Examination of cytokine profile and therapeutic response in inflammatory bowel disease using patient-derived epithelial organoids

Summary: In this project, our aims were to characterize the cytokine profiles of the IBD colon organoids and assess the utility of organoid cultures (OCs) to predict response to therapy. Colonic OCs were established from IBD and non-IBD patients and cytokine expression patterns in different passages were determined by Proteome Profiler assay, qPCR, ELISA and immunostaining. Our results showed no significant difference between the biopsies and the organoid's cytokine expression until the first passage. We detected major pro-inflammatory cytokines such as TNF-α, IL-6, PAI-1 and IL-8, both in biopsies and organoids. After second passage the cytokine expression decreased or disappeared in the OCs. After anti-TNF-α treatment we detected a decrease in the IL-6 gene expression in treated OCs. In summary we successfully established OCs from inflamed colonic biopsies obtained from IBD patients. Our results suggest that colon OCs could be used for *ex vivo* investigations until the 2nd passage. Additionally, we identified and characterized PAI-1 as a novel marker for IBD activity, which was also tested in OCs. The patient samples and data obtained during the study were utilized in several subsequent studies. Based on the know-how generated, we have filed a patent application with the National Intellectual Property Office to exploit the results and are further developing the diagnostic kit with our industrial partner EpiPharma Pharmaceutical Research Ltd. We also plan to patent the use of organoids in the prediction of therapeutic response if the predictive utility of organoids could be verified in a larger patient population. Even though the Covid pandemic significantly hindered the scientific activity and the collection of patient samples, during the reporting period, we published 51 manuscripts (44 international and 7 Hungarian). Additionally, two other manuscripts that summarizes our findings with the colonic organoids are currently under submission.

Introduction. Inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing disorders of the gastrointestinal tract that are characterized by intestinal inflammation and epithelial injury. Both disorders are associated with marked morbidity and can have a major impact on an individual's quality of life and their ability to work, frequently require hospitalization and surgery which highlights the need for optimized anti-inflammatory therapy. At present, the exact pathogenesis of IBD remains unknown. Several studies highlighted that the balance between pro-inflammatory and anti-inflammatory cytokines in the mucosa regulates the development and potential perpetuation of mucosal inflammation in patients with IBD. Without definitive therapy, non-specific anti-inflammatory treatment is applied in IBD involving corticosteroids and antibodies specific for pro-inflammatory cytokines, such as anti-TNF antibodies. Anti-TNF therapy resulted in marked clinical and endoscopic improvement both in CD and UC leading to a new era of anti-cytokine

therapies. However, despite significant attempts, none of the presently acceptable biological therapy is considered as a "magic bullet". Our data on 911 IBD patients revealed that the rate of intestinal resection in CD was 66% and rate of colectomy in UC was 16%. These data highlighted that parallel to the increasing incidence of IBD, the requirement for surgery remains unacceptably high even in this biologic era. The severe complications develop due to initial non-respond, or loss of response during the treatment. 16-20% of IBD cases exposed to corticosteroids will presumably not response to the therapy. Moreover, around 10–30% of patients do not respond to the initial treatment with TNF blockers and 23–46% of patients lose response over time. In addition, in several cases TNFα is not the major mediator of inflammation eventually leading to non-response to anti-TNF therapy. Despite significant efforts, there is no reliable approach available to predict the resistance, or non-responsiveness of patients to steroid, or biological treatment. Based on these data, there is an unmet need to develop such approaches that would optimize and personalize the selection of treatment for IBD patients.

In vitro organoid cultures (OCs) may be utilized as ex vivo disease models that also recapitulate the biological features of the individual IBD patients. OCs can be generated from Lgr5-positive adult stem cells; they have capabilities for long-term growth, maintain the original cellular diversity, function, and spatial organization specific to the organ they represent. OCs have been successfully implicated to model malignancy and inflammation in the gastrointestinal tract. Because of the ability to grow robust and diverse patient-derived organoids, these tissues can be used to correlate gene expression patterns with drug responses and thus identify personalized approaches for treating diseases.

