MASARYK UNIVERSITY

FACULTY OF MEDICINE

Modern approaches in diagnostics of inflammatory bowel disease in a pediatric population

HABILITATION THESIS

In Pediatrics

(Collection of previously published scholarly works)

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I hereby declare that I wrote this habilitation thesis on my own, using the relevant resources listed in the references.

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Signature

Acknowledgment

I would like to thank very sincerely all my mentors who stimulated my interest in research questions, namely Prof. Ondřej Slabý and Prof. Ajay Goel.

Many thanks are due to my research collaborators and colleagues from the Department of Pediatrics, University Hospital Brno; Faculty of Medicine, Masaryk University; and Central European Institute of Technology.

Last but not least, I would like to express my sincere thanks to my whole family, my always supportive wife Hana, and our beloved children.

Commentary

Inflammatory bowel disease (IBD) is an umbrella term describing disorders that cause chronic inflammation of the gastrointestinal tract. IBD affects both children and adults, but children have their specificities. IBD is classically divided into Crohn's disease and ulcerative colitis. In the current view, pediatric IBD encompasses a continuum of clinical categories, including typical ulcerative colitis, atypical ulcerative colitis, Crohn's colitis, and Crohn's disease. Where none of the above can be determined, we refer to inflammatory bowel disease unclassified. The incidence of IBD has been increasing in recent decades worldwide, and IBD has moved from being previously at the periphery of interest among gastroenterologists into focus as one of the most studied diseases. The pathogenesis of IBD involves an interaction of genetic and environmental variables that disrupt the relationship between the immune system and the gut microbiota. The clinical manifestations of IBD can be not only intestinal but also extraintestinal, and more aggressive forms of the disease are often present in children compared to adults. The course of IBD is unpredictable, with alternating periods of flare-up and remission. IBD in childhood can disrupt somatic and psychological development and has relevant social and economic consequences. Therefore, accurate and timely diagnosis and adequate therapy are essential. The presented habilitation thesis documents the author's experience in managing pediatric patients with various gastroenterological problems, but at its core is a focus on IBD and description of modern diagnostic approaches. This habilitation thesis is conceived as a collection of 10 articles previously published by the author and his colleagues. It contains individual chapters dealing with the basic aspects of IBD. Where relevant, it is followed by commentaries introducing the topic of each publication, describing the current state of knowledge and how the author has contributed to knowledge in this field. The work is based on research activities at the authors' workplaces, the Department of Pediatrics, University Hospital Brno; the Faculty of Medicine, Masaryk University; and Central European Institute of Technology.

Content of the work

1. Introduction

Inflammatory bowel disease (IBD) is a term encompassing a range of chronic inflammatory conditions affecting the gastrointestinal tract and is traditionally subdivided into Crohn's disease (CD) and ulcerative colitis (UC).[1] CD is an inflammatory disease affecting any part of the gastrointestinal tract from the oral cavity to the rectum, but the most typical site of involvement is the terminal ileum. Clinical manifestations of CD in children are varied and may include fatigue, weight loss, abdominal pain, diarrhea, growth impairment, and delayed puberty.[2] UC is characterized by inflammatory involvement of the colon. Typical symptoms depend on the extent of the disease and include crampy abdominal pain and diarrhea, often with blood.[3] According to various sources, up to one-quarter of patients with IBD are diagnosed in childhood or adolescence.[4, 5] The incidence of IBD has been increasing in recent decades in all populations (pediatric and adult) and worldwide.[6, 7] The causes of this phenomenon are not clearly understood and this is probably one of the reasons why IBD has moved from the periphery of interest among gastroenterologists to one of the most studied diseases.[8] The pathogenesis of IBD involves an interaction of genetic and environmental variables that disrupt the relationship between the immune system and the gut microbiota.[9] There are significant differences between the child and adult populations, and patients diagnosed during childhood have differential characteristics (e.g., higher prevalence of CD, more extensive disease, and more frequent family history).[10-13] In addition, the use of immunomodulators and biologic agents is greater in childhood-onset patients, suggesting a more aggressive course of the disease.[14] The course of IBD is unpredictable, with alternating periods of flare-up and remission. The variety of symptoms, both intestinal and extraintestinal; the presence of complications; often challenging diagnostic procedures; and the therapy itself, including potential side effects, make the disease challenging for young patients and their families. Proper somatic development may be impaired, while quality of life, social life, and education also are affected.[15] The main elements of diagnostic workup include upper and lower endoscopy with mucosal biopsies and imaging procedures, and, in the differential diagnosis, it is necessary to exclude many IBD-mimicking diseases.[16] Accurate and timely diagnosis and adequate therapy are essential, because diagnostic delay is associated with complicated disease course.[17, 18] Therapeutic options include medication, nutritional therapy, and surgery.[19-22] The goal of IBD treatment is achieving deep remission of the disease, which means elimination of the patient's symptoms, normalization of laboratory parameters, induction of mucosal healing, restoration of normal growth, and prevention of

surgical complications.[23] Thus, an interdisciplinary approach involving different health professions is essential in the management of patients with such complex medical problems.[24] Moreover, the professionals caring for these patients must have a broad understanding of the topic because there is increasing interest in such additional treatment modalities as complementary and alternative medicine.[25-27]

2. Classification

2.1. Types of IBD

Even though IBD in children and adults is similar in many ways, pediatric IBD (PIBD) often has atypical features making classification of PIBD challenging.[11, 28, 29] Moreover, accurate phenotype classification of IBD is crucial for appropriate management and prognostication.[30] Consensus-based criteria for the diagnosis of PIBD were published in 2005.[31] These included a defined diagnostic work-up for new PIBD patient that enabled standardized diagnostics for CD, UC, and indeterminate colitis.[31] This was an important achievement for both clinical practice and clinical research. In 2014, the revised Porto criteria were released and identified subtypes of PIBD as UC, atypical UC (atypical phenotypes such as macroscopic rectal sparing, isolated non-serpiginous gastric ulcers, normal crypt architecture, absence of chronicity in biopsies, or a cecal patch.), IBD unclassified (IBDU; inflammation is limited to the colon with features that make differentiation between UC and CD uncertain even after a complete workup), and CD.[16] Consequently, the Paediatric IBD Porto Group of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) published the ‘‘PIBD-Classes’’ criteria that standardized the differentiation of PIBD into five categories: typical UC, atypical UC, IBDU, Crohn’s colitis, and CD.[32]

2.2. Disease location

The previously used Montreal classification for disease characterization had several weaknesses regarding the classification of children.[33, 34] For this reason, an international group of pediatric IBD experts developed evidence-based consensus recommendations for a pediatric modification of the Montreal classification (the so-called Paris classification) in an effort to facilitate research in PIBD and create uniform standards for defining IBD phenotypes.[35] Table 1 and Table 2 summarize Paris classification for CD and UC, respectively.[35]

Table 1. Paris classification for Crohn's disease.

Age at diagnosis	**A1a**: 0–<10 years **A1b**: 10–<17 years **A2**: 17–40 years **A3**: >40 years
Location	**L1**: distal 1/3 ileum ± limited cecal disease **L2**: colonic **L3**: ileocolonic **L4**a: upper disease proximal to ligament of Treitz **L4b**: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum
Behavior	**B1**: nonstricturing nonpenetrating **B2**: stricturing **B3**: penetrating **B2B3**: both penetrating and stricturing disease, either at the same or different times **p**: perianal disease modifier
Growth	**G0**: no evidence of growth delay **G1**: growth delay

Table 2. Paris classification for ulcerative colitis.

Extent	**E1**: ulcerative proctitis **E2**: left-sided UC (distal to splenic flexure) **E3**: extensive (hepatic flexure distally) **E4**: pancolitis (proximal to hepatic flexure)
Severity	**S0**: never severe **S1**: ever severe

It should be added that this classification has more use in research than in clinical practice. Nevertheless, the Paris classification for PIBD could, for example, have a predictive value for long-term worse outcome.[36]

2.3. Age of onset

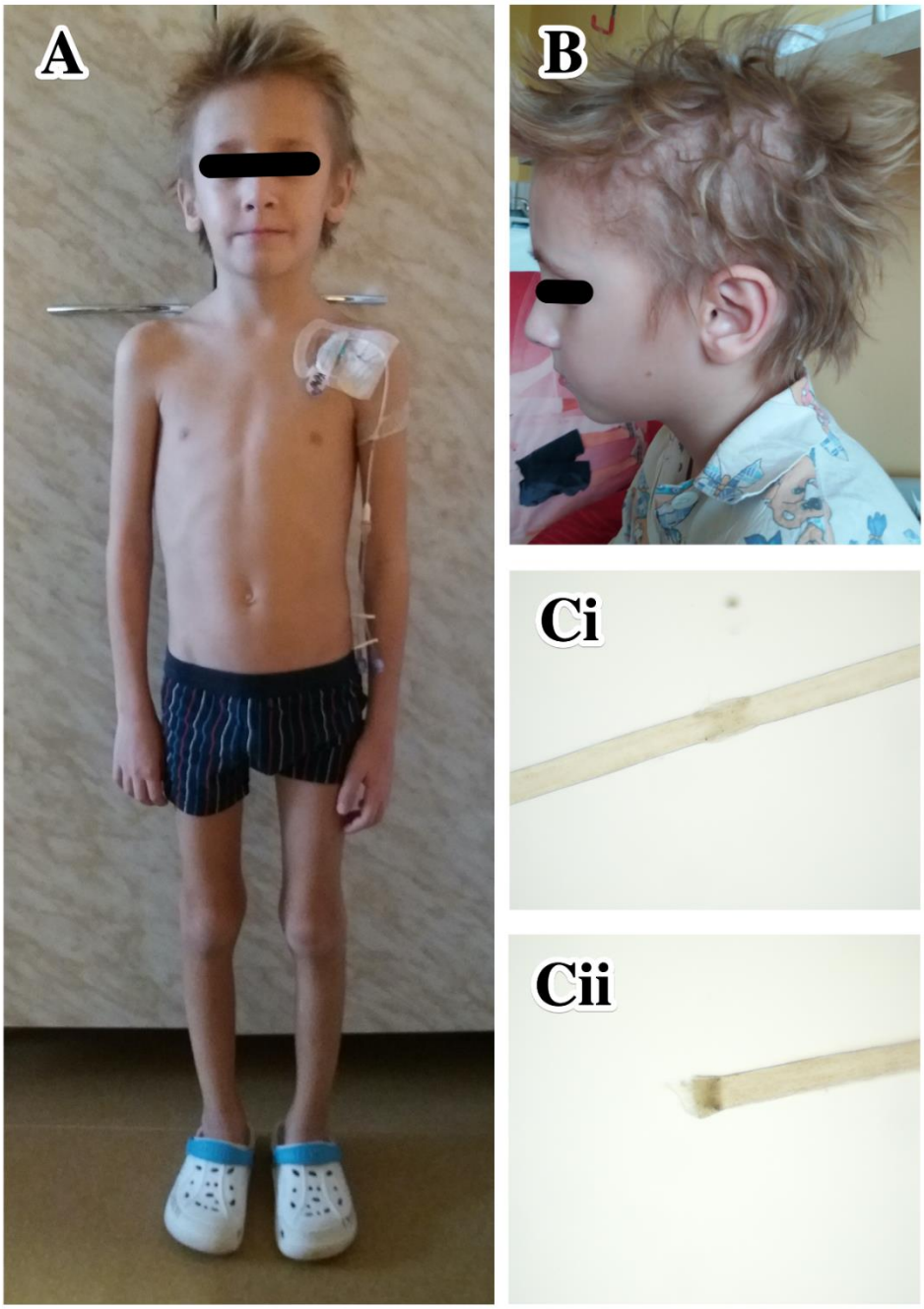
PIBD patients can also be classified in terms of age. Montreal classification distinguished pediatric-onset IBD as cases diagnosed before 17 years of age and labeled them A1.[33] In the Paris classification,[35] A1 was further divided into A1a (diagnosed before 10 years of age) and A1b (diagnosed between 10 and 17 years of age). Among A1a cases, those diagnosed before 6 years of age are classified as very-early-onset IBD (VEO-IBD).[37] Furthermore, IBD cases with onset by 2 years of age were classified as infantile-IBD and by 28 days of age as neonatal-onset IBDs, respectively.[37, 38] Now, therefore, we have subgroups of pediatric-onset IBD (<17 years), early-onset IBD (<10 years), VEO-IBD (<6 years), infantile-onset IBD (<2 years), and neonatal-onset IBD (<28 days).[37-39]

IBD generally is regarded as a polygenic disease, and a genome-wide association study has revealed more than 240 known disease-associated genes.[40] Pediatric-onset IBD seems to have a greater genetic component compared to adult-onset IBD due to lower cumulative exposure to environmental factors.[41] In pediatrics, the name IBD is also used for a group of diseases that have similar symptomatology but are caused by rare genetic defects. Other diseases that involve the symptoms of IBD have a separate name, often based on molecular classification, and their manifestations are referred to as "IBD-like."[38, 39] Rare monogenic IBD and IBD-like syndromes therefore present the genetic extreme where a single highly pathogenic variant causes IBD symptoms. As such, pediatric-onset IBD represents a spectrum ranging from extreme monogenic variants (typically VEO-IBD) to adolescent complex variants (as in adults).[41] Evaluating and managing children with VEO-IBD is a challenging field for pediatric gastroenterologists worldwide.[37, 42] Specifics in diagnostics of VEO-IBD will be further described in another chapter.

2.4. Authors' contribution to the knowledge

My colleagues and I described a unique diagnosis of trichohepatoenteric syndrome (THES) (**Annex 1**). To our knowledge, this was the first case diagnosed in the Czech Republic. THES can have a clinical presentation similar to that of VEO-IBD and is often assigned to this group (or to VEO-IBD-like). The described patient had somatic retardation and woolly hair appearance and suffered from recurring episodes of watery mucous diarrhea, impaired liver functions, and failure to thrive (Figure 1).[42]

Figure 1. Clinical data of the patient with trichohepatoenteric syndrome.



(A) Overall habitus of the patient. (B) Detail of the hairy part of the head. Brittle, easily breakable hair. (Ci) Hair analysis using light microscopy showing trichorrhexis nodosa. Magnification 40×. (Cii) Bristly split hair ends. Magnification 40×. The figure had been published in Jabandziev et al.[42]

Esophagogastroduodenoscopy and colonoscopy were performed at 4 years to rule out possible IBD, but only signs of nonspecific colitis were evident. Massive parallel sequencing

targeting a panel of primary immunodeficiency-related genes was used to examine the patient's DNA. Next-generation sequencing (NGS) analysis revealed two heterozygous variants in the *TTC37* gene. Nonsense p.Arg1201* and missense p.Leu1505Ser variants in exons 34 and 42, respectively, were evaluated as pathogenic based on *in silico* predictions, their rare occurrence in the general population, and the fact that both mutations had already been described in patients with THES. THES could be a life-threatening condition, particularly in children who develop liver disease or severe infection courses, and it must be considered in the differential diagnosis in children for whom a diagnosis of VEO-IBD is being considered.[42]

3. Epidemiology

IBD has undoubtedly become a global disease.[43, 44] Depending upon individual regions, we can find regions with low, medium, and high incidences.[7] Differences in incidence could also be found by race[45] and by age (children vs. adults).[11] There is a general consensus within the gastroenterologist community that the incidence of IBD is increasing worldwide,[7, 44] and especially in low-income countries.[46, 47] With these trends, a significant burden on health systems and economic impacts can be expected.[48-50] It is not easy to compare and standardize data from different parts of the world, however, due to heterogeneity among study designs. Diagnostic criteria can differ. Some studies use hospital records, while others use surveys and administrative data.[51] The age limit is a vital inclusion criterion with a significant impact on reported incidence rates, as individual studies differ significantly in their definitions of childhood or upper age limit (15, 18, or 20 years respectively).[6] Most data are retrospective; only a few prospective population-based studies have been conducted, those being particularly from developing countries. Furthermore, the incidence rates are often an extrapolation from one or more regions of a country.[51] It cannot be ruled out that better diagnostics of IBD is at least partly responsible for the increase in incidence.[52] In connection with the differences in incidence in individual countries, a so-called north–south gradient is discussed, with the highest incidence of IBD being found in the north.[4] In adult patients, a positive association with a country's wealth (as measured by gross domestic product) has been discussed.[53, 54] It is also necessary to mention the fact that we do not have recent data from a large number of countries, and most countries lack accurate estimates for the incidence of pediatric IBD.[51, 55]

The most recent systematic review studying the incidence of IBD in the pediatric population worldwide was published in 2018,[6] and it is interesting to compare that with the previous systematic review from 2011.[4] The authors of the 2011 systematic review stated that the incidence of CD was increasing especially in some countries and, conversely, that the incidence of UC remained the same in most countries.[4] In 2018, Sykora et al. reviewed 140 studies reporting data from 38 countries.[6] The highest annual pediatric incidences of IBD were 23/100,000 person-years in Europe; 15.2/100,000 in North America; and 11.4/100,000 in Asia, the Middle East, and Oceania. The highest annual incidences of CD were 13.9/100,000 in North America and 12.3/100,000 in Europe. The highest annual incidences of UC were 15.0/100,000 in Europe and 10.6/100,000 in North America. The highest annual incidences of IBD-U were 3.6/100,000 in Europe and 2.1/100,000 in North America. Overall,

67% of CD, 46% of UC, and 11% of IBD-U studies reported increasing incidence in the trend analyses.[6, 56] From a general perspective, it is not very clear how incidence trends will continue to evolve. It is questionable whether, for example, developed countries are still experiencing further increase in incidence. In a recently published systemic review and meta-analysis, Roberts et al. stated that the incidence of pediatric IBD continues to increase throughout Europe, with more robust evidence of a north–south than of an east–west gradient.[57]

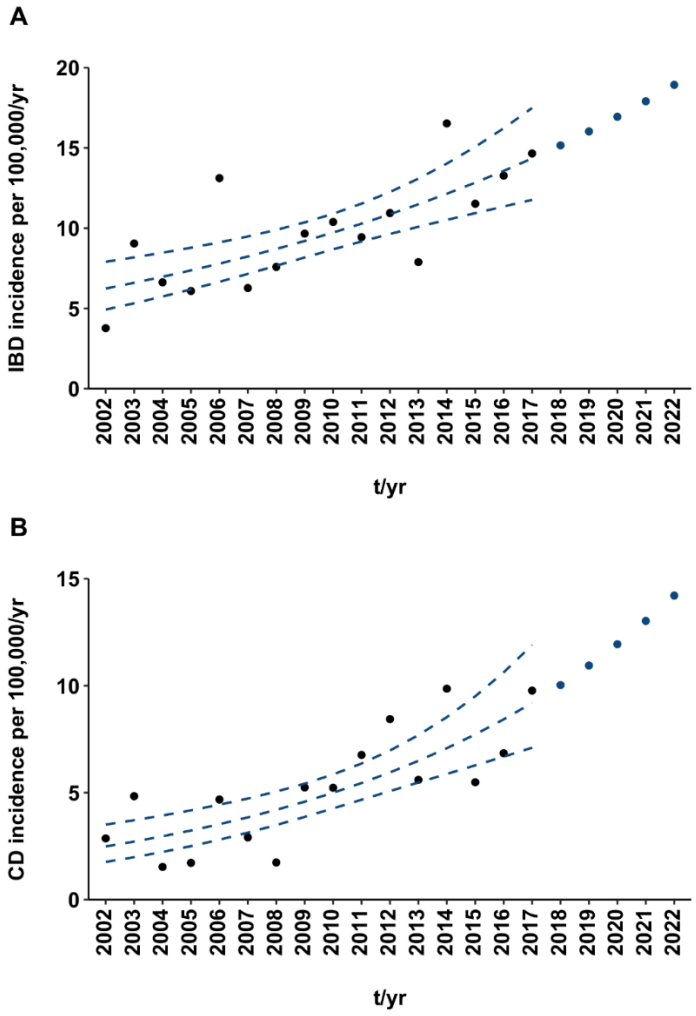
3.1. Authors' contribution to the knowledge

Until recently, we did not have much information about the epidemiology of IBD in children within the Czech Republic. The first Czech national survey was conducted and published by Pozler et al. in 2006.[58] This paper described how the incidence of CD had increased from 0.25/100,000 in 1990 to 1.25/100,000 in 2001. Schwarz et al.[59] rather more recently (2017) published a 16-year prospective study of pediatric IBD patients in the Pilsen Region of the Czech Republic showing that a group of 170 pediatric patients (study period 2000–2015) represented an average incidence of IBD per 100,000/year of 10.0 (6.2 for CD, 2.8 for UC, and 1.0 for IBD-U).[56, 59]

Our study (**Annex 2**) aimed to determine the incidence and trends of IBD in the population of Czech children.[56] The study included a 16-year period in the South Moravian Region. It characterized differences by sex and age and projected the incidence of IBD for future years. Overall, 358 pediatric patients with newly diagnosed IBD at the Department of Pediatrics, University Hospital Brno, were evaluated. Diagnosed were 192 children (53.6%) with CD, 123 (34.4%) with UC, and 43 (12.0%) with IBD-U. The incidence of IBD increased from 3.8 (CD 2.9, UC 0.9, and IBD-U 0.0) per 100,000/year in 2002 to 14.7 (CD 9.8, UC 4.0, and IBD-U 0.9) per 100,000/year in 2017 ($p<0.001$) (Figure 2). The overall IBD incidence per 100,000/year was 9.8 (95% confidence interval [CI]: 8.8–10.9). Constituent incidences per 100,000/year were CD 5.2 (95% CI: 4.5–6.0), UC 3.4 (95% CI: 2.8–4.0), and IBD-U 1.2 (95% CI: 0.9–1.6). IBD incidence was projected to reach 18.9 per 100,000/year in 2022 (Figure 2). The incidence of CD, UC, and U-IBD found in our study, including the proportions among them, were practically identical to the results from Schwarz et al.[56, 59] Both studies showed that the overall incidence of IBD is increasing in Czech children, and especially the incidence of CD, while the trends for UC and IBD-U seem to be constant.

Despite some limitations, we would therefore suggest that our results are potentially similar to the incidence to be found in pediatric IBD patients across the Czech Republic.[56]

Figure 2. Incidence rates per 100,000/year among children (0–18 years of age) newly diagnosed with IBD (A) and CD (B) in South Moravian Region, 2002 to 2017 and 2018 to 2022.



Black points represent actual data. Broken blue lines indicate trend over the observed period based on Poisson regression along with a 95% confidence interval. Blue points are future projections. The figure had been published in Jabandziev et al.[56]

These results therefore suggest that incidence rates of pediatric IBD and its subtypes in the Czech Republic are among the highest in the literature. Moreover, these data further emphasize the need to identify risk factors that contribute to the increasing incidence of IBD.[56, 60]

4. Etiopathogenesis

Knowing IBD's pathogenesis is key to understanding the causes of IBD, and a better understanding of these processes could lead to the development of novel IBD therapies.[61] Despite significant achievements, we do not yet have a comprehensive theory describing all the pathogenetic processes leading to IBD. There is growing evidence, however, that the pathogenesis of IBD results from an interplay among host genetic susceptibility, environmental factors, immunological abnormalities, and intestinal barrier alteration (Figure 3).[62, 63]

Figure 3. Mechanisms involved in the pathogenesis of inflammatory bowel disease.

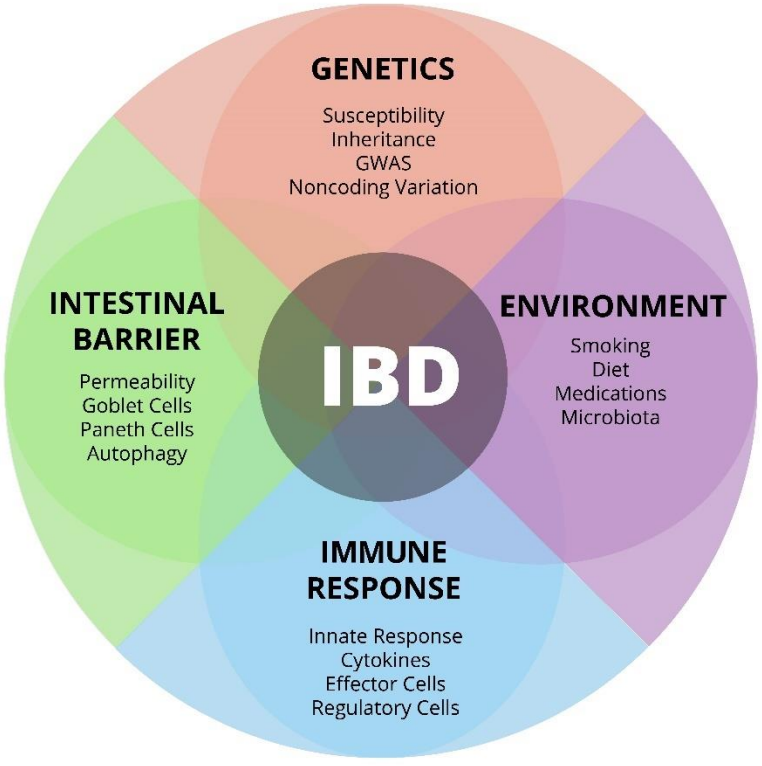


Figure was prepared in accordance with Ramos et al.[63] and created in collaboration with the Service Center for E-Learning at Masaryk University, Faculty of Informatics.

