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DOKTORSKA DISERTACIJA

GREGOR NOVAK

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DOKTORSKA DISERTACIJA
DOCTORAL THESIS

GREGOR NOVAK

OCENJEVANJE MESTA ODVZEMA BIOPSIJ PRI AKTIVNI CROHNOVI BOLEZNI

EVALUATING LOCATION OF BIOPSY PROCUREMENT IN ACTIVE CROHN'S DISEASE

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Ljubljana, 5. junij 2019 Podpis avtorja: _____

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List of abbreviations (in alphabetical order)

CD - Crohn's disease

CI - confidence interval

GHAS - Global Histologic Disease Activity Score

IL - interleukin

MPO - myeloperoxidase

NHI - Nancy Histological Index

qPCR - quantitative Polymerase Chain Reaction

RHI - Robarts Histopathology Index

SD - standard deviation

SES-CD - Simple endoscopic score for Crohn’s disease

TNFα - tumor necrosis factor-α

UC - ulcerative colitis

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1 IZVLEČEK

Izhodišča in namen: Histološka remisija je možen terapevtski cilj pri zdravljenju bolnikov s Crohnovo boleznijo (CB), vendar do danes ni določenih meril za odvzem biopsij črevesne sluznice. Namen naše raziskave je določiti mesto za odvzem biopsij v prisotnosti razjed pri CB, ki daje najvišje histopatološko točkovanje, največjo sluznično mRNK izražanje pro-vnetnih genov in največje število vnetnih celic.

Hipoteza in cilji: Naša hipoteza je, da obstajajo razlike v histopatoloških točkovanjih, izražanju genov in številu vnetnih celic v biopsijah črevesne sluznice na različnih razdaljah od razjed pri CB.
Cilji študije so določitev mesta odvzema biopsij pri CB, pridobljenih s treh različnih razdalj od razjed v debelem in tankem črevesu, ki ima:

- najvišjo histopatološko točkovanje z uporabo več točkovnikov histološke aktivnosti bolezni,
- najvišjo izražanje mRNK izbranih genov,
- največje število vnetnih celic, dokazanih imunohistokemično.

Metode: V prospektivno multicentrično študijo v treh centrih smo vključili 51 bolnikov s histološko potrjeno diagnozo CB in z razjedami > 0,5 cm v debelem in/ali tankem črevesu. Biopsije smo odvzeli na treh različnih razdaljah od razjede. Histopatološko aktivnost smo ocenili s tremi točkovniki histološke aktivnosti bolezni. Izražanje mRNK petih pro-vnetnih genov smo določili s kvantitativno verižno reakcijo s polimerazo. Število vnetnih celic v lamini proprii smo določili z imunohistokemičnim barvanjem. Za statistično analizo smo uporabili modele mešanih učinkov.

Rezultati: Potrdili smo hipotezo, da so prisotne razlike v histopatoloških točkovanjih, izražanju pro-vnetnih genov in številu MPO-pozitivnih celic v biopsijah črevesne sluznice na različnih razdaljah od razjede pri CB. Biopsije odvzete iz roba razjede imajo večjo histopatološko vnetno aktivnost, izražanje mRNK pro-vnetnih genov in število MPO-pozitivnih celic v lamini proprii v primerjavi z biopsijami odvzetimi v bližini ulkusa tako v debelem kot tankem črevesju ($p<0,005$).

Zaključki: Mesto odvzema biopsije pri CB, ki doseže največjo histopatološko vnetno aktivnost bolezni in najvišje sluznično izražanje mRNK pro-vnetnih genov, je rob ulkusa.

1 ABSTRACT

Background and aim: Histological remission is a potential therapeutic target in Crohn's disease (CD) but no standards exist to guide the sampling of mucosal biopsies. We aim to determine the location for biopsy procurement in the presence of ulcers which yields the highest histopathological score, the greatest mRNA expression of pro-inflammatory genes and the highest number of inflammatory cells.

Hypothesis and objectives: Our hypothesis is that differences in histopathological scores, mucosal gene expression and number of inflammatory cells of mucosal biopsies at various distances from ulcers in CD exist.
The objective of the study is to determine the location of biopsy, procured from three different distances from ulcers in the colon and ileum, which yields:

- the highest histopathological scores using several indices
- the greatest mucosal mRNA expression of selected genes
- the highest number of inflammatory cells as detected by immunohistochemistry.

Methods: This prospective multicentric study enrolled 51 patients from three centers with a histologically confirmed diagnosis of CD and ulcers > 0.5 cm in the colon or/and ileum. Biopsy specimens were obtained at three different distances from ulcers. Histopathological disease activity was assessed using three histological scores. mRNA expression was measured by quantitative polymerase chain reaction. Number of inflammatory cells was determined by immunohistochemical staining. Statistical analysis was performed using mixed effects models.

Results: We confirmed our hypothesis that differences in histopathological scores, mucosal gene expression and number of MPO-positive cells of biopsies at various distances from ulcers exist. Biopsies from the edge of the ulcer yield a higher histopathological score, higher mRNA expression of pro-inflammatory genes and higher number of MPO-positive cells compared to biopsies procured next to the ulcer in the colon and ileum ($p<0.005$).

Conclusion: The location for biopsy procurement which yields the highest histopathological score and highest mRNA expression of pro-inflammatory genes is the ulcer edge.

2 BACKGROUND

GENERAL BACKGROUND

CROHN'S DISEASE

The term inflammatory bowel disease represents a group of chronic relapsing inflammatory intestinal disorders and principally refers to two major categories: Crohn's disease (CD) and ulcerative colitis (UC).[1] In CD inflammation typically involves the whole bowel wall, whereas in UC it is usually confined to the mucosa. Inflammation in CD is usually patchy, discontinuous and segmental.[2] CD can affect any part of the gastrointestinal tract from the mouth to anus and perianal skin area, but most commonly involves the terminal ileum and colon. In a rough estimation, 25% of the patients have colon involved only, 25% terminal ileum only and 50% have ileocolitis.[1]

EPIDEMIOLOGY OF CROHN'S DISEASE

CD occurs globally, but there are important variations in both incidence and prevalence within geographical regions.[3,4] The highest incidence rates of CD are reported in Northern Europe (especially Faroe Islands) and North America.[4] The highest prevalence values were found in Europe (CD 322 per 100000 in Germany) and North America (CD 319 per 100000 in Canada).[3] In Europe, incidence rates range up to 11.5 per 100,000 person-years for CD and prevalence of CD ranges from 1.5 to 213 cases per 100,000 persons. The incidence of CD continues to increase worldwide. In Europe, the incidence of CD increased from 1.0 per 100,000 person-years in 1962 to 6.3 per 100,000 person-years in 2010. Importantly, incidence of CD is increasing especially in once low-incidence regions, such as Eastern Europe and Asia.[4]
Similar trends are noticed in Slovenia. A Slovenian pediatric epidemiological study found a mean annual incidence of inflammatory bowel disease of 4.03 cases per 100,000 children with 60% of cases being CD (2.42 cases per 100,000). The incidences of CD rose from 1.99 cases per 100,000 children between 1994 to 1999 to 2.88 cases per 100,000 children between 2000 and 2005.[5] A more recent pediatric epidemiological study confirms a high incidence of CD

(4.6 cases per 100,000 children) which is comparable to the reported rates from western Europe. Interestingly, the phenotype of disease in Slovenian children has changed from more benign to complicated disease behavior. Stricturing and penetrating phenotype had doubled. A total of 19% of children with CD required surgery.[6] A recent epidemiological study in the adult population estimated an incidence of CD of 6.1 cases per 100,000 in the period from 2010 to 2012 with a prevalence of 150 cases per 100,000 in 2012. Similar to the pediatric population an increase in the incidence of CD was found in the adult population. Median age at diagnosis was 34 years. One in three patients had immunosuppressive drugs, one in six had a previous operation.[7]

SOCIOECONOMIC ASPECTS OF CROHN'S DISEASE

CD usually affects adolescents and young adults who are at their most productive and creative years. Frequent relapses and symptoms of active disease, disturbing extraintestinal manifestations, chronic and unpredictable nature of the disease, medical and surgical treatments and their side effects have a huge impact on daily lives of patients with CD and cause significant reduction in quality of life.[8] CD may affect many aspects of life, including bowel control, fatigue, social isolation, and a fear of developing cancer or needing surgery.[9] Depression and anxiety are more common among patients with CD than the general population.[10] CD adversely impacts work life, resulting in reduction in working hours, absenteeism, and changes in career choices.[9] CD is associated with a significant psychosocial burden and beside work affects education, social life and spare time. It was estimated that 2.5 -3 million people in Europe are affected by inflammatory bowel disease, which carries an estimated direct cost of 4.6-5.6 billion Euro per year.[11] However, in a study from Germany the direct medical costs were only 32% costs of the disease. The majority of the cost (64%) were due to indirect costs such as early retirement or sick leave.[12] An average cost of a patient with CD in Germany is 1425 Euro per month[12] manifesting the significant economic aspect the disease has on societies.

ETIOPATHOGENESIS

Despite intensive research in the field the etiopathogenesis of inflammatory bowel disease is not completely known. It is a complex and multifactorial disease which is a result of an

interplay between immune dysregulation, genetic predisposition, gut microbiota and environmental factors.[13] According to the most prevalent hypothesis, inflammatory bowel disease is an intestinal disease which develops in genetically predisposed individuals in whom environmental factors trigger an exaggerated immune response to luminal antigens.[14]

The role of genetics in the pathogenesis was manifested by clustering of CD patients within families and in some ethnical groups. Since 1980s several studies found familial aggregation in CD with a positive family history ranging up to 25%.[15] Significantly higher concordance rate for CD among monozygotic than among dizygotic twin pairs strongly points to a genetic influence.[16] CD is more common in Jews than non-Jews.[17] To date more than 163 genetic loci are known which are associated with inflammatory bowel disease. Among them 30 are specific for CD and 110 are associated with both CD and UC. Many of the loci are implicated in pathogenesis of CD, including microbe recognition, lymphocyte activation, cytokine signaling, epithelial defense etc. Many IBD loci are also involved in other immune-mediated disorders (ankylosing spondylitis and psoriasis).[18] Carriers of two alleles of NOD2/CARD15 have a 17-times increased risk to develop CD, but with at least one allele the risk of stenosing phenotype and small bowel involvement increases.[19]