The **aim** of the present study is to 1) establish human colonic epithelial organoids from CD and UC patients. Using these organoids we will 2) determine and compare the cytokine profile produced by IBD organoids with the patient serum samples, and in the next step to 3) analyse changes of cytokine production before and after corticosteroid and/or biological therapy (etc. anti TNF-α, anti IL-6, anti IL-12/23, JAK inhibitors) to assess therapeutic response and 4) to correlate with clinical and endoscopic outcomes after treatment.

Results

Optimization of human colonic OC from IBD patients

During the generation of human colonic OCs we experienced several difficulties, as the organoids were prone to infection, whereas the growth of the organoids were less effective than

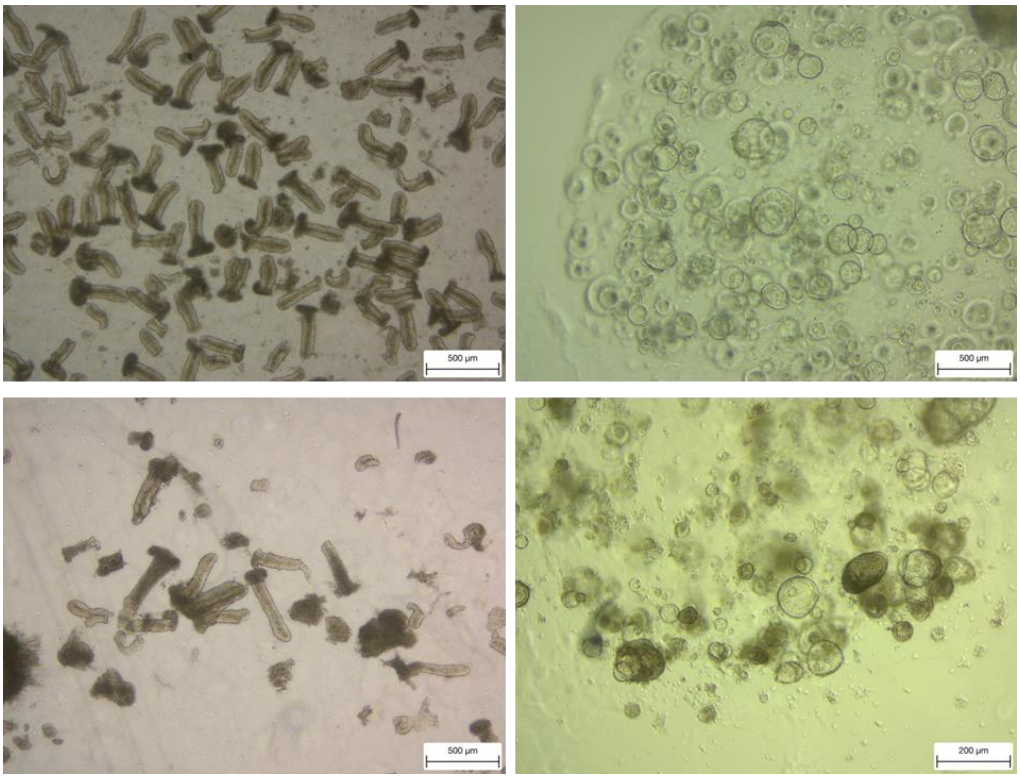

Figure 1. Isolation of crypts and organoid cultures. The upper images show isolated colonic crypts and generated OCs from control patients, whereas the lower images show crpyts and organoids from active IBD patients.

expected. After several steps of troubleshooting, we set up the final protocol, which we currently use. In this protocol human colonic OCs are generated from 3-4 colon biopsy samples. First colonic crypts are isolated as previously described (Farkas K at el., Inflamm Bowel Dis 2011;17(4):884–98.). Briefly, biopsy samples are washed 3 times with Hanks' Balanced Salt Solution (HBSS, Sigma, H9269) which was supplemented with antibiotic and antimycotic mix. Then biopsies are minced into ~1mm^3 pieces and transferred to the sterile, 30 ml centrifuge tube (Greiner, 201170) and washed with HBSS 8-10 times. The tissue pieces are placed into 3 ml 10 mM Dithiothreitol (DTT, Roche, 11583786001) in competed HBSS to reduce the disulfide bonds and are incubated in shaking incubator at 165 rpm on 37°C for 15 minutes. Next, DTT is removed, and the tissue is washed with HBSS 3 times. The samples are put into 3 ml completed HBSS containing 0.8 mg/ml Collagenase A (Roche, 37170821) and incubated for 50 minutes at 165 rpm on 37°C in shaking incubator. At the end of the incubation, the samples are resuspended and the isolated crypts in the supernatant are checked with Primovert light microscope (Zeiss). If necessary, 15 minutes digestion step is repeated one or two times to maximize the number of isolated crypts. Then the supernatant is collected into a sterile 1.5