4.1. Intestinal barrier and immune response

The mucosal barrier and its proper functioning, are essential in protecting against adhesion and invasion of luminal microorganisms.[64] This is made possible by intact epithelium, synthesis of various antimicrobial peptides, and mucus layer formation.[65] Damages to the intestinal mucosal barrier include, among others, defective production of antimicrobial peptides, changes in the thickness or composition of the intestinal mucus layer, changes in pattern recognition receptors, and defects in the autophagy process.[66] The intestinal epithelium is in a functional equilibrium with the luminal contents. Disturbance of this equilibrium can lead to dysbiosis and further to the development of a pathological condition such as IBD.[67, 68] In CD patients, for example, there have been described abnormal intestinal permeability; abnormities of tight junctions;[69] and dysfunction of goblet cells, Paneth cells,[70] and M cells.[63] Upon encountering antigen and microbial products gaining access through the intestinal barrier, dendritic cells and other antigen-presenting cells initiate a cascade of pro- and anti-inflammatory signals.[71] This results in the activation of various subsets of local and circulating lymphocytes migrating to effector sites where inflammation occurs.[63] All these processes influence the innate response and production of various cytokines while affecting the function of effector and regulatory cells. Dysregulated immunological reactions in the gut that lead to imbalance in pro- and anti-inflammatory pathways involved in innate and adaptive immunity are regarded as fundamental to the development and persistence of inflammation in IBD.[72]

4.2. Environment

The available evidence suggests that environmental exposures have variable effects on individuals with IBD.[73] In a study by Elten et al., for example, greater exposure to residential greenspace during childhood was associated with reduced IBD risk, thus suggesting a novel avenue to IBD prevention in children.[74] The same study group revealed associations between maternal and early-life exposures to air pollutants and risk of pediatric-onset IBD diagnosis.[75] In a national case-control study in Sweden, higher cumulative exposure to systemic and particularly broad-spectrum antibiotic therapy was associated with greater risk of new-onset IBD and its subtypes.[76] Moreover, greater adherence to a Mediterranean diet was associated with significantly lower risk of later-onset CD in two prospective studies by Khalili et al.[77] Among environmental factors, diet is widely thought to play a crucial role in IBD

development. A positive explanation could be that dietary habits have an essential role in defining the composition of the human gut microbiota and patients suffering from IBD show a generalized decrease in bacterial biodiversity and reduction in specific taxa, including *Firmicutes*, *Bacteroidetes*, *Lactobacillus*, and *Eubacterium*.[78] An umbrella review of available meta-analyses identified nine factors that increase the risk of IBD: smoking (CD), urban living (CD and IBD), appendectomy (CD), tonsillectomy (CD), antibiotic exposure (IBD), oral contraceptive use (IBD), consumption of soft drinks (UC), vitamin D deficiency (IBD), and non-Helicobacter pylori-like enterohepatic Helicobacter species (IBD). Furthermore, seven factors reduce the risk of IBD: physical activity (CD), breastfeeding (IBD), bed-sharing (CD), tea consumption (UC), high levels of folate (IBD), high levels of vitamin D (CD), and Helicobacter pylori infection (CD, UC, and IBD).[79] Nevertheless, the impact of modifying specific environmental factors on risk of disease, risk of disease progression, and risk of relapse remains inadequately studied, there being only limited high-quality data available from interventional studies.[80, 81]

4.3. Genetics

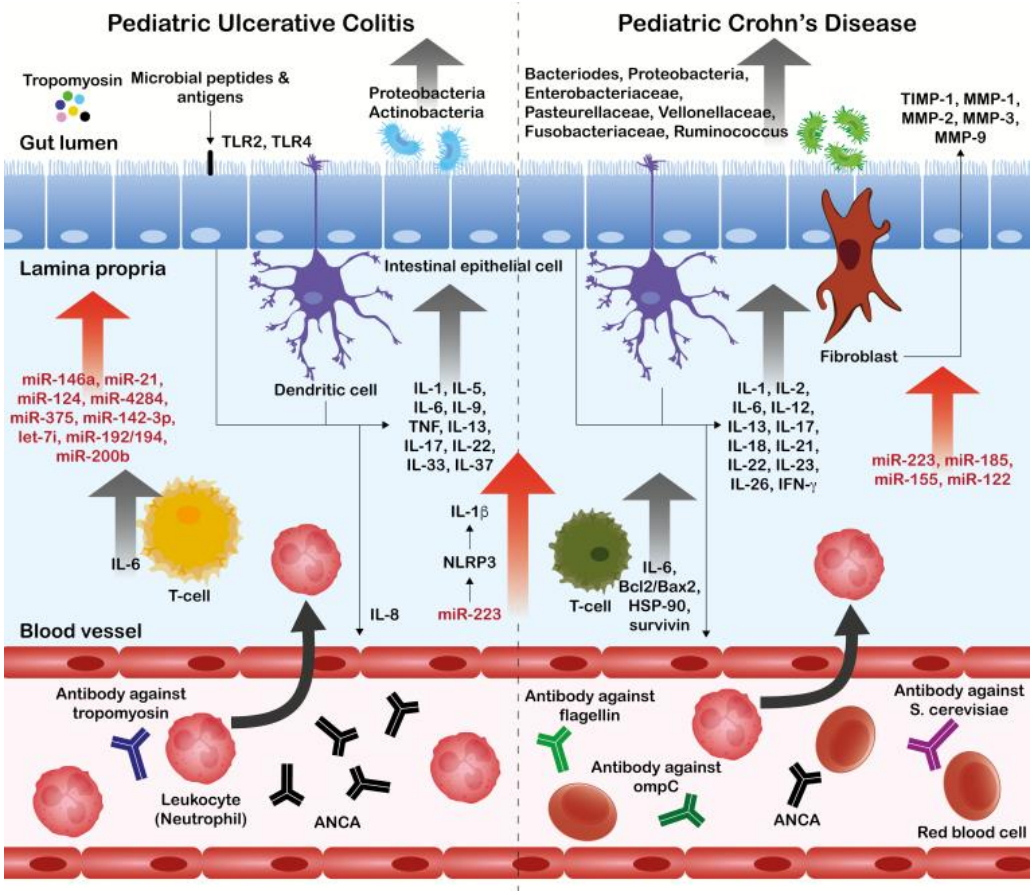
Studies of genetic context in IBD have gradually used various approaches reflecting advances in genetic testing (family and twin studies, linkage studies, candidate gene association studies, genome-wide association studies).[82] Genome-wide association studies (GWAS) alone have revealed the majority of information related to the genetics of IBD, showing that complex genetic disorders like IBD are polygenic, being driven by multiple, common genetic polymorphisms.[63, 83] To date, GWAS and post-GWAS deep resequencing studies have identified 240 IBD-associated loci.[41, 84] Nevertheless, as many as 80–90% of GWAS-identified loci are confined to noncoding variation that exerts its pathogenic effects through modulation of gene expression. Recent studies have focused upon small intranuclear molecules that can broadly regulate gene expression, such as epigenetic markers, microRNAs, and non-coding RNAs, all of which have been implicated in the pathogenesis of IBD through different pathways and will be discussed later.[63, 85]

4.4. Authors' contribution to the knowledge

The family of noncoding RNAs (ncRNAs) exhibits a variety of biological functions.[86] Noncoding RNAs can be divided according to their function into two groups: housekeeping ncRNAs (e.g., tRNAs, rRNAs, snRNAs, snoRNAs) and regulatory ncRNAs. Transcripts

shorter than 200 nucleotides are termed short noncoding RNAs and transcripts exceeding 200 nucleotides are called long noncoding RNAs. Both groups are involved in regulating gene expression and operate on several levels depending on their types. The short ncRNAs, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs), are involved mostly in post-transcriptional regulation but also in many other specific processes such as transposon silencing or ribosomal RNA maturation.[85, 87] The ncRNAs have emerged as potential biomarkers for several diseases, as these are generally stable and abundantly present in a variety of clinical specimens, including tissues and bodily fluids; are highly tissue-specific, cell type-specific, and condition-specific; and can be readily detected by routine and inexpensive laboratory techniques.[85, 88, 89] Several miRNAs and specific miRNA signatures have been identified in IBD-associated tissues, including serum,[90] intestinal mucosa,[91, 92] and stools.[93, 94] Among many other cellular processes, it has been shown that miRNAs play a significant role in intestinal immunity. In our article (**Annex 3**), we provided an overview of current knowledge on ncRNAs, their altered expression profiles in pediatric IBD patients, and how these are emerging as potentially valuable clinical biomarkers.[85] Figure 4 depicts tissue miRNAs involved in the development of PIBD.[85]

Figure 4. Tissue miRNAs involved in the development of pediatric IBD.



Abbreviations: TLR, toll-like receptor; TNF, tumor necrosis factor; ANCA, anti-neutrophil cytoplasmic antibodies; IFN, interferon; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; Bcl2, B-cell lymphoma 2; BAX, BCL2 associated X; CCL, CC chemokine ligand; CCR, CC chemokine receptor; ompC, outer membrane protein C precursor. The figure is modified from Park et al.[95] *and had been published in Jabandziev et al.*[85]

5. Diagnostics

Diagnosis of IBD is based on the Revised Porto criteria.[16, 31] In the Czech Republic, national recommendations based upon these criteria are available.[96, 97] Accurate diagnosis should be based on a combination of history, physical and laboratory examination, esophagogastroduodenoscopy, and ileo-coloscopy with obtaining mucosal samples for histological examination and imaging of the small bowel, if indicated.[16] It is necessary to exclude other diseases mimicking the symptoms of IBD.[16] Thus, we should rule out possible intestinal infection, allergic diseases, and many others; determine the basic subtype of IBD (UC, CD, UIBD), extent of the disease (Paris classification),[35] activity of the disease (using Pediatric Crohn's Disease Activity Index [PCDAI][98-100] and the Pediatric Ulcerative Colitis Activity Index [PUCAI]);[101, 102] and the behavior of the disease.[96] Table 3 presents the overall comparative characteristics of CD and UC.[103]

Table 3. Multiple characteristics of Crohn's disease and ulcerative colitis.

Clinical features	Crohn's disease	Ulcerative colitis
Symptoms and signs	Abdominal pain, diarrhea, weight loss, anorexia, growth failure	Bloody diarrhea, abdominal pain
Location	Mouth to anus; involves all layers of the gut: mucosa to serosa; most common: ileocolonic	Colon; involves only mucosa; most common: pancolitis
Endoscopic findings	Segmental distribution, aphthous ulcers, deep fissuring ulcers, cobblestoning, perianal disease, strictures, fistulas	Diffuse and continuous erythema, friability, granularity, loss of vascular pattern from rectum to variable extent
Histological findings	Pathognomonic non-caseating granulomas; patchy cryptitis, crypt abscesses, ileitis	Cryptitis, crypt abscesses, crypt architectural distortion, basal lymphocytosis, distal Paneth cell metaplasia
Radiologic findings	Rigid stenotic segments, skip areas, and sinus tracts or fistulas	Dilatation of colon in toxic megacolon

Figure was prepared in accordance with Oliveira et al.[103]

5.1. Patient's history, symptoms, and clinical examination

Only few aspects are given greater emphasis in the literature than the importance of a detailed evaluation of the patient's history and a thorough clinical examination, including a careful examination of the perianal area. This is in contrast with the fact that this often is not done in routine clinical practice.[104] IBD is manifested by a wide range of symptoms, both intestinal and extraintestinal.[105] Although typical symptoms from gastrointestinal tract affection are usually at the forefront, IBD could first present as extra-intestinal manifestations.[106] These include erythema nodosum, pyoderma gangrenosum, episcleritis, uveitis, arthritis, and others.[107] Clinical manifestations of CD in children may include fatigue, weight loss, abdominal pain, diarrhea, anemia, growth failure, and delayed puberty.[2] UC is characterized by inflammatory affection of the colon. Typical symptoms include crampy abdominal pain, diarrhea, and rectal bleeding. The clinical symptomatology also depends on the extent of the disease; according to the Paris classification,[35] the disease can be divided into isolated proctitis, left-sided colitis, extended colitis, and pancolitis. Pancolitis is very common in children and affects about three-quarters of patients.[108]

5.2. Laboratory tests

Baseline tests include erythrocyte sedimentation rate, complete blood count, liver enzymes, albumin, and C-reactive protein (CRP).[16, 96] Use of serum antibodies remains complementary in clinical practice, and serology may have a role in assessing prognosis (e.g., p-ANCA, ASCA).[109] Nevertheless, normal blood tests cannot exclude PIBD. Almost one-tenth of all IBD patients diagnosed in a study by Ashton et al. had normal values for all widely used blood tests, and even more than 20% of patients had normal CRP.[110] In comparing widely used laboratory tests in UC and CD, normal inflammatory markers were more common in UC. UC was more likely to have abnormal liver enzymes and all normal results. CD was more likely to have abnormal ESR, CRP, hemoglobin, platelets, and albumin.[110]

In recent years, fecal calprotectin (FCP) testing has gained relatively high clinical popularity and widespread use.[111] In a recent study by Walker et al., FCP testing of children with suspected IBD accurately distinguished IBD from a functional gut disorder, reducing secondary care referrals and associated utilization of diagnostic health care.[112] Nevertheless, clinicians must also be aware of the potential methodological limitations of FCP examination in routine practice.[113, 114] Evidence regarding the clinical use and value of FCP measurements

in various gastrointestinal disorders in children were reviewed in a recently published position paper from ESPGHAN. Currently, FCP's main use lies in the diagnosis and monitoring of IBD and its differentiation from functional gastrointestinal disorders.[115]

Consensus statements have recently been published regarding prognostic laboratory (and clinical) factors for such important clinical questions, among others, as need for surgery and disease complications with regard to CD[116] and UC.[117]

5.3. Endoscopic examination

Endoscopy has an essential and irreplaceable role in the diagnosis and management of PIBD. It is used to differentiate IBD subtypes, monitor disease activity, assess response to therapy, and treat possible complications.[118, 119] Various endoscopic scoring systems are used in both research and clinical practice,[120] including, among others, Simple Endoscopic Score for Crohn's Disease (SES-CD),[121] Ulcerative Colitis Endoscopic Index of Severity (UCEIS),[122] and Rutgeerts score[123] for predicting the postoperative recurrence in CD patients after ileocolonic resection. Revised Porto criteria recommend performing upper gastrointestinal endoscopy and ileo-colonoscopy with multiple biopsies for all suspected patients with PIBD, as well as small bowel imaging (unless typical UC is determined after endoscopy and histology) by magnetic resonance enterography or wireless capsule endoscopy.[16] Currently, recommendations are generally available for endoscopy in pediatrics, including indications for diagnostic and therapeutic esophagogastroduodenoscopy and ileo-colonoscopy, stricture/stenosis endoscopic management, endoscopic solving of upper and lower gastrointestinal bleeding; and others.[124, 125] Recommendations for training and ongoing skills maintenance in pediatric endoscopy have recently been published.[126] In pediatrics, performing an endoscopic examination means, in most cases, undergoing general anesthesia. It is essential to determine the optimal examination strategy in the diagnosis of patients with chronic conditions such as non-bloody diarrhea and abdominal pain. In a study from de Vijver et al., evaluating symptoms and blood and stool markers in patients with non-bloody diarrhea was found to be the optimal test strategy that allowed for reserving diagnostic endoscopy only for children at high risk for IBD. This approach allowed minimizing exposure to endoscopy in children.[127]

5.4. Histomorphologic examination

Collaboration with a pathologist is essential, and the pathologist is part of the multidisciplinary team caring for pediatric IBD patients. Very important is to share clinical data with the pathologist. Differential diagnosis between CD and UC can be particularly challenging, such as when there are atypical presentation patterns for UC and Crohn's colitis.[128] Extremely important is obtaining mucosal biopsies from all parts of the gastrointestinal tract in IBD patients, and missing biopsies from grossly normal tissue would have missed abnormal histology.[129] It should be emphasized that diagnostic gastrointestinal biopsies in VEO-IBD patients differ from those occurring with older onset of PIBD and include broader differential diagnosis (e.g., autoimmune enteropathy).[130] Supplemental (special) stains based upon a patient's clinical history are needed in special situations and must take into account the local differential diagnostic situation, such as in low-resource countries.[131, 132]

5.5. Imaging methods

The noninvasive nature of imaging makes it an ideal tool for serial assessment of disease activity and treatment response. The choice of imaging (bowel ultrasonography, computed tomography, magnetic resonance imaging, etc.) should be based on the specific advantages and disadvantages of each (ionizing radiation, availability, reproducibility, length of examination, cost).[103] For example, while ultrasound is a widely used, safe, fast, and low-cost method both for children suspected of IBD and for monitoring children with IBD,[133] the diagnostic accuracy of ultrasound in detecting intestinal inflammation remains inconclusive.[134]

5.6. Examples of modern diagnostic methods potentially useful in clinical practice

Although we have a fairly wide range of tools at our disposal for adequate diagnosis of IBD, several alternative diagnostic techniques have been developed recently. Various noninvasive markers are currently available, but they have limitations and do not provide ideal utility.[135] In pediatric and adult patients, we can often encounter diagnostic delays.[17, 18] Thus, it would be highly advantageous to have tools to predict whether patients will develop IBD at all. For example, Torres et al. identified a panel of 51 protein biomarkers (e.g., complement cascade proteins, lysosomes, glycosaminoglycans) that were predictive of Crohn's disease within 5

years. On the other hand, predictive factors for the development of UC could not be found.[136] It is clear that further research efforts are needed in this field. Recently, several studies have investigated serum dipeptidyl peptidase-4 as a biomarker of IBD severity and also as a predictor of subclinical disease activity, but with conflicting results.[137-139] Serum profiling and non-coding RNAs are just starting to become widely used but reveal great promise for future clinical practice. Non-coding RNAs and their role in IBD are among the author's main scientific focus areas and are discussed elsewhere.[85, 140] In any case, combining different serum biomarkers can be valuable in improving the performance of disease evaluation.[141]

Studies have recently revealed as potentially promising new fecal biomarkers of intestinal inflammation calgranulin-C (S100A12), tumor pyruvate kinase isoenzyme type M2 (TuM2-PK), and fecal osteoprotegerin (FOPG).[142] Fecal calgranulin-C and FCP had excellent test characteristics to predict IBD and justify endoscopy in a study by Heida et al.[143] Moreover, calgranulin C appears to be a more suitable maker for predicting mucosal healing in children with IBD.[142] Other, less well-established fecal biomarkers include high mobility group box 1 (HMGB1), chitinase 3-like 1, defensins, matrix metalloproteinases, and human nucleic acid. Most of these still require further extensive evaluations and validation.[135]

New endoscopic methods can be expected to emerge and be applied in clinical practice. In the case of UC, for example, magnification colonoscopy, endocytoscopy, and confocal laser endomicroscopy (CLE) enable assessing histological inflammation without the need for biopsy.[144] Concretely, CLE allows visualization of mucosal abnormalities and thus in vivo histology during ongoing endoscopic evaluation by identifying macroscopically normal-appearing mucosa, assessing intestinal epithelial barrier function and vascular permeability, and characterizing potential mucosal lesions, including dysplastic lesions. In particular, CLE used in conventional endoscopy could facilitate the assessment of mucosal healing in IBD.[145] In a pilot study by our study group, we evaluated CLE's usefulness in diagnosing esophageal diseases and came to promising results.[146]

Massively parallel sequencing, also termed next-generation sequencing (NGS), is increasingly used in clinical practice. Its high capacity creates new opportunities for NGS's clinical application for establishing disease diagnoses and prognoses and in making therapeutic decisions.[42, 147]

Targeted sequencing is the NGS assay most commonly used. Typically, it interrogates tens or hundreds of genes presumed to be associated with a particular clinical phenotype or group of diseases. Targeted NGS panels are today very time- and cost-effective.

A specific type of targeted NGS assay, known as whole-exome sequencing (WES), analyzes all of a genome's protein-coding regions. It is particularly beneficial in cases of disorders that are phenotypically heterogeneous and when it is difficult to select an appropriate panel of candidate causative genes. Despite its clear advantages, WES also has some limitations, such as that intronic and noncoding regions remain uncovered and sequencing quality (often expressed as sequencing depth) is insufficient for particular genes, precisely because the range of targeted regions is so very large. Because NGS technology produces huge amounts of data, the processing and storage of that information represents a considerable bioinformatic challenge.[148] When properly selected and used, NGS technologies provide excellent tools that greatly expand the range of genes analyzed and can contribute importantly to ensuring adequate preventive and therapeutic procedures for patients at risk.[39, 42, 147] This is evidenced by use of the methodology in our patients, as documented in the articles discussed in this work.[42, 149]

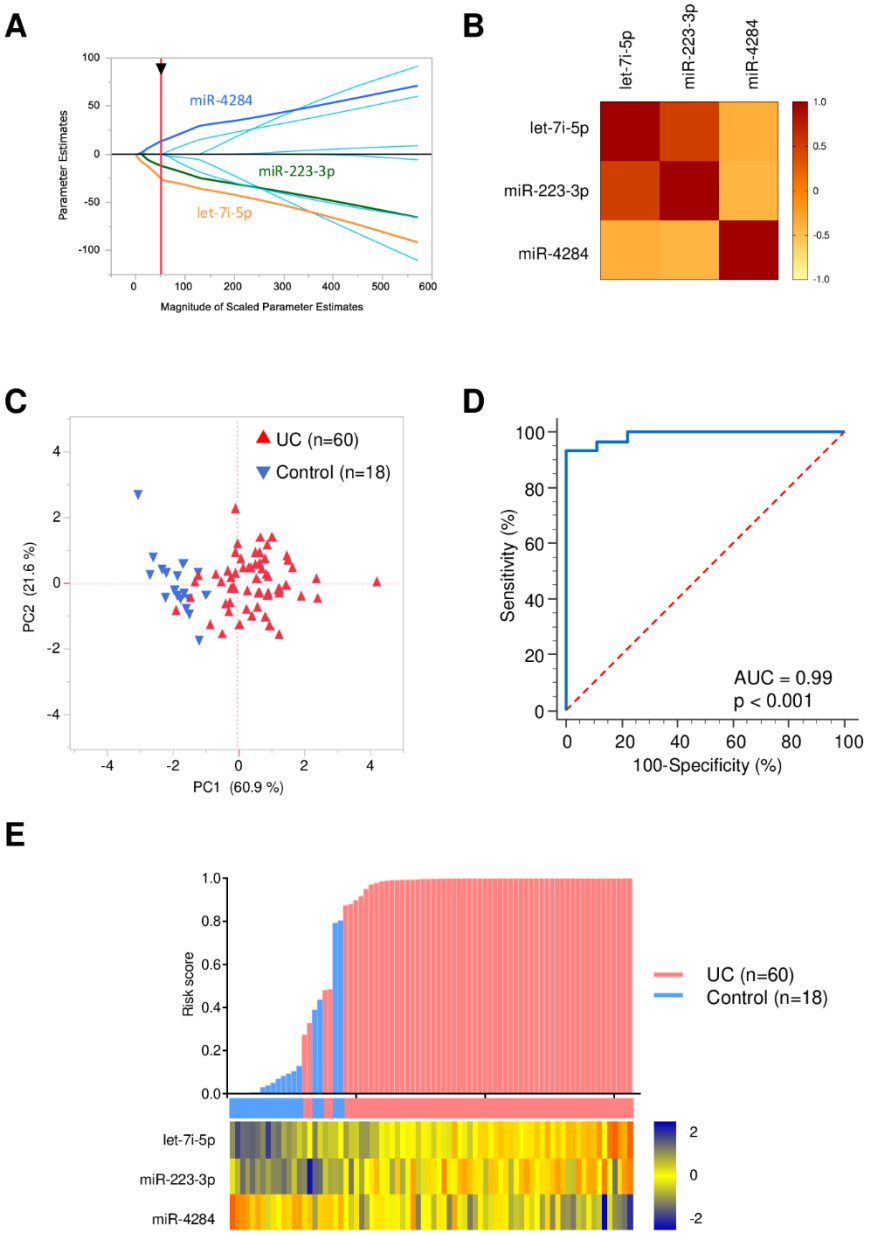
5.7. Authors' contribution to the knowledge

Despite advances in IBD diagnostics and therapy, there is still a need for biomarkers to accurately identify IBD patients and predict treatment response.[85] This knowledge could ultimately lead to more individualized approaches to patients. The identification of patients at high risk for a complicated disease course could then be crucial.[150, 151]

In the article by Jabandziev et al. (**Annex 4**),[140] we stratified therapeutically naive pediatric patients diagnosed with UC pancolitis according to the severity of their condition and prediction for standard treatment according to the specific expression of 10 candidate miRNAs that we identified in a previous review paper.[85] The work was created during the author's time in the laboratory of Prof. Ajay Goel, at the Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute of City of Hope, Los Angeles, CA, USA. The study enrolled therapeutically naïve, pediatric UC patients only with confirmed pancolitis. We examined formalin-fixed paraffin-embedded specimens of colonic tissue for expression of 10 selected candidate miRNAs. We performed receiver operating characteristic curve analysis, using area under the curve and a logistic regression model, to

evaluate the diagnostic and predictive power of the miRNA panels. The final analysis included 60 patients. As a control group, 18 children without macroscopic and microscopic signs of inflammatory bowel disease were examined. A combination of three candidate miRNAs (let-7i-5p, miR-223-3p, and miR-4284) enabled accurate detection of pediatric UC patients and controls (Figure 5). A panel of four candidate miRNAs (miR-375-3p, miR-146a-5p, miR-223-3p, and miR-200b-3p) was associated with UC severity in pediatric patients, and another combination of three miRNAs (miR-21-5p, miR-192-5p and miR-194-5p) was associated with early relapse of the disease. Nine patients out of the total were diagnosed with primary sclerosing cholangitis simultaneously with ulcerative colitis. A panel of 6 candidate miRNAs (miR-142-3p, miR-146a-5p, miR-223-3p, let-7i-5p, miR-192-5p, and miR-194-5p) identified those patients with primary sclerosing cholangitis. Specific combinations of miRNAs show promise as tools for potential use in precise disease identification and severity and prognostic stratification in pediatric patients with ulcerative pancolitis.[140]

Figure 5. Diagnostic accuracy of 3-miRNA panel for identifying UC patients.



(A) The adaptive LASSO model. (B) A correlation matrix displaying Spearman's rank correlation coefficient for each pair of three selected miRNAs. (C) Principal component analysis illustrating the good separation of UC-patient group and control group. (D) ROC curves for detecting UC patients using the 3-miRNA panel. (E) A waterfall plot representing risk score of each patient. Red and blue columns indicate UC patients and controls, respectively. A heat map illustrating expression levels of the three candidate miRNAs expressed differentially between UC patients and controls. The figure had been published in Jabandziev et al.[140]

6. Differential diagnostics

In the diagnostics of children with suspected IBD, it is necessary to differentiate a relatively wide range of diseases that may be similar in their symptoms to the manifestations of IBD.[30] Patients' history and specific clinical signs should always be taken into account. In thinking about differential diagnostics, it is necessary to consider (and also to exclude if possible) infectious causes, immune-inflammatory disorders, vascular ischemic disorders, drug-induced colitis, and many others.[152] Possible IBD-mimicking diseases are summarized in Table 4.[153, 154] Particularly very challenging in differential diagnosis can be to manage patients with VEO-IBD. Therefore, this topic is discussed in a following separate chapter.

Table 4. Differential diagnostics of IBD-mimicking diseases.

Infection	bacterial	Campylobacter jejuni, Salmonella sp., Shigella dysenteriae, Yersinia enterocolitica, enteroinvasive E. coli, Clostridium difficile etc.
	viral	Norovirus, Rotavirus, Coronavirus, Adenovirus, Echovirus, Cytomegalovirus, Herpes simplex virus type 2 etc.
	parasitic	Giardia lamblia, Entamoeba histolytica, etc.
	fungal	Candida crusei, Candida glabrata etc. (especially in immunocompromised patients)
	toxins	Vibrio cholerae (choleragen), Staphylococcus aureus (enterotoxin A-E), Clostridium difficile (toxin A, B) etc.
Drugs (drug-induced colitis)	microscopic colitis	non-steroidal anti-inflammatory drugs, proton pumps inhibitors, statins, β-blockers, etc.
	macroscopic colitis	chemotherapeutic drugs (e.g., taxane, platinum), immunomodulators (e.g., infliximab, adalimumab), laxatives, antibiotics etc.
	inflammatory colitis	non-steroidal anti-inflammatory drugs, immune checkpoint inhibitors (nivolumab) etc.
Vascular-ischemic disorders		Henoch-Schönlein purpura, systemic vasculitis (dermatomyositis, systemic lupus erythematodes), granulomatosis with angiitis
Immune-inflammatory (including monogenetic diseases)		eosinophilic gastroenteritis, severe combined immunodeficiency syndrome, common variable immunodeficiency diseases, interleukin-10 signaling defects, chronic granulomatous disease, X-linked lymphoproliferative syndrome type 1,2, Wiskott-Aldrich syndrome, Omenn syndrome, congenital neutropenia, Behcet's disease, autoimmune enteropathy, agammaglobulinemia, mevalonate kinase deficiency, etc.
Other		coeliac disease, lactose intolerance, irritable colon syndrome, glycogen storage disease type b, radiation colitis, intestinal lymphoma, Hermansky-Pudlak syndrome, trichohepatoenteric syndrome, sarcoidosis, laxative abuse, etc.