The role of environmental factors is manifested by the fact that individuals that migrate from a low-incidence region to a high-incidence region for CD have an increased risk of developing CD, particularly among first generation children.[20,21] Incidence of CD is increasing in the developing countries; many speculate it is due to environmental factors associated with better socioeconomical situation, improved hygienic conditions and changes in diet. Several theories of environmental causes of developing CD were postulated.[21] Among the most predominant hypothesis is the hygiene theory which proposes that the rising incidence of immune-mediated diseases is attributed to a lack of exposure to enteric pathogens in childhood. This can lead to an increased susceptibility of an inappropriate immunologic response upon exposure to new antigens later in life.[21] Smoking is a well-known environmental risk factor which increases the risk for acquiring CD.[22]

Many microbes have been implicated as possible causes of inflammatory bowel disease, including Mycobacterium avium subspecies paratuberculosis and adherent-invasive strains of Escherichia coli.[21] However, more probably the crucial role in the etiopathogenesis is played by the interaction of luminal microbiota and intestinal immune system. In inflammatory bowel disease, the intestinal immune system is totally disrupted.[23] Due to a leaky barrier microbial antigens from commensals gain access to the underlying mucosal tissue. In genetically predisposed individuals these usually harmless antigens trigger an inflammatory response

because dendritic cells falsely recognize commensals as pathogens and promote differentiation of naive T cells into effector T cells (predominantly to Th1 in CD) and natural killer T cells. The proinflammatory cytokines from activated T cells stimulate macrophages (part of the innate immune response) to secrete numerous cytokines, including tumor necrosis factor α (TNFα). Inflammation attracts numerous leucocytes which enter from the systemic circulation and release chemokines that attract even more inflammatory cells which further increases the inflammatory process. Tissue damage results from the release of numerous noxious mediators secreted by inflammatory cells.[23]

CLINICAL PRESENTATION

CD may manifest very acutely with severe abdominal pain and intestinal obstruction, but more often it presents with long-lasting symptoms. First symptoms of CD are usually subtle and atypical which may postpone the correct diagnosis (diagnostic delay) and appropriate management. Symptoms are heterogeneous because CD can involve any part of the gastrointestinal tract, but mostly include abdominal pain, diarrhoea, and weight loss. Systemic symptoms, including fever, malaise and anorexia may be present as well.[24,25] Abdominal pain may be sustained or intermittent and colicky. It is due to abscesses, transmural inflammation with peritoneal pain receptors irritation, and strictures with excitation of stretch receptors in the bowel wall.[25] Diarrhoea and blood in the stools are less common than with UC. Because of catabolic state induced by inflammation, poor oral intake and inadequate intestinal absorption of inflamed gastrointestinal tract patients with CD may be malnourished and lose weight. A typical feature of CD is a stricture or stenosis which is often asymptomatic. However, when the intestinal luminal diameter progressively shortens strictures may cause bowel obstruction which manifests with colicky postprandial abdominal pain, vomiting, bloating and abdominal distention. If the inflammation involves the entire intestinal wall, fistulas and abscesses may form. Symptoms of fistulas depend on the location. If a fistula has a cutaneous opening drainage of pus, stool, or blood is typical. Abscesses usually manifest with abdominal or perianal pain, spiking fever and local tenderness.[25]

In general, clinical presentation of CD is dependent on the disease location, extent, disease behaviour, intensity of inflammation, complications of the intestine and extraintestinal manifestations. For example, ileal CD typically manifests with pain in the right lower quadrant,

usually exacerbated by meals, whereas colonic CD with diarrhoea, rectal bleeding, and urgency.[25]

CD primarily affects the gastrointestinal tract. However, in up to 47% it may manifest in other organ systems as well.[26] Extraintestinal manifestations can occur in each organ system of the human body, but are most often involve mouth (oral ulcers), eyes (uveitis, episcleritis), skin (erythema nodosum, pyoderma gangrenosum) and joints (peripheral arthropathies, sacroiliitis, ankylosing spondylitis). Some extraintestinal manifestations can be have even a bigger impact on quality of life of patients than the intestinal disease. Some extraintestinal manifestations correlate with the intestinal disease activity and normally improve with the treatment of CD, whereas other have a course independent of the bowel disease activity.[25,26]

DIAGNOSIS OF CROHN'S DISEASE

CD is a very heterogeneous disease with a variety of phenotypes in terms of disease location, behavior and age of onset. Therefore, it is not surprising that no gold standard for diagnosis of CD exists. Diagnosis of CD is established by a non-precisely defined combination of clinical presentation, endoscopic appearance, radiology, histology, surgical and biochemical findings. According to the current ECCO guidelines genetic and serological testing is not recommended for the diagnosis to date.[24]

In the every-day clinical practice the first line procedure to diagnose CD is ileocolonoscopy and biopsies from the terminal ileum as well as colonic segments. Irrespective of the findings at ileocolonoscopy, further diagnostic investigations are recommended to determine the disease location, extent and behaviour. To assess the involvement of small bowel cross-sectional imaging (MR-enterography and CT-enterography) and trans-abdominal ultrasound are recommended. Small bowel capsule endoscopy is reserved for patients with a high clinical suspicion for CD in the small bowel despite negative findings with endoscopy and cross-sectional imaging.[24]

TREATMENT OF CROHN'S DISEASE

In general, first line treatment of CD is medicamental with drugs of various modes of action. Surgical therapy is indicated in case of complications and when medicamental therapy is ineffective.[24]

The therapeutic landscape for CD is rapidly evolving in recent years. For many years, TNF antagonists (infliximab and adalimumab) were the mainstay for treatment of patients with moderate-to-severe CD. TNF antagonist were the only available biological drug for inflammatory bowel disease at that time. However, recently two new classes of biological therapy were added to the therapeutic armamentarium of CD; the integrin antagonist vedolizumab and the interleukin (IL)-12/23 antagonist ustekinumab. The first janus kinase inhibitor tofacitinib entered the market just this year, but is to date registered only for UC. Moreover, several new agents with different mode of action are in the pipeline to enter the market for treatment of CD in the years to come, many completing phase 2 and enrolling in phase 3 trials.[27]

In the last century, no biological therapy was available. We relied on the so-called conventional therapy which consists of aminosalicylates, antibiotics, local and systemic corticosteroids, thiopurins (azathioprin, 6-mercaptopurin), methotrexate and exclusive enteral nutrition (in children).[24]

In general, treatment of CD consists of 2 phases. First is the induction of remission, then is the maintenance of remission.[24] Systemic corticosteroid are very effective drugs for induction of remission with rates of clinical remission after 3 weeks up to 90%.[28] However, use of corticosteroids is limited by a high incidence of numerous serious side effects (cataracts, osteoporosis, osteonecrosis of the femoral head, myopathy, increased susceptibility to infection, arterial hypertension, diabetes mellitus, sleep disturbance, adrenal insufficiency, acne, moon face, etc).[24] Systemic steroids should be used only short-term for induction of remission. Besides, majority of patients have a clinical relapse after cessation of corticosteroids.[28] Therefore, drugs for maintenance of remission are needed. Traditionally, immunomodulators such as thiopurines and methotrexate were used for maintenance of remission in CD. A Cochrane review found an odd ratio for maintenance of remission with thiopurines of 2.32, with a number needed to treat of 6.[29] In another meta-analysis, 60% of patients on methotrexate maintained remission compared to 39% of placebo patients, with 4

patients needed to be treated to prevent a relapse.[30] However, the modern treatment goal of mucosal healing was reached in only 17% with thiopurines[31] and 11% with methotrexate.[32] First biological drugs (TNF antagonists) became available at the beginning of this century and proof themselves to be more effective than conventional drugs.[31,32] All available biological drugs are indicated for induction and maintenance of remission of CD (infliximab[31,33], adalimumab[34,35], ustekinumab[36], vedolizumab[37]). With more effective therapeutical options better control of disease activity is possible, which translates into long-term remission, mucosal healing, improved quality of life and better prognosis of patients with CD. Despite the fact that biological agents interfere with the immune system of patients and supress it, their safety profile is satisfactory.[38] With more targeted mode of action as seen with vedolizumab the safety profile is even superior.[39]

SPECIAL BACKGROUND

TREATMENT TARGETS IN CROHN'S DISEASE

Historically, the treatment goal for CD has been symptom elimination with as few side effects as possible, but it is now known that persistent subclinical inflammation may remain which is believed to lead to progressive bowel damage. This can over time lead to complications of CD, such as intestinal strictures, fistulae and abscesses.[40] These complications often translate to loss of organ function and need for surgical intervention (usually bowel resection), which leads to disability of patients with CD.[41] Up to 80% of patients require surgery at some point during the course of CD.[42] As a result, with the introduction of more effective medical therapies over the last decade there is an evolution in therapeutic endpoints from clinical symptom resolution to more strict endpoints, such as mucosal healing.[43–45] Accumulating data in the literature has demonstrated a correlation between endoscopic response and/or remission and decreased rates of clinical relapse, reduced need for hospitalisation and surgery.[45–49] With this convincing pile of evidence it is not surprising that endoscopic remission (absence of ulceration at ileo-colonoscopy) was proposed as a treatment target for regular clinical practice in the STRIDE consensus recommendations.[50] However, the presence of endoscopic healing does not necessarily correlate with an absence of histological inflammation, since up to one-third of biopsies from CD patients with endoscopically-healed mucosa may demonstrate evidence of ongoing histological disease.[51–53] With the introduction of several new medical therapies histologic remission is a reachable target. Histological remission has been achieved with several drugs, including azathioprine[54], methotrexate[55], and infliximab[51]. Additionally, more than 50 years ago Truelove et al already described histological endpoints in clinical trials of inflammatory bowel disease.[56] Thus, the essential question is whether histological remission has any additional advantages over endoscopic remission alone for favourable long-term outcomes of patient with CD (prevention of disease relapse, reduced need for hospitalisation and surgery). Is endoscopic remission sufficient or should histological remission additionally be used to define mucosal healing?[52]