ml tube and centrifuged for 5 minutes at 2000 rpm on 4°C. The isolated crypts are resuspended in Matrigel (Corning, 356232) diluted with HBSS in a 1:4 ratio and 10 µl domes are placed into a 24 well plate (Greiner, 662160), 2 domes per well (Figure 1.). After the polymerization of Matrigel 1 ml feeding media (Supp. Table 2.), which completed with 10 µM Rho kinase inhibitor (Tocris, 1254), is added per well. The media is changed every other day. OCs are passaged after 7 days culturing using TrypLE Express Enzyme completed with 10 µM Rho kinase inhibitor.

Cytokine expression of human colin OCs from IBD patients

To compare the possible changes of the cytokine expression profiles of colonic biopsy samples and the organoids generated from the biopsy, we performed a comprehensive analysis of the cytokine expression using a Proteome Array kit. In this series of experiments biopsies were captured from the inflamed part of the colon and one biopsy was used to determine the cytokine expression, whereas the other were used to establish colonic OCs. Before passages a smaller group of organoids were used to isolate proteins to detect cytokines, whereas the rest was passaged further. As demonstrated on Figure 2. we found that the expression of cytokines in the OCs after the first passage recapitulated the cytokine expression pattern of the original biopsy. In contrast further increase of the passage numbers triggered a decrease of the cytokine expression, which was almost completely lost in passage number 3.

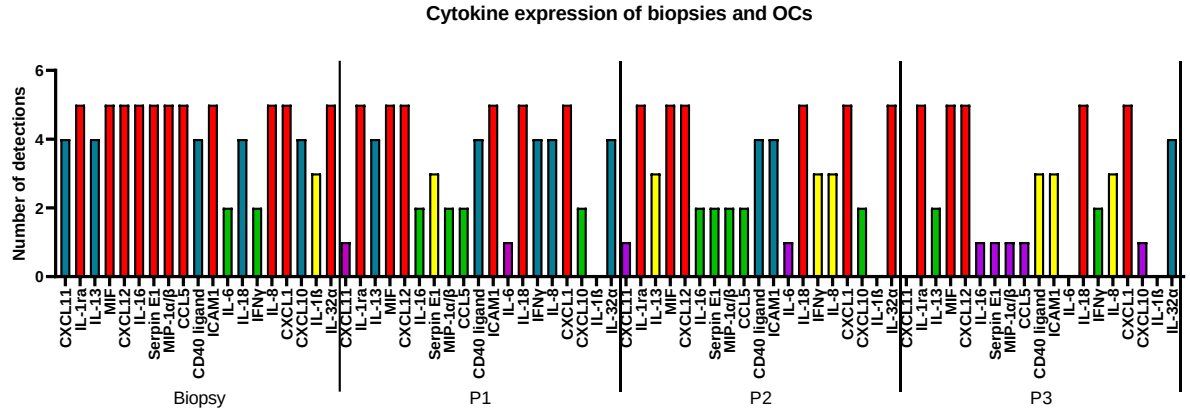

Figure 2. Cytokine expression profiles of colonic biopsies and OCs. The bar chart shows the number of detections in each group. P1-3: passage number 1-3.

The expression of TNFα in human colonic OCs

The elevated level of TNFα is a hallmark in IBD, therefore we also assessed the expression of this cytokine in the colonic OC samples. Immunofluorescent staining revealed that the colonic OCs express TNFα, which was detectable until passage number 3 (Figure 3.).