Table was prepared in accordance with Kliegman et al.[153] and Hamdeh et al.[154]

6.1. Very-early-onset IBD

In VEO-IBD patients who do not respond to standard therapy, we must consider the possible presence of primary immunodeficiency. This possibility should be considered especially in children under 2 years of age. A phenotypic aide-memoire summarizing key findings to ensure that a careful clinical history for VEO-IBD and examination are made to narrow the search for an underlying monogenetic defect is: YOUNG AGE MATTERS MOST (YOUNG AGE onset, **m**ultiple family members and consanguinity, **a**utoimmunity, **t**hriving failure, **t**reatment with conventional medication fails, **e**ndocrine concerns, **r**ecurrent infections or unexplained fever, **s**evere perianal disease, **m**acrophage activation syndrome and hemophagocytic lymphohistiocytosis, **o**bstruction and atresia of intestine, **s**kin lesions and dental and hair abnormalities, and **t**umors).[38] Warning signs that could ideally lead to suspicion of monogenic VEO-IBD or IBD-like disease are summarized in Table 5.[38]

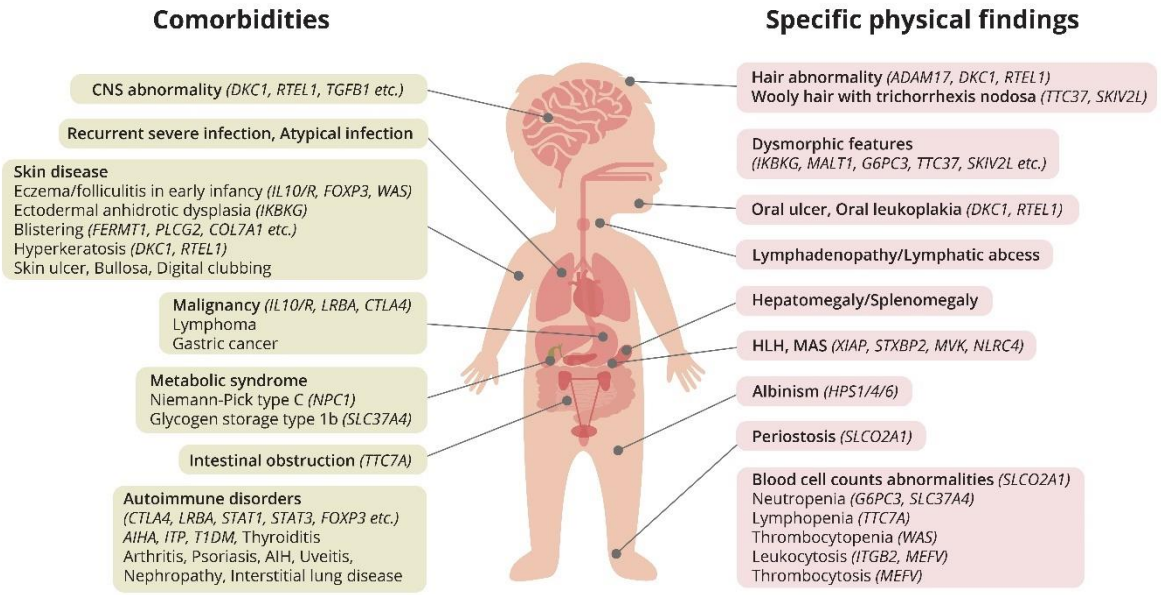
Table 5. Warning signs for suspecting monogenic IBD.

Key points	Comments
Very early age onset of IBD-like immunopathology	Likelihood increases with very early onset, particularly in those younger than 2 years of age at diagnosis
Family history	In particular, consanguinity, predominance of affected males in families, or multiple family members affected
Atypical endoscopic or histological findings	For example, extreme epithelial apoptosis or loss of germinal centers
Resistance to conventional therapies	Such as exclusive enteral nutrition, corticosteroids, and/or biological therapy
Skin lesions, nail dystrophy, or hair abnormalities	For example, epidermolysis bullosa, eczema, folliculitis, pyoderma or abscesses, woolly hair, or trichorrhexis nodosa
Severe or very early onset perianal disease	Fistulas and abscesses
Lymphoid organ abnormalities	For example, lymph node abscesses, splenomegaly
Recurrent or atypical infections	Intestinal and non-intestinal
Hemophagocytic lymphohistiocytosis	Induced by viral infections such as Epstein-Barr virus or cytomegalovirus, or macrophage activation syndrome
Associated autoimmunity	For example, arthritis, serositis, sclerosing cholangitis, anemia, and endocrine dysfunction, such as thyroiditis and type 1 diabetes mellitus
Early development of tumors	For example, non-Hodgkin lymphoma, skin tumors, hamartoma, thyroid tumors

Table was prepared in accordance with Uhlig et al.[38]

Physical findings and comorbidities of which physicians should be aware in suspicion of monogenic VEO-IBD or VEO-IBD-like syndromes at the initial physical examination and during follow-up are depicted in Figure 6.[155]

Figure 6. Key indicators of monogenic IBD in clinical practice.



In parentheses after the listed comorbidities and specific clinical findings are genes whose mutations are responsible for the disease manifestations. Figure was prepared in accordance with Nambu et al.[155] and created in collaboration with the Service Center for E-Learning at Masaryk University, Faculty of Informatics.

Certainly, a subset of the more aggressive, therapy-resistant VEO-IBDs, which have recently been regarded as associated with inborn errors of immunity (IEI), should be mentioned.[28, 156] The updated IEI list from the International Union of Immunological Societies phenotypic classification includes 406 IEI disorders with 430 gene defects.[157] To date, more than 50 monogenic defects associated with IBD or IBD-like phenotype have already been identified.[158] Gastrointestinal manifestation of IEI is common and may precede the primary diagnosis of IEI.[156, 158] From a pathophysiological point of view, VEO-IBD related to IEI manifests as a result of impaired barrier function of the intestinal epithelium, defects in bacterial killing by phagocytes, increased hyper- or autoimmune inflammatory pathways, or

impaired development and function of the adaptive immune system.[42, 97, 156, 158] The subgroups of IEI-associated VEO-IBD are summarized in Table 6.[28]

Table 6. Inborn error of immunity-associated VEO-IBD groups

• Genetic variants influencing the integrity of intestinal barrier • Genetic variants influencing bacterial recognition and clearance • Genetic variants in the IL-10-IL-10R pathway and related cytokine family members • Genetic variants impairing regulatory T cells • Genetic variants impairing development of the adaptive immune system • Genetic variants resulting in autoinflammatory disorders

Table was prepared in accordance with Kelsen et al.[28]

Because the management of monogenic IBD is different from that for classical IBD, it is crucial to identify these patients. The Paediatric IBD Porto Group of ESPGHAN recently published a position paper covering indications, technologies (targeted panel, exome and genome sequencing), gene panel setup, cost-effectiveness of genetic screening, and requirements for the clinical care setting.[39]

It is clear from the above that the differential diagnosis of IBD and especially of the VEO-IBD group is highly complex and challenging. Therefore, it should ideally be performed in IBD centers having sufficient experience and capability to perform adequate diagnosis.[159]

6.2. Authors' contribution to the knowledge

Phosphomannomutase-2 deficiency (PMM2-CDG)

Congenital disorders of glycosylation (CDG) comprise a large and heterogeneous group of more than 130 monogenic diseases.[160] CDG is caused by defects in the synthesis of glycans and in the attachment of glycans to proteins and lipids. Clinical manifestation is multisystemic.[161, 162] The N-linked protein glycosylation defect PMM2-CDG (phosphomannomutase-2 deficiency), previously known as CDG type Ia, was the first reported CDG and remains the most common CDG to date.[160] PMM2-CDG patients can be divided into those with neurological and those with neurovisceral phenotype.[163] Neurovisceral phenotype includes heart (pericardial effusion and cardiomyopathy), liver (high serum transaminases), and gastrointestinal (chronic diarrhea, feeding tube, and gastroesophageal reflux) involvement.[164, 165] In a retrospectively analyzed group of 96 French patients with PMM2-CDG, the presenting signs were mostly neurological (hypotonia, intellectual

disability, cerebellar syndrome) and observed in almost all the patients. In addition to neurological signs, a total of 38 patients exhibited visceral features including at least one of the following: feeding difficulty requiring nutritional support (n=23), cardiac features (n=20; pericarditis), hepato-gastrointestinal features (n=12; chronic diarrhea: 7, protein-losing enteropathy: 1, ascites: 3, liver failure: 1, portal hypertension: 1), kidney features (n=4), and hydrops fetalis (n=1).[164]

We described (**Annex 5**) an infant boy with PMM2-CDG and novel splicing mutation, who presented with pericardial effusion, typical dysmorphic facial features, inverted nipples, failure to thrive, and psychomotor retardation. Screening for CDG performed using isoelectric focusing of serum transferrin showed a typical PMM2-CDG pattern. Exome sequencing revealed one common pathogenic variant (c.691G > A/p.Val231Met) and one novel variant (c.447 C 3dupA) in the PMM2 gene. Both PMM2 variants were further confirmed by Sanger sequencing in both the proband and the parents' DNA. The novel variant was predicted to result in loss of donor splice site. Analysis at mRNA level confirmed that it leads to exon five skipping (r.348_447del) and causes premature termination of translation to the protein (p.G117Kfs-4).[149] Although CGD-MM2 is a relatively rare disorder, it can have gastrointestinal symptomatology and thus should be considered, especially in the differential diagnosis of patients with suspected VEO-IBD or VEO-IBD-like syndromes.

MIRAGE syndrome

A 2016 paper reported on a group of 11 patients with a newly identified syndrome that was termed MIRAGE.[166] MIRAGE is an acronym for the significant findings of **m**yelodysplasia, **i**nfection, **r**estriction of growth, **a**drenal hypoplasia, **g**enital phenotypes, and **e**nteropathy. Gastrointestinal complications include chronic diarrhea and esophageal dysfunction.[166] MIRAGE syndrome is an extremely rare disease. Several dozen patients have been described so far.[167-170]

We described the case report of a patient with MIRAGE syndrome (**Annex 6**).[171] The author cared for this patient for a long time at our department in the intensive care unit and ambulatory care. The reported patient had a novel mutation in *SAMD9* (c.2471 G>A, p.R824Q), manifesting with prominent gastrointestinal tract involvement and immunodeficiency. Among other major symptoms, he had difficulty swallowing, requiring percutaneous endoscopic gastrostomy, frequent gastrointestinal infections, and perianal

erosions. He suffered from repeated infections and periodic recurring fevers with elevation of inflammatory markers. At 26 months of age, he underwent hematopoietic stem cell transplantation that significantly improved hematological and immunological laboratory parameters. Nevertheless, he died at day 440 post-transplant due to sepsis. Even though it is a sporadic disease, SAMD9 mutations should be considered as a cause of enteropathy in pediatric patients, especially in combination with other described symptoms.[171]

Eosinophilic esophagitis

The finding of eosinophilic infiltration accompanies inflammatory diseases of the gastrointestinal tract of various origins, and the differentiation of primary and secondary forms is crucial for further diagnosis and treatment.[172] Primary eosinophilic gastrointestinal disorders (EGID) are a relatively new group of diseases. They can affect any part of the gastrointestinal tract and are characterized by mucosal infiltration of eosinophils in the absence of other causes for eosinophilia.[173, 174] We can diagnose eosinophilic esophagitis, eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic enteritis, and eosinophilic colitis, or a combined involvement of different parts of the gastrointestinal tract.[173] In populations of Western countries, eosinophilic esophagitis is by far the most common form.[172] These diseases present with a broad spectrum of often non-specific symptoms and with differential diagnosis ranging from functional gastrointestinal diseases to severe organic disorders, including IBD. Generally, clinical manifestation depends upon the location of eosinophilic infiltration. The pathophysiology of EGID is still unclear. It seems that food and aeroallergens play an essential role. The endoscopic appearance of EGID is not specific. A combination of serious clinical suspicion and histopathologically proven dense eosinophilic infiltration of the intestine are essential for diagnosis.[174] Unfortunately, with the exception of the esophagus, eosinophilic infiltration of the gastrointestinal wall is common.[175] Furthermore, we can find eosinophils in mucosal specimens in the cases of many inflammatory diseases, such as celiac disease, IBD, and parasitic infections. Moreover, eosinophilic infiltration of the gastrointestinal tract may accompany hypereosinophilic syndrome, vasculitis, as well as post-medicamentous and malignant diseases.[176]

In our observational survey (**Annex 7**),[177] we aimed to characterize features of eosinophilic esophagitis (EoE) diagnosed in five pediatric endoscopy centers and to describe local strategies for its treatment. This analysis focused on describing their general situation (age, gender, symptoms) and also aimed to investigate any possible linkage between age and

symptoms or length of diagnosis period. Demographic features; clinical symptoms; laboratory, endoscopic, and histopathological findings; and chosen treatment of patients were recorded and analyzed. We precisely described 33 new cases of children with EoE and their clinical characteristics. To our best knowledge, this was the first retrospective study on pediatric patients with EoE in the Czech Republic. We have proposed collecting long-term prospective observational data into a national EoE register of patients in the Czech Republic, as doing so would significantly improve our knowledge of this disease.[177]

Herpetic esophagitis

Endoscopic examination of the upper gastrointestinal tract comprises an integral part of the diagnostics process in patients with suspected IBD.[16, 125] Inflammation in upper gastrointestinal tract is described in approximately half of the children with IBD during the initial assessment. In a study by Castellaneta et al., the sites most frequently involved were the stomach (67%) followed by the esophagus (54%) and duodenum (22%).[178] From the pathological point of view, among the manifestations of pediatric IBD in upper gastrointestinal tract include lymphocytic esophagitis, focally enhanced gastritis, duodenal inflammation, and epithelioid granulomas.[179] In addition to IBD, differential diagnosis of ulcerative esophagitis must consider esophageal infections (herpes simplex, cytomegalovirus, varicella-zoster virus, candida, or various bacterial agents), trauma, esophageal burns, and Behcet's disease.[180, 181]

We described (**Annex 8**)[181] the case report of a 7-year-old immunocompetent boy with a suddenly occurring triad of symptoms: odynophagia, chest pain, and fever. In addition to other standard examinations, we performed esophagogastroduodenoscopy, where significant inflammatory changes transitioning to longitudinal ulcerations in the distal third of the esophagus were revealed.[181] In addition to the standard sampling of esophageal tissue, biopsy material was also sent for bacteriological and mycological examination as well as for the detection of herpes simplex virus (HSV) and cytomegalovirus by polymerase chain reaction (PCR). The result of HSV DNA detection by PCR from the esophageal mucosa was positive. Herpesvirus infections, in general, can complicate the course of IBD. Immunosuppressive therapy appears to increase the risk of herpesvirus reactivation and predispose to more severe infections. While cytomegalovirus colitis and systemic Epstein-Barr virus reactivation are well known, HSV is rarely involved. When HSV infections do occur, they are usually confined to sites of local reactivation, such as mucosal surfaces (e.g., the oropharynx,

anogenital region, and eyes) and sites on the skin. However, case reports describing disseminated HSV infection with colonic involvement have been published.[181, 182] The presented work demonstrates the necessity to consider the possible presence of HSV infection in differential diagnosis of endoscopic findings of gastrointestinal ulcerations.[181]

7. Therapy

The goals of treatment include to relieve disease symptoms, achieve intestinal healing, reach growth potential, and optimize the quality of life while limiting drug toxicity.[183] IBD therapy has come a long way in recent decades, and it will probably continue to undergo intensive development. There are national recommendations for the treatment of pediatric IBD patients,[96, 97] as well as recent international recommendations for the treatment of both UC and CD.[19-22] In an ideal situation, therapy of pediatric patients with IBD should be individualized, using a risk-stratification approach and predictors of poor outcome.[184] Treatment modalities could be divided into several groups, specifically medical therapy, nutritional therapy, and endoscopic and surgical therapy. Experimental therapeutic modalities, such as fecal microbial transplantation, plasma exchange, using of mesenchymal stromal cells, and others, have also been studied.[185-187] There also are some alternative approaches, which some patients require and about which the treating physician should be informed.[188]

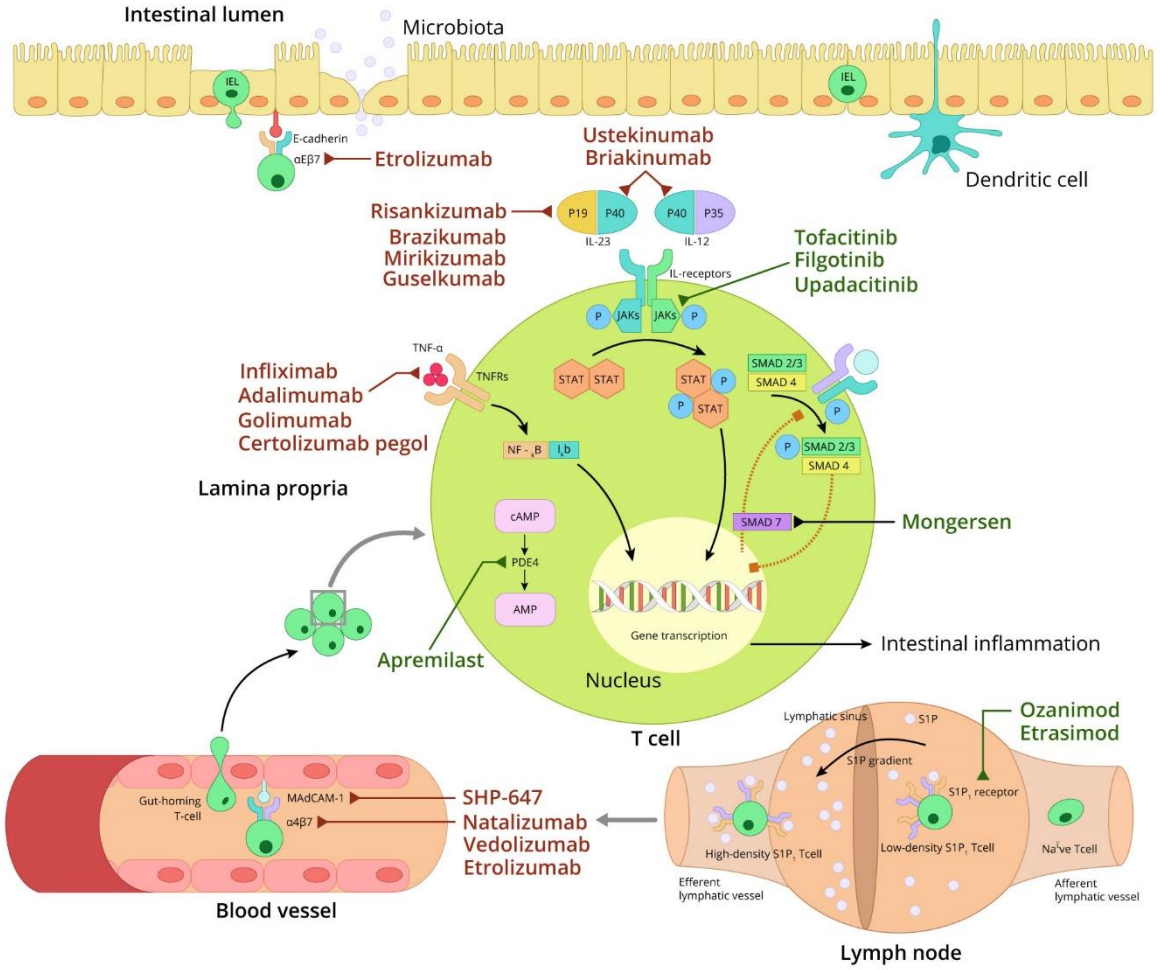
7.1. Medical therapy

Medical therapy has long been limited to so-called non-biological therapies (aminosalicylates, thiopurines, and steroids). Together with a better understanding of the etiopathogenesis of IBD, the development of biological therapy has been a significant advance in treatment.[189] Currently, tumor necrosis factor-alpha inhibitors infliximab and adalimumab are approved for use in pediatric patients in the Czech Republic. The use of other agents, such as ustekinumab and vedolizumab, is only possible in the so-called “off label” regimen after approval by the relevant health insurance company.[97] It can be expected, however, that other and novel therapies could enter the diagnostic repertoire of pediatric gastroenterologists. Generally, this occurs after experience has been gained with new drugs in the treatment of adult patients.[190] The new biologics and small molecule drugs block immune cell communication or migration. Novel small molecules include Janus kinase (JAK) inhibitors (tofacitinib), small mothers against decapentaplegic homolog (SMAD)7 antisense oligonucleotides (mongersen), sphingosine-1-phosphate (S1P) receptor modulators (ozanimod, etrasimod), and phosphodiesterase (PDE)4 inhibitors (apremilast). Other novel biologics include anti-integrins (vedolizumab, natalizumab), anti-cytokines (ustekinumab, risankizumab, brazikumab, mirikizumab, guselkumab), and anti-mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) (etrolizumab).[183, 191] Some of these drugs are already part of clinical practice, while others are being tested in various phases of clinical trials.

Cases of children being treated with combinations of biological agents with relatively promising results have already been described in the literature. Dual biological therapy could thus potentially expand the range of treatment options.[192]

Figure 7 summarizes the different therapeutic agents and their site of action within the pathophysiology of IBD.[191]

Figure 7. Therapeutic targets of novel biologics and small molecules for the treatment of inflammatory bowel disease.



Biologics and small molecules are indicated by red and green colors, respectively. AMP, adenosine monophosphate; cAMP, cyclic adenosine monophosphate; IEL, intraepithelial lymphocyte; Iκb, inhibitor of κB; IL, interleukin; JAK, Janus kinase; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; NF-κB, nuclear factor-κB; P, phosphorylation; PDE, phosphodiesterase; S1P, sphingosine-1-phosphate; SMAD, small mothers against decapentaplegic homolog; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TGF-βR, TGF-β receptor; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor. The figure was prepared in accordance with Na et al.[191] and created in collaboration with the Service Center for E-Learning at Masaryk University, Faculty of Informatics.

7.2. Nutritional therapy

In pediatrics, nutritional therapy can be used for two reasons: to improve the nutritional status of both UC and CD patients or as a means to induce remission in patients with a defined type of CD (exclusive enteral nutrition).[193] Recently, evidence has been published on the possibility of combining enteral nutrition and a special diet in the treatment of CD (Crohn's Disease Exclusion Diet, CDED), suggesting very promising results.[194, 195] At our department, we have relatively good experience with this treatment modality. In the context of IBD treatment, the effect of other diets has also been investigated, but with inconclusive results.[196-199]

7.3. Endoscopic and surgical therapy

Endoscopic examination and follow-up is a common modality in managing patients with IBD.[125] Endoscopic instrumentation allows us, for example, to dilate possible stenoses.[200] Surgical treatment has become a common part of comprehensive IBD treatment. In UC, this involves mainly subtotal or total colectomy with the construction of ileo-anal pouch anastomosis.[201] In CD, it is often an ileocecal resection performed because of irreducible inflammatory activity in this area, or resolution of symptomatic stenosis of the ileocecal valve.[202] The role of the surgeon in managing complications of perianal involvement (i.e., fistulas, abscesses) is indispensable.[203, 204]

7.4. Authors' contribution to the knowledge

Ileocecal resection is an integral part of treating selected pediatric and adult patients with CD,[201, 205] despite the fact that, concurrently with the development of biological therapies, the need for surgery is decreasing.[206] The issue of predicting postoperative disease recurrence, however, was not entirely clear. The aim of the study by Poredska et al. (**Annex 9**)[207] was to determine whether the histological activity of CD in resection margins after ileocecal resection is associated with early endoscopic recurrence of the disease. This study was conducted with adult patients, but some of these patients had been previously treated at the at the authors' workplace (i.e., the Department of Pediatrics). The resection line was always up in macroscopically healthy tissue. Ileocecal resected specimens were histologically examined for the presence of microscopic signs of CD, including both resection margins, that is, the small and large intestine. At 6 months postoperatively, patients underwent a follow-up

colonoscopy during which endoscopic recurrence was assessed according to the Rudgeerts score.[208] We investigated whether histological findings in the resection margins correlated with endoscopic recurrence of CD. Furthermore, the effects of preoperative therapy and other risk factors associated with endoscopic recurrence were evaluated. Endoscopic recurrence of CD at 6 months after surgery was observed in 23 patients out of a total of 107 enrolled. Microscopic evidence of CD in the resection margins was associated with significantly higher endoscopic recurrence in the anastomosis (56.5% versus 4.8%, $p<0.001$). Duration of disease from diagnosis to surgery ($p=0.006$) and length of resected bowel ($p=0.019$) were significantly longer in patients with proven endoscopic recurrence. Thus, it was shown that microscopic evidence of CD in the resection line in cases of ileocecal resection was significantly associated with a higher risk of early postoperative endoscopic recurrence. The results of a recent meta-analysis, where our work was included, showed that positive resection margins and myenteric plexitis and granulomas in the resection margins significantly increased the risk for postoperative recurrence of CD.[209] This is consistent with the results of our study.[207]

8. Complications

This chapter will discuss complications, both within and outside the digestive tract, that are so-called extraintestinal complications. It should be said that complications include here, from a somewhat broader point of view, manifestations that may be present as initial manifestations of the disease in individual patients (e.g., perianal fistulation). In the following text, the issue of potential adverse effects of medicament therapy will not be discussed further.

