonstrated the lack of specificity of FC for IBD, and it is increased in colorectal cancer, gastroenteritis, irritable bowel syndrome, diverticulitis, food intolerance, and nonsteroidal enteropathy as well. In our patient cohort, faecal PAI-1 demonstrated remarkable selectivity, as it was elevated only in active IBD patients. In contrast, in other GI diseases, such as adenoma, colorectal cancer, or diverticulosis, it was not significantly higher than in the control patients. In addition, FC concentration is affected by disease-independent factors such as age and comorbidities and shows considerable day-to-day variability. There are no optimal cut-off values to define active and inactive disease or to predict clinical and endoscopic remission or treatment response. FC

level is localization dependent as it frequently shows normal value in CD located in the small bowel and cannot differentiate between CD and UC. Our preliminary results showed that faecal PAI-1 was higher in patients with colonic and ileocolonic disease localization. Additionally, it was also higher in patients with ileal localization, but the difference was not statistically significant. Therefore, this parameter requires further investigation. A recent study highlighted that FC is unstable at room temperature, which may lead to false negative results and undertreatment in children with IBD. In contrast, faecal PAI-1 showed remarkable stability for up to one week.

There are some limitations to this study. First, the sample size did not allow subgroup analysis by disease type, location, and treatment type. To confirm the predictive value of faecal PAI-1 in predicting relapses, longitudinal sample collection will be needed, which is currently ongoing. To further validate the results, multicentric clinical trials with large patient populations will be required. On the other hand, there are several strengths of the current work. First, this is the first comprehensive evaluation of PAI-1 in different human biological samples (serum, colon mucosa, and faeces) on gene and protein expression levels. In contrast, the previous report focused solely on colonic mucosal gene expression. Another strength is the identification of a potential selective faecal biomarker, which could be utilized in patient follow-up in IBD.

Novel observations

- **The protein level of PAI-1 is increased in the patients with active IBD:** We found that the PAI-1 concentration was elevated in the serum, mucosal, and faecal samples of endoscopically and clinically active IBD patients compared to the healthy controls and inactive IBD.
- **The effective therapies reduce the concentration of PAI-1:** Our results suggested that the serum, mucosal, and faecal PAI-1 levels decreased significantly in the responders after the efficient treatment.
- **The gene expression of Serpin E1 (gene encoding PAI-1) reflects the disease activity and therapeutic response:** The mucosal gene expression pattern of Serpin E1 was similar to the protein level.
- **The epithelial cells can be the source of the PAI-1 in the intestinal mucosa:** The PAI-1 concentration in the organoids and the secreted PAI-1 level in the media was higher in the IBD organoids compared to the control.
- **The serum, mucosa, and faecal PAI-1 level correlate with the disease activity:** The study revealed that there is a positive correlation between the PAI-1 concentration and

endoscopically activity in the serum, mucosa, and faecal samples of IBD patients. Moreover, ROC curves demonstrated that PAI-1 level showed a relatively high power to distinguish between active and inactive IBD or control patients.

- **The PAI-1 protein is stable for up to 7 days at room temperature and 4°C in the faecal sample:** The PAI-1 protein was detectable from the stool when it was incubated at room temperature and 4°C.
- **Faecal PAI-1 concentration is selectively elevated in active IBD patients:** This thesis demonstrated that the faecal PAI-1 was elevated only in active IBD. Therefore, it could distinguish patients with active IBD from patients with other gastrointestinal diseases.

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