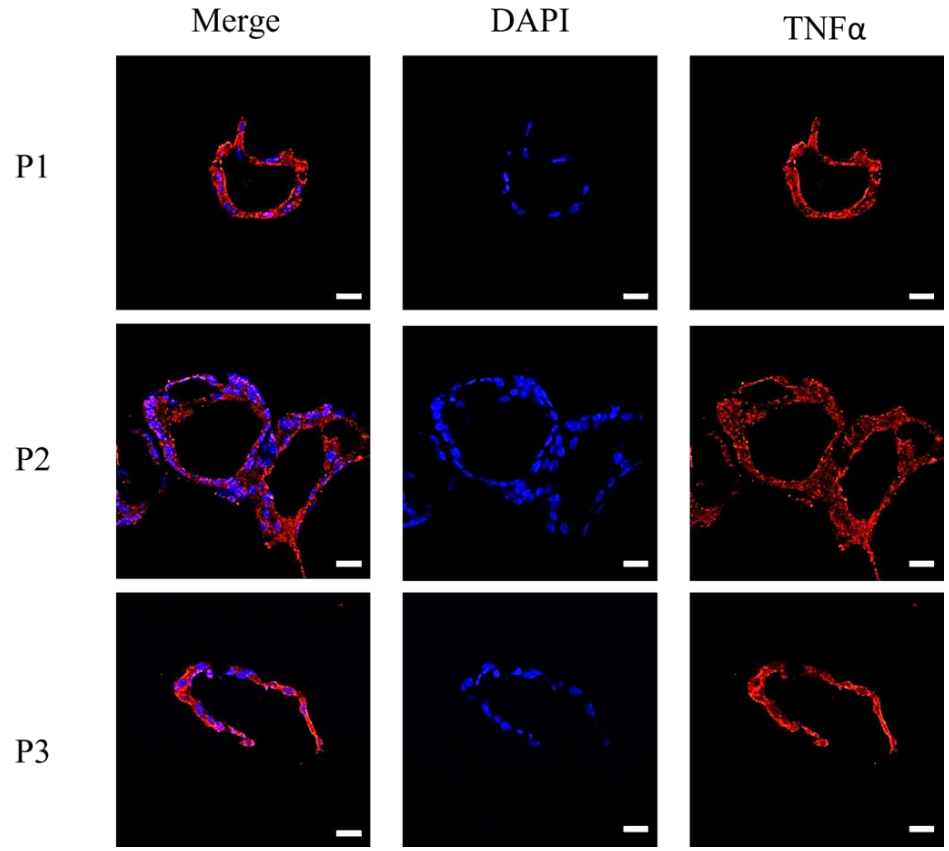

Figure 3. Expression of TNFα in colonic OCs. Colonic OCs were generated from biopsies collected from the inflamed part of the colon of active IBD patients. The immunostaining showed the expression of this cytokine in all samples. Scale bar: 10 μm.

Measurement of the protein concentration of TNFα in human colonic OCs

As the immunostainings revealed the expression of TNFα in colonic OCs, but the concentration was not determined, we used ELISA to quantify the expression levels. This measurement showed that the TNFα concentration was the highest in the biopsy samples, however OCs in the first passage also expressed TNFα in a relatively high concentration (Figure 4.). As expected from the previous results, TNFα expression was lower in P2-3 organoids.

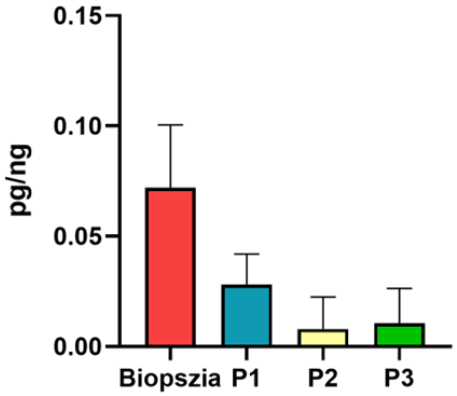

Figure 4. TNFα expression in biopsies and colonic OCs. ELISA measurements revealed that colonic OCs in the first passage produce TNFα, although the expression is lower than in the biopsy samples. In P2-3 the expression is further decreased.

Ex vivo treatment of colonic OCs with anti-TNFα therapy

As our results suggested that the colonic OCs maintain the cytokine expression until the first passage, we performed a proof-of-concept experiment and treated the organoids with infliximab (5µg/mL) or adalimumab (8µg/mL) *in vitro* and measured the expression of inflammatory genes. In these series of experiments we detected the significant decrease of the IL-6 gene expression suggesting that the in vitro treatment had an effect on the colonic OCs (Figure 5.). Based on these we will perform further experiments to assess the effects of the in vitro treatment on colonic OCs. Currently we are also following up patients to compare the *in vitro* results with the real-life clinical response to the therapy.