8.1. Complications from within the gastrointestinal tract

Strictures can develop practically anywhere in the gastrointestinal tract, and especially in patients with CD. These lesions may be asymptomatic or may require some type of intervention (dilatation or surgical resection).[210]

Gastrointestinal fistulas are abnormal connections between the bowel and neighboring organs. The occurrence of CD in childhood is associated with more aggressive development of perianal fistulas. Fistulas can occur in almost one-third of patients within 5–7 years after diagnosis.[211] Fistula types include perianal, entero-cutaneous, entero-enteric, entero-mesenteric, recto-vaginal, and entero-vesical.[212] Perianal fistulas are most common and commonly cause severe infections, fecal incontinence, perianal discharge, negative self-image, and social isolation. Perianal fistulas significantly reduce the quality of life of patients and are relatively difficult to treat,[213] but the introduction of anti-TNF has improved patients outcomes.[183, 214]

Toxic megacolon (TM) is one of the most feared complications of IBD, especially UC, and should be considered in these patients.[215] It is characterized by a combination of systemic toxicity (fever, tachycardia, leukocytosis, altered mental status) and segmental or total dilatation of the colon.[216] TM is fraught with high morbidity and mortality and can require surgical treatment. Various infections, especially *Clostridium difficile*, may also be a causative factor in the development of TM.[21, 212]

Other complications include primary sclerosing cholangitis, orofacial granulomatosis, and cholecystolithiasis.[217-220] A feared long-run complication is malignancy, inasmuch as the onset of IBD in childhood is associated with increased risk of any cancer, and especially of gastrointestinal cancers, both in childhood and later in life.[221]

8.2. Extraintestinal and systemic complications

Approximately one-third of children with CD and as many as 10% of children with UC suffer from growth impairment. The cause of growth failure is multifactorial and includes, for example, decreased appetite and decreased peroral intake, increased metabolic demand, malabsorption due to mucosal inflammation, growth hormone resistance due to inflammation, and use of corticosteroids. Therefore, thorough monitoring of linear growth is extremely important, and overall treatment effort should be directed to restoring normal growth.[12, 222] Along with the above factors, even lower physical activity can lead to lower bone mineral density,[223] as a higher incidence of low bone mineral density in pediatric IBD patients has been observed in children with CD. A higher rate of bone fracture in children with IBD was reported to follow corticosteroid treatment.[224] Patients with IBD also are at risk for deficiencies of various micronutrients, such as iron, folate, vitamin B_{12}, and vitamin D.[12] Psychosocial influences are not negligible, as children with IBD can have higher rates of depressive and anxiety disorders.[225] Various body systems can be affected, including musculoskeletal (arthritis, ankylosing spondylitis),[226] integumentary system (erythema nodosum, pyoderma gangrenosum),[227] ophthalmological (iritis, episcleritis),[228] urogenital (calcium oxalate stones),[229] hematological (anemia, thromboembolism)[230, 231] and immunological (infections).[212, 232, 233]

8.3. Authors' contribution to the knowledge

In general, IBD and its treatment are associated with significant morbidity and highly probable hospitalization.[234, 235] Hospitalization alone increases the risk of infectious complications.[236] IBD patients, and especially those with more severe disease courses, often require corticosteroids, biologics, and/or immunosuppressive agents. Underlying active disease and treatment with immune-suppressing therapy contributes to an increased risk of serious and opportunistic infections in these patients.[235, 237] Moreover, beyond immunological dysregulations commonly underlying IBD, risk factors for infections in children with IBD are represented by rare monogenic disorders, including primary immunodeficiencies, poor nutritional status, and, above all, in comparison to adults, more frequent need for early and/or combined immunosuppressive and biologic therapies.[232, 233]

Sepsis is a syndrome shaped by pathogen factors and host factors (e.g., sex, race and other genetic determinants, age, comorbidities, environment) with characteristics that evolve over time.[238, 239] What differentiates sepsis from infection is an aberrant or dysregulated host response and the presence of organ dysfunction. The clinical and biological phenotype of

sepsis can be modified by preexisting acute illness, long-standing comorbidities, medication, and interventions.[240] Despite significant advances in research and treatment strategies, sepsis remains among the most threatening conditions in intensive care units for children and adults.[241, 242] Considerable variabilities in the course of sepsis can be caused by, among other things, variants of the genes encoding the individual components of the immune system.[243] Only limited data was available describing the influence of single nucleotide polymorphisms and their combinations on the risk for development and severity of the course of sepsis in the pediatric population.[244]

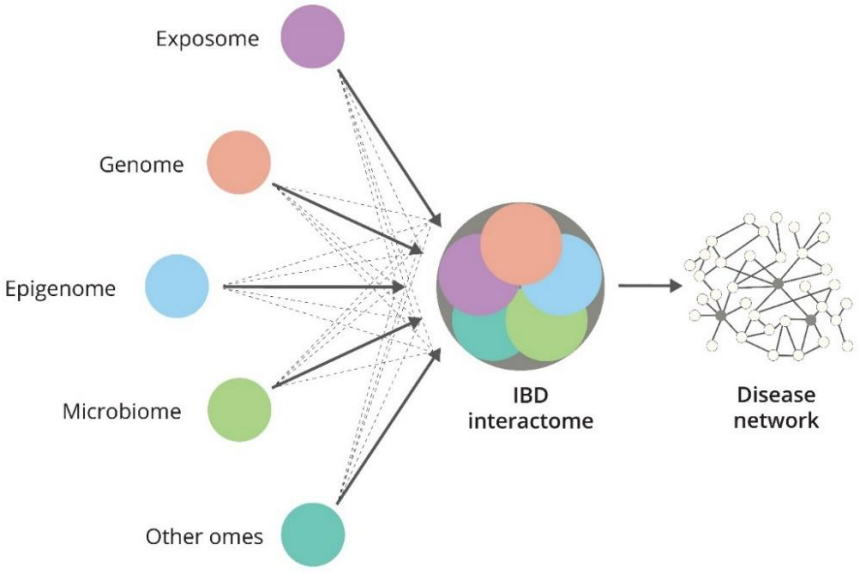
In our study (**Annex 10**), we revealed statistically significant differences in the structure of single nucleotide polymorphism combinations between the group of patients with septic conditions and the control group, as well as between the group of patients with the most severe conditions (severe sepsis, septic shock, and multiorgan dysfunction syndrome) and the control group. This approach has made it possible to describe low-, medium- and high-risk combinations of genetic polymorphisms in the development and subsequent severity of septic conditions. The study described a total of 23 pediatric patients who died due to a septic condition. One of these patients was treated for Crohn's disease and died due to septic shock.[244] Septic complications are relatively rare in pediatric patients with IBD, but the possibility for their occurrence should be kept in mind.[245]

9. Potential perspectives on further research

9.1. General perspectives on research in the field of IBD

Currently, research in the field of IBD is very intensive and the interest in this issue is constantly increasing. Barash et al. mapped IBD research from the MEDLINE/PubMed database for the 25-year period 1992 and 2016. A total of 18,653 relevant publications were classified as IBD-related and further analyzed. The annual number of publications increased almost 4-fold (from 354 to 1361) during the studied period.[246] Despite recent advances in research, however, the complexity of IBD still creates enormous challenges for researchers.[247] Current research is not fully able to satisfactorily address important research questions.[248] Further and deeper understanding of the pathophysiological processes in IBD would help to discover relevant biomarkers that could be used in both diagnosis and treatment.[81] A promising concept could relate to functional integration of those -omes relevant to the pathogenesis of IBD, such as the exposome, genome, epigenome, microbiome, and others, forming the so-called IBD interactome.[249, 250] This could be defined as a disease network within which dysregulation of individual -omes causes intestinal inflammation mediated by dysfunctional molecular networks controlling all biological events (Figure 8).[250]

Figure 8. Functional integration of -omes relevant to the pathogenesis of the IBD-forming IBD interactome.



The hypothetical disease network on the right shows the various molecular hubs (white nodes) of the network, which is regulated by central regulatory hubs (gray nodes). The figure was prepared in accordance with Souza et al.[250] and created in collaboration with the Service Center for E-Learning at Masaryk University, Faculty of Informatics.

In step with a better understanding of the pathogenesis of IBD, the portfolio of therapeutic options continues to expand. Nevertheless, new therapeutics can be costly, and patients may stop responding to therapies or not respond to them in the first place. Therefore, there is a growing need for more personalized therapy based on insights into the biology of the underlying disease and a drive to change our approach from reactive treatment driven by disease complications to proactive care to prevent disease sequelae.[72] This thinking embraces a clear desire to make medicine as individualized – or, more precisely, as personalized – as possible. Personalized medicine involves identifying patients at high risk of progression and complications and better characterizing patients who may respond preferentially to specific treatments.[251] In an even narrower sense, we have recently encountered the term precision medicine. The concepts of personalized medicine and precision medicine are very similar, but precision medicine also includes a multidisciplinary, data-driven approach to support better clinical decision-making through a clear understanding of the molecular basis of an individual's disease.[72] Precision medicine in this sense means tailoring treatment to the individual patient and incorporating different data-driven (and multi-omics) approaches to support accurate clinical decision-making. In the case of IBD, precision medicine would be of significant benefit, as it would allow for early treatment that is both effective and appropriate for the individual.[72] In summary, there is a need to understand and predict the natural course of IBD-disease susceptibility, activity, and behavior; to predict disease progression and response to therapy; and to optimize current and develop new molecular technologies.[252] Precision medicine could provide the ways to do just that. To achieve this, prospective longitudinal cohort studies are needed to identify and validate precision medicine biomarkers for predicting disease progression and for predicting and monitoring treatment response. Methodological harmonization across studies together with the development of standardized methods and infrastructure are key to achieving this goal.[252] It should be borne in mind that the overall situation can change quite a lot. After all, who among caregivers and patients could have guessed the substantial changes to the routine management of IBD that we experienced during the unexpected coronavirus disease 2019 (COVID-19) pandemic?[253]

9.2. Author's perspectives on research in the field of IBD

Microbial dysbiosis and microRNA

Together with colleagues from the Department of Pathology, University Hospital Brno and Central European Institute of Technology, we obtained grant funds from the *Czech Health*

Research Council for carrying out our research project entitled: *Study on microbial dysbiosis and microRNA deregulation as a basis of the therapy individualization in pediatric patients with inflammatory bowel disease*. Work on the project is already fully underway. As part of this research, we will work with our biobank, where we already have material from more than 100 pediatric patients with newly diagnosed IBD. The specific aims of the project are to:

- identify miRNAs specific for IBD patients by global profiling of miRNA expression in the tissues and stools of IBD patients compared to healthy control subjects and miRNAs specific for different IBD subtypes (classes) by comparing miRNA expression profiles in therapeutically naïve patients;

- establish an miRNA predictive panel to identify patients with good and bad response to a standard therapeutic regimen;

- validate and analytically characterize candidate diagnostic, prognostic, and predictive miRNAs on an independent cohort of IBD patients;

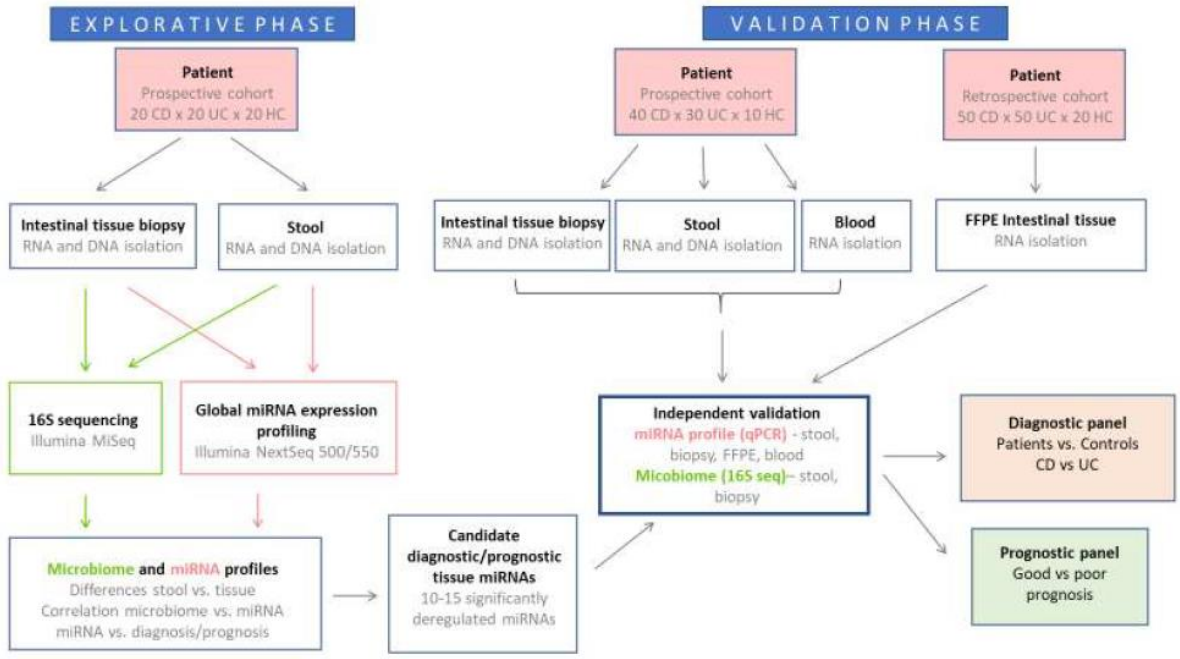
- analyze microbiome composition in stool and tissue biopsy samples and identify specific changes in microbiome composition in IBD patients compared to healthy controls and in patients with different IBD subtypes;

- compare levels of candidate diagnostic, prognostic, and predictive miRNAs in tissue, stool and plasma and correlate these with specific changes in the composition of the intestinal microbiome; and

- propose a prospective clinical trial verifying the clinical benefit of identified miRNAs in improving the diagnostic and therapeutic approach to pediatric patients with IBD.

A schematic representation of the study design is depicted in Figure 9.

Figure 9. Schematic representation of the study design.



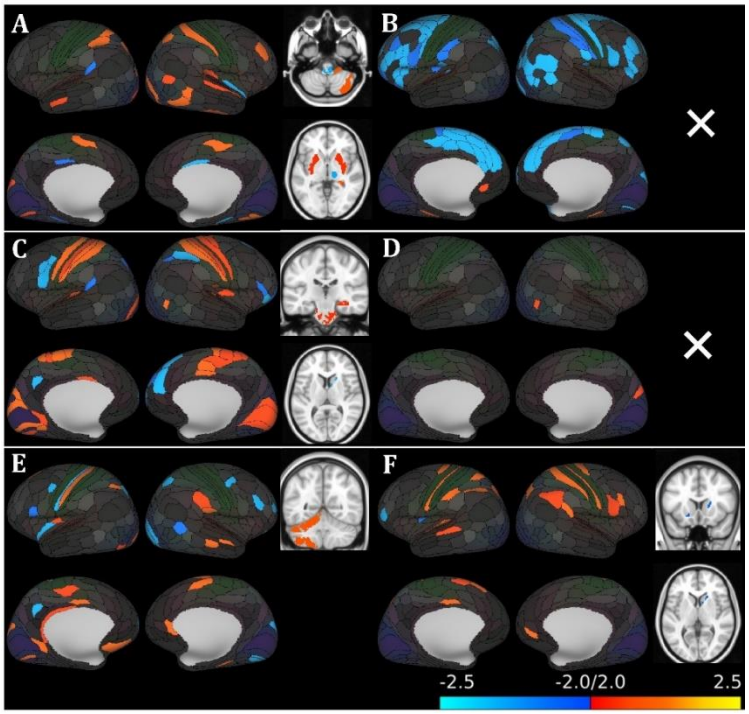
CD – Crohn's disease; UC – ulcerative colitis; HC – healthy control.

Brain alterations in pediatric patients with IBD

Central nervous system (CNS) involvement has been reported in pediatric and adult IBD patients, with both neurological and psychiatric phenomena.[254-259] To date, there are not many studies investigating directly affection of the CNS in CD, especially in children. Research on possible cerebral involvement in IBD generally and CD specifically has been largely marginalized and failed to capitalize on recent developments in magnetic resonance imaging (MRI). In a cross-sectional pilot study, we searched for eventual macrostructural, microstructural, and functional brain affection in children with CD early after the disease's onset. Nine pediatric CD patients within 2 years of disease development and at the same time with their disease in remission (according to FCP and PCDAI) and nine healthy controls underwent structural, diffusion-weighted imaging and resting-state functional MRI acquisition in combination with extensive neuropsychological testing. While no differences in cortical thickness between CD patients and healthy controls were found, alterations were detected in diffusion tensor imaging parameters over vast cortical regions essential for regulation of the autonomous nervous system, sensorimotor processing, cognition, and behavior. These alternations were accompanied by generally increased functional and structural connectivity

(Figure 10). Although still requiring further validation in longitudinal projects enrolling more significant numbers of subjects, this study sets out possible directions for further research. The discussed data are from an article that is now under review at the journal Frontiers in Pediatrics. In the future, we would like to seek grant funding to conduct a prospective study on microstructural changes in the brain of pediatric patients after the diagnosis of IBD.

Figure 10. Results of parcellated analysis comparing Crohn's disease patients and healthy controls.



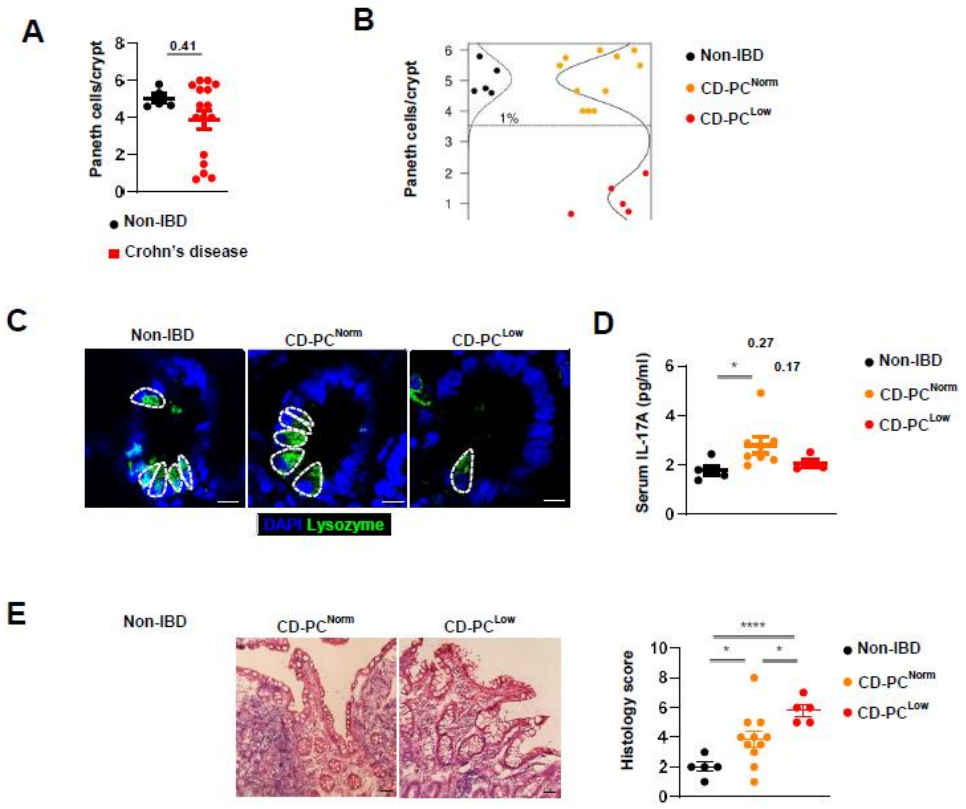
(A) fractional anisotropy, (B) mean diffusivity, (C) degree centrality, (D) amplitude of low-frequency fluctuations (no significant results), (E) structural connectivity, and (F) partial functional connectivity regularized using ridge regression. Absence of significant results (subcortical outcomes for mean diffusivity amplitude of low frequency fluctuations and structural connectivity) is marked with "×".

Paneth cells and interleukin 17

Toward better understanding IBD pathophysiology, we investigated, in cooperation with colleagues from the Institute of Molecular Genetics of the Czech Academy of Sciences (IMG CAS), the role of interleukin 17 (IL-17) on Paneth cells (PCs). Paneth cells are a highly specialized cell type with many functions in intestinal physiology.[260] Recently, there is increasing evidence for a potential role of PCs in the development of ileal CD.[261] As

mentioned above, microbiota composition regulation is essential for intestinal homeostasis when it is known that IL-17 regulates antimicrobial peptide production on epithelial surfaces.[262] The effect of IL-17 on PCs is nevertheless still unclear. We showed that PCs express high levels of surface IL-17 receptor (IL-17R), and targeted ablation of IL-17R in PCs decreases the cellularity of ileal PCs and the expression of their enteric α-defensins. Mice with PCs lacking IL-17R showed upregulated inflammatory pathways and higher severity of induced ileitis. These changes were associated with lower gut microbiota diversity, capable of inflicting death upon its transfer to genetically susceptible mice. Strikingly, a sub-cohort of pediatric patients with newly diagnosed Crohn's disease displayed a low number of ileal PCs, high ileitis severity score, and diminished serum levels of IL-17, thereby resembling the phenotype of mice with IL-17R-deficient PCs. Our study identified IL-17 signaling in PCs as an essential contributor to ileal homeostasis acting via the prevention of dysbiosis (Figure 11). The discussed data are from an article that is now under review at the journal Cell Reports. We will continue our cooperation with IMG CAS on research in this field.

Figure 11. Patients with CD manifest low ileum PC numbers, which correlate with low serum IL-17 and terminal ileitis.



*(A–C) Immunofluorescence microscopy analysis of frozen ileum biopsies from CD and non-IBD patients. Sections were stained with lysozyme (PCs, green) and DAPI (nuclei, blue). (A) Graph shows average numbers of PCs in patient/biopsy per crypt. (B) The PC number shows bimodal distribution in CD patients. The number of PCs per crypt is plotted arbitrarily for non-IBD and CD individuals, together with the best normal (dashed curve) and bimodal normal (full line) fits to the data, respectively. All samples in the CD-PCLow group of CD patients lie below the first percentile of non-IBD individuals and CD-PCNorm patients (horizontal dashed line). Non-IBD n=5, CD-PCNorm n=11, and CD-PCLow n=5. (C) Representative images of ileum crypts from non-IBD patients (left), CD-PCNorm (middle), and CD-PCLow patients (right). Scale bar represents 10 μm. (D) Level of IL-17A measured by ELISA in the serum of patients analyzed and stratified in B (sera were not available from 3 patients in CD-PCNorm and 1 patient in the CD-PCLow cohort). Data shown was tested by Student's t-test. (E) Representative ilea hematoxylin and eosin staining (left) and histopathology scoring (right) from biopsies of patients analyzed and stratified in B. Scale bar represents 50 μm. Data in (A), (D), and (E) have been tested by Student's t-test. p-values >0.05 are shown. *, p<0.05; ****, p<0.0001. Horizontal lines show mean±SEM.*

10. Conclusions

Comprehensive management of IBD patients, especially in children, is a major challenge for gastroenterologists and other health care professionals worldwide. In addition to its complexity, however, it represents a fascinating and dynamically changing field of medicine to which it is worth devoting one's efforts. The approach to diagnosis, follow-up, and management of IBD has undergone a major transformation in recent decades and can be expected to continue dynamically to evolve. Together with colleagues, we intend to continue research in this area. We would be delighted if our findings would, at least in part, contribute to better care for pediatric IBD patients.

List of abbreviations

CD	Crohn’s disease
CDED	Crohn’s disease exclusion diet
CDG	congenital disorders of glycosylation
CLE	confocal laser endomicroscopy
CRP	C-reactive protein
EGID	eosinophilic gastrointestinal disorders
EoE	eosinophilic esophagitis
ESPGHAN	European Society of Paediatric Gastroenterology, Hepatology and Nutrition
FCP	fecal calprotectin
FOPG	fecal osteoprotegerin
GWAS	genome-wide association studies
HMGB1	high mobility group box 1
HSV	herpes simplex virus
IBD	inflammatory bowel disease
IBDU	inflammatory bowel disease unclassified
IEI	inborn errors of immunity
IL-17	interleukin 17
JAK	Janus kinase
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
miRNAs	microRNAs
MRI	magnetic resonance imaging
ncRNAs	noncoding RNAs
NGS	next-generation sequencing
PCDAI	Pediatric Crohn’s Disease Activity Index
PCR	polymerase chain reaction
PCs	Paneth cells
PDE	phosphodiesterase
PIBD	pediatric inflammatory bowel disease
piRNAs	PIWI-interacting RNAs

PMM2	phosphomannomutase 2
PUCAI	Pediatric Ulcerative Colitis Activity Index
RNA	ribonucleic acid
S1P	sphingosine-1-phosphate
siRNAs	small interfering RNAs
SMAD	small mothers against decapentaplegic homolog
THES	trichohepatoenteric syndrome
TM	toxic megacolon
TuM2-PK	tumor pyruvate kinase isoenzyme type M2
UC	ulcerative colitis
VEO-IBD	very-early-onset inflammatory bowel disease
WES	whole-exome sequencing

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List of Annexes

(Selected publications arranged in order of appearance in the text)

Annex 1

Jabandziev P, Hlavackova E, Bily V, Karaskova E, Kunovsky L, Ravcukova B, Grombirikova H, Kozumplikova R, Buckova H, Slaba K, Pinkasova T, Jouza M, Pecl J, Jezova, M, Curtisova V, Freiberger T. Trichohepatoenteric syndrome in a patient with TTC37 mutations - case report. Gastroent Hepatol. 2020; 74 (6), 481–487.

Annex 2

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Annex 1

doi: 10.48095/ccgh2020481

Trichohepatoenteric syndrome in a patient with *TTC37* mutations – a case report

Trichohepatoenterický syndrom u pacienta s mutacemi genu *TTC37* – kazuistika

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Summary: We report a patient with somatic retardation and woolly hair appearance who suffered from recurring episodes of watery mucous diarrhea, impaired liver functions, and failure to thrive. He manifested with severe infection courses, including hepatitis of unknown origin complicated by liver failure at 4 months, bronchopneumonia at 4 years, and life-threatening sepsis with septic shock at 8 years of age. Esophagogastroduodenoscopy and colonoscopy were performed at 4 years to rule out inflammatory bowel disease (IBD), and only signs of nonspecific colitis were evident. Immunology workup revealed slight reduction in CD4+ naive subsets and impaired T cell response to mitogens. Massive parallel sequencing (also termed next-generation sequencing – NGS) targeting a panel of primary immunodeficiency-related genes was used to examine the patient's DNA. NGS analysis revealed two heterozygous variants in the *TTC37* gene. Nonsense p.Arg1201* and missense p.Leu1505Ser variants in exons 34 and 42, respectively, were evaluated as pathogenic based on *in silico* predictions, their rare occurrence in the general population, and the fact that both mutations had already been described in patients with trichohepatoenteric syndrome (THES). As clinical features in our patient were in accordance with this diagnosis, we consider our findings as causative. THES could be a life-threatening condition, particularly in children who develop liver disease or severe infection courses. THES can have a similar clinical presentation as does very early-onset inflammatory bowel disease (VEO-IBD) and is often assigned to this group. Although IBD is generally regarded as a polygenic disease, some children with VEO-IBD are known also to have diseases with monogenic etiologies, as in THES. Targeted NGS is an efficient tool for establishing an accurate diagnosis in VEO-IBD patients.

Key words: trichohepatoenteric syndrome – very early-onset inflammatory bowel disease – children – next-generation sequencing

Souhrn: Prezentujeme kazuistiku pacienta se somatickou retardací a abnormálním vzhledem vlasů, který trpěl opakovanými epizodami vodnatého hlenovitého průjmu, postihem jaterních funkcí a neprospíváním. U pacienta byly pozorovány těžké průběhy infekcí včetně hepatitidy neznámého původu komplikované selháním jater ve 4 měsících, bronchopneumonie ve 4 letech a septického šoku ve věku 8 let. Ezofagogastroduodenoskopie a koloskopie byly provedeny ve 4 letech, k vyloučení zánětlivého onemocnění střev (IBD). Histologicky však byly patrné pouze známky nespecifické kolitidy. Imunologické vyšetření odhalilo snížený počet naivních CD4+ lymfocytů a sníženou T-lymfoproliferativní odpověď na mitogeny. K vyšetření vzorku DNA pacienta bylo použito masivní paralelní sekvenování (nazývané také sekvenování nové generace, next generation sequencing – NGS) zaměřené na panel genů souvisejících s primárními imunodeficiencemi. NGS analýza odhalila dvě heterozygotní varianty v genu *TTC37*. Nonsense varianta p.Arg1201* a missense varianta p.Leu1505Ser v exonu 34, resp. 42, byly vyhodnoceny jako patogenní na základě predikce *in silico* a jejich vzácného výskytu v obecné populaci. Vzhledem k tomu, že obě mutace již byly popsány u pacientů s trichohepatoenterickým syndromem (THES) a klinické známky u našeho pacienta byly v souladu s touto diagnózou, považujeme naše zjištění za příčinné. THES je v dětství často život ohrožujícím onemocněním, zejména u pacientů, u kterých se rozvine onemocnění jater nebo trpí závažným průběhem infekčních onemocnění. THES může mít podobný klinický obraz jako zánětlivé onemocnění střev s velmi časným začátkem (very early-onset inflammatory bowel disease – VEO-IBD) a je často do této skupiny přiřazován. IBD jsou obecně považována za polygenní onemocnění, nicméně u některých dětí s VEO-IBD je známo, že jejich onemocnění má monogenní etiologii, tak jako je tomu u THES. Cílený NGS je účinným nástrojem pro jednoznačné stanovení diagnózy u pacientů s VEO-IBD.