HISTOLOGICAL ASSESSMENT IN CROHN'S DISEASE - BIOPSY SAMPLING

CD by definition is a segmental process that may involve any part of the gut. Consequently, as CD can be patchy in nature, with regions of intervening normal-appearing mucosa between areas of ulcerations and inflammation, it is challenging to collect endoscopic mucosal biopsies for histopathological assessment in a consistent and reproducible manner. The optimal biopsy sampling is crucial for histological assessment of disease activity in CD.[52,53] Without standardized sampling of biopsies it is not possible to determine the potential value of histological evaluation in management of CD and assessment of response to therapy in clinical trials of CD.[53,57] The attendees of the STRIDE consensus felt that to date the level of evidence was insufficient to recommend histologic remission as a treatment target in CD in every-day clinical practice.[50] According to the STRIDE consensus paper, the main obstacles for implementation of this concept (beside a lack of a validated definition of histological remission) was the risk of sampling error.[50] To date there is no clear guidance on biopsy sampling or the location for biopsy procurement in the presence of patchy CD, such as ulcers. In previous studies biopsies were procured according to various protocols with different number of biopsies at various locations. In most studies biopsies were taken from the most macroscopically inflamed areas.[57] A lack of a standardized biopsy sampling protocol was obvious also in recent clinical trials for assessment of response to therapy that included histological disease activity assessment as an trial endpoint.[58,59] In the post-hoc analysis of the EXTEND study, up to 10 biopsy specimens were collected (two from each segment). If areas of active disease were present, the samples were procured from those locations.[58] In the Fitzroy phase two trial of filgotinib for CD, 12 biopsies were procured from the least and worst affected site in six intestinal segments. In segments with endoscopically normal mucosa, biopsies were obtained at the investigator's discretion.[59] The need for standardization of biopsy sampling can be noticed in the literature.[53,57,60] It was suggested that proper sampling of patchy lesions could be improved by taking biopsies from the edge of ulcers, if present.[53,60] This strategy seems sensible, but there are no empirical evidence to support and justify this strategy.[57]

HISTOLOGICAL REMISSION IN CROHN'S DISEASE

Based on the STRIDE consensus paper, the second main reason for histological remission not being a treatment target is a lack of a validated definition of histological remission.[50] Several definitions of histological remission in CD were suggested, but the most appropriate one is yet to be determined.[50,52,61] In order to evaluate histological mucosal healing as a treatment target, a histological scoring index is required to objectively quantify the degree of histological inflammation.[57] In multiple histological scoring systems for inflammatory bowel disease definition of remission ranged from residual inflammation with persistent architectural distortion, to normalization of mucosa. However, the vast majority of scoring indices generally accepts that histological remission means at least a lack of active mucosal inflammation caused by neutrophils.[52] Histological disease activity scoring systems in IBD vary in histological features, terminology and classification of severity.[52,57] To date it is not known which histological features are most relevant for assessment of disease activity in CD.[57] The first histological index used in inflammatory bowel disease was described in 1956.[56] Several new CD-specific histological scoring systems have been described since then which can be broadly categorized as either stepwise or numerical. Stepwise indices grade inflammation into categories such as mild, moderate or severe. Whereas, numerical indices assign a point score for each histological feature evaluated which are summed to determine the final score of histological disease activity.[57] A recent Cochrane systematical review evaluated the development and operating characteristics of available numeric histological disease activity indices for CD. The main finding was that to date there are no fully validated histological scoring indices for assessment of histological disease activity in CD.[57] The most widely used histological index in clinical studies is Global Histologic Disease Activity Score (GHAS)[51,62] which was only partly validated.[57] It is a numerical instrument which allows for separate grading of both ileal and colonic biopsies. The GHAS consists of 8 histological features assessing acute and chronic inflammation, extent of inflammation and epithelial damage.[51,62] The authors of the Cochrane review concluded that the development of a validated histological scoring system for CD is a research priority.[57]

RNA EXPRESSION OF INFLAMMATORY GENES AND IMMUNOHISTOCHEMISTRY

The STRIDE consensus suggested that other histopathological and molecular methods of assessing inflammatory activity should ideally be developed and titrated against histopathology.[50] Analysing mucosal gene expression, their translation, post-translational modifications and proteomic expression has long been a mainstay of basic medical research and translational medicine. Several clinical studies have investigated the role of various histologic biomarkers detected by immunohistochemistry as an indicator of disease severity.[63,64] Similarly to histopathology, no standards to guide the biopsy sampling of the intestine for assessment of immunohistochemistry in patients with CD are known.

Measuring the expression of inflammatory genes from mucosal intestinal biopsies is commonly performed in clinical studies of patients with CD. For example, expression of some inflammatory cytokines from intestinal biopsies was shown to be increased not only in active areas of disease but also in endoscopically unaffected mucosa.[65] Inflammatory genes that were over-expressed in the setting of active CD normalize after successful treatment.[66] Gene expression profiles can predict response to treatment with infliximab in patients with CD.[67] However, there are no current standards to guide the sampling of the intestine for gene expression studies in patients with CD.[68]

3 AIM, HYPOTHESIS AND OBJECTIVES

Despite the increasing importance of histological disease activity assessment and mRNA expression in mucosal biopsies from patients with CD, it is not known where to procure mucosal biopsies. In this work, we aim to determine the location for biopsy procurement in the presence of ulcers in patients with CD which yields the highest histopathological score, the greatest mucosal mRNA expression of selected pro-inflammatory genes and the highest number of inflammatory cells as detected by immunohistochemistry. These results will allow us to answer the main scientific question; where to procure mucosal biopsies in the presence of ulcers in CD.

Our hypothesis is that there are differences in histopathological disease activity scores, mucosal gene expression of pro-inflammatory genes and number of inflammatory cells as detected by immunohistochemistry in mucosal biopsies at various distances from ulcer edge in CD.

The objectives of the study are:

- To determine the location for biopsy, procured from three different distances from ulcers in the colon and ileum in CD, which yields the highest histopathological scores using several histological disease activity indices.
- To determine the location for biopsy, procured from three different distances from ulcers in the colon and ileum in CD, which yields the greatest mucosal mRNA expression of selected genes.
- To determine the location for biopsy, procured from three different distances from ulcers in the colon and ileum in CD, with the highest number of inflammatory cells in lamina propria as detected by immunohistochemistry.

4 METHODS

STUDY DESIGN

A prospective multi-centric cross-sectional study was performed at three centers in Slovenia (University Medical Centre Ljubljana, Ljubljana), Belgium (Imelda General Hospital, Bonheiden) and the Netherlands (Academic Medical Centre, Amsterdam) from June 2016 to January 2017.

The local or national ethical committees at each of these centers approved the protocol of the study. The National Medical Ethics Committee of Republic of Slovenia assessed the study at its meeting on 17th may 2016 and decided that it is ethically acceptable and thus gave consent (KME 103/05/16). All patients gave a written informed consent before enrollment in the study.

PARTICIPANTS

We defined eligible patients as those who were 18 years of age or older (male or female); who had a histologically confirmed CD of the terminal ileum (L1), colon (L2), or ileocolon (L3) according to the Montreal classification[69] prior to the study ileo-colonoscopy; who were not on systemic anticoagulation therapy including warfarin or novel oral anticoagulants; who were able to participate fully in all aspects of the clinical trial; and most importantly who had at least a single ulcer > 0.5 cm at the study ileo-colonoscopy. We excluded all pregnant women.

PROCEDURES

Before the ileo-colonoscopy, all potentially eligible patients underwent a physical examination with measurement of vital signs, and demographical data and medical history were collected. Patients with at least a single ulcer > 0.5 cm in the colon or/and (neo-)terminal ileum at ileo-colonoscopy performed as part of routine clinical care for their CD were enrolled in the study. All study ileo-colonoscopies were video-recorded using a central image management system. Endoscopic disease activity was assessed by a single blinded central reader using the Simple endoscopic score for Crohn's disease (SES-CD)[70]. Besides eventual procurement of biopsies

as part of routine care (if needed), six biopsy specimens (3 for histopathological assessment and immunohistochemistry, and 3 for gene expression analysis) were obtained adjacent to an ulcer for the purpose of the study in the following manner: two biopsies perpendicular to the edge of the largest ulcer present in the colon and/or (neo-) terminal ileum (referred to as location 1); two biopsies at a distance of 1 open forceps diameter (7-8 mm) from the edge of the ulcer in non-ulcerated mucosa (referred to as location 2); and two biopsies at a distance of 3 open forceps diameters (21-24 mm) from the edge of the ulcer in non-ulcerated mucosa (referred to as location 3). All endoscopists performing study ileo-colonoscopies were instructed to measure the distance from an ulcer edge with a standard open biopsy forceps (7-8 mm in diameter). To standardize the procedure identical biopsy forceps were provided to all three sites (EndoJaw Alligator Jaw-step fenestrated with needle, FB220-U, Olympus). If ulcers were present in both the (neo-)terminal ileum and colon, 6 biopsies adjacent to the largest ulcer in the (neo-)terminal ileum and 6 adjacent to the largest ulcer in the colon (total of 12 biopsies) were obtained. If ulcers were present only in the (neo-) terminal ileum or colon, 6 biopsies adjacent to the largest ulcer were obtained (total of 6 biopsies). Endoscopists were instructed to avoid biopsies of ulcers at the anastomosis in the setting of post-operative recurrence of CD (possible ischemic etiology). Correct biopsy procurement (distance from the ulcer edge) was confirmed by the blinded central reader. His confirmation stood as the final assessment for eligibility of the samples in the statistical analysis.

All individual biopsies were collected into separate labelled tubes. Half of the biopsies planned for histopathology and immunohistochemistry was gently put in prefilled 10 ml 10% buffered formalin tubes, the residual biopsies planned for mRNA gene expression analysis were immersed in a 2 ml Eppendorf tubes prefilled with RNA stabilization solution (RNAlater®). Biopsies from all sites were shipped at room temperature to the central laboratory (Tytgat Institute for Liver and Intestinal Research, Amsterdam UMC, The Netherlands) within 48 hours of collection.

HISTOPATHOLOGICAL ASSESSMENT

After routine processing of biopsies at the central laboratory (Tytgat Institute for Liver and Intestinal Research, Amsterdam UMC, The Netherlands), biopsies were sectioned at 4.5 μm, stained with hematoxylin and eosin, and digitised. All steps involved in creating a digital histopathology image were routinely assessed for quality control to ensure that high quality images were provided for central reading. A single blinded expert gastrointestinal pathologist trained in the scoring of histological indices reviewed each of the digitalized histopathological specimens and assigned numerical scores to quantify the degree of histological activity in each specimen. Of the current histopathological indices developed to evaluate severity of disease activity the Global Histological Disease Activity Score (GHAS)[62], Robarts Histopathology Index (RHI)[71] and Nancy Histological Index (NHI)[72] were used. GHAS was used because it is among the most comprehensive and widely used scores in patients with CD.[53] The GHAS is a numerical histological disease activity score that assesses eight items.[51] In our study a modification of the H subscore (number of biopsy specimens affected) of the GHAS was performed to use percentage of affected surface in one biopsy rather than number of biopsies affected. The reason for that was that as only 1 biopsy per location was available for assessment as opposed to multiple biopsies in the original study. The RHI is a modification of the Geboes Score and assesses four histological features. Scores range from zero (no disease activity) to 33 (severe disease activity). RHI was shown to be reproducible, responsive and valid in UC.[71] NHI is a simple stepwise scoring system that assesses three items, and ranges from a grade of zero (absence of histological disease activity) to a grade of four (severely active disease). NHI was shown to be valid with good reliability and responsiveness in UC patients.[72] However, neither RHI nor NHI have been validated in CD patients.