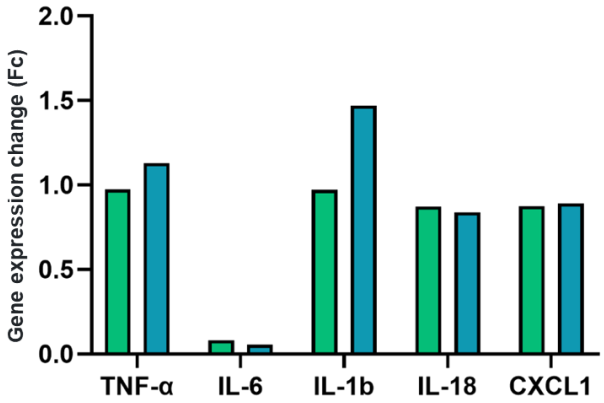

Figure 5. Expression of inflammatory genes in the colonic OCs after anti-TNFα therapy. *In vitro* treatment of the colonic OCs with infliximab (green bars) or adalimumab (blue bars) induced a decrease of IL-6 gene expression suggesting that the treatment was efficient.

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

In another study we identified PAI-1 as a potential biomarker in IBD. To further investigate whether epithelial cells could be the primary sources of PAI-1 in the colonic tissue, we established colonic organoid cultures from biopsy samples obtained from control subjects and from IBD patients with active disease. In these experiments, organoids were used for analysis after the first passage. The analysis of Serpin E1 gene expression revealed that the relative gene expression Fc was higher in the inflamed IBD organoids, however the difference was not significant (Figure 6. A.). On the other hand, immunstaining revealed that the expression of PAI-1 was higher in IBD organoids (Figure 6. B.). Moreover, the PAI-1 concentration in the organoids (Figure 6. C.) and the secreted PAI-1 concentration in the media (Figure 6. D.) were significantly higher in the IBD organoids compared to the control (Figure 6. D.). These results confirmed that the epithelial cells could be a major source of the expressed PAI-1 in the colon in IBD.