Klíčová slova: trichohepatoenterický syndrom – zánětlivá onemocnění střev s velmi časným začátkem – děti – sekvenování nové generace

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder the incidence of which has been increasing rapidly in the past decade, and especially in child populations [1,2]. Very early-onset IBD (VEO-IBD) comprises a group of children who are diagnosed before 6 years of age and includes a diverse spectrum of rare genetic disorders. Some of these monogenic disorders do not respond to conventional therapy and demand effective targeted therapies due to their association with high morbidity and mortality. Because these diseases are rare and can manifest with heterogeneous phenotypes, a correct diagnosis is often delayed [3]. The key component of diagnosis is a combination of clinical history, evaluation of immune functions, and molecular genetic analysis using targeted sequencing panels or whole-exome sequencing (WES). Sequencing today plays an increasingly important diagnostic role. A large number of immunodeficiencies and epithelial cell defects can be associated with VEO-IBD and severe phenotypes. Early identification of VEO-IBD with a genetic basis and, in particular, immunodeficiencies is crucial for assessing prognosis and targeting therapy to the dysfunctional pathway [4,5].

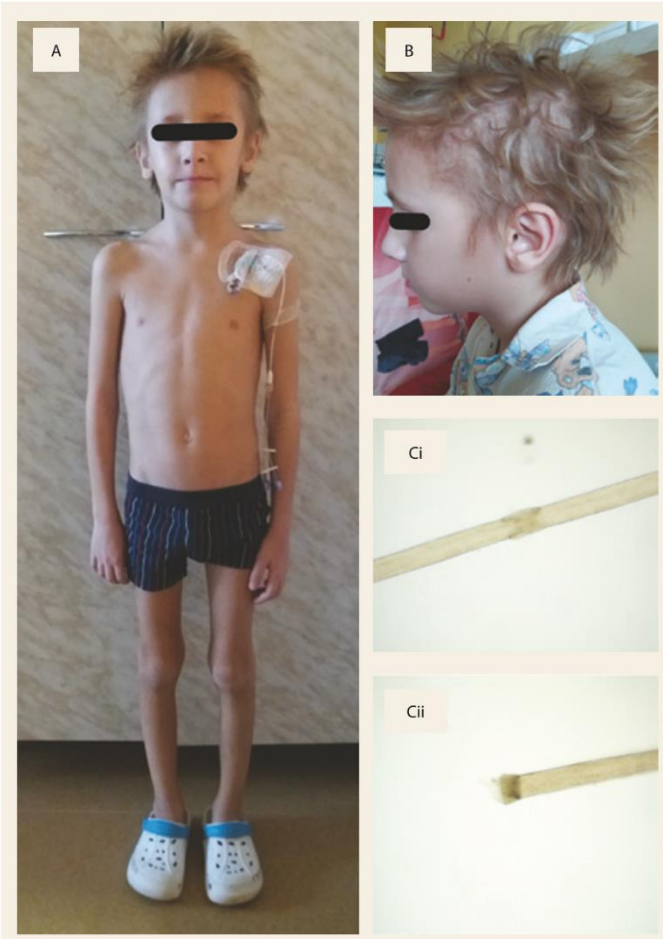

Fig. 1. Clinical data of the patient.
A – Overall habitus of the patient. B – Detail of the hairy part of the head. Brittle, easily breakable hair. Ci – Hair analysis using light microscopy showing trichorrhexis nodosa. Magnification 40×. Cii – Bristly split hair ends. Magnification 40×.
Obr. 1. Klinická data pacienta.
A – Celkový habitus pacienta. B – Detail vlasaté části hlavy. Křehké, snadno lomivé vlasy. Ci – Analýza vlasu pomocí světelného mikroskopu. Trichorrhexis nodosa. Zvětšení 40×. Cii – Roztřepené štětkovité konečky vlasů. Zvětšení 40×.

Case report

We present the case report of a 9-year-old boy with chronic watery stools with mucus, failure to thrive, growth impairment, and medical history of several severe infection courses at the time of diagnosis.

This first child from a first, uncomplicated pregnancy was born in the 40th week of gestation to Caucasian nonconsanguineous parents. Delivery was spontaneous, birth weight was 3,000 grams, birth length was 49 cm, and the Apgar score was of no physiological concern (10-10-10). The patient was fully breastfed, and total time of breastfeeding was 4 months. At 4 months of age, he was admitted to the hospital with hepatitis of unknown origin complicated by liver failure. The patient had an episode of rotavirus gastroenteritis at 2 years of age. *Streptococcus pneumoniae* pneumonia manifested at 4 years of age. Failure to thrive and frequent chronic watery stools with mucus and without blood was evident from the second year of life. Esophagogastroduodenoscopy and colonoscopy were carried out at the age of 4 years without signs of macroscopic pathology. Histologically, only nodular hyperplasia of the terminal ileum was confirmed, and signs of nonspecific colitis were evident. Due to the patient's persistent clinical symptoms, it was de-

cided to perform a control colonoscopy at 8 years of age. During a routine standard bowel emptying before examination, the patient developed septic shock with pleuropneumonia leading to lung failure with the need for artificial ventilation. Throughout the follow-up, the patient received no specific therapy other than symptomatic.

The patient's appearance was marked by a short, asthenic stature, tiny face, and brittle blond hair (Fig. 1). There was no skin or joint pathology present. Mental impairment was not recognized. Because there were signs of gastrointestinal tract impairment and medical history of severe infection courses, an inborn error of immunity (IEI) was suspected. Complement or phagocytoses deficiency was ruled out. The patient revealed no laboratory sign of antibody deficiency, and therefore cellular deficiency was suspected. Slight IgA hypergammaglobulinemia without any sign of IgG or IgM hypogammaglobulinemia was present. Routine post-vaccination antibody levels of IgG against pneumococcal capsular polysaccharide, tetanus toxoid, and *Haemophilus influenzae* type b antigen were protective. Polysaccharide antigen response was not evaluated. The absolute counts and percentage numbers of T CD3+CD4+, NK CD16/56+, and B CD19+ lymphocyte subsets remained within the reference range. Slight T CD3+CD8+ lymphocytosis and mild reduction of CD3+CD4+ naive subsets were present. T cell lymphoproliferative response to mitogens was impaired (ConA, PHE, antiCD3antiCD28). Interpretation of decreased T cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) could be questionable at this age.

Next-generation sequencing (NGS) targeting a panel of primary immunodeficiency-related genes was used to examine the patient's DNA. The NGS analysis revealed two heterozygous variants in the *TTC37* gene (Fig. 2). No additional relevant pathogenic or likely pathogenic mutations were identified by NGS. Both nonsense p.Arg1201* and missense p.Leu1505Ser variants in exons 34 and 42, respectively, were evaluated as pathogenic based on *in silico* predictions and/or their rare occurrence in the general population. Considering the facts that both mutations had already been described in patients with trichohepatoenteric syndrome (THES) and that clinical features in our patient were in accordance with this diagnosis, we considered our finding as causative, even though it could not be confirmed that both alleles were affected due to the impossibility to investigate the DNA of the patient's parents.

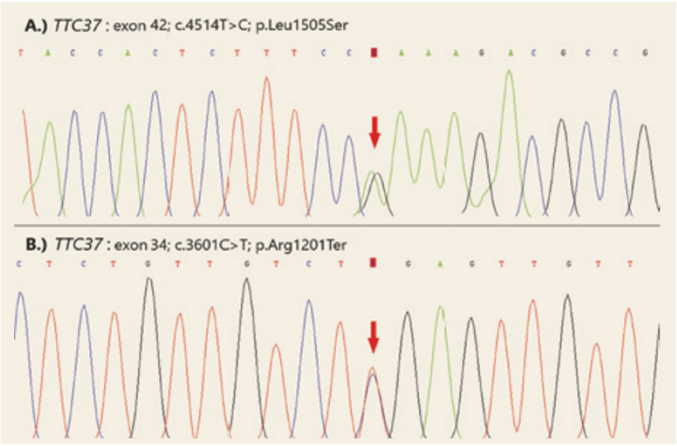

Fig. 2. Confirmation of *TTC37* variants by Sanger sequencing.
Chromatograms display: A – Part of exon 42 reverse sequence containing c.4514T>C variant. B – Part of exon 34 sequence containing c.3601C>T variant. Both variants indicated by red arrow.
Obr. 2. Potvrzení variant *TTC37* metodou Sangerova sekvenování.
Chromatogramy zobrazují: A – Část reverzní sekvence exonu 42 obsahující variantu c.4514T> C. B – Část sekvence exonu 34 obsahující variantu c.3601C> T; obě varianty jsou označeny červenou šipkou.

From the age of 8 years, the patient was stable without manifestation of any severe infectious course with persistent diarrhea. Sadly, the patient died tragically at age 10 for reasons unrelated to this main diagnosis.

Discussion

THES is a rare autosomal recessive syndromic enteropathy with a recently described molecular basis. To date, only about 100 affected individuals have been reported in the literature [6]. THES is a potentially life-threatening pediatric condition, particularly in children with liver disease or severe infection courses. Our patient also went through several life-threatening events, although his death in a car accident was not related to this condition. THES is generally considered to be a neonatal enteropathy and is characterized by intractable diarrhea (seen in almost all affected children), woolly hair (seen in all), intrauterine growth restriction, facial dysmorphism, and short stature. Additional findings include recurrent or severe infections, skin abnormalities, and liver disease. Mild intellectual disability is seen in about 50% of affected individuals. Less common findings include congenital heart defects and platelet anomalies [6–8]. Similarly to VEO-IBD, THES is characterized as a combined immunodeficiency with associated syndromic features according to the 2019 International Union of Immunological Societies (IUIS) updated phenotypical classification [9]. Immu-

Tab. 1. Inborn error of immunity-associated VEO-IBD groups (adapted according to [13]).
Tab. 1. Vrozené poruchy imunity asociované s VEO-IBD (upraveno podle [13]).

Genetic variants influencing the integrity of intestinal barrier
Genetic variants influencing bacterial recognition and clearance
Genetic variants in the IL-10-IL-10R pathway and related cytokine family members
Genetic variants impairing regulatory T cells
Genetic variants impairing development of the adaptive immune system
Genetic variants resulting in autoinflammatory disorders

nology workup in such cases could reveal hypogammaglobulinemia (IgG, IgA), IgA monoclonal gammopathy, impaired post-vaccination response, and/or impaired interferon gamma (IFNγ) production [6,9].

THES is caused by mutation of either *TTC37* or *SKIV2L*, two genes that encode two components of the human SKI complex [6,8,10]. The SKI complex is a tetraprotein complex including an RNA helicase subunit encoded by *SKIV2L*, a subunit made of a tetratrico peptide repeats (TPR) protein encoded by *TTC37*, and two subunits of a WD40 (Trp/Asp repeats) domain containing protein encoded by *WDR61*. The SKI complex is the cofactor of the RNA exosome and is involved in such specific functions of the RNA exosome as cytoplasmic mRNA degradation, particularly in case of STOP loss and/or gain and post-endoribonucleolytic cleavage residues decay. The SKI complex is also known to play a role in antiviral responses [6,11].

To date, THES is the only known Mendelian disorder associated with these two *TTC37* and *SKIV2L* genes. It is inherited as an autosomal recessive trait, with a penetrance of 100%. With the exceptions of a few patients evidencing only one mutation, only strict homozygous or compound heterozygous cases have been reported, and digenic inheritance has never been observed. Generally, THES is characterized by the association of nine main clinical signs: intractable diarrhea, hair abnormalities, facial dysmorphism, intrauterine growth restriction (IUGR), immunodeficiency, skin abnormalities, liver disease, congenital cardiac defects, and platelet anomalies. Bourgeois et al stated that patients mutated in *SKIV2L* seem to be more severely affected than are patients with *TTC37* mutations, with several signs being noticed earlier in life or more serious. In fact, 37% of *TTC37* defective patients have been observed to have cardiac defects, 39% skin abnormalities, 51% liver disease, 56% immune defect, 70% IUGR, 84% facial dysmorphia, 95% hair abnormalities, and 100% diarrhea [6,7]. Clinical features of our patient were in full accordance with the diagnosis of THES (liver disease, immune defect, facial dysmorphia, hair abnormalities, and diarrhea).

Very-early-onset IBD

THES can have a clinical presentation similar to that of VEO-IBD and is often assigned to this group. Evaluating and managing children with VEO-IBD is a challenging field for pediatric gastroenterologists worldwide [12]. The incidence of childhood IBD is increasing and constitutes 8–25% of all IBD cases, with 6–15% presenting under the age of 6 years [1–3,13,14]. Childhood IBD is understood as IBD onset at younger than 17 years of age, where IBD diagnosed before 6 years of age constitutes VEO-IBD. Infantile IBD sets before 2 years of age, and neonatal IBD manifests before 28 days of life [14].

IBD in general is regarded as a polygenic disease, and a genome-wide association study has revealed more than 230 known disease-associated genes. However, some children with VEO-IBD are known also to have diseases with monogenic etiologies. Recent advances in genetic evaluation have enabled the identification of gene mutations responsible for some forms of IBD. Monogenic or Mendelian disorder-associated IBD (MD-IBD) is a term used to represent IBD caused by genetic mutations [12].

Inborn errors of immunity

A subset of more aggressive, therapy-resistant VEO-IBD with early onset and frequently with positive family history is recently understood to be associated with inborn errors of immunity (IEI) [3]. The updated list of IEI from the IUIS phenotypical classification comprises 406 IEI disorders with 430 gene defects [9]. More than 50 IBD or IBD-like associated monogenic defects have been identified [15]. VEO-IBD related to IEI manifests due to impairment of intestinal epithelial barrier function, defects in phagocyte bacterial killing, increased hyper- or autoimmune inflammatory pathways, or impaired development and function of the adaptive immune system (Tab. 1). A gastrointestinal manifestation of IEI is common and may precede the principal IEI diagnosis [3,15]. Failure to thrive and diarrhea could be the first signs of IEI in general. Consanguinity, positive family history (IBD vs. IEI), multiple miscarriages, personal history of severe infectious disease courses, opportunistic infection, fungal infection or recurrent or persistent viral infection (e. g., Epstein-Barr virus, cytomegalovirus, papillomavirus, herpes simplex virus), recurrent deep skin or internal organ abscesses, mucocutaneous candidiasis, recurrent sinopulmonary infection, autoimmunity (type 1 diabetes mellitus, various forms of cytopenia, autoimmune endocrinopathy, etc.), benign lymphoproliferation with or without hepatosplenomegaly, and failure to thrive constitute the main IEI warning signs [14,16]. Also, skin lesions, malignancy, or onset of hemophagocytic lymphohistiocytosis in an IBD patient refractory to conventional therapy raises suspicion of monogenic IBD origin [15].

Tab. 2. Warning signs for suspecting monogenic IBD [4].
Tab. 2. Varovné příznaky vedoucí k podezření na monogenní IBD.

Key points	Comments
Very early age onset of IBD-like immunopathology	Likelihood increases with very early onset, particularly in those younger than 2 years of age at diagnosis
Family history	In particular, consanguinity, predominance of affected males in families, or multiple family members affected
Atypical endoscopic or histological findings	For example, extreme epithelial apoptosis or loss of germinal centers
Resistance to conventional therapies	Such as exclusive enteral nutrition, corticosteroids, and/or biological therapy
Skin lesions, nail dystrophy, or hair abnormalities	For example, epidermolysis bullosa, eczema, folliculitis, pyoderma or abscesses, woolly hair, or trichorrhexis nodosa
Severe or very early onset perianal disease	Fistulas and abscesses
Lymphoid organ abnormalities	For example, lymph node abscesses, splenomegaly
Recurrent or atypical infections	Intestinal and non-intestinal
Hemophagocytic lymphohistiocytosis	Induced by viral infections such as Epstein-Barr virus or cytomegalovirus or macrophage activation syndrome
Associated autoimmunity	For example, arthritis, serositis, sclerosing cholangitis, anemia, and endocrine dysfunction, such as thyroiditis and type 1 diabetes mellitus
Early development of tumors	For example, non-Hodgkin lymphoma, skin tumors, hamartoma, thyroid tumors

Impairment of the intestinal barrier epithelial surface leads to a transepithelial transformation of commensal bacteria, inflammation, and dysbiosis increasing the inflammation loop [17]. Chronic granulomatosis disease with impaired NADPH oxidase function could mimic IBD [13]. VEO-IBD is understood as the main sign of genetic variants in the IL-10-IL-10R pathway and related cytokine family member disorders [8].

Underlying immune dysregulation dominates in a group of genetic variants impairing T regulatory cell (Treg) function. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) should always be considered if IBD is accompanied by type I diabetes mellitus manifesting during the first year of life. IPEX-like disorders include disturbed IL2-IL2R interaction, signal transducer and activator of transcription 5b (STAT5b) deficiency, lipopolysaccharide-responsive beige-like anchor protein (LRBA) deficiency, and cytotoxic T lymphocyte antigen 4 (CTLA4) deficiency. Complex central immune dysregulation represents autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED) [9,13].

A broad group of genetic variants impairing development of the adaptive immune system consist of severe combined immunodeficiency (SCID), including Omenn syndrome, combined immunodeficiencies with syndromic features (Wiskott-Aldrich syndrome, THES), and predominantly antibody deficiencies (common variable immunodeficiency – CVID) [3,13].

IBD could also be a sign of autoinflammatory disorders, such as mevalonate-kinase deficiency (hyper IgD syndrome, HIDS), familial Mediterranean fever, or NLRC4-associated inflammatory disease [13].

Clinical warning signs leading to suspicion of IBD-like or monogenic VEO-IBD are summarized in Tab. 2. Leukopenia, lymphocytopenia, and neutropenia should be ruled out in the immunology workup [16]. Autoimmune cytopenias could signal underlying immune dysregulation. To exclude antibody deficiency or combined immunodeficiency, IgG, IgM, and IgA levels and/or IgG and IgA subclasses compared with age-adjusted levels should be evaluated [16,19]. Hyper IgM syndromes should be considered if IgG and IgA are decreased or absent but IgM is elevated or normal [20]. Elevated IgE could be together with other characteristic features a sign of hyper IgE syndromes. Decreased post-vaccination titers or impaired response to polysaccharide and/or protein antigen is a routine diagnostic tool in antibody deficiencies settings [16,19]. Flow cytometry phenotyping of main T CD3+, CD3+CD4+, CD3+CD8+, B CD19+, and NK CD16/56 lymphocyte subsets have diagnostic and prognostic value [20]. Impaired T or B cell lymphoproliferative response to mitogens, reduced naive T cell, reduced recent thymic immigrants, and reduced or absent TRECs underline cellular or combined immunodeficiency [20,21]. Dihydrorhodamine burst test must be evaluated when chronic granulomatous disease is suspected. In cases of potential hemophagocytic lymphohistiocytosis, NK cell cytotoxicity tests are part of a diagnostic algorithm [9]. Flow cytometry protein expression (LRBA, CTLA4), protein signaling (STAT3), and interleukin expression (IL10) help in confirming diagnoses [20].

Next-generation sequencing

Whole-exome sequencing or IBD sequencing panels provide diagnostic as-

surance [13]. Massive parallel sequencing, also termed next-generation sequencing (NGS), is a technology for detecting gene variants and enables us to analyze hundreds and thousands of genes, or even an entire genome, in a short period of time. The capacity of NGS offers new opportunities for clinical application and is increasingly used for establishing disease diagnosis and prognosis and in making therapeutic decisions [22].

The most commonly used NGS assay is targeted sequencing, which typically interrogates tens or hundreds of genes presumed to be associated with a particular clinical phenotype or group of diseases (i.e., primary immunodeficiencies). Targeted NGS panels are gaining popularity due to their time- and cost-effectiveness. A specific type of targeted NGS is an assay analyzing all protein-coding regions of the genome. Known as whole-exome sequencing (WES), it is particularly beneficial in cases of disorders that are phenotypically heterogeneous and in which it is difficult to select an appropriate panel of candidate causative genes. Despite its apparent advantages, however, WES also has some limitations, such as that intronic and noncoding regions remain uncovered or that sequencing quality (often expressed as sequencing depth) is insufficient for particular genes, simply because the range of targeted regions is too large. Finally, it should be said that NGS technology produces huge amounts of data such that their processing and storage presents a great bioinformatic challenge [23].

When properly selected and used, NGS technologies constitute an excellent tool that greatly expands the range of genes analyzed and can contribute importantly to ensuring adequate preventive and therapeutic procedures for at-risk patients [22].

Conclusion

VEO-IBD is often a severe and debilitating form of IBD and frequently overlaps with immune deficiencies and other rare diseases. The majority of IBD are thought to be polygenic, but rare cases can be attributed to disease-causing variants within a single gene, including *TTC37* in the case of THES. Targeted NGS is an efficient tool for establishing an accurate diagnosis in VEO-IBD or VEO-IBD-like patients.

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Conflict of Interest: The authors declare that the article/manuscript complies with ethical standards, patient anonymity has been respected, and they state that they have no financial, advisory or other commercial interests in relation to the subject matter.

Publication Ethics: This article/manuscript has not been published or is currently being submitted for another review. The authors agree to publish their name and e-mail in the published article/ manuscript.

Dedication: This work was supported by Ministry of Health, Czech Republic – conceptual development of research organization (FNBr, 65269705) and by Masaryk University Specific research project MUNI/A/1099/2019: Innate immunity factors in some immunopathology conditions..

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

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Publikační etika: Příspěvek nebyl dosud publikován ani není v současnosti zaslán do jiného časopisu pro posouzení. Autoři souhlasí s uveřejněním svého jména a e-mailového kontaktu v publikovaném textu.

Dedikace: Podpořeno MZ ČR – RVO (FNBr, 65269705) a projektem specifického výzkumu Masarykovy univerzity MUNI/A/1099/2019: Faktory nespecifické imunity u některých imunopatologických stavů.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/Doručeno: 30. 9. 2020
Accepted/Přijato: 15. 11. 2020

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Annex 2

OPEN

Regional Incidence of Inflammatory Bowel Disease in a Czech Pediatric Population: 16 Years of Experience (2002–2017)

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ABSTRACT

Objectives: Inflammatory bowel disease (IBD) is today a global disease, the incidence of which is growing in the pediatric population. This prospective study aims to decipher IBD incidence and its trend in a pediatric population through 16 years in the South Moravian Region of the Czech Republic.

Methods: We evaluated data concerning 358 pediatric patients with newly diagnosed IBD at University Hospital Brno, which is a gastroenterology center for the entire pediatric population (0–18 years) and cares for all pediatric IBD patients in the South Moravian Region (1,187,667 inhabitants).

Results: The study encompassed 3,488,907 children during 16 years. We diagnosed 192 children (53.6%) with Crohn disease (CD), 123 (34.4%) with ulcerative colitis (UC), and 43 (12.0%) with IBD-unclassified (IBD-U). The incidence of IBD increased from 3.8 (CD 2.9, UC 0.9, and IBD-U 0.0) per 100 000/year in 2002 to 14.7 (CD 9.8, UC 4.0, and IBD-U 0.9) per 100,000/year in 2017 ($P < 0.001$). The overall IBD incidence per 100,000/year was 9.8 (95% confidence interval [CI]: 8.8–10.9). Constituent incidences per 100,000/year were CD 5.2 (95% CI: 4.5–6.0), UC 3.4 (95% CI: 2.8–4.0), and IBD-U 1.2 (95% CI: 0.9–1.6). IBD incidence was projected to reach 18.9 per 100,000/year in 2022.

Conclusions: The overall incidence of pediatric IBD in the Czech Republic is increasing, and especially that of CD, whereas trends in UC and IBD-U appear to be constant. These data highlight the need to identify risk factors involved in the rising incidence of IBD.

Key Words: children, Crohn disease, Czech Republic, incidence, inflammatory bowel disease, ulcerative colitis

(*JPGN* 2020;70: 586–592)

Received September 19, 2019; accepted February 2, 2020.

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The authors report no conflicts of interest.

DOI: 10.1097/MPG.0000000000002660

What Is Known

- The incidence of pediatric-onset inflammatory bowel disease shows geographical variability worldwide.
- The incidence and prevalence of childhood-onset inflammatory bowel disease have risen rapidly in recent decades.

What Is New

- This research describes an increase in the incidence of inflammatory bowel disease, mainly because of a rise in the incidence of Crohn disease, between 2002 and 2017 in a population of Czech children.
- Prospectively evaluated incidence rates of pediatric inflammatory bowel disease and its subtypes in the Czech Republic are among the highest in the literature.

Inflammatory bowel disease (IBD), consisting of Crohn disease (CD), ulcerative colitis (UC), and inflammatory bowel disease unclassified (IBD-U), is a chronic relapsing inflammatory disorder with a multifactorial etiology. Both genetic aspects and environmental factors are important for IBD pathogenesis (1,2). Approximately 8% of IBD patients have an early onset of the disease in childhood (2). Reviews in the past decade have concluded that the incidence of pediatric-onset IBD has been rising globally, albeit with great geographic variations (3,4). By analyzing studies of IBD incidence and prevalence from around the world, it has been shown that the incidence is rising not only in high-income, but also in low-income and middle-income countries (5). Nevertheless, that incidence remains much lower in the latter countries (6,7). IBD is associated with higher morbidity and decreased quality of life in patients, results in significantly more frequent use of health care resources, and has become a major health concern (8,9). In the case of children, it is key to realize that the forms of the disease are often more aggressive than in adults, as evidenced by higher rates of immunomodulatory drugs and biologic therapies use compared with adults (10).

We have had relatively limited data on the incidence of IBD in Czech children. Therefore, it would be highly desirable to have more precise data in this field, to describe the exact trends in current developments, and to compare these data with those from other regions, especially within Central Europe.

FIGURE 1. Location of South Moravian Region (orange) within the Czech Republic (yellow) and Europe (gray).

The aim of our study was to determine the IBD incidence and trends in the Czech pediatric population. The study takes in a 16-year period in the South Moravian Region within the Czech Republic (Fig. 1). It characterizes differences by sex and age, anticipates the incidence of IBD for future years in this region, and, by extension, for the whole of the Czech Republic (11).

METHODS

Study Design

We conducted the study in the South Moravian Region, one of the 14 regional administrative units in the Czech Republic. The region has 1,187,667 inhabitants (2018, Czech statistical office), constituting 11% of the Czech Republic's total population. Children account for 19% of this number, or 229,375. The region covers an area of 7188 km^2. The health system in the Czech Republic is tax-funded and offers universal access.

The diagnosis and treatment of pediatric patients with IBD in the Czech Republic is limited only to highly specialized centers, which are strategically located and provide comprehensive care to IBD patients. In the South Moravian Region, all regional hospitals are referring pediatric patients for a comprehensive examination to 1 center in the regional capital, University Hospital in Brno. This referral pathway is rigid and has not changed throughout the study period.

Diagnosis of patients under 18 years of age by gastroenterologists serving adults is very unlikely, as in the Czech Republic, this would not be reimbursed by health insurance. The only reason for potential drop-out from our study is diagnosis of a local inhabitant outside of our region. We consider this to be very unlikely, but it does constitute a potential bias in our study. We believe the absolute majority of pediatric IBD patients from the South Moravian region are referred to our center.

The inclusion criteria consisted of children having been diagnosed with IBD according to relevant guidelines (12,13) (clinical history, physical examination, laboratory and serological testing, radiologic findings, and endoscopic appearance with stepwise biopsy for review by clinical pathologists) in a period between January 1, 2002 and December 31, 2017.