MUCOSAL GENE EXPRESSION

Biopsy samples for mRNA analysis from the RNA stabilisation solution (RNAlater®) were homogenised using TissueLyser LT (Qiagen). RNA was extracted from the biopsy specimens using the Bioline Isolate II RNA Mini Kit (GC Biotech), according to the manufacturer's instructions. Total RNA concentration was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). First strand complementary DNA was synthesised from mRNA using RevertAid reverse transcriptase (Fermentas, Thermo Fisher Scientific), according to the

manufacturer's instructions. The expression of five genes previously shown to be significantly up-regulated in colonic and/or ileal mucosa of CD patients in the setting of acute inflammation and known to be implicated in the pathogenesis of CD was analysed:

- **IL–6**, a pro-inflammatory cytokine involved in the pathogenesis of CD,[68,73]
- **IL–8**, a potent pro-inflammatory chemokine, which both attracts and activates granulocytes,[66,68,74]
- **IL–23 (p19 + p40 subunits)**, a pro-inflammatory cytokine secreted by innate immune cells that activates Th17-type T cells and has pathogenic relevance for CD,[73,75,76]
- Platelet endothelial cell adhesion molecule (**PECAM-1 or CD31)**, which mediates transendothelial migration and recruitment of inflammatory cells,[66,77]
- **S100A9***,* antimicrobial peptides which form calprotectin, a fecal biomarker in inflammatory bowel disease.[68,78]

Expression levels of mRNA for the genes of interest as well as reference genes (β-actin and cyclophillin) were determined in duplicate by quantitative real-time reverse transcriptase PCR using SensiFAST SYBR® No-ROX Kit (GC-Biotech) according to the manufacturer's instructions on a LightCycler instrument (Roche) and gene specific primers (See **Table 1** for primers used). Relative mRNA expression levels for the genes of interest were calculated as a ratio relative to the geometric mean of the endogenous reference genes (β-actin and cyclophillin).

Table 1. Primers used for qPCR analysis.

Marker	**Sense**	**Antisense**
β-actin	AATGTGGCCGAGGACTTTGA	TGGCTTTTAGGATGGCAAGG
Cyclophyllin	ACGGCGAGCCCTTGG	TTTCTGCTGTCTTTGGGACCT
IL-6	AGTGAGGAACAAGCCAGAGC	GTCAGGGGTGGTTATTGCAT
IL-8	AAATTTGGGGTGGAAAGGTT	TCCTGATTTCTGCAGCTCTGT
IL-23 p19	CAGGGACAACAGTCAGTTCTGC	CCTCAGGCTGCAGGAGTTG
IL-12/23 p40	Quantitect primer assays (Qiagen)	
S100A9	Quantitect primer assays (Qiagen)	
PECAM-1	Quantitect primer assays (Qiagen)	

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Immunohistochemical staining was used to detect the presence of inflammatory cells in the biopsy specimens which are increased in the colonic and ileal mucosa during active inflammation in CD. Specific monoclonal antibodies directed against **CD68,**[60,64,65] a marker of monocytes/macrophages, **CD3**, a marker of T cells, and **MPO (myeloperoxidase)**, a marker of neutrophils, but also present in monocytes/macrophages, were used. Immunohistochemical staining of slides containing 4.5 μm biopsy sections with antibodies against CD68 (Dako/Agilent, M0876 mouse monoclonal αCD68 clone RPM1), CD3 (Thermo Scientific, RM-9107, rabbit monoclonal αCD3e clone SP7) and MPO (Dako/Agilent, A0398 rabbit polyclonal αMPO), was performed on a Roche Ventana Benchmark Ultra IHC autostainer (Roche Diagnostics Nederland BV) using a Roche Optiview DAB detection kit (Roche Diagnostics Nederland BV) as follows: (1) antigen retrieval for 24 minutes (CD68 and MPO) or 48 minutes (CD3) at 100° C in Roche CC1 buffer (pH 8.5), followed by (2) incubation with αCD3, αCD68, or αMPO at dilutions of 1:100, 1:200 and 1:4000, respectively for 32 minutes at room temperature. Counterstaining of sections with hematoxylin was performed automatically on the Benchmark Ultra stainer.

Stained slides were subsequently scanned on a Philips Intellisite Ultrafast scanner (Philips) at 40x magnification. Whole slide images in Philips TIFF format were converted for processing into standard TIFF images using the GDAL/OGR Geospatial Data Abstraction software Library, version 2.3.2 (Open Source Geospatial Foundation), and loaded into Definiens Tissue Studio 4.3.1 (Definiens AG) for brightfield analysis (0.25 μm per pixel, 40x magnification). Images were preprocessed to identify relevant regions of interest on each slide and manual correction was performed to exclude various artifacts, tissue folds, regions that were out of focus or exhibited poor scanning quality, completely unstained large tissue fragments, and regions with stained epithelial cells. Colour deconvolution was used to isolate the hematoxylin and DAB stain channels, and nuclei were segmented in the tissue region of interest using a hematoxylin stain intensity threshold of 0.05 and an average nuclear size of 30 μm^2. Cell simulation was performed by growing the nuclear regions. Positive cells were detected using DAB intensity thresholds in the cell, where intensities less than 0.3, between 0.3 and 0.4, and greater than 0.4 were denoted as negative, “low,” and “high” for the marker of interest. Thresholds were chosen to classify the cellular areas, set by visual inspection of multiple tissue sections with positive and negative cells present. Three thresholds

corresponding to more permissive (low stain intensity) to more restrictive (high stain intensity) were chosen.

Total number of marker positive cells per mm^2 of analysed tissue area was calculated. Positive cells included those classified as both "low" and "high." Analysed tissue area was determined by adding areas of the tissue and stained epithelium regions of interest.

STATISTICAL ANALYSIS

Since multiple biopsies were taken from each patient (possible clustering effect), the mixed effects model approach was chosen to adjust for within subject correlations among location (1, 2 and 3) and segments (colon and ileum).[79] This mixed effects model approach was adopted to analyze each of the histopathological indices (GHAS, NHI and RHI), mRNA expression levels of the selected genes, and number of positive inflammatory cells in the lamina propria. mRNA expression levels and numbers of positive inflammatory cells were analyzed on the log scale to account for non-normality of the data. In this model biopsy location and segment were treated as a fixed effect and patients were regarded as random effects.
The least square contrasts were used to compare locations in terms of histological scorings, mRNA expression levels for the genes of interest, and numbers of inflammatory cells. Statistical significance was defined with a two-sided p value of 0.05. The corresponding 95% confidence intervals were used to present results.

Due to the lack of relevant data in the literature, the sample size was calculated using Cohen's standardized effect size (mean difference divided by standard deviation). Assuming a comparison of scores between any two locations within a patient with a paired t-test, a sample of 50 subjects (pairs) will be sufficient to detect an effect size 0.4 with 80% power at the 5% significant level, which was from a clinical perspective an important effect size for each objective to make a biopsy location recommendation. This sample size was conservative as the mixed effects model approach is likely to be more powerful than paired t-test.

5 RESULTS

BASELINE CHARACTERISTICS

In all three centres a total of 78 patients with CD were screened. The eligibility criteria were met by 51 who were enrolled in the study. The reason for all 27 screen failures was a lack of at least a single ulcer >0.5 cm in diameter detected on ileo-colonoscopy.

The mean age of enrolled patients was slightly above 42 years with a mean disease duration of 13 years. Most enrolled patients had ileocolonic disease. The mean SES-CD score of the study patients was 11 (standard deviation [SD] = 7.5). Biopsies were procured from the ileum in 26, the colon in 18, and from both segments in 7 patients.

More details on baseline patient and disease characteristics are summarised in **Table 2**.

Table 2. Baseline patient and disease characteristics

Characteristic	
Female gender, n (%)	25 (49)
Mean age, years (SD)	42.2 (16.7)
Caucasian, n (%) *	49 (96.1)
Mean disease duration, years (SD)	13.1 (10.8)
Disease location, n (%)	
L1	16 (31.4)
L2	8 (15.7)
L3	22 (43.1)
L3 + L4	5 (9.8)
Location from which biopsies were taken, n (%)	
Ileum	26 (51.0)
Colon	18 (35.3)
Both	7 (13.7)
Current medications for CD, n (%)	
Vedolizumab	7 (13.7)
TNF antagonists	13 (25.5)
5-ASA	4 (7.8)
Corticosteroids and budesonide	8 (15.7)
Immunosuppressives (thiopurines, methotrexate)	16 (31.4)
Ustekinumab	1 (2.0)
Patients with previous surgeries for CD, n (%)	
Abdominal	18 (35.3)
Perianal	8 (15.7)
Mean SES-CD score (SD)	11.0 (7.5)

*Data missing for 2 patients.
Disease location: L1 ileal; L2 colonic; L3 ileocolonic; L4 upper GI tract.

HISTOPATHOLOGICAL DISEASE ACTIVITY SCORES

All histological disease activity scores were highest at the ulcer edge and significant decreasing trends with distance from the ulcer edge were observed in all histological disease activity scores ($p<0.05$). Same decreasing trends were found in both the ileum and the colon ($p<0.05$).

For all histological disease activity scores comparisons significant differences in both the ileum and the colon were observed between the ulcer edge and locations 2 and 3 ($p<0.0001$). Significant differences in histological disease activity scores were also observed between locations 2 and 3 in the ileum ($p<0.05$). No statistically significant difference between locations 2 and 3 in the colon was found, but there was a trend for decreased histological disease activity scores in location 3 relative to location 2.

Mean histological disease activity scores of all three histological scoring indices used (GHAS, RHI and NHI) according to biopsy location and segment (ileum, colon) are shown in **Table 3.**

Table 3. Mean histological disease activity scores according to biopsy location and segment.