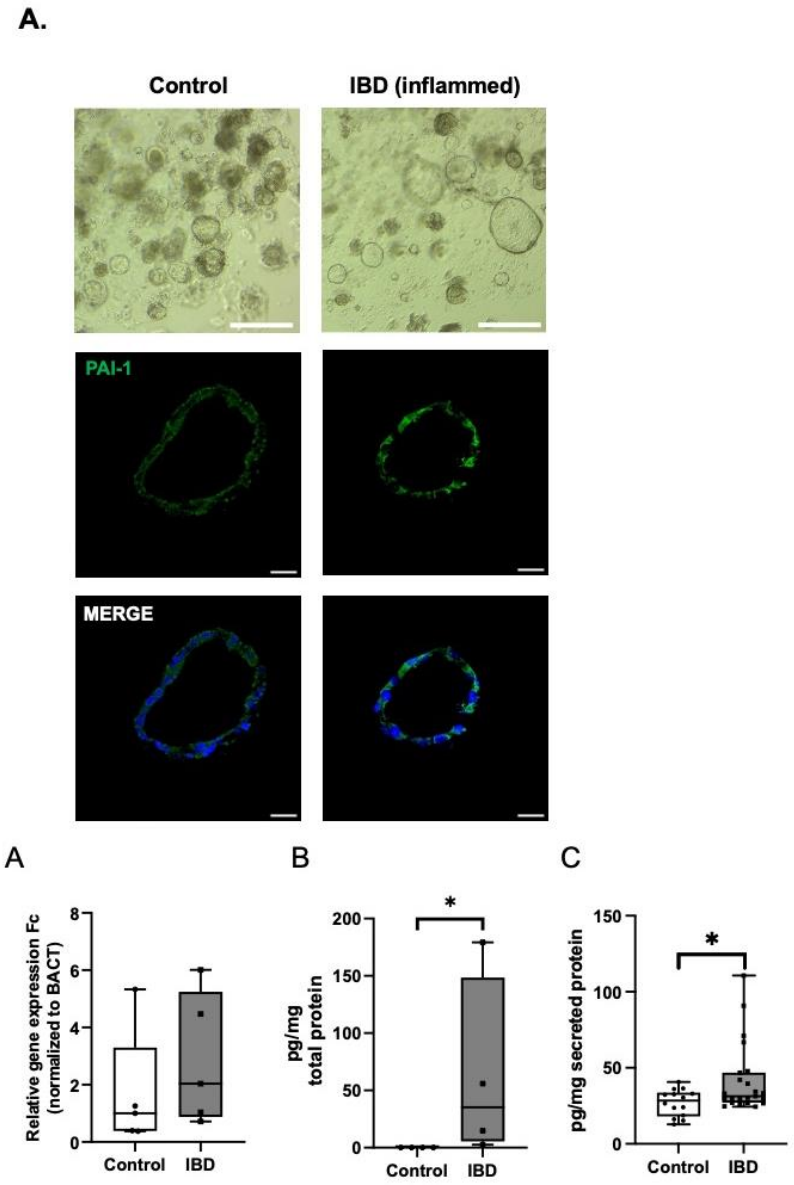

Figure 6. The expression of PAI-1 in colonic organoids. PAI-1 expression is higher in organoids established from active IBD. **A-C.** The gene expression (A), intracellular (B) and secreted PAI-1 levels (C) were higher in IBD organoids.

The generation of colonic OCs required the collection of large amount of biological samples and clinical data, which we also capitalized in subsequent studies to gain further information about the imbalanced cytokine homeostasis and the possible interactions with other factors, such as the type of treatment or the composition of the fecal microbiome.

- The relationship between clinical outcomes and serum anti-TNF levels is controversial, however the detection of the presence of anti-TNF antibodies in OCs may be used to predict the response to therapy. Therefore, in a study we aimed to perform simultaneous

analyses of serum, mucosal, and fecal anti-TNF-α levels. Consecutive IBD patients who received maintenance anti-TNF-α therapy were enrolled. The number of TNF-α positive cells in the mucosa was detected using immunofluorescent labeling on biopsy samples. Serum, mucosal and fecal anti-TNF-α, serum anti-drug antibody, and fecal calprotectin levels were determined using ELISA. Each patient underwent body composition analysis as well. Data of 50 patients were analyzed. The number TNF-α positive cells was significantly higher in the inflamed part of the colon than in the un-inflamed part of the colon. Tissue and fecal drug levels did not show any association with serum drug levels; moreover, serum anti-TNF concentration did not correlate with endoscopic activity. Mucosal anti-TNF levels were higher only in IFX-treated patients in remission and IFX-treated patients with detectable fecal anti-TNF had lower tissue drug levels. Presence of the drug in the feces was significantly different according to disease activity. Fecal drug concentration is suggested to be a better predictor of endoscopic activity and loss of response, and fecal drug monitoring may improve the estimation accuracy of tissue drug levels. ***Szántó K. et al., Expert Opin Biol Ther. 2021 Apr;21(4):539-548***

- Utilization of the colonic OCs in the study of microbial-epithelial interactions is a promising future direction of our research. To gain more insight into this area, in a focused study we also investigated the gut microbial composition in UC after total proctocolectomy and ileal pouch-anal anastomosis (IPAA) surgery. Clinical data of patients, blood and faecal samples were collected. Faecal microbiota structure was determined by sequencing the V4 hypervariable region of the 16S rRNA gene. Overall, 56 patients were enrolled. Compared to the Healthy group, both the Pouch active and UC active groups had higher *Enterobacteriaceae, Enterococcaceae* and *Pasteurellaceae* abundance. The Pouch and UC groups showed distinct separation based on their alpha and beta bacterial diversities. The UC group had higher *Prevotellaceae, Rikenellaceae, Ruminococcaceae* abundance compared to the Pouch active group. Pouch and FAP participants showed similar bacterial community composition. There was no significant difference in the bacterial abundance between the active and inactive subgroups of the Pouch or UC groups. Gut microbiome and anatomical status together construct a functional unit that has influence on diversity, in addition to intestinal inflammation that is a part of the pathomechanism in UC. ***Bálint A. et al., Pharmaceuticals (Basel). 2020 Oct 28;13(11):346.***

The COVID-19 pandemic was a significant challenge to the whole healthcare system, including the gastroenterology departments. In addition, the patient care was reorganized and the endoscopic interventions were limited to the emergency cases. These issues together significantly impacted our studies. To overcome the challenges, we collected data and published our experiences in three manuscripts.