All patients underwent upper gastrointestinal endoscopy and ileocolonoscopy, with small bowel imaging (unless typical UC was determined after endoscopy and histology) by magnetic resonance enterography. All children were 0–18 years of age at the time of diagnosis and were resident in the South Moravian Region. The newly diagnosed IBD patients were subdivided into 3 main clinical types: CD, UC, and IBD-U. If a differentiation between UC and CD could not be determined after a complete workup, these patients were designated as IBD-U (11,13). The data were prospectively collected by experienced gastroenterologists into a study database administered by the Institute of Biostatistics and Analyses. Only unequivocal IBD cases were enrolled into the study. The patients without indisputable diagnosis according to the Porto criteria were excluded from further analyses. During the study, data was validated by other experienced gastroenterologists by blindly selecting 10 cases from each year and validating patient records by comparison with original data in the hospital information system. The Institutional Ethical Committee approved the study at University Hospital Brno in accordance with the 1964 Declaration of Helsinki.

Statistical Methods

Standard statistical methods were adopted for data description. Count data are summarized using absolute and relative frequencies. Median and interquartile range of nonmissing observations are reported for continuous data. Kruskal-Wallis test was used to examine between-group differences for continuous data, whereas Fisher exact test and exact rate ratio test (14), assuming Poisson counts, were applied for count data.

Age-gender adjusted incidence rates are expressed as newly diagnosed children per 100,000 pediatric population per year (100,000/year) and reported with 95% confidence intervals based on a gamma distribution. Data regarding the size of the pediatric population were obtained from the Czech Statistical Office, which counts the number of inhabitants either in actual years or accounts for all regions' redistributions and adjusts past numbers with regard to the regions' present sizes. For this study, we chose to work with figures related to actual years. Incidence trends and future projections were estimated using Poisson regression. All statistical significances were evaluated on a level of $\alpha = 0.05$. The entire analysis was conducted in the statistical software R. Poisson models were estimated using the *glm* function from the built-in *stats* package.

RESULTS

Demographics and Inflammatory Bowel Disease Incidence

The basic demographic characteristics of 358 children (<19 years of age) diagnosed for IBD between 2002 and 2017 in the South Moravian Region are shown in Table 1. Over the 16 years, the Czech Statistical Office accounted for 3,488,907 children in the area, and of those diagnosed with IBD, 192 (53.6%) were diagnosed as CD, 123 (34.4%) as UC, and 43 (12.0%) as IBD-U. Among all the IBD cases, 53.1% were boys. The median age of a diagnosed child was 13.9 years (interquartile range: 4.9), the median time between the first symptoms and diagnosis was 4.0 months (interquartile range: 6.3), and no significant differences were found in basic demographic characteristics between diagnoses (all $P > 0.05$).

Incidence rates per 100,000/year for the given period 2002 to 2017 are captured in Figure 2. Except for the year 2015, when CD and UC incidences were comparable, the incidence of CD outgrew those of UC and IBD-U after 2009, which had not been the case prior to

TABLE 1. Demographics of children (0–18 years of age) newly diagnosed with inflammatory bowel disease, Crohn disease, ulcerative colitis, and inflammatory bowel disease-unclassified in the South Moravian Region, 2002 to 2017

Parameter	IBD	CD	UC	IBD-U
Sex, N (%)				
N	358	192	123	43
Male	190 (53.1%)	104 (54.2%)	65 (52.8%)	21 (48.8%)
Female	168 (46.9%)	88 (45.8%)	58 (47.2%)	22 (51.2%)
Age at diagnosis (years)				
N	358	192	123	43
Median (interquartile range)	13.9 (4.9)	14.4 (4.7)	13.6 (4.3)	12.4 (6.7)
Time from first symptoms to diagnosis (months)				
N	301*	156	112	33
Median (interquartile range)	4.0 (6.3)	4.0 (6.6)	3.0 (6.2)	5.0 (6.0)

CD = Crohn disease; IBD = inflammatory bowel disease; IBD-U = inflammatory bowel disease-unclassified; UC = ulcerative colitis.
*Discrepancy between number of patients and total N is because of missing data.

2009. Before 2009, no obvious predominance of any diagnosis was observable. A rising trend in IBD incidence is nevertheless noticeable for the entire 16-year period. Although in 2002, the IBD incidence was only 3.8 (CD 2.9, UC 0.9, and IBD-U 0.0) per 100,000/year, in 2017 it reached 14.7 (CD 9.8, UC 4.0, and IBD-U 0.9) per 100,000/year. Although there is no significant difference in CD and IBD-U incidences between years 2002 and 2017 (both $P > 0.05$), this is not the case for IBD ($P < 0.001$) and CD ($P = 0.003$). The predominance of the CD diagnosis after 2009 is confirmed by its being significantly higher than the UC and IBD-U incidences ($P = 0.029$ and $P < 0.001$, respectively) in 2017, but its incidence was only higher than that of IBD-U ($P = 0.016$) in 2002.

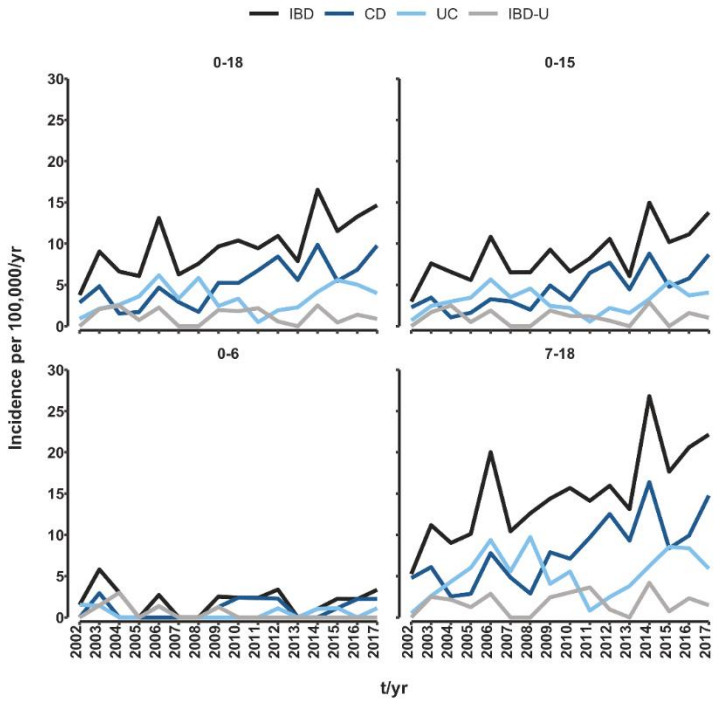

FIGURE 2. Incidence rates per 100,000/year in different age groups of children (0–18, 0–15, 0–6, 7–18 years of age) newly diagnosed with inflammatory bowel disease (IBD), Crohn disease (CD), ulcerative colitis (UC), and inflammatory bowel disease-unclassified (IBD-U) in the South Moravian Region, 2002 to 2017.

TABLE 2. Inflammatory bowel disease incidence by age and sex in newly diagnosed children (0–18 years of age) with inflammatory bowel disease, Crohn disease, ulcerative colitis, and inflammatory bowel disease-unclassified in the South Moravian Region, 2002 to 2017

Incidence per 100,000/year	IBD	CD	UC	IBD-U
Age				
0–18				
N	358	192	123	43
Mean (95% CI)	9.8 (8.8–10,9)	5.2 (4.5–6.0)	3.4 (2.8–4.0)	1.2 (0.9–1.6)
0–15				
N	257	135	92	30
Mean (95% CI)	8.6 (7.6–9.8)	4.5 (3.8–5.4)	3.1 (2.5–3.8)	1.1 (0.7–1.5)
0–6				
N	26	14	7	5
Mean (95% CI)	2.0 (1.3–3.0)	1.1 (0.6–1.8)	0.5 (0.2–1.1)	0.4 (0.1–0.9)
7–18				
N	332	178	116	38
Mean (95% CI)	14.7 (13.1–16.3)	7.8 (6.6–9.0)	5.2 (4.3–6.2)	1.7 (1.2–2.3)
Gender				
Male				
N	190	104	65	21
Mean (95% CI)	10.1 (8.7–11.7)	5.5 (4.4–6.7)	3.4 (2.6–4.4)	1.2 (0.7–1.8)
Female				
N	168	88	58	22
Mean (95% CI)	9.5 (8.1–11.1)	4.9 (3.9–6.1)	3.3 (2.5–4.3)	1.3 (0.8–1.9)

CD = Crohn disease; CI = confidence interval; IBD = inflammatory bowel disease; IBD-U = inflammatory bowel disease-unclassified; UC = ulcerative colitis.

A detailed overview of overall incidences by diagnosis, age, and sex is provided in Table 2. The overall IBD incidence per 100,000/year for the 16-year period for children up to 18 years of age was 9.8 (95% CI: 8.8; 10.9), and for children up to 15 years of age, it was 8.6 (95% CI: 7.6–9.8). The overall CD and UC incidences were found to be significantly higher than that of IBD-U (both $P < 0.001$). The difference in CD versus UC incidences also was significant ($P < 0.001$).

Paris Classification

According to the Paris Classification (15), there were 53 patients (27.6%) in the CD group under 10 years of age (A1a), 115 (59.9%) were between 10 and 17 years of age (A1b), and 28 patients (12.5%) were over 17 years of age (A2). Occurring most frequently was the ileocolic localization (L3) in 134 (69.8%) patients. Upper GIT involvement (L4a and L4b) was found in 28 (14.5%) patients. Most patients (145 [75.5%]), had the nonstricturing and nonpenetrating form of CD (B1), 24 (12.5%) the stricturing form (B2), 16 (8.3%) the penetrating form (B3), and only 1 patient (0.5%) the stricturing and penetrating form (B2, B3). In 6 patients (3.1%) the behavior of the disease could not be validly evaluated. Perianal disease was found in 22 (11.5%) patients. Growth delay at the time of diagnosis was present in 57 (29.7%) CD patients.

Among 123 patients diagnosed with UC, there were 6 (4.9%) patients only with proctitis (E1), 16 (13.0%) with left-sided (distal to splenic flexure) colitis (E2), and 8 (6.5%) with extensive (distal to hepatic flexure) colitis (E3). Pancolitis was present in 90 (73.2%) patients. In 3 patients (2.4%), the extent of the disease could not be validated.

Inflammatory Bowel Disease by Age

The IBD incidence begins to grow quickly from the age of 8. The peak occurs at 17 years of age, with decrease thereafter. Of those diagnosed with IBD, only 26 (7.3%) were children under 7 years of age, whereas the majority of child patients were at least 7 years of age at diagnosis. Early presentation of CD, UC, and IBD-U at age 6 and earlier occurred in 14 (3.9%), 7 (2.0%), and 5 (1.4%) children, respectively. The overall IBD incidence per 100,000/year for children ages up to 6 years was 2.0 (95% CI: 1.3–3.0), whereas significantly higher ($P < 0.001$) incidence of 14.7 (95% CI: 13.1–16.3) occurred for children aged 7 years and older. Likewise CD, UC, and IBD-U incidences among young children (<7 years) were significantly lower (all $P < 0.001$) than were those for older children (≥ 7 years). No significant between-diagnoses differences were found for patients up to 6 years of age. For patients aged 7 and older, the CD incidence was found to be significantly greater than were those for UC and IBD-U ($P = 0.001$ and $P < 0.001$, respectively), which was true also for incidences of UC vs. IBD-U ($P < 0.001$).

Inflammatory Bowel Disease by Sex

Of the 358 children diagnosed with IBD, 53.1% were boys (CD 54.2%, UC 52.8%, and IBD-U 48.8%). Overall male and female IBD incidences per 100,000/year were 10.1 (95% CI: 8.7–11.7) and 9.5 (95% CI: 8.1–11.1), respectively, with no statistically significant difference between the 2 ($P = 0.606$). No significant sex-related differences were found between incidences of CD, UC, and IBD-U (all $P > 0.05$). Among both boys and girls, CD and UC incidences were proven to be significantly higher compared with those for IBD-U (all $P < 0.001$) as well as CD vs. UC incidence ($P = 0.005$ and $P = 0.022$ for boys and girls, respectively).

Trends in Inflammatory Bowel Disease Incidence

Figure 3 shows actual IBD and CD incidences per 100,000/year, an estimated trend over the observed 16 years, 95% CI, and

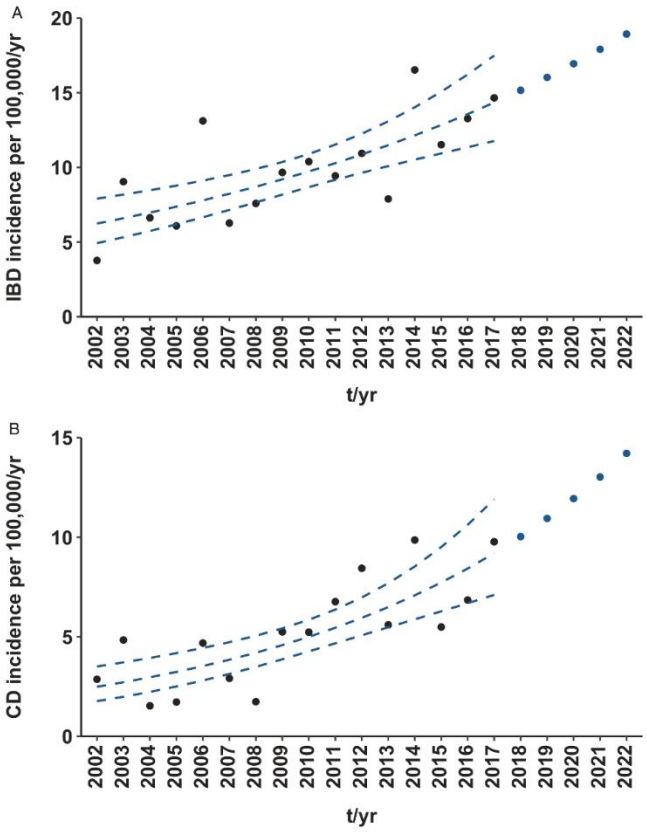

FIGURE 3. Incidence rates per 100,000/year among children (0–18 years of age) newly diagnosed with inflammatory bowel disease (IBD) (A) and Crohn disease (CD) (B) in South Moravian Region, 2002 to 2017 and 2018 to 2022. Black points represent actual data. Broken blue lines indicate trend over the observed period based on Poisson regression along with a 95% confidence interval. Blue points are future projections.

future projections for the next 5 years (2018–2022). Overall, the IBD incidence per 100,000/year has risen significantly, with the relative risk (RR) of being diagnosed with IBD increasing by 5.7% each year (RR = 1.057, $P < 0.001$). The rising trend in IBD mainly reflects significant increase in newly diagnosed CD cases (RR = 1.091, $P < 0.001$), whereas neither UC (RR = 1.030, $P = 0.133$) nor IBD-U incidence (RR = 0.988, $P = 0.723$) showed any significant changes. The IBD incidence is projected to reach 18.9 per 100,000/year in 2022 (14.2 for CD).

The relative risk per year of being diagnosed with IBD was 2.7% greater for girls (RR = 1.072, $P < 0.001$) than for boys (RR = 1.045, $P = 0.005$). The difference is substantially smaller for the CD diagnosis, with girls facing relative risk of being diagnosed that is 1.2% higher than in the case of boys (RR = 1.088 [$P < 0.001$], RR = 1.100 [$P < 0.001$] for boys and girls, respectively). There appeared a significant increase in incidence over the 16-year period of girls being diagnosed with UC (RR = 1.065, $P = 0.026$).

DISCUSSION

We provide a detailed longitudinal data set describing IBD incidence and its trends in pediatric patients through the 16 years between 2002 and 2017 within a well-defined geographical area of the Czech Republic. To our knowledge, this is the most recent and comprehensive study in this field. Our results provide important insights into the high incidence of IBD and its increasing trend, which are due mainly to the rise in rates of CD. The overall IBD incidence per 100,000/year shows IBD incidence in children within the Czech Republic currently to be among the highest in the world (3–6,16,17). These data show considerably higher incidences than were determined from the first Czech national survey conducted about 2 decades earlier by Pozler et al (18). In another, smaller Czech study from the Olomouc Region, an overall increase in the incidence of IBD was also confirmed (19). Schwarz et al recently published a 16-year prospective study of pediatric IBD patients in the Pilsen Region of the Czech Republic showing that a group of 170 pediatric patients (study period 2000–2015) represented an

average incidence of IBD per 100,000/year of 10.0 (6.2 for CD, 2.8 for UC, and 1.0 for IBD-U). That study also projected increasing future incidence (11). Our study is based on data from a region of almost similar size but with more than 2 times the number of pediatric patients with IBD. The incidence of IBD subtypes revealed by our group, and including the proportions among them, is practically identical to the results from the Pilsen Region. Moreover, the incidence trends are very similar. Although acknowledging certain limitations, we therefore, can presume that our findings are potentially similar to incidences to be found in pediatric IBD patients throughout the Czech Republic. We clearly demonstrate an overall increase in IBD incidence within the population of Czech children, with the overall incidence rising more than 3 times when comparing data from the first and final year in our data set. The overall IBD incidence per 100,000/year rose significantly over the study period, that trend reflecting mainly the statistically significant increase in CD incidence even as the UC and IBD-U incidences showed no statistically significant changes. Our future projections put the IBD incidence at 18.9 per 100,000/year in 2022 (14.2 per 100,000/year for CD). In the future, we will be able to compare these projections with real data obtained from our patients. In comparison to neighboring countries in Central and Eastern Europe, our data suggests that the increase in the incidence of IBD is particularly noticeable in Hungary (20,21) and Slovenia (22) whereas it seems to be stable in Germany (23). In Austria, on the other hand, an overall increase was observed from 1997 to 2007 in both CD and UC, primarily in the largest urban areas (24). The overall incidence of IBD cases was surprisingly very low in Poland, at 2.7 per 100,000/year (0.6 for CD, 1.3 for UC, and 0.8 for IBD-U) (6,25). No current data on the incidence of IBD in neighboring Slovakia is known at this time. Globally, CD predominates over UC and IBD-U in areas having high IBD incidence. Recent data indicate higher rates of pediatric CD than UC in Europe and North America, except in northern California (26), Finland (27), Poland (25), and Italy (28), where the incidence of UC exceeds that of CD (6). The reasons for these notable differences remain uncertain (4,6,29). In a recent systematic review, Sykora et al analyzed 140 pediatric incidence studies. They demonstrated substantial increase in the incidence of pediatric IBD as well as great geographic variation. The incidence of IBD remains highest in the northern populations of Europe and America but has remained stable or even decreased. Rising rates of pediatric IBD have been observed in previously low-incidence areas and much of the developing world, as well as among children of immigrants. The incidence rates of CD and UC vary worldwide between 0.2/100,000 and 13.9/100,000 and between 0.1/100,000 and 15/100,000, respectively. In time-trend analyses, 67% of CD and 46% of UC studies have reported significant increases (6). Variation in IBD incidence may reflect differences in the distribution of various environmental triggers for a given disease in specific areas. Rapidly changing IBD incidence in such areas create an opportunity for future studies of genetic-environmental interactions (6,30,31). Exposure to environmental factors in childhood appears to be essential for the later development of IBD. In rapidly developing areas, such as Asia, the food composition of traditional human diets is changing. People are shifting from homemade to processed foods, and lifestyles are changing also in other ways. All this may affect the composition of the human intestinal microbiota and potentially be related to increasing IBD (31–35). We can hypothesize that this reflects a certain similarity to the significant rise in the socioeconomic level within the Czech Republic after the close of the communist era, and thus, an influence on the increase in IBD. One of the prerequisites for developing a proper understanding of IBD's pathogenetic context and next steps in better care for pediatric patients is to find accurate and relevant data, including data for IBD's incidence and prevalence in these patients (36).

Acknowledgment: This work was supported by the Ministry of Health of the Czech Republic, grant no. 17-29389A and MH CZ - DRO (FNBr, 65269705).

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Annex 3

The Emerging Role of Noncoding RNAs in Pediatric Inflammatory Bowel Disease

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Prevalence of inflammatory bowel disease (IBD), a chronic inflammatory disorder of the gut, has been on the rise in recent years—not only in the adult population but also especially in pediatric patients. Despite the absence of curative treatments, current therapeutic options are able to achieve long-term remission in a significant proportion of cases. To this end, however, there is a need for biomarkers enabling accurate diagnosis, prognosis, and prediction of response to therapies to facilitate a more individualized approach to pediatric IBD patients. In recent years, evidence has continued to evolve concerning noncoding RNAs (ncRNAs) and their roles as integral factors in key immune-related cellular pathways. Specific deregulation patterns of ncRNAs have been linked to pathogenesis of various diseases, including pediatric IBD. In this article, we provide an overview of current knowledge on ncRNAs, their altered expression profiles in pediatric IBD patients, and how these are emerging as potentially valuable clinical biomarkers as we enter an era of personalized medicine.

Key Words: pediatrics, inflammatory bowel disease, Crohn's disease, ulcerative colitis, noncoding RNA, microRNA

INTRODUCTION

Inflammatory bowel disease (IBD) is an umbrella term for ulcerative colitis (UC) and Crohn's disease (CD). These chronic inflammatory disorders of the gastrointestinal tract often are diagnosed in adolescence and young adulthood. Some 8%–25% of IBD patients have early onset of the disease in childhood.[1, 2] These cases are more severe,[3] with many extraintestinal issues such as delayed growth and development.[4] The prevalence of these diseases is steadily rising worldwide, and the increase is particularly rapid in the pediatric population.[5–7] Current diagnostic routine includes symptom assessment, endoscopic examination and biopsy, histology, serology, and radiology.[8, 9] No standard diagnostic routine and reliable direct biomarkers are currently available. The biomarkers we have now reflect only general inflammation rather than specific pathogenesis associated with ongoing IBD or a specific subtype of IBD. A time-consuming and often painful diagnostic process eventually leading to surgical intervention is a particularly traumatic experience for young children, but this could very well be avoided by the use of noninvasive or minimally invasive biomarkers for diagnostics and therapeutic disease monitoring.

Although novel therapeutic strategies are effective in managing symptoms and achieving long-term remission, these approaches are not curative, and in some patients, no or only poor response is observed.[10, 11] Early identification of such patients by innovative diagnostic approaches and their redirection to other therapeutic options is therefore essential for improving therapeutic outcomes. Moreover, novel discoveries in IBD pathogenesis are necessary to identify the targets and to develop novel therapeutic strategies. Noncoding RNAs (ncRNAs) are currently being studied intensively in pediatric IBD patients because they constitute a promising, novel class of biomarkers and therapeutic targets.

NONCODING RNAs, THEIR CLASSIFICATION, FUNCTION, AND BIOGENESIS

After the Human Genome Project revealed that only 1.5% of the genome is protein-coding,[12] it became clear that

Received for publications September 4, 2019; Editorial Decision January 2, 2020.

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Author contribution: PJ, JB, TP, and LK wrote the manuscript. OS and AG critically revised the manuscript. All authors reviewed and approved the final manuscript.

Supported by: This work was supported by the Ministry of Health, Czech Republic, Conceptual Development of Research Organization (FNBr, 65269705) and National Cancer Institute (CA184792 and CA202797).

Conflicts of interest: The authors declare that they have no conflict of interest.

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doi: 10.1093/ibd/izaa009
Published online 03 February 2020

there is more to DNA than mere proteins. Even earlier, there had been some sparse information available about the existence of RNAs untranslated to the proteins, but this was long considered an exception rather than a rule. In addition to transfer and ribosomal RNA, the first observations of small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) were made in the 1980s.[13–16] Later, cancer-related deregulation of ncRNAs RNA H19,[17] growth arrest-specific transcript 5 (GAS5),[18] and prostate cancer antigen (PCA3/DD3)[19] pointed to the now well-documented phenomena as to the involvement of noncoding genome in the development of many complex diseases. Only after the discovery of gene expression regulation through RNA interference facilitated by short noncoding RNAs,[20–22] however, did exploration of noncoding genome really begin to take off.

Noncoding RNAs can be divided according to their function into 2 groups: housekeeping ncRNAs (e.g., tRNAs, rRNAs, snRNAs, snoRNAs) and regulatory ncRNAs. The latter of these is historically subdivided into 2 large groups according to the arbitrary dividing line of 200 nucleotides in length. Transcripts shorter than 200 nucleotides are termed short noncoding RNAs (sncRNAs), and transcripts exceeding 200 nucleotides are called long noncoding RNAs (lncRNAs). Both groups are involved in regulating gene expression and operate on several levels depending on the type of ncRNA. The sncRNAs, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs), are involved mostly in post-transcriptional regulation but also in many other specific processes such as transposon silencing or rRNA maturation.[23] The lncRNAs are much longer by definition and comprise a more diverse group of transcripts. They are known to affect many cellular processes on transcriptional, post-transcriptional, and translational levels. Although both short and long noncoding transcripts usually possess no protein coding capacity, there has been some evidence of cryptic reading frames and translation in to shorter micropeptides in lncRNAs formerly regarded as noncoding.[24–26]

MicroRNAs

Among all ncRNAs, miRNAs have been studied thoroughly and have claimed the most attention in recent decades. Along with the discovery of RNA interference in the early 2000s, miRNAs were observed first in *Caenorhabditis elegans* as master regulators of developmental timing[20, 21, 27–30] and later in many other species, including humans.[30, 31] Their distinct length of 18 to 25 nucleotides makes them a very specific group of transcripts, currently encompassing about 2000 different mature miRNAs.[32] Naturally produced endogenously, miRNAs constitute a pivotal cellular mechanism for regulating expression in as many as 60% of human genes[31] by complementary binding to their target messenger RNAs (mRNAs). Of the 18 to 25 nucleotides, 8 are essential and make up what is termed the "seed" region, which binds to the 3′ untranslated region of the target, thereby leading to repressed translation of the target mRNA, either by its destabilization or degradation.[33, 34] As the seed region is fairly short, many different mRNAs can contain a complementary sequence and be affected by a single miRNA, thus making it a pleiotropic regulator of several targets.[33] Stemming from either individual miRNA genes or intergenic and intragenic regions of protein-coding genes,[35] miRNAs are canonically transcribed by RNA polymerase II,[36] thereby creating polyadenylated and capped pri-miRNAs.[37, 38] This primary transcript is usually several hundred nucleotides long and contains a future mature miRNA sequence in the stem of the secondary hairpin structures of pri-miRNA. Next, splicing of the pri-miRNA is facilitated by a microprocessor complex consisting of RNase III Drosha[39] and a dimer of DiGeorge critical region 8 (DGCR8).[40, 41] The microprocessor cleaves the pri-miRNA transcript, creating 1 or several hairpin structures—pre-miRNAs—that each contain one future miRNA. Pre-miRNAs are transported by nuclear transporter protein exportin 5[42] to the cytoplasm, where they are processed further. The RNase III–type enzyme Dicer,[43] together with other cooperating proteins (dependant on the species; in humans it is trans-activation-responsive RNA binding protein [TRBP]),[44] cleaves pre-miRNA close to the terminal loop and creates a double-stranded RNA intermediate. One of the strands is recruited into an RNA-induced silencing complex (RISC) with proteins from the Argonaute family (AGO).[44] The strand that is recruited is termed "leading," and the other one, called the "passenger strand," is usually degraded; although in some cases, it also can be recruited into the RISC.[45]

The canonical pathway of miRNA biogenesis can be overcome, as some miRNAs have been observed to be produced alternatively in noncanonical ways that exclude certain steps and also give rise to other types of sncRNAs.[46–49]

LONG NONCODING RNAs

Long ncRNAs were first regarded as nonfunctional because their roles in the cells have been unknown and their sequences are less conserved than are those of protein-coding genes.[50, 51] In comparison with miRNAs, lncRNAs encompass a much broader group due to their definition by length. Though miRNAs encompass only a specific 18 to 25 nucleotides in length of the spectrum, everything from 200 nucleotides and larger is considered an lncRNA unless it is a coding sequence.[52–56] Next-generation sequencing revealed that lncRNAs originate from more than 59,000 genes.[57] That number was expanded even further by the NONCODE database to more than 96,000 genes producing over 172,000 transcripts.[58] Not many of these, however, have been experimentally validated to date.[59] Nevertheless, structural and functional variability makes it difficult to create a meaningful and useful classification system;[60] currently, several systems are being used based upon localization in the genome in relation to the protein-coding genes, according to their function or depending upon the means of their origin.