Segment	Colon			Ileum		
Location	1	2	3	1	2	3
GHAS	10.1 (8.5, 11.7)[1,2]	5.2 (3.4, 6.9)	4.2 (2.5, 5.8)	8.3 (6.9, 9.6)[1,2]	5.1 (3.6, 6.6)[3]	3.1 (1.6, 4.6)
RHI	19.5 (14.7, 24.4)[1,2]	5.8 (2.6, 9.0)	4.8 (2.3, 7.4)	14.2 (10, 18.3)[1,2]	6.6 (3.7, 9.5)[3]	3.4 (1.1, 5.7)
NHI	3.3 (2.7, 3.8)[1,2]	1.2 (0.7, 1.8)	0.9 (0.4, 1.4)	2.7 (2.1, 3.2)[1,2]	1.3 (0.8, 1.8)[3]	0.7 (0.3, 1.2)

Location: 1, edge of largest ulcer; 2, 7-8 mm from ulcer edge; 3, 21-24 mm from ulcer edge.
Within segment pairwise comparison with location 2, [1]$p<0.0001$; or location 3, [2]$p<0.0001$, [3]$p<0.05$.
Values in parentheses represent 95% confidence interval (CI).

mRNA EXPRESSION OF MUCOSAL INFLAMMATORY GENES

Similar to histological disease activity scores, highest mRNA expression of pro-inflammatory genes in mucosal biopsies was observed at the ulcer edge with decreasing trends with distance from the ulcer edge ($p<0.05$). Same significant decreasing trends were found in both the ileum and the colon ($p<0.05$).

For all mRNA expression levels comparisons significant differences in both the ileum and the colon were observed between the ulcer edge and locations 2 and 3 ($p<0.005$).

Log-transformed mRNA expression levels of genes of interest (IL-6, -8, -23 [p19 and p40 subunits], CD31 and S100A9) according to biopsy location and segment are shown in **Table 4.** Relative mRNA expression levels for all genes of interest were calculated as a ratio relative to the geometric mean of the endogenous reference genes (β-actin and cyclophillin).

Table 4. Log-transformed mRNA expression levels for genes of interest according to biopsy location and segment.

Segment	Colon			Ileum		
Location	1	2	3	1	2	3
IL-6	-4.3 (-4.9, -3.6)[1,3]	-7.1 (-7.7, -6.5)	-7.2 (-7.6, -6.7)	-4.9 (-5.5, -4.4)[1,3]	-7.3 (-7.8, -6.8)	-7.6 (-8.0, -7.2)
IL-8	-2.6 (-3.2, -2.0)[1,3]	-5.3 (-5.8, -4.7)[4]	-5.9 (-6.4, -5.3)	-2.7 (-3.2, -2.1)[1,3]	-5.2 (-5.7, -4.7)	-5.6 (-6.1, -5.1)
IL-23 (p19)	-5.7 (-6.1, -5.3)[1,3]	-6.6 (-6.9, -6.3)	-6.7 (-7.0, -6.4)	-5.7 (-6.0, -5.3)[1,3]	-6.8 (-7.0, -6.5)	-6.7 (-7.0, -6.5)
IL-23 (p40)	-8.7 (-9.2, -8.2)[1,4]	-9.8 (-10.2, -9.4)	-9.9 (-10.3, -9.5)	-8.6 (-9.0, -8.2)[1,4]	-9.7 (-10.1, -9.3)	-9.5 (-9.9, -9.1)
CD31	-2.1 (-2.3, -1.8)[1,3]	-2.7 (-2.9, -2.5)	-2.7 (-3.0, -2.5)	-2.2 (-2.4, -1.9)[2,3]	-2.6 (-2.8, -2.4)[4]	-2.9 (-3.1, -2.7)
S100A9	-1.4 (-1.8, -0.9)[1,3]	-3.2 (-3.6, -2.8)	-3.4 (-3.8, -3.0)	-1.6 (-2.0, -1.2)[1,3]	-3.0 (-3.3, -2.7)[4]	-3.3 (-3.6, -3.0)

Within segment pairwise comparison with location 2, [1]$p<0.0001$, [2]$p<0.001$; or location 3, [3]$p<0.0001$, [4]$p<0.005$.
Values in parentheses represent 95% CI.

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Number of MPO-positive cells in both ileum and colon was highest at the ulcer edge and differed significantly compared to locations 2 and 3 ($p<0.0001$). A decreasing trend was observed in the number of these cells with distance from the ulcer edge in both the colon and ileum ($p<0.05$).

However, these trends were not observed for CD3+ or CD68+ cells. No differences in the number of CD3+ or CD68+ cells were found according to biopsy location next the ulcer except of significant differences for the number of CD68+ cells at the ulcer edge and location 3 as well as between locations 2 and 3 in the ileum ($p<0.05$).

Log-transformed numbers of CD3+, CD68+ and MPO-positive cells per mm^2 of tissue area according to biopsy location and segment are shown in **Table 5**.

Table 5. Log-transformed number of inflammatory cells according to biopsy location and segment.

Segment	Colon			Ileum		
Location	1	2	3	1	2	3
CD3+	7.3 (7.0, 7.7)	7.3 (7.0, 7.6)	7.1 (6.7, 7.5)	7.3 (7.0, 7.6)	7.3 (7.0, 7.6)	7.3 (7.0, 7.6)
CD68+	6 (5.6, 6.4)	5.9 (5.4, 6.4)	5.9 (5.4, 6.4)	6.1 (5.8, 6.5)[3]	6.1 (5.7, 6.5)[3]	5.5 (5.1, 5.9)
MPO+	6.9 (6.3,7.5)[1,2]	4.5 (3.7, 5.2)	3.9 (3.2, 4.6)	6.6 (6.1, 7.2)[1,2]	4.7 (4.0, 5.3)	4.1 (3.5, 4.6)

Within segment pairwise comparison with location 2, [1]$p<0.0001$; or location 3, [2]$p<0.0001$, [3]$p<0.05$.
Values in parentheses represent 95% CI.

6 DISCUSSION

The optimal location for biopsy procurement for assessment of histological disease activity in inflammatory bowel disease has to date not been defined despite the increasing importance of histology in clinical trials in recent years. This fact is surprising since the first study to use a histological disease activity index in an inflammatory bowel disease trial was published more than 60 years ago.[56] We believe this is the first study in patients with CD to assess the relationship between biopsy location according to ulcerations and histological disease activity, mucosal mRNA expression of proinflammatory genes, and number of inflammatory cells which are implicated in the pathogenesis of CD.

We confirmed our hypothesis that differences in histopathological disease activity scores, mucosal gene expression of proinflammatory genes and number of MPO-positive cells in mucosal biopsies at various distances from ulcer edge in CD exist. These findings were not consistently observed for CD3+ or CD68+ cells as detected by immunohistochemistry. According to our objectives, the most important finding was that biopsies collected from the ulcer edge in both the colon and ileum yield the highest histological disease activity scores (irrespective of the histological scoring index used), mRNA expression levels of proinflammatory genes, and number of MPO-positive cells in the lamina propria. The existence of a gradient of inflammation from the ulcer edge to the most distant location evaluated in our study (21-24 mm which corresponds to 3 open forceps diameters) was observed. These main findings indicate that when assessing maximal disease activity in patients with CD with pathohistological and molecular methods biopsies should be procured from the edge of the ulcer. If this is not possible due to various reasons (stenosis, looping of the endoscope etc.), biopsies should be procured as close to the ulcer edge as possible to minimise sampling error.

In the last decade several new agents with different mechanisms of action have been introduced for treatment of inflammatory bowel disease,[36,37,80] with many more in the pipeline.[27] Besides, new treatment algorithms have been implicated in the clinical management of patients with CD in the past years.[47,81,82] These improvements have allowed consideration of more rigorous endpoints beyond endoscopic mucosal healing, such as histological mucosal healing or histological remission.[52] The premise for histological remission in CD is that persistent histological inflammation may confer an increased risk of clinical symptoms, clinical relapse, hospitalisation, resection and neoplasia. However, to date

there is insufficient evidence to consider histological remission as a potential treatment target for CD in everyday clinical practice.[50] Besides, there are several challenges to be addressed before histological remission could be considered a treatment target, including a lack of validated histological disease activity indices for CD, no determined definition of histological remission in CD and the risk of sampling error when procuring mucosal biopsies.[50]
In order to evaluate the benefit of histologic remission as a potential treatment target in CD, a validated histological scoring index is required to objectively quantify the degree of histologic inflammation.[57] However, limited data exist regarding which histologic features are most relevant for assessing active inflammation in CD.[53]

Before we have designed our study, we assessed operating properties of currently existing histological scoring indices for evaluation of disease activity in CD to identify the most suitable index for our study. For that reason, we performed a Cochrane systemic review to evaluate the development and operating characteristics of available histologic disease activity indices in CD.[57] Electronic searches of MEDLINE, EMBASE, PubMed, and the Cochrane Library databases were performed from inception to July 2016 and supplemented by manual reviews of bibliographies and abstracts submitted to major gastroenterology meetings (Digestive Disease Week, United European Gastroenterology Week, European Crohn's and Colitis Organisation). Any study design that evaluated a histologic disease activity score in adult (> 16 years) patients with CD was considered for inclusion in the systemic review. All titles and abstracts of the studies identified from the search were independently reviewed by two authors. A full text of eventually relevant studies was reviewed for inclusion. The investigators of the original study were contacted as needed for any clarification. From each eligible study we extracted and recorded data on the number of patients enrolled in the study, patient characteristics (age, gender, etc.), description of histologic disease activity index utilized; and outcomes such as content, construct and criterion validity (the extent to which an instrument truly measures the outcome that it is intended to assess), responsiveness (the ability to detect a meaningful change in health status), intra-rater and inter-rater reliability (the consistency or reproducibility of an instrument), and feasibility (the ease with which an instrument can be utilized in a given setting).[57] Histologic scoring indices can be broadly categorized as either stepwise or numerical. Stepwise instruments separate disease activity into few categories (for example: mild, moderate, and severe). On the other hand, numerical instruments assign a point scale to biopsy findings which can then be summed to determine an total score.[53] In our Cochrane systemic review stepwise histological scoring indices were

excluded. We believe that stepwise scoring indices are easy to use, but are likely to be less responsive to clinically meaningful changes in disease activity.[57]

The literature search identified 3520 publications and 4 additional publications were identified through other sources. After duplicates removal 2573 records were screened for inclusion. Of these, 102 reports were selected for full text review. Eighty-six reports were excluded. In total we identified 16 reports of 14 studies describing 14 different numerical histological indices which fulfilled the inclusion criteria.[57]

Only one study specifically aimed to develop and validate a histological index. However, this Naini and Corina score is not specific to assess disease activity in CD, but rather to diagnose inflammatory bowel disease. None of the remaining histological disease activity indices identified in the systemic review (including GHAS, the most widely used index in clinical trials and studies) has been formally fully-validated. Besides, most validation studies were of poor methodological quality. The conclusion of our systemic review was that there are to date no available fully-validated histological scoring indices to evaluate disease activity in CD and that validation of existing indices or development of a new one according to currently accepted standards is a research priority.[57]