- To determine the effect of the COVID-19 pandemic on the workflow and infection prevention and control strategies of endoscopy units in real-life setting, we invited the members of Hungarian Society of Gastroenterology to participate in this cross-section survey study and to complete an online, anonymous questionnaire. We enrolled a total of 120 endoscopists from 83 institutes. Only 33.33% of them had undergone training about infection prevention in their workplace. 95.83% of endoscopists regularly used risk stratification of patients for infection prior endoscopy. While indications of examinations in low-risk patients varied widely, in high-risk or positive patients endoscopy was limited to gastrointestinal bleeding (95.00%), removal of foreign body from esophagus (87.50%), management of obstructive jaundice (72.50%) and biliary pancreatitis (67.50%). Appropriate amount of personal protective equipment was available in 60.85% of endoscopy units. In high-risk or positive patients, surgical mask, filtering facepiece mask, protective eyewear and two pairs of gloves were applied in 30.83%, 76.67%, 90.00% and 87.50% of cases, respectively. Personal protective equipment fully complied with European guideline only in 67.50% of cases. ***Bor R. et al. BMC Gastroenterol. 2021 Mar 3;21(1):98.***

- We also compared the national experiences with the international, therefore we performed an observational, cross-sectional, questionnaire-based study. Responds came from many countries, and the participation was voluntary. The survey contained 40 questions, which evaluated the effect of the COVID-19 pandemic on the endoscopy units and assessed the infection control. A total of 312 questionnaires were filled, 120 from Hungary, and 192 internationally, and 54 questionnaires (17.3%) were sent from high-risk countries; 84.9% of the gastroenterologists declared that they read the European Society of Gastrointestinal Endoscopy (ESGE) statement, while only 32.1% participated in any advanced training at their workplace. Overall, 92.1% of gastroenterologists realized risk stratification, and 72.1% claimed to have enough protective equipment. In 52.6% of the endoscopy units, at least one endoscopist had to

discontinue the work due to any risk factor, while 40.6% reported that the reduced staff did not affect the workflow. Gastroenterologists considered that the five most important examinations both in low and high-risk patients are the following: lower/upper gastrointestinal (GI) bleeding with hemodynamic instability, endoscopic retrograde cholangiopancreatography (ERCP) in obstructive jaundice, foreign body in the esophagus, ERCP in acute biliary pancreatitis, and iron deficiency anemia with hemodynamic instability, which correlates well with the ESGE recommendation. Significant correlation was found in the usage of the necessary protective equipment in high-risk patients depending on the countries ($p < 0.001$). ***Resal T.. et al. Therap Adv Gastroenterol. 2021 Apr 22;14:17562848211006678.***

- IBD patients are likely to be more susceptible to viral infections, and this is significantly influenced by the type of therapy they receive. To provide information to physicians treating IBD patients, we summarized the available evidence regarding viral infections and IBD, focusing on SARS-CoV infections, and we provided practical recommendations related to patient management during the COVID-19 pandemic era. ***Farkas K et al. Therap Adv Gastroenterol. 2021 Apr 12;14:1756284820988198.***

Conclusions

In conclusion in the reporting period, we successfully established the generation of colonic organoid cultures from biopsy samples. Of note, we were also able to generate organoids form the inflamed tissue, which was rather difficult. Using these organoids, we demonstrated that the samples maintain the cytokine expression until the first passage, which is comparable with the original tissue. We also performed proof-of-concept experiments and demonstrated that the organoids could be used to detect the drug response *in vitro*. During this period, we published several manuscripts, in which we utilized the data generated in this project. Two other papers are currently under submission from the results of the experiments with the colonic organoids. Moreover, we were able to submit a patent and we are currently working on the further development of the patented idea. Moreover, the generated unique knowledge about the colonic organoids in IBD patients could be utilized in several subsequent studies.