Although sharing many similarities with mRNAs, lncRNAs are more tissue- and time-specific and operate in much lower concentrations.[52, 60–62] They are localized both in cell nucleus and in the cytoplasm in 1 or more copies, but nuclear localization, especially close to the chromatin, is their preferential one.[52, 63] Close to the chromatin, they affect gene expression by facilitating chromatin interactions and guiding chromatin-remodeling complexes,[64, 65] thus activating or repressing transcription. Other ways of transcriptional regulation include cooperation with transcription factors,[66, 67] binding to regulatory sequences[68–70] and promoting splicing of mRNAs in complexes with other splicing molecules.[71, 72]

When translocated to the cytoplasm, lncRNAs are involved in post-transcriptional and post-translational regulation of gene expression while acting in cooperation with RNA-binding proteins, or they affect the stability and degradation of such proteins and thus facilitate protein turnover.[73] The mRNA stability is affected by the binding of RNA–protein complexes containing lncRNAs as guiding molecules, which causes either degradation or enhanced translation of the target mRNA.[74, 75] The RNA–protein complexes are also involved in various signaling pathways,[76] fulfill certain roles in cellular organelles, or help transport other molecules into organelles.[77] A separate category of lncRNAs, so-called "competing endogenous RNAs" (ceRNAs), serve as decoys or sponges for miRNAs and so alter the relative availability of miRNAs.[78] Similarly, lncRNAs serve also as protein decoys, averting proteins from binding to other transcripts.[79]

In contrast to the well-described canonical pathway of miRNAs, a general biogenetic pathway for lncRNAs is difficult to trace, as lncRNAs present a diverse group of transcripts produced in several ways. Nevertheless, an initial part of the biogenesis is shared not only among lncRNAs but also by all transcripts. This consists of transcription by polymerase II, polyadenylation, 5′ capping, and chromatin modifications typical for protein-coding sequences.[52, 80] However, lncRNA genes usually contain fewer but longer exons, and their expression is more time- and tissue-specific. Enormous variability exists on the post-transcriptional level, which includes such specific modifications as cleaving of the 3′ end by RNAse P or back-splicing to creating a circular lncRNA (circRNA).[81, 82] There is also some evidence that miRNA transcriptional apparatus is somewhat involved in lncRNA biogenesis. After all, sncRNAs, including for example miRNAs themselves, arise from formerly long primary transcripts classifiable as lncRNAs and only later are processed by specific biogenetic pathways.[83, 84]

NONCODING RNAs AS BIOMARKERS

Great demand exists for a precise, possibly noninvasive biomarker that can provide a faster, simpler, and more efficient way of characterizing patients and personalizing management of the disease. The ncRNAs have emerged as potential biomarkers for several diseases, as these are generally stable and abundantly present in a variety of clinical specimens, including tissues and bodily fluids; are highly tissue-specific, cell type-specific, and condition-specific; and can be readily detected by routine and inexpensive laboratory techniques.[85, 86]

The majority of the human genome encodes RNAs that do not code for proteins.[20, 21, 87] These ncRNAs affect normal expression of the genes, including genes involved in the immune system, inflammation, and IBD pathogenesis. Although miRNAs are the most studied regulatory ncRNAs to date and miRNA-targeted diagnostics and therapeutics have already reached clinical development,[28, 85, 86, 88] the importance of lncRNAs as potential biomarkers and therapeutic targets is increasingly recognized.[85, 86, 89, 90]

Both short and long ncRNAs function mostly as regulators and fine-tuners of gene expression. Although miRNAs share a simple structure and, in the majority of cases, bind to their target mRNAs through their 8-nucleotide long seed region to post-transcriptionally regulate gene expression,[27] lncRNAs use many different molecular mechanisms depending on the length and structure of a given transcript. This enables a wide variety of functions, spanning from transcription regulation and acting as miRNA sponges to orchestrating epigenetic modifications.[82] Several miRNAs[89, 90] and specific miRNA signatures[91, 92] have been identified in IBD-associated tissues. It has been shown that among many other cellular processes, miRNAs play a significant role in intestinal immunity.[93] Nevertheless, there exists only sparse information on ncRNA profiles and their diagnostic potential in pediatric IBD patients (summarized in Tables 1 and 2). Given that adult and pediatric IBD have some differences in manifestation, etiology, and genetic background,[4] it is expected that ncRNA profiles may reflect these differences. To examine these aspects thoroughly, we searched the PubMed database for relevant studies according to the following strategy: "miRNA" and "pediatric" and ("ulcerative" and "colitis") or ("crohn" and "disease") or "IBD." When we excluded nonclinical studies and chose only studies carried out on pediatric patients, the remaining 11 articles (Tables 1 and 2) were relevant for our discussion.

NONCODING RNAs IN TISSUES OF PEDIATRIC IBD PATIENTS

The research group of Koukos et al focused on differences in ncRNA expression profiles in pediatric and adult IBD patients and published its study in 2013.[94] In addition to showing that the IL6/STAT3 pathway is a critical factor in the development and progression of IBD, those authors identified 5 miRNAs suppressing activity of STAT3. These are miR-125, miR-101, miR-26, miR-124, and let-7, with miR-124 probably being a central regulator of STAT3 due to its greater than 90% inhibitory effect on STAT3 in human colonocytes.[95] Further investigation using real-time quantitative polymerase chain reaction (RT-qPCR) on adult and pediatric samples revealed that

TABLE 1. Summary of Tissue ncRNAs Associated With Various Aspects of Pediatric IBD

Study	ncRNA	Change in expression (patients)	Compared groups	Number of patients, sample type	Statistical parameters			Technological platform
					Best *P*- achieved	AUC	Sensitivity/ Specificity (%)	
Koukos et al., 2013 [40]	miR-101	Down	IBD vs. non-IBD	45 biopsies	—	—	—	MicroRNA-library screen, RT-qPCR
	miR-26	Down			—			
	miR-124	Down	pUC vs. pCD/non-IBD		<0.0001/<0.01			
Koukos et al., 2015 [42]	miR-4284	Down	pIBD vs. non-IBD	37 biopsies	<0.05	—	—	minCURY microRNA array, RT-qPCR
Zahm et al., 2014 [43]	miR-192	Down	pUC vs. controls	50 biopsies	0.0006	—	—	nCounter, TaqMan low density array, RT-qPCR
	miR-194				0.0019			
	miR-200b				0.0056			
	miR-375				0.0001			
	miR-142-3p	Up			0.0048	—	—	
	miR-146a				0.0027			
	miR-21				0.0003			
	let-7i				0.0007			
	miR-24	—	pUC vs. pCD		—	0.83	83.3/85.7	
Béres et al., 2016 [44]	miR-122	Up	ipCD vs. C/pUC	28 FF samples,	<0.01	—	—	RT-qPCR
	miR-146a	Up	pUC vs. C	71 FFPE samples	<0.001			
	miR-155	UP	ipCD vs. C		<0.001			
Szücs et al., 2016 [45]	miR-146a	Up	ipCD vs. inpCD vs. C	30 FFPE samples	<0.001	—	—	RT-qPCR
	miR-155							
Béres et al., 2017 [33]	miR-185	Up	ipCD vs. C	44 FF samples	<0.05	0.81	62.5/100	NGS, RT-qPCR
	miR-223				<0.001	1	100/100	
	miR-146a	—	pCD vs. pUC		<0.01	0.838	80/76.92	
	miR-142–3p				<0.01	0.888	77.78/90.31	
Tang et al., 2018 [48]	miR-15a	Down	CDre vs. CDac	54 FF samples	<0.05	—	—	RT-qPCR

Abbreviations: AUC, area under the curve; C, control; FFPE, formalin-fixed paraffin-embedded; CDac, active Crohn's disease; CDre, Crohn's disease in remission; FF, fresh frozen tissue; ipCD, histologically intact tissue of pediatric patients with Crohn's disease; inpCD, histologically inflamed tissue of pediatric patients with Crohn's disease; NGS, next-generation sequencing; pCD, pediatric patients with Crohn's disease; pIBD, pediatric patients with inflammatory bowel disease; pUC, pediatric patients with ulcerative colitis; non-IBD, control patients without inflammation typical for IBD; RT-qPCR, real-time quantitative polymerase chain reaction.

TABLE 2. Serum ncRNAs With Successfully Validated Biomarker Potential for Various Aspects of Pediatric IBD

Study	ncRNA	Change in expression (patients)	Compared groups	Number of patients, sample type	Statistical parameters			Technological platform
					Best *P*- achieved	AUC	Sensitivity/ Specificity (%)	
Zahm et al., 2011[49]	miR-484 miR-16	Up	pCD vs. control vs. celiac	102 blood serum samples	<0.0001	0.917 0.912	82.61/84.38 73.91/100	TaqMan human microRNA array, RT-qPCR
Zahm et al., 2014[43]	miR-192 miR-142–3p miR-21	Up	pUC vs. control	47 blood serum samples	0.0045 0.0078 —	0.757 0.723 0.718	79.31/77.78 75.86/66.67 75.86/66.67	TaqMan low density array human microRNA panel, RT-qPCR
Heier et al., 2016[50]	miR-146a miR-146b miR-320 miR-486	Down	pIBD pharmaco-dynamics	19 PBMC samples	<0.05 <0.01 <0.01 <0.01	—	—	RT-qPCR
De Iuciidibus et al., 2018[51]	miR-29c-3p	Up	pIBD pharmaco-dynamics	10 PBMC samples	<0.01	—	—	NGS, RT-qPCR
Lucafò et al., 2018[52]	GAS5	Up	pIBD pharmaco-dynamics	19 PBMC samples	<0.05	—	—	RT-qPCR

Abbreviations: AUC, area under the curve; C, control; GAS5, growth arrest-specific transcript 5; NGS, next-generation sequencing; PBMC, peripheral blood mononuclear cells; pCD, pediatric patients with Crohn's disease; pIBD, pediatric patients with inflammatory bowel disease; pUC, pediatric patients with ulcerative colitis; RT-qPCR, real-time quantitative polymerase chain reaction.

let-7 and miR-125 were downregulated specifically in adult patients, miR-101 and miR-26 in pediatric and adult patients, and miR-124 only in pediatric patients with active UC. Thus, these are potential diagnostic biomarkers depicting disease activity. Downregulation of miR-124 was due to methylation of miR-124 promoter, which provided the first evidence as to a role of epigenetic regulation in pediatric IBDs.[94] The Koukos team continued its research efforts and 2 years later published another study on pediatric IBD patients,[96] again comparing active and inactive disease vs healthy controls and adult UC patients. They discovered a 24-miRNA signature that was deregulated in colonic tissue, with miR-4284 being the most downregulated ncRNA in pediatric UC patients. Its expression was also downregulated in patients with active vs inactive UC. Further in vitro experiments showed that miR-4284 is present in colonic epithelial cells and regulates expression of C-X-C motif chemokine 5 (CXCL5) by binding to its 3′UTR. Correspondingly, CXCL5 levels are increased in pediatric patients with UC due to miR-4284 downregulation.[96] The CXCL5 is known for its expression in colonic epithelial cells, and as a facilitator of neutrophil recruitment, it is one of the major players in the development of UC.[93, 95]

In the study by Zahm et al,[97] tissue expression profiles from rectal biopsies revealed specific miRNA patterns associated with pediatric UC and CD compared with controls. Four miRNAs that were enriched in epithelial cells (miR-192, miR-194, miR-200b, and miR-375) were significantly downregulated in UC patients compared with controls. Contrarily, 4 miRNAs that were overexpressed in inflammatory cells (miR-142–3p, miR-146a, miR-21, and let-7i) were upregulated in UC patients compared with controls. Only miR-375 and miR-21 were significantly altered in pediatric CD patients in comparison with controls. In UC patients receiving the immunomodulator 6-mercaptopurine or methotrexate, significant elevation was observed of miR-375 and miR-192 compared with in UC patients not receiving immunomodulators. A single miRNA, miR-24, was differentially expressed between UC and CD patients and enabled correct classification of 84% of patients, with a sensitivity of 83% and specificity of 86%.[97]

Another study focusing on both pediatric CD and UC came from the group of Béres et al.[98] They selected for their study miR-146a, miR-122, and miR-155, which previously had been shown to play an important role in immune processes and immune-mediated diseases. MiR-146a and miR-155 levels were significantly higher in the inflamed mucosa of children with CD and UC compared with the intact mucosa.[98] Moreover, the authors demonstrated induction of miR-146a and miR-155 after treatment with TNF-α (a potent inflammatory cytokine and effective therapeutic target in IBD)—and hence, their potential involvement in TNF-α proinflammatory signaling. The same team achieved similar results when comparing expression of miR-146a, miR-155, and miR-122 in inflamed duodenal tissue of CD patients, intact duodenal tissue of CD patients, and healthy controls.[99]

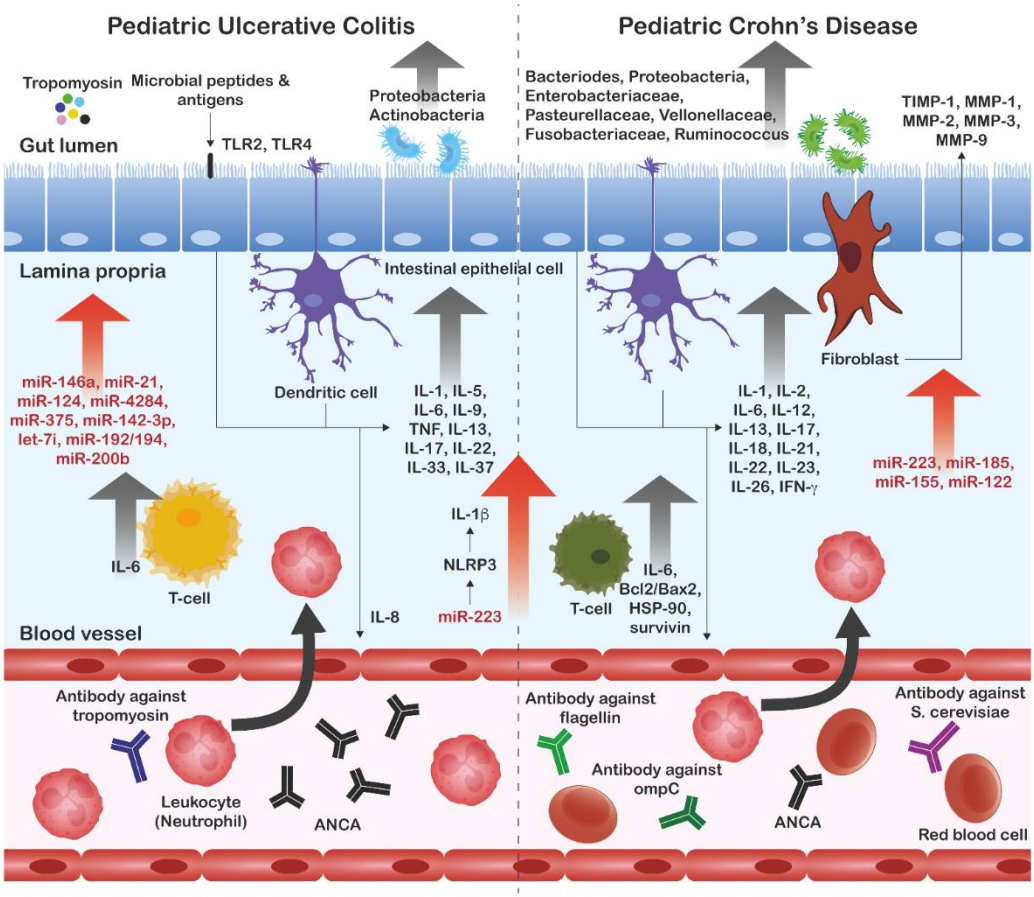

FIGURE 1. Tissue miRNAs involved in the development of pediatric IBD (modified from Park et al., 2017).[107] Abbreviations: TLR, toll-like receptor; TNF, tumor necrosis factor; ANCA, anti-neutrophil cytoplasmic antibodies; IFN, interferon; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; Bcl2, B-cell lymphoma 2; BAX, BCL2 associated X; CCL, CC chemokine ligand; CCR, CC chemokine receptor; ompC, outer membrane protein C precursor.

Their follow-up publication describes the most robust biomarker study to date concerning ncRNAs in pediatric IBD patients.[89] Using small RNA sequencing of fresh-frozen tissue biopsies, the authors obtained specific miRNA profiles of CD patients with inflamed and intact histology and also those of healthy controls. The validation phase of the study by Béres et al[89] was conducted not only in CD but also in pediatric UC patients, thereby providing additional information on the discovered miRNAs. The most interesting results from a diagnostic perspective are summarized in Table 1. There was significant overlap between the miRNA expression profiles differentiating CD and UC from healthy patients (upregulation of miR-18a, miR-21, miR-31, miR-99a, miR-99b, miR-125a, miR-126, miR-142-5p, miR-146a, and miR-223 and downregulation of miR-141 and miR-204 in diseased tissue). Nevertheless, there were some miRNAs upregulated in UC compared with CD, namely miR-21, miR-31, miR-125, miR-142-3p, and miR-146a; on the other hand, the expression levels of miR-100, miR-150, and miR-185 were increased in CD patients compared with UC patients. Through combined pathway analysis of miRNAs and their target mRNAs identified in CD, those authors revealed a strong association of these miRNAs and mRNAs with

inflammation, fibrosis, and response to microbiome, in addition to immune and inflammatory response. Five miRNAs differentially expressed in UC patients (miR-20a, miR-126, miR-141, miR-142, and miR-223) were connected to the ABCG2 and ABCB1 efflux transport proteins important in intestinal barrier protection against external stimuli.[89]

It seems that some of these miRNAs are probably not specific for pediatric IBD patients but more likely are important for IBD in general or inflammation as such. Similar profiles have been detected in studies performed on samples from adult IBD patients,[90] and some miRNAs are well-known players in inflammatory processes (eg, miR-146a, miR-155, and miR-21),[100] regardless of whether these are in adult or pediatric patients.

However, there are some examples showing just the opposite. Particularly noteworthy is that miR-223, identified in the Béres study as one of the most significantly upregulated miRNAs in UC and CD (in both inflamed and intact tissue),[89] has been described in adult IBD as a negative regulator of inflammation.[101] Results from Neudecker et al relating to adult IBD patients and an animal model showed that overexpression of miR-223 can attenuate inflammation. Moreover, they observed the release of proinflammatory cytokines and chemokines in myeloid-derived cells through the miR-223–NOD-like receptor 3 (NLRP3)–interleukin-1β (IL-1β) regulatory circuit as a critical component of intestinal inflammation and homeostasis.[101] MiR-223 is probably one of the examples indicating the differences between the underlying IBD pathogenesis in adult and pediatric patients.

The most recent work from Tang et al[102] was focused on miR-15 as a regulator of Cdc4, a potent regulator of the cell cycle. The miR-15 level was quantified in 33 pediatric IBD patients and 21 controls, and moderate increase in miR-15 expression was observed in CD patients. Unfortunately, the variability of miR-15 expression was too high, thus precluding its use as a reliable biomarker of CD. Testing for potential correlation between miR-15 expression and PCDAI score also was unsuccessful.[102]

NONCODING RNAs IN BODILY FLUIDS OF PEDIATRIC IBD PATIENTS

Concerning ncRNAs in bodily fluids (Table 2), Zahm et al provided initial promising findings.[97, 103] In their early work, these authors revealed that levels of miR-484 and miR-16 were significantly deregulated in blood serum of CD patients compared with healthy controls. Clinical testing achieved 83% sensitivity and 84% specificity for miR-484 and 74% sensitivity and 100% specificity for miR-16; these levels are indisputably higher than those for such laboratory markers currently used, such as C-reactive protein or anti-*Saccharomyces cerevisiae* antibody.[103] In addition to the tissue miRNA profiles from rectal biopsies of pediatric UC and CD patients described previously, they identified in their further work miRNA biomarkers in blood serum. Circulating miR-192, miR-142-3p, and miR-21 were confirmed to be elevated in both UC and CD samples relative to controls, and they correctly classified 79%, 72%, and 72% of IBD patients, respectively. In patients from whom both serum and rectum miRNAs were measured, circulating miRNA levels did not correlate with those of the tissue. There were also no differences in circulating miRNAs that would enable differentiating between CD and UC patients.[97]

Heier et al performed expression profiling of 24 circulating miRNAs involved in inflammation or steroid response to examine their responsiveness to anti-inflammatory treatments (eg, prednisone, infliximab).[104] They identified that 3 miRNAs (miR-146a, miR-146b, and miR-320a) known to be induced by inflammatory signaling were responsive to—or downregulated by—both drugs. A fourth miRNA, miR-486, showed a significant change in response to prednisone but not to infliximab. Together, measuring levels of these miRNAs could potentially help in assessing inflammatory disease and therapeutic response.[104] A similar study evaluated differentially expressed miRNAs during glucocorticoid treatment in blood cells (specifically peripheral blood mononuclear cells [PBMCs]) of pediatric patients with IBD (8 UC, 2 CD) enrolled at diagnosis and followed for the first weeks of steroid therapy.[105] Peripheral blood was obtained at diagnosis (T0) and after 4 weeks of prednisone treatment (T4). Among the 18 miRNAs differentially expressed from T0 to T4, 16 were upregulated and 2 were downregulated. Three miRNAs (miR-144, miR-142, and miR-96) could putatively recognize the 3′UTR of the glucocorticoid receptor gene, and 3 miRNAs (miR-363, miR-96, miR-142) contained glucocorticoid responsive element sequences, thereby potentially enabling direct regulation by the glucocorticoid receptor.[105]

The only study in pediatric IBD patients focusing on lncRNAs thus far was related to glucocorticoid therapy response and GAS5 levels in PBMCs. Clinical activity was assessed using the Pediatric Crohn's Disease Activity Index (PCDAI) for patients with CD and the Pediatric Ulcerative Colitis Activity Index (PUCAI) for patients with UC. Clinical remission was defined as PCDAI <10 or PUCAI <10, whereas clinical improvement was defined as a reduction of at least 15 points from the baseline score for PCDAI and at least 20 points from baseline for PUCAI.[106] Growth arrest-specific 5 levels were measured in PBMCs of 19 pediatric IBD patients at diagnosis and after the first cycle of glucocorticoids. This demonstrated upregulation of the lncRNA in patients with unfavorable steroid response, indicating that GAS5 can be considered a novel pharmacogenomic marker useful for personalizing glucocorticoid therapy.[106]

CONCLUSION AND FUTURE PERSPECTIVES

Not many studies to date have been focused on ncRNAs in pediatric IBD patients, very little knowledge exists as to the underlying biology of miRNAs involved in pediatric IBD patients (Fig. 1),[107] and the current descriptive observations are

often derived by extrapolation of discoveries from adult IBD experimental studies. Existing results show promise, however, as there is significant overlap in miRNA profiles across independent studies. Specifically, miR-146a, miR-142-3p, and miR-223 seem to be emerging as potential noninvasive biomarkers of pediatric IBD in the near future. Some of these miRNAs are specific for pediatric IBDs when compared with adult counterparts. There also are miRNA biomarkers (eg, miR-24), enabling accurate differentiation between UC and CD cases and tissue miRNA expression changes reflecting successful glucocorticoid treatment. In bodily fluids, miRNAs have been observed to differ by their levels in blood serum of IBD patients and controls. In PBMCs, miRNAs and lncRNA GAS5 have been shown responsive to the anti-inflammatory agents prednisone and infliximab. A variety of study designs are found in the current literature, however. These need to be unified and include independent validation cohorts of patients to provide solid and more convincing results. Also, high-throughput technologies for ncRNA profiling are not as common, and a majority of the studies are based on preselected groups of ncRNA candidates. A higher level of methodological standardization is necessary also to develop reliable clinical-level biomarkers.

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Annex 4

Journal of *Clinical Medicine*

MDPI

Article

MicroRNAs in Colon Tissue of Pediatric Ulcerative Pancolitis Patients Allow Detection and Prognostic Stratification

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Abstract: Prevalence of inflammatory bowel disease has been on the rise in recent years, especially in pediatric populations. This study aimed to provide precise identification and stratification of pediatric patients with diagnosed ulcerative colitis (UC) according to the severity of their condition and the prediction for standard treatment according to the specific expression of candidate miRNAs. We enrolled consecutive, therapeutically naïve, pediatric UC patients with confirmed pancolitis. We examined formalin-fixed paraffin-embedded specimens of colonic tissue for the expression of 10 selected candidate miRNAs. We performed receiver operating characteristic curve analysis, using area under the curve and a logistic regression model to evaluate the diagnostic and predictive power of the miRNA panels. Sixty patients were included in the final analysis. As a control group, 18 children without macroscopic and microscopic signs of inflammatory bowel disease were examined. The combination of three candidate miRNAs (let-7i-5p, miR-223-3p and miR-4284) enabled accurate detection of pediatric UC patients and controls. A panel of four candidate miRNAs (miR-375-3p, miR-146a-5p, miR-223-3p and miR-200b-3p) was associated with severity of UC in pediatric patients and a combination of three miRNAs (miR-21-5p, miR-192-5p and miR-194-5p) was associated with early relapse of the disease. Nine patients out of the total were diagnosed with primary sclerosing cholangitis (PSC) simultaneously with ulcerative colitis. A panel of 6 candidate miRNAs (miR-142-3p, miR-146a-5p, miR-223-3p, let-7i-5p, miR-192-5p and miR-194-5p) identified those patients with PSC. Specific combinations of miRNAs are promising tools for potential use in precise disease identification and severity and prognostic stratification in pediatric patients with ulcerative pancolitis.

Keywords: pediatrics; inflammatory bowel disease; ulcerative colitis; microrna; primary sclerosing cholangitis

Citation: Jabandziev, P.; Kakisaka, T.; Bohosova, J.; Pinkasova, T.; Kunovsky, L.; Slaby, O.; Goel, A. MicroRNAs in Colon Tissue of Pediatric Ulcerative Pancolitis Patients Allow Detection and Prognostic Stratification. *J. Clin. Med.* **2021**, *10*, 1325. https://doi.org/10.3390/jcm10061325

Academic Editor: Ewa Małecka-Panas

Received: 26 February 2021
Accepted: 18 March 2021
Published: 23 March 2021

1. Introduction

Inflammatory bowel disease (IBD) is a chronic complex disorder of the digestive system caused by multiple factors, including genetics, epigenetics, gut microbiota, environmental factors and altered immune system, although the precise mechanisms underlying the pathogenesis of this disease remain unclear [1–3]. Owing to chronic inflammation, patients with ulcerative colitis (UC) and Crohn's disease (CD) involving the colon have an

increased risk for colon cancer. The risk increases with time from diagnosis and higher-risk groups include those younger at diagnosis, with pancolitis, or with associated primary sclerosing cholangitis (PSC) [4,5].