Among existing histological disease activity indices for CD, the partially validated and widely used GHAS[51,60,62] was chosen in our study to assess histological inflammation because it is among the most comprehensive. However, a modification to assess percentage of affected surface in one biopsy rather than number of biopsies affected was applied. The reason for that was that as only 1 biopsy per location was available for assessment of disease activity as opposed to multiple biopsies in the original study by D'Haens. Despite this important modification of the GHAS, we believe it has a minor impact on the final score. Besides, we decided to use two additional histological scoring indices which were recently developed and validated for assessment of disease activity in UC (the RHI[71] and NHI[72]). It is important to stress out that neither index has been validated for assessment of disease activity in patients with CD. However, both CD and UC belong to inflammatory bowel disease and disease activity in both is defined by the degree of neutrophilic inflammation and surface injury (ulcers and erosions). Further studies are needed to assess operating characteristics of these two indices in the setting of CD. Our study may provide a preliminary basis for this research.[83]

Another important challenge in assessment of histological disease activity is the risk of

sampling error when procuring mucosal biopsies. Up to now, there is no guidance on biopsy sampling or the location for biopsy collection in presence of ulcers. The Cochrane systemic review we have performed has revealed that biopsies were procured according to various protocols with different numbers of biopsies at various locations. In majority of cases, biopsies were procured from macroscopically most involved areas,[57] but evidence to support this strategy is lacking. Our study clearly shows that biopsies from the edge of an ulcer in both the colon or ileum yield significantly higher histological disease activity scores than biopsies from non-ulcerated mucosa ($p<0.0001$) suggesting that the inflammatory activity in CD is highest at the ulcer edge. These findings could be anticipated to some degree since erosions/ulcerations are items included in all three histological indices used in our study and as such contribute considerably to the final index score. Although our study was not intended to determine appropriate biopsy location in non-ulcerated mucosa, we observed significantly higher histological disease activity scores in biopsies taken from non-ulcerated mucosa closer to ulcers (location 2) than in biopsies further away (location 3) in the ileum and a similar non-significant trend was observed in the colon. However, additional studies are needed to determine the most appropriate biopsy location in non-ulcerated mucosa. Findings of our study may become the core stone to develop standardised and optimised biopsy sampling protocols in CD while taking into account the anatomic patchiness to reduce sampling error.

In the immunohistochemistry part of our study, we confirmed the highest number of MPO-positive cells in biopsies from the ulcer edge in both the colon and ileum and a gradient of MPO-positive cells from the ulcer edge to the most distant location evaluated. MPO-positive cells mostly represent neutrophils[84,85] which are associated with active inflammation in inflammatory bowel disease[72] and are included as items of numerical histological scoring indices.[53,57] However, the same was not true for CD3+ (T lymphocytes)[86] or CD68+ (macrophages/monocytes)[87] cells which are also important in the pathogenesis of CD. These cells were not more prevalent at the edge of an ulcer. Interestingly, no consistent differences between various biopsy locations were observed. The reason for these results is not known and deserves further research.

Highest mRNA expression in mucosal biopsies of selected proinflammatory genes was observed at the ulcer edge ($p<0.005$) with decreasing trends with distance from the ulcer edge ($p<0.05$) in both the ileum and the colon. Indeed, assessment of mRNA expression of selected genes may become an important objective tool/method for management of patient with CD in everyday clinical practice and clinical trials. In the STRIDE consensus, authors suggested

that molecular methods of assessing histological remission should be developed and titrated against histopathology.[50] It was proven that measuring pro-inflammatory gene expression from mucosal biopsies during colonoscopy is a feasible and reproducible method to measure site-specific inflammation in CD.[68] No more than 2 to 5 biopsies are needed to reduce sampling error to <25% for most genes. Besides, expression of some pro-inflammatory genes correlates with endoscopic disease activity (SES-CD).[68] Over-expressed pro-inflammatory genes in the setting of active CD may be down-regulated or even normalised after successful medical treatment with infliximab[66,88] and adalimumab[75]. Additionally, normalisation of pro-inflammatory gene expression was shown to predict long-term remission after discontinuation of successful treatment with TNF antagonists.[76] Even before treatment is initiated, gene expression profiles can predict response to infliximab in patients with CD.[67]
Interestingly, it was suggested that in patients with Crohn's colitis and rectal inflammation analysis of gene expression in biopsies sampled from the distal colon (rectum and descending colon) could be used as a proxy for more proximal disease activity, which could potentially lead to avoiding complete colonoscopy.[68] These results are consistent with our study with no significant differences in expression levels of pro-inflammatory genes in colon and ileum (data not shown).

There are several strengths and limitations to our study. To best of our knowledge, this is the first study in patients with CD to assess the relationship between biopsy location according to ulcerations and outcomes of interest. Our findings are important evidence for standardisation of biopsy procurement in CD, especially in presence of ulcerations and may become a foundation for development of standardised biopsy sampling protocols in CD with the intent to reduce sampling errors. Two relatively new histological scoring indices (RHI and NHI) were tried out for assessment of disease activity in CD for the first time. Their use supports further validation and assessment of their operating characteristics in this disease. The very robust prospective study design supports the high reliability of our results. The multi-centric character of our study increases the generalisability of the findings. Importantly, all study colonoscopies were video-recorded and a blinded central reader confirmed the correct biopsy procurement next to the ulcer before biopsies were included in the final statistical analyses.[83]

The fist limitation of our study is that concomitant medications were not accounted for in the statistical analysis. The results for mRNA expression of certain genes could have been influenced by drug-specific mechanisms of action. For example, mRNA expression of IL-23 could have been depressed by treatment with ustekinumab[36], an IL-12/23 antagonist.

However, endoscopically active CD with at least one ulcer >0.5 cm was confirmed in all included patients by a blinded central reader irrespective of their treatment. Secondly, data from patients with post-operative recurrence of CD in the neo-terminal ileum were pooled with data from patients with ileal CD without previous surgery. These two settings of ileal CD might have different etiopathogenesis and consequently different readouts. In our study, we evaluated 33 ileal biopsy samples in total, of which 13 were from the neo-terminal ileum. In a post hoc analysis no differences were observed for any of the outcomes in biopsies collected from the ileum or neo-terminal ileum (data not shown). Thirdly, data for ileal and colonic biopsies were pooled from patients with different disease location phenotype according to Montreal classification. To be more precise, ileal biopsies from patients with ileal (L1) and ileocolonic (L3) disease and colonic biopsies from patients with colonic (L2) and ileocolonic (L3) disease were pooled and analysed together. However, a post hoc analysis did not find any statistical difference in any of the study outcomes between ileal or colonic biopsies collected in ileocolonic disease (L3) versus purely ileal (L1) or purely colonic (L2) disease (data not shown). Finally, ileocolonoscopy with biopsy collection is an invasive procedure which can cause complications such as bleeding and perforation. In our study, two to four biopsies from the edge of ulcers in active CD were safe and did not cause any complications. However, the safety of taking multiple (more than 2 to 4) biopsies from the edge of ulcers in patients with active CD was not studies and may add to the risk of the procedural complications.

7 CONCLUSION

Based on the results of our study we can conclude that biopsy procurement from the edge of an ulcer in CD consistently yielded the highest histological disease activity scores, mRNA expression levels of selected proinflammatory genes and numbers of neutrophils. These results will contribute to standardisation of procurement of biopsy samples for pathohistological and molecular methods in CD and reduce sampling error. These finding could be used in clinical trials and everyday clinical practice. Additionally, our findings add important evidence for development of standardised biopsy sampling protocols in CD.

8 OBSEŽEN POVZETEK V SLOVENSKEM JEZIKU

IZHODIŠČA

Crohnova bolezen (CB) je napredujoča bolezen, za katero je značilno kronično vnetje prebavnega trakta. V preteklosti je bil cilj zdravljenja CB odpravljanje simptomov s čim manj stranskimi učinki. Danes je znano, da lahko sub-klinično vnetje vztraja in poveča tveganje za razvoj zapletov, kot so stenoze, fistule in ognojki, ki zahtevajo operativno zdravljenje. Posledično je prišlo do premika cilja zdravljenja pri CB od odprave kliničnih simptomov do zazdravljenja sluznice (angl. mucosal healing). Endoskopsko zazdravljenje sluznice, ki danes velja za cilj zdravljenja CB, ni nujno povezano z odsotnostjo histološkega vnetja. Do tretjina biopsij črevesne sluznice pri bolnikih s CB z endoskopsko zazdravljeno sluznico kaže znake vztrajajoče histološke aktivnosti. Ključno vprašanje je torej, ali je endoskopska zazdravljenje sluznice dovolj za preprečitev ponovitev bolezni in prihodnjih zapletov, ali je treba vključiti tudi histološko remisijo.

CB je po definiciji segmentni proces, ki lahko neenakomerno prizadene črevo, kjer se izmenjujejo področja sluznice normalnega izgleda s področji vnetja, zato je dosleden odvzem biopsij za oceno histološke vnetne aktivnosti težaven. Ni jasnih smernic glede mesta odvzema biopsij za oceno histološke vnetne aktivnosti pri CB. V preteklih raziskavah natančno mesto ni bilo nikoli določeno; biopsije so običajno bile odvzete iz roba razjede, v bližini razjedenih območij ali naključno. Potrebo po standardizaciji odvzema biopsij je bila v literaturi večkrat izpostavljena.

Različni histološki markerji, ki jih določamo s pomočjo imunohistokemičnih metod, so se izkazali kot kriterij vnetne aktivnosti pri CB. Ni znanih standardov za odvzem/vzorčenje biopsij črevesne sluznice za imunohistokemično analizo pri bolnikih s CB.

Meritve izraženosti vnetnih genov v biopsijah črevesne sluznice se pogosto uporabljajo v raziskovalne namene pri bolnikih s CB. Dokazano je bilo, da je izražanje vnetnih citokinov v biopsijah črevesne sluznice povečano tako v področjih aktivnega vnetja kot tudi v endoskopsko normalni sluznici. Vnetni geni, ki so bili prekomerno izraženi pri aktivni CB, so se normalizirali po uspešnem zdravljenju. Profili izražanja genov v biopsiji črevesne sluznice lahko napovedo odziv na zdravljenje pri bolnikih s CB. Mesto odvzema biopsij za določanje genskega izražanja pri bolnikih s CB ni znano.

NAMEN, HIPOTEZA IN CILJI

Čeprav postaja pomen določanja histološke aktivnosti bolezni in izražanja mRNK v biopsijah črevesne sluznice pri bolnikih s CB vse večji, zaenkrat ni znano, kje je najprimerneje odvzeti biopsije. Namen naše raziskave je določiti mesto za odvzem biopsij v prisotnosti razjed pri bolnikih s CB, ki daje najvišje točkovanje histološke vnetne aktivnosti, največjo sluznično izražanje mRNK izbranih genov in največje število vnetnih celic, dokazanih imunohistokemično. Ti rezultati nam bodo omogočili odgovoriti na glavno znanstveno vprašanje; kje odvzeti biopsije črevesne sluznice pri bolnikih s CB v prisotnosti razjed.

Naša hipoteza je, da obstajajo razlike v histopatoloških točkovanjih, izražanju genov in številu vnetnih celic v biopsijah črevesne sluznice na različnih razdaljah od razjed pri bolnikih s CB. Cilji študije so določitev mesta odvzema biopsij pri CB, pridobljenih s treh različnih razdalj od razjed v debelem in tankem črevesu, ki daje:

- najvišjo histopatološko točkovanje z uporabo več točkovnikov histološke aktivnosti bolezni,
- največje izražanje mRNK izbranih genov,
- največje število vnetnih celic, dokazanih imunohistokemično.

METODE

ZASNOVA ŠTUDIJE IN POPULACIJA

Izvedli smo prospektivno multi-centrično študijo v treh centrih v Sloveniji, Belgiji in na Nizozemskem, ki je vključila 51 bolnikov s CB. Odbori za raziskovalno dejavnost/lokalne etične komisije v vsakem od centrov so odobrili preiskavo. Vsi bolniki so pred vključitvijo v študijo dali pisno soglasje za sodelovanje v študiji. Primerne bolnike smo definirali kot osebe: ki so stare 18 let ali več (moški ali ženske); ki so že pred študijsko ileokolonoskopijo imeli histološko potrjeno diagnozo CB terminalnega ileuma (L1), kolona (L2) ali ileokolona (L3); ki niso na sistemski antikoagulacijski terapiji, vključno z varfarinom ali novimi peroralni antikoagulanti; ki so v celoti zmožni sodelovati v vseh vidikih klinične študije; in najpomembneje, ki imajo pri študijski ileokolonoskopiji najmanj eno razjedo večjo od 0,5 cm. Nosečnice smo izključili.

Pred preiskavo smo vključene bolnike klinično pregledali, izmerili vitalne funkcije, zbrali demografske podatke in naredili natančno anamnezo.

POSTOPKI

V študijo so vključeni bolniki z vsaj eno razjedo > 0,5 cm v debelem črevesu in/ali (neo-) terminalnem ileumu pri ileokolonoskopiji, opravljeni kot del rutinske obravnave za CB. Poleg morebitnih odvzemov biopsij kot dela rutinske obravnave smo za namene študije odvzeli šest biopsij zraven razjede (3 za histopatološko in imunohistokemično analizo, 3 za analizo genskega izražanja): dve biopsiji pravokotni na rob največje razjede v debelem črevesu in/ali (neo-) terminalnem ileumu, dve biopsiji na razdalji enih odprtih biopsijskih kleščic od roba razjeda (7-8 mm) v sluznici brez razjed in dve biopsiji na razdalji treh odprtih biopsijskih kleščic od roba razjede (21-24 mm) v sluznici brez razjed. Če so bile razjede prisotne tako v (neo-)terminalnem ileumu kot v debelem črevesu, smo odvzeli 6 biopsij v bližini največje razjede (neo-)terminalnega ileuma in 6 v bližini največje razjede v debelem črevesu (skupaj 12 biopsij). Če so bile razjede prisotne bodisi v (neo-) terminalnem ileumu bodisi v debelem črevesu, smo odvzeli 6 biopsij v bližini največje razjede (skupaj 6 biopsij). Vsem študijskim centrom smo priskrbeli biopsijske kleščice, da smo standardizirali postopek odvzema biopsij. Vse biopsije so bile ločeno zbrane v označene posodice. Polovica biopsij (za histopatologijo in imunohistokemijo) je bila vstavljena v prednapolnjene 10 ml posodice z 10% pufriranim formalinom, preostale biopsije (za analizo mRNK) so bile takoj potopljene v prednapolnjen posodice (10 ml) z RNA stabilizacijsko raztopino (RNAlater®). Biopsije iz vseh študijskih centrov so bile na sobni temperaturi odposlane do osrednjega laboratorija še istega dne.
Napravili smo video-posnetke vseh študijskih ileo-kolonoskopij. Pregled ileo-kolonoskopij s strani centralnega ocenjevalca (ang. blinded central reader), ki ni imel nobenih informacij o bolnikih, je vključeval oceno endoskopske aktivnosti bolezni z uporabo točkovnika SES-CD (Simple Endoscopic Score for Crohn's Disease) in potrditev pravilnega odvzema biopsij (prava razdalja od razjede). Potrditev pravilnega odvzema biopsij s strani centralnega ocenjevalca je predstavljala pogoj za vključitev bolnika v statistično analizo.

HISTOPATOLOŠKA OCENA

Vsak biopsijski vzorec je bil zbran v ločeni posodici. Po rutinskem procesiranju in barvanju vzorcev s H&E v centralnem laboratoriju (Inštitut Tytgat, Amsterdam, Nizozemska) smo diapozitive digitalizirali. Patolog, ki ni imel nobenih podatkov o bolnikih in endoskopiji, je pregledal vsakega od digitaliziranih vzorcev biopsij in določil številčni/točkovni rezultat histološke vnetne aktivnosti bolezni z uporabo histoloških točkovnikov.
Od obstoječih histopatoloških točkovnikov, razvitih za oceno aktivnosti bolezni, smo uporabili GHAS (Global Histological Disease Activity Score), RHI (Robarts Histopathology

Index) in NHI (Nancy Histological Index). GHAS smo uporabili, ker je med najbolj celovitimi in pogosto uporabljenimi točkovniki pri bolnikih s CB. Podtočkovanje H v točkovniku GHAS smo prilagodili tako, da smo namesto števila prizadetih biopsij uporabili odstotek prizadete površine v eni biopsiji. Točkovnik RHI je modifikacija Geboesovega točkovnika, ki je ponovljiv (angl. reproducible), odziven (angl. responsive) in validiran pri bolnikih s ulceroznim kolitisom (UC). NHI je preprost sistem točkovanja, ki je validiran z visoko stopnjo odzivnosti in zanesljivosti (angl. reliability) pri UC. Ne RHI ne NHI sicer nista bila validirana pri bolnikih s CB.

ANALIZA MRNK

Izražanje mRNK pro-vnetnih genov smo določili s kvantitativno verižno reakcijo s polimerazo. Analizirali smo izražanje petih genov, ki je pomembno povečano v sluznici debelega črevesja in ileuma pri bolnikih s CB pri akutnem vnetju. Ti geni so:

- **Interlevkin (IL)-8 (IL–8)**, potenten pro-vnetni kemokin, ki privablja in aktivira granulocite
- **PECAM-1 ali CD31** (angl. Platelet endothelial cell adhesion molecule), adhezijska molekula, ki posreduje transendotelijski prehod vnetnih celic
- **S100A9**, antimikrobni peptid, ki tvori kalprotektin, fekalni biomarker pri kronični vnetni črevesni bolezni
- **IL–6**, pro-vnetni citokin, vključen v patogenezo CB
- **IL–23 (podenoti p19 in p40)**, pro-vnetni citokin, ki aktivira T limfocite tipa-Th17 in je vpet v patogenezo CB

Relativno izražanje mRNK opisanih genov je bilo izračunano kot razmerje glede na povprečje endogenih referenčnih genov (beta aktin in ciklofilin A).

IMUNOHISTOKEMIČNA ANALIZA

Imunohistokemično barvanje v biopsijskih vzorcih je bilo uporabljeno za detekcijo vnetnih celic, katerih število je povečano v sluznici debelega črevesja in ileuma pri bolnikih s CB pri aktivnem vnetju. Uporabili smo specifična monoklonska protitelesa proti **CD68**, markerju monocitov/makrofagov, **CD3**, markerju T limfocitov, in **MPO (mieloperoksidazi),** markerju nevtrofilcev.

STATISTIČNA ANALIZA

Vsakemu preiskovancu je bilo odvzetih več biopsij, zato so bili podatki statistično analizirani z uporabo modelov mešanih učinkov (angl. mixed effects models), ki so upoštevali potencialne učinke grozdenja. Model mešanih učinkov je bil uporabljen za statistično analizo vsakega od točkovnikov histološke aktivnosti bolezni (GHAS, NHI in RHI), izražanja mRNK izbranih genov in števila vnetnih celic, dokazanih imunohistokemično. Nivo izražanja mRNK in število vnetnih celic smo analizirali na logaritemski skali, da bi upoštevali možno nenormalno razporeditev podatkov. V teh modelih smo mesto odvzema biopsij (lokacija 1, 2 in 3) in segment (kolon ali ileum) obravnavali kot stalni učinek (angl. fixed effects), bolniki pa so bili obravnavani kot naključni učinek (angl. random effects).
Za primerjavo histopatoloških točkovanj, izražanja mRNK izbranih genov in števila vnetnih celic smo uporabili kontraste med različnimi lokacijami. Razlike so bile opredeljene kot statistično pomembne pri dvostranski p vrednosti <0,05. Za predstavitev rezultatov smo uporabili 95-odstotni interval zaupanja.

Določitev velikosti vzorca

Zaradi pomanjkanja relevantnih podatkov v literaturi je bila velikost vzorca izračunana z uporabo Cohenove standardizirane velikosti učinka (povprečna razlika deljena s standardno deviacijo). Če primerjamo rezultate med dvema lokacijama pri preiskovancu s parnim t-testom, je vzorec 50 oseb (parov) zadostoval za odkrivanje velikosti učinka 0,4 z 80% statistično močjo pri 5% stopnji statistične pomembnosti. To bi bila iz kliničnega zornega kota zadostna velikost učinka za vsakega od zastavljenih ciljev, da bi priporočili lokacijo odvzema biopsije. Izračunana velikost vzorca je konzervativna, saj je model mešanih učinkov verjetno statistično močnejši od parnega t-testa.

REZULTATI

Skupno smo v vseh treh centrih pregledali 78 bolnikov s CB, od katerih jih je 51 izpolnjevalo vse vključitvene kriterije. Razlog izključitve 27 bolnikov iz študije je bila odsotnost vsaj ene razjede >0,5 cm na ileokolonoskopiji. Osnovne demografske značilnosti bolnikov in karakteristike CB so predstavljene v **tabeli 6.**
Biopsije smo pridobili iz ileuma pri 19, debelega črevesja pri 15 in iz obeh segmentov črevesja pri 17 bolnikih. Vsi so imeli aktivno CB s povprečno endoskopsko aktivnostjo 11 (SD = 7,5) po SES-CD točkovniku.