Although prevalence of the disease peaks in the fourth and fifth decades of life, a substantial number of patients have onset of IBD during early childhood [6,7]. Significant differences in clinical manifestation, possible pathophysiology and treatment between children and adults prevent extrapolation of knowledge from one group to the other [8]. Studies have shown that phenotypes of pediatric patients with IBD are more aggressive and with more damaging impact disrupting normal growth and overall development of the child [9]. Moreover, therapeutically naïve patients often show quite different responses to the treatment given [10,11]. Despite outstanding achievements in therapeutic interventions, there is a clear need for biomarkers enabling accurate identification, prognosis and prediction of response to therapies to facilitate a more individualized approach. In recent years, evidence has continued to evolve concerning non-coding RNAs (ncRNAs) and their roles as integral factors in key immune-related cellular pathways. Specific deregulation patterns of ncRNAs have been linked to the pathogenesis of adult and pediatric IBD [12]. Several microRNAs (miRNAs) and specific miRNA signatures have been identified in IBD-associated tissues. It has been shown that, among many other cellular processes, miRNAs play a significant role in intestinal immunity. Nevertheless, there exists only sparse information on ncRNA profiles and their diagnostic and prognostic potential in pediatric IBD patients [12]. In this article, we stratify therapeutically naïve pediatric patients with diagnosed UC according to the severity of their condition and the prediction for standard treatment according to the specific expression of 10 candidate miRNAs.

2. Materials and Methods

2.1. Study Design

Subjects were enrolled at the Department of Pediatrics, University Hospital Brno, Czech Republic. Eligible subjects consisted of children having been diagnosed with UC (only pancolitis) according to relevant guidelines [13,14] (clinical history, physical examination, laboratory results, serological testing, radiologic findings and endoscopic appearance with stepwise biopsy for review by clinical pathologists) in a period between 1 January 2012 and 31 December 2018. All patients underwent upper gastrointestinal endoscopy and ileocolonoscopy. All were 0–18 years of age at the time of diagnosis and were resident in the South Moravian Region, one of 14 regional administrative units in the Czech Republic [6]. Enrolled into the study were only unequivocal UC cases with pancolitis (with proven macroscopic and microscopic disease affection) as determined by the Paris classification (E4) [15]. Patients without indisputable diagnosis of UC according to the Porto criteria were excluded from further analyses [14]. Initial severity of the disease was evaluated by an experienced gastroenterologist using the pediatric ulcerative colitis activity index (PUCAI) score [16]. In our other analyses, we focused only on patients with mild (PUCAI 10–34) and moderate (PUCAI 35–64) ulcerative pancolitis. Patients with severe colitis (PUCAI > 65) were excluded from the study for a low number of patients (n = 3) and lack of representative material for an examination. We examined formalin-fixed paraffin-embedded specimens of colonic tissue (rectosigmoid colon).

We focused on precise identification of pediatric UC patients and, thereafter, on possible stratification for severity of disease according to specific patterns of candidate miRNAs' expression in colonic tissue. We then undertook to create a candidate model for predicting early relapse of the disease. Patients received initial standardized treatment with mesalazine and/or corticosteroids guided by the PUCAI according to relevant national guidelines [17] and were evaluated for adherence to therapy by use of structured interview. We monitored whether after standard treatment a patient experienced an early relapse during the first year of treatment despite significant initial remission of the disease, defined as PUCAI < 10 during the first 3 months after diagnosis and fecal calprotectin <100 ug/L. Moreover, we compared patients with diagnosed PSC and patients without this diagnosis.

As a control group, we used children indicated for the endoscopic examination mostly due to chronic abdominal pain, but these patients did not show neither macroscopic nor microscopic signs of IBD and fecal calprotectin was normal. None of the patients were diagnosed with IBD one year after enrollment in the study.

The Institutional Ethical Committee approved the study at University Hospital Brno in accordance with the Declaration of Helsinki.

2.2. Candidate miRNAs Selection

A reasonable number of candidate miRNAs were selected based on a literature review published elsewhere [12]. These were only demonstrably dysregulated miRNAs in terms of downregulation and upregulation in pediatric patients: 5 upregulated (miR-21-5p, miR-142-3p, miR-146a-5p, miR-223-3p and let-7i-5p) and 5 downregulated (miR-192-5p, miR-194-5p, miR-200b-3p, miR-375-3p and miR-4284).

2.3. Nucleic Acid Isolation and miRNA Expression Analysis

Total RNA was isolated from formalin-fixed paraffin-embedded biopsy tissues from rectosigmoideum using ALL Prep DNA/RNA FFPE kit (Qiagen, Valencia, CA, USA). Synthesis of complementary DNA (cDNA) was conducted using miRCURY LNA RT Kit (Qiagen, Valencia, CA, USA). miRCURY LNA miRNA PCR Assays (primer mix) for the following miRNAs were used for quantitative real-time reverse transcription analysis (qRT-PCR): miR-16-5p, miR-21-5p, miR-142-3p, miR-146a-5p, miR-192-5p, miR-194-5p, miR-200b-3p, miR-223-3p, miR-375-3p, miR-4284 and let-7i-5p (catalog no.: YP00205702, YP00204230, YP00204291, YP00204688, YP00204099, YP00204080, YP00206071, YP00205986, YP00204362, YP02114835, YP00204394; product no.: 339306, Qiagen). qRT-PCR was performed using the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, London, UK) on the Quantstudio 6 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and we used miR-16-5p as an endogenous control for data normalization. The relative expression levels of target genes were normalized against beta-actin using the 2-ΔCT method.

2.4. Statistical Analysis

We performed receiver operating characteristic (ROC) curve analysis and used area under the curve (AUC) values to evaluate the diagnostic power of the miRNA panel. For ROC analysis, we used a risk score calculated by a logistic regression model. The categorical factors were analyzed by Fisher's exact test or chi-squared test and continuous factors were analyzed by Mann–Whitney U test. Spearman's correlation coefficient was used to determine statistical dependence between variables. All statistical tests were two-sided and p-value < 0.05 was considered significant. All statistical analyses were performed using MedCalc statistical software Version 19.1 (Medcalc Software bvba, Ostend, Belgium), JMP software 14.0.0 (SAS Institute, Cary, NC, USA) and GraphPad Prism version 8.2.0 (GraphPad Software, San Diego, CA, USA). To identify the most robust miRNA panel for the diagnosis of UC patients, the adaptive LASSO model was applied to the qPCR data using JMP 14.0.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Clinical Characteristics of Patients

A total of 60 patients with complete clinical data and with a sufficient amount of material from colonic tissue for further analysis were included in the final analysis. The basic demographic and clinical characteristics of patients are shown in Table 1.

As a controls, we used a group of 18 children without macroscopic and microscopic signs of IBD. The control group consisted of 12 girls (66.7%) and 6 boys (33.3%), with mean age of 14.6 years.

3.2. Expression of 10 Selected miRNAs

Differences in expression of selected 10 miRNAs in patients with mild and moderate UC (based on PUCAI score) and healthy controls are depicted in Figure 1.

Table 1. Demografic and clinical characteristics of UC patients.

Severity of the Disease (PUCAI Score)	Mild	Moderate	Total
	29 (48.3%)	31 (51.7%)	60 (100%)
Sex, N (%)			
Male	13 (44.8%)	13 (41.9%)	26
Female	16 (55.2%)	18 (58.1%)	34
Mean age at diagnosis (years)	13.7	13.9	13.7
Mean PUCAI:			
At diagnosis	20.7	43.7	32.3
After 3 months	2.2	5.0	3.7
Treatment, N (%)			
Mesalazine	29 (100%)	31 (100%)	60 (100%)
Corticosteroids	18 (62%)	25 (80.6%)	43 (71.7%)
Response rate to initial treatment, N (%)	26 (89.7%)	25 (80.6%)	51 (85%)
Early relapse, N (%)	6 (20.7%)	7 (22.6%)	13 (21.7%)
Primary sclerosing cholangitis, N (%)	6 (20.7%)	3 (9.7%)	9 (15%)

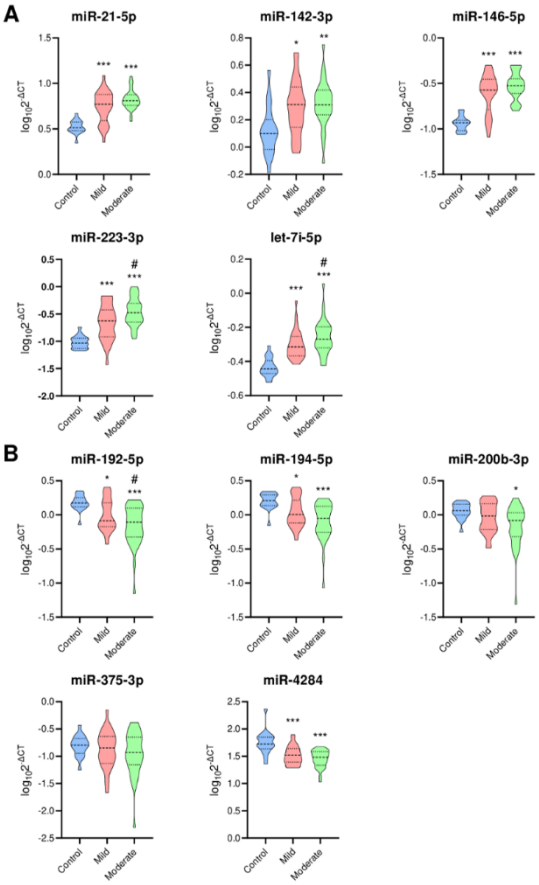

Figure 1. Expression of selected 10 miRNAs. (**A**) Upregulated miRNAs; (**B**) Downregulated miRNAs. Violin plots indicating expression of each miRNA. Blue, red and green violins indicate controls, mild UC patients and moderate UC patients, respectively. Thick dotted line, median; thin dotted line 25% and 75% quartiles. Y-axis is defined as log10(2-ΔCT). * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ versus control, # $p < 0.05$ versus mild UC patient.

In the Supplementary materials, we provide the expression of 10 selected miRNAs (Supplementary Figure S1), ROC curves for the 10 individual miRNAs for distinguishing UC patients from controls (Supplementary Figure S2) and a model for diagnostic accuracy of 10-miRNA panel for identification of pediatric UC patients (Supplementary Figure S3).

3.3. Identification of Robust miRNA Panel for UC Diagnosis

To identify the most robust miRNA panel for the diagnosis of UC patients, the adaptive LASSO model was applied to the qPCR data using JMP 14.0.0 (SAS Institute, Cary, NC, USA). Three candidate miRNAs (let-7i-5p, miR-223-3p and miR-4284) were distinguished for accurate identification of pediatric UC patients (Figure 2). A risk score for detection of UC patients was calculated as follows: logit (risk score) = 37.84 + 38.89 × let-7i-5p + 5.46 × miR-223-3p − 10.65 × miR-4284.

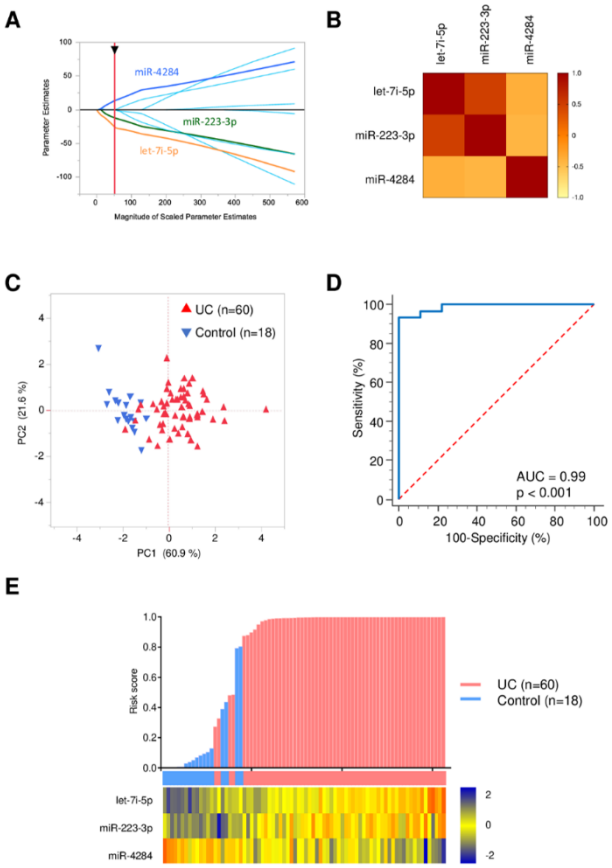

Figure 2. Diagnostic accuracy of 3-miRNA panel for identifying UC patients. (**A**) The adaptive LASSO model; (**B**) A correlation matrix displaying the Spearman's rank correlation coefficient for each pair of three selected miRNAs; (**C**) Principal component analysis illustrating the good separation of UC-patient group and control group; (**D**) ROC curves for detecting UC patients using 3-miRNA panel; (**E**) A waterfall plot representing risk score of each patient. Red and blue columns indicate UC patients and controls, respectively. A heat map illustrating expression levels of the three candidate miRNAs expressed differentially between UC patients and controls.

3.4. Severity Stratification Panel

In the quest to find the most robust miRNA panel for identifying UC severity, an adaptive LASSO model was applied to the qPCR data using JMP 14.0.0 (SAS Institute, Cary, NC, USA). Four candidate miRNAs (miR-375-3p, miR-146a-5p, miR-223-3p and miR-200b-3p) were prioritized for identifying severity of UC in pediatric patients (Figure 3).

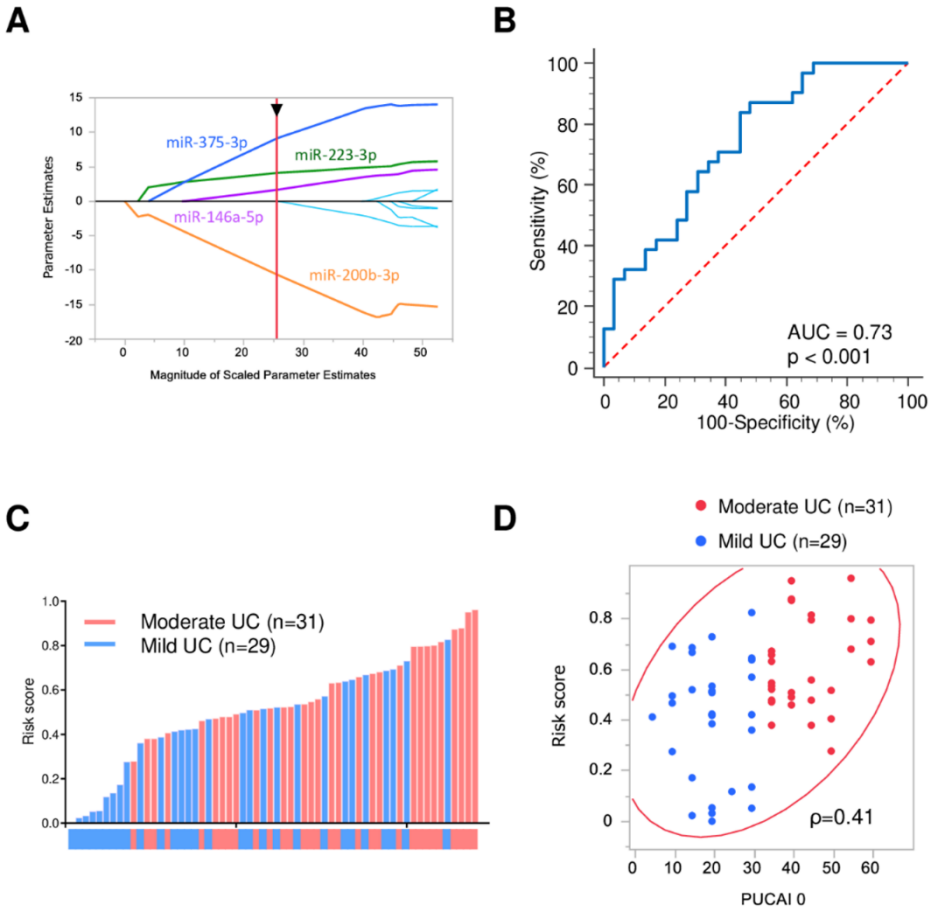

Figure 3. Diagnostic accuracy of 4-miRNA panel for identifying severity of UC patients. (**A**) Adaptive LASSO model; (**B**) ROC curves for detecting UC patients using 4-miRNA panel; (**C**) A waterfall plot representing risk score of each patient. Red and blue columns indicate moderate and mild UC patients, respectively; (**D**) A scatter plot showing the correlation of PUCAI with risk score. Red and blue circles indicate moderate and mild UC patients, respectively. ρ is the Spearman's rank correlation coefficient.

We built a risk score for quantifying the severity of the UC patients as follows: logit (risk score) = 6.99 − 8.32 × miR-200b-3p + 2.45 × miR-223-3p + 5.61 × miR-375-3p + 2.50 × miR-146a-5p.

3.5. Early Relapse Prediction

Endeavoring to stratify patients according to their response to the standard therapeutic regime, we identified in the same manner three specific miRNAs (upregulated miR-21-5p and downregulated miR-192-5p and miR-194-5p) that appear to be associated with early disease relapse, where early relapse was assessed as a significant worsening of the patient's condition during the first year of treatment (based on clinical score, laboratory results and elevated fecal calprotectin levels) (Figure 4).

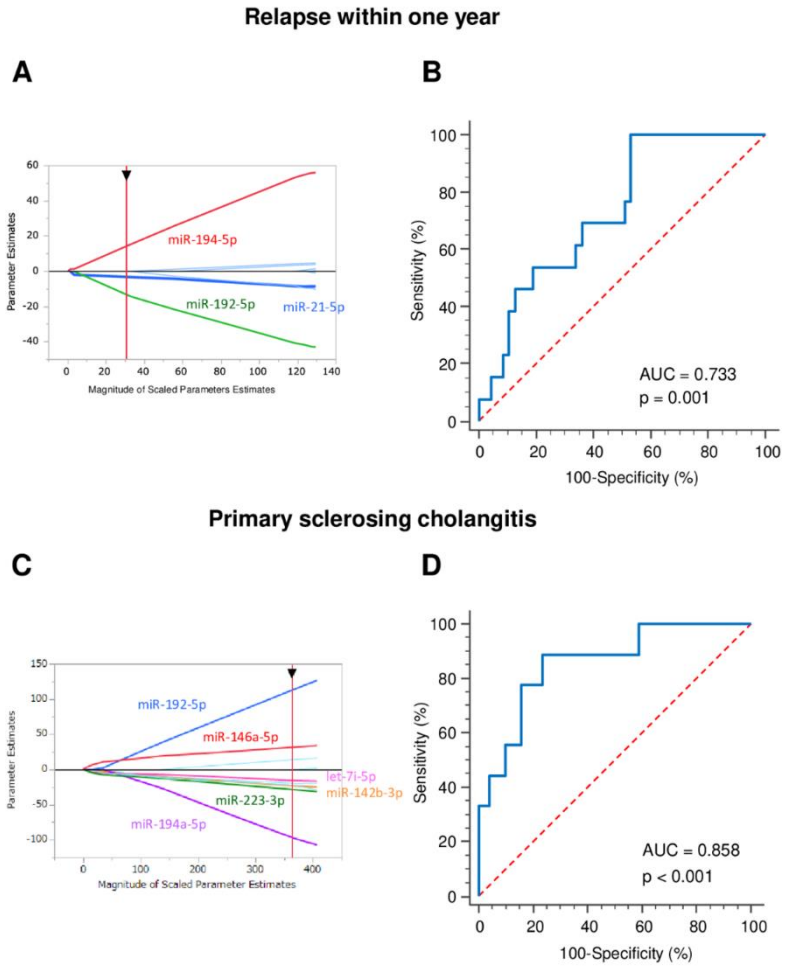

Figure 4. Diagnostic accuracy of miRNAs for identifying relapse within 1 year and primary sclerosing cholangitis. (**A**) Adaptive LASSO model; (**B**) ROC curves for detecting early relapse in UC patients using 3-miRNA panel. Logit (risk score) = 1.26 − 4.44 × miR-21-5p − 15.83 × miR-192-5p + 17.60 × miR-194-5p; (**C**) Adaptive LASSO model was applied to the qPCR data; (**D**) ROC curves for detecting primary sclerosing cholangitis in UC patients using 6-miRNA panel. Logit (risk score) = − 0.24 − 5.43 × miR-142-3p + 13.00 × miR-146a-5p + 14.59 × miR-192-5p − 19.57 × miR-194-5p − 5.23 × miR-223-3p − 14.42 × let-7i-5p.

3.6. Primary Sclerosing Cholangitis Identification

To determine the most robust miRNA panel for the identification of patients with UC and primary sclerosing cholangitis, an adaptive LASSO model was applied to the qPCR data using JMP 14.0.0 (SAS Institute, Cary, NC, USA). Four upregulated (miR-142-3p, miR-146a-5p, miR-223-3p and let-7i-5p) and two downregulated (miR-192-5p and miR-194-5p) miRNAs were identified (Figure 4).

4. Discussion

A knowledge of non-coding RNAs, mainly regarding miRNAs, has grown rapidly in recent years, mostly since the development of next-generation sequencing. Expression levels of miRNAs are closely related to many vital cellular processes and their regulation, as miRNAs act as fine-tuners of those processes. By complementary binding to the 3′-untranslated region of their target mRNA, miRNAs destabilize or activate the degradation of their target mRNA and thus preclude its translation. Being involved in a complex regulatory network consisting of not only other miRNAs but also of many other regulatory molecules, some miRNAs can regulate multiple pathways even as other miRNAs are simultaneously regulated by several pathways. Thus, a complex network exists and is involved also in IBD. Evidence on the involvement of miRNAs in the development of IBD is based mostly on studies performed on the adult population. Studies performed on cohorts of pediatric patients are rather sparse and of limited reliability, as most of the studies work with several dozen patients at most. Despite all the limitations, the results are mutually confirming [12], at least in the cases of several miRNAs, namely miR-21, miR-146a and miR-142-3p [18–20].

Most of the previously published studies were focused on differentiating IBD cases from healthy controls, thus identifying so-called diagnostic biomarkers. We tested, also, the ability of chosen miRNAs to differentiate UC cases from healthy patients, which was feasible using a combination of let-7i, miR-223 and miR-4284. There exist, however, many more specific clinical challenges, such as responsiveness to therapy, prediction of relapse, differentiation of IBD subtypes and preventing overtreatment. We therefore focused our effort also on other biomarker aspects and successfully identified a combination of miR-375, miR-146a, miR-223 and miR-200b that was able to identify patients with a more severe course of the disease. Moreover, we identified that the upregulation of miR-21 and downregulation of miR-192 and miR-194 are indicative of early relapse after treatment and more precisely relapse within 1 year after administering treatment. In order to find a biomarker discerning patients with IBD associated with PSC (PSC-IBD), we showed for the first time that a combination of 3 upregulated miRNAs (miR-142-3p, miR-146a-5p and miR-223-3p) and 3 downregulated miRNAs (miR-192-5p, miR-194-5p and miR-375-3p) is typical for PSC-IBD patients.

Although IBD's exact pathogenesis remains unclear, its inflammatory nature is evident also from miRNAs that are repeatedly identified as deregulated in IBD patients of either subtype [12]. Potential involvement of those miRNAs can be traced to the most essential pathways and agents of systemic inflammatory response, such as TLR/NF-κB, TNF-α and other pro- and anti-inflammatory factors, either as their regulators (as in the case of miR-375,3 miR-223 [21,22], let-7i [23], miR-200b and the entire miR-200 family [24,25] and miR-146a [26]) or as the molecules regulated by such pro- and anti-inflammatory agents, such as miR-142 [27,28]. Closely related to inflammation seems to be autophagy, a natural process of removing certain cellular components. Among others, it is involved in maintaining homeostasis and survival of such inflammatory cells as lymphocytes, neutrophils and macrophages [29] miR-223, miR-146a [26,30], miR-142 [28], miR-192 [31] and miR-194 [26] are all involved in regulation of autophagy and thus, in inflammation through NOD2, ATG16L1 and mTOR pathways [29]. A specific spot is held by miR-21, notoriously known as a potent inflammatory switch and a key regulator of both pro- and anti-inflammatory factors, which acts as a "molecular rheostat" [32]. Moreover, persistent inflammation is a prerequisite for carcinogenesis and consistent with this are findings of

miRNAs upregulated in IBD and their involvement in the development of cancer, such as miR-21 through PCDC4 downregulation [33] and miR-4284 through the CXCL5 pathway and regulation of chemotaxis and proliferation [34–36].

In our study, we have successfully validated results and confirmed biomarker potential in several miRNAs identified in studies of similar design. Moreover, to the best of our knowledge, we have evaluated for the first time in a Central European representative population of children with ulcerative pancolitis expression of selected miRNAs and their association with key clinical questions. Our study cohort is relatively homogenous, consisting exclusively of well-described pancolitic disease patients, thus avoiding any inconsistency stemming from an uneven range of the disease among patients. The results demonstrate that the specific combinations of expressed miRNAs in colonic mucosa are associated with important clinical parameters for more precise diagnosis, prediction of prognosis and clinical outcome. The uniqueness of our study lies in an unusually large study cohort. No previous study has been carried out on more than 20 patients with UC, and therefore, the reliability of the information has been somewhat limited. Independent validation of the results on a cohort including as many as 78 pediatric patients has never been published to date. Moreover, we have identified miRNAs and their combinations that not only can differentiate patients from controls but also patients with early relapse of the disease. This is of great diagnostic and prognostic importance. Due to our stratification of patients is based solely on the PUCAI score, however and that score itself has some diagnostic limitations, our results should be verified on a cohort described more precisely by other clinical and endoscopic (Mayo score) parameters.

In addition, unique is the identification of patients with PSC-IBD. Although typical for middle-aged males, this condition also affects children. In the majority of the childhood cases, it is associated with IBD, typically with UC, while association with CD is less common [5,37]. The course of PSC-IBD and IBD alone is similar, but colon dysplasia may occur in patients with PSC-IBD and this predisposes them to the development of colon cancer later in life. Those patients are therefore at greater risk and need more frequent follow-up [4,38]. Early identification of PSC-IBD patients could lead to more effective care.

As current therapeutic options do not allow for early identification of patients who would not profit from a standard therapy either for non-responsiveness or for other causes, identification of potential biomarkers is of utmost interest for physicians. It should be noted, though, that the miRNAs validated in our study and successfully confirmed as deregulated are involved in inflammation as such, and therefore, do not reflect specific IBD changes other than overall inflammation of the gut. Moreover, this study validates previously published results. The main limitation of our study is a lack of the CD patients cohort disabling determination of the specificity of tested biomarkers.

While this study undeniably brings value to the existing knowledge, independent validation in combination with profiling that uses such currently available high-throughput techniques as next-generation sequencing would be even more significant.

5. Conclusions

In conclusion, we independently verified the biomarker potential of miRNAs feasible for assessing detection and prognosis and discovered that their combinations could distinguish not only UC patients from controls without signs of IBD but even more specific conditions such as disease severity, early relapse and even association with PSC. A larger cohort of patients with not only UC but also CD and a study based on expression profiling using high-throughput platforms and independent validation is greatly needed in this field.

Supplementary Materials: The following are available online at https://www.mdpi.com/2077-0383/10/6/1325/s1, Supplementary Figure S1: Expression of 10 selected miRNAs, Supplementary Figure S2: Receiver operating characteristic (ROC) curves for 10 individual miRNAs for distinguishing UC patients