Tabela 6: Osnovne značilnosti bolnikov in Crohnove bolezni.

Značilnost, n	51
Ženski spol, n (%)	25 (49)
Povprečna starost, leta (SD)	42,2 (16,7)
Povprečno trajanje bolezni, leta (SD)	13,1 (10,8)
Lokacija bolezni, n (%)	
L1	16 (31,4)
L2	8 (15,7)
L3	22 (43,1)
L3 + L4	5 (9,8)
Lokacija odvzema biopsij, n (%)	
Ileum	19 (37,3)
Kolon	15 (29,4)
Oba segmenta	17 (33,3)
Zdavilo za CB, n (%)	
Vedolizumab	7 (13,7)
TNF antagonisti	13 (25,5)
5-aminosalicilati (mesalazin)	4 (7,8)
Kortikosteroidi ali budezonid	8 (15,7)
Imunosupresivi (tiopurini, metotreksat)	16 (31,4)
Ustekinumab	1 (2,0)
Bolniki s predhodnimi operacijami CB, n (%)	
Abdominalne operacije	18 (35,3)
Perianalne operacije	8 (15,7)
Povprečna endoskopska aktivnost CB - SES-CD (SD)	11.0 (7,5)

Lokacija bolezni: L1 ilealna, L2 kolonična, L3 ileokolonična, L4 zgornja prebavila.

HISTOPATOLOŠKO TOČKOVANJE

Povprečne vrednosti histološke aktivnosti bolezni izračunane s histološkimi točkovniki (GHAS, RHI in NHI) glede na razdaljo od razjede in glede na segment (ileum ali kolon) so predstavljene v **tabeli 7.**

Trend padanja histološke aktivnosti bolezni glede na oddaljenost od razjede je bil opazen tako v tankem kot v debelem črevesju ($p<0,05$), pri čemer je najvišja histološka aktivnost CB opažena v biopsijah, odvzetih iz roba razjede. Statistično pomembe razlike ($p<0,0001$) so bile dokazane pri vseh primerjavah med robom razjede in lokacijama 2 oz. 3 tako v ileumu kot v kolonu.

Tabela 7: Histološka aktivnost bolezni glede na lokacijo in segment.

	Kolon			Ileum		
Lokacija	1	2	3	1	2	3
GHAS	10,1 (8,5, 11,7)[1,2]	5,2 (3.4, 6.9)	4,2 (2,5, 5,8)	8,3 (6,9, 9,6)[1,2]	5,1 (3,6, 6,6)[3]	3,1 (1,6, 4,6)
RHI	19,5 (14,7, 24,4)[1,2]	5,8 (2,6, 9,0)	4,8 (2,3, 7,4)	14,2 (10, 18,3)[1,2]	6,6 (3,7, 9,5)[3]	3,4 (1,1, 5,7)
NHI	3,3 (2,7, 3,8)[1,2]	1,2 (0,7, 1,8)	0,9 (0,4, 1,4)	2,7 (2,1, 3,2)[1,2]	1,3 (0,8, 1,8)[3]	0,7 (0,3, 1,2)

Parne primerjave znotraj segmenta z lokacijo 2, [1]p<0,0001, ali lokacijo 3, [2]p<0,0001, [3]p<0,05
Lokacija: 1, rob največje razjede; 2, 7-8 mm od roba razjede; 3, 21-24 mm od roba razjede.
Vrednosti v oklepajih predstavljajo 95% interval zaupanja.

mRNK IZRAŽANJE PRO-VNETNIH GENOV

Logaritmično transformirani nivoji izražanja analiziranih pro-vnetnih genov glede na razdaljo od razjede in glede na segment (ileum ali kolon) so predstavljeni v **tabeli 8**.

Podobno kot pri histopatološki aktivnosti smo opažali trend nižjega izražanja mRNK pro-vnetnih genov glede na oddaljenost od razjede tako v tankem kot v debelem črevesju (p<0,05), pri čemer je bila najvišja izraženost genov v biopsijah, odvzetih iz roba razjede. Statistično pomembe razlike (p<0,005) so bile dokazane pri vseh primerjavah med robom razjede in lokacijama 2 oz. 3 tako v tankem kot v debelem črevesju.

Tabela 8: Nivo izražanja (logaritmično transformiran) izbranih pro-vnetnih genov glede na lokacijo in segment.

	Kolon			Ileum		
Lokacija	1	2	3	1	2	3
IL-6	-4,3 (-4,9, -3,6)[1,3]	-7,1 (-7,7, -6,5)	-7,2 (-7,6, -6,7)	-4,9 (-5,5, -4,4)[1,3]	-7,3 (-7,8, -6,8)	-7,6 (-8,0, -7,2)
IL-8	-2,6 (-3,2, -2,0)[1,3]	-5,3 (-5,8, -4,7)[4]	-5,9 (-6,4, -5,3)	-2,7 (-3,2, -2,1)[1,3]	-5,2 (-5,7, -4,7)	-5,6 (-6,1, -5,1)
IL-23 (p19)	-5,7 (-6,1, -5,3)[1,3]	-6,6 (-6,9, -6,3)	-6,7 (-7,0, -6,4)	-5,7 (-6,0, -5,3)[1,3]	-6,8 (-7,0, -6,5)	-6,7 (-7,0, -6,5)
IL-23 (p40)	-8,7 (-9,2, -8,2)[1,4]	-9,8 (-10,2, -9,4)	-9,9 (-10,3, -9,5)	-8,6 (-9,0, -8,2)[1,4]	-9,7 (-10,1, -9,3)	-9,5 (-9,9, -9,1)
CD31	-2,1 (-2,3, -1,8)[1,3]	-2,7 (-2,9, -2,5)	-2,7 (-3,0, -2,5)	-2,2 (-2,4, -1,9)[2,3]	-2,6 (-2,8, -2,4)[4]	-2,9 (-3,1, -2,7)
S100A9	-1,4 (-1,8, -0,9)[1,3]	-3,2 (-3,6, -2,8)	-3,4 (-3,8, -3,0)	-1,6 (-2,0, -1,2)[1,3]	-3,0 (-3,3, -2,7)[4]	-3,3 (-3,6, -3,0)

Parne primerjave znotraj segmenta z lokacijo 2, [1]p<0,0001, [2]p<0,001; ali lokacijo 3, [3]p<0,0001, [4]p<0,005. Vrednosti v oklepajih predstavljajo 95% interval zaupanja.

IMUNOHISTOKEMIČNA ANALIZA

Logaritmično transformirana števila CD3+, CD68+ in MPO-pozitivnih celic na mm^2 glede na razdaljo od razjede in glede na segment (ileum ali kolon) so prikazana v **tabeli 9**.

Opažali smo gradient števila MPO-pozitivnih celic glede na oddaljenost od razjede tako v tankem kot v debelem črevesju ($p<0,05$). Število MPO-pozitivnih celic (nevtrofilci) je bilo najvišje na robu razjede in se je pomembno razlikovalo od lokacije 2 in 3 ($p<0,0001$) tako v tankem kot v debelem črevesju. Slednje ne velja za CD3+ in CD68+-pozitivne celice.

Tabela 9: Logaritmično transformirano število CD3+, CD68+ in MPO-pozitivnih celic na mm^2 glede na lokacijo in segment.

	Kolon			Ileum		
Lokacija	1	2	3	1	2	3
CD3+	7,3 (7,0, 7,7)	7,3 (7,0, 7,6)	7,1 (6,7, 7,5)	7,3 (7,0, 7,6)	7,3 (7,0, 7,6)	7,3 (7,0, 7,6)
CD68+	6 (5,6, 6,4)	5,9 (5,4, 6,4)	5,9 (5,4, 6,4)	6,1 (5,8, 6,5)[3]	6,1 (5,7, 6,5)[3]	5,5 (5,1, 5,9)
MPO+	6,9 (6,3,7,5)[1,2]	4,5 (3,7, 5,2)	3,9 (3,2, 4,6)	6,6 (6,1, 7,2)[1,2]	4,7 (4,0, 5,3)	4,1 (3,5, 4,6)

Parne primerjave znotraj segmenta z lokacijo 2, [1]$p<0,0001$, ali lokacijo 3, [2]$p<0,0001$, [3]$p<0,05$. Vrednosti v oklepajih predstavljajo 95% interval zaupanja.

ZAKLJUČKI

Gre za prvo študijo, ki je ocenjevala razmerje med mestom odvzema biopsije glede na razjedo in histološko aktivnostjo bolezni, nivojem izražanja pro-vnetnih genov ter številom vnetnih celic pri CB. Slednje je presenetljivo, saj so prve točkovnike histološke aktivnosti kronične vnetne črevesne bolezni opisovali že pred več kot 60 leti.

Potrdili smo hipotezo, da obstajajo razlike v histološki vnetni aktivnosti, izražanju izbranih genov in številu MPO-pozitivnih celic (predstavljajo v glavnem nevtrofilce) v biopsijah črevesne sluznice na različnih razdaljah od razjede pri bolnikih s CB.

Naša glavna ugotovitev je, da biopsije odvzete iz roba razjede tako v debelem črevesju kot ileumu dosežejo največjo histopatološko vnetno aktivnost bolezni, največje sluznično izražanje mRNK pro-vnetnih genov in največje število MPO-pozitivnih celic v lamini proprii. Obstaja gradient vnetja od roba razjede proti najbolj oddeljenemu mestu ocenjevanja biopsij (21-24 mm). MPO-pozitivne celice predstavljajo nevtrofilce, ki so povezani z aktivnim vnetjem pri kronični vnetni črevesni bolezni. Podobnega trenda pri CD3+ (T limfociti) in

CD68+ (makrofagi/monociti)-pozitivnih celicah nismo opažali, saj med biopsijami med različnimi lokacijami ni bilo pomembnih razlik v številu teh celic.

Naše ugotovitve bodo prispevale k poenotenju odvzema biopsij za analizo s patohistološkimi in molekularnimi metodami pri bolnikih s CB, kar bo vodilo v zmanjšanje napak zaradi vzorčenja biopsij. Naše ugotovitve bodo uporabne tako v kliničnih študijah kot v vsakodnevni klinični praksi.

Menimo, da bodo naše ugotovitve osnova za standardizacijo in optimizacijo protokolov odvzema biopsij pri CB. Slednje bi lahko pospešilo vključitev histoloških ciljev in ciljev translacijske medicine v klinične študije in vsakodnevno klinično prakso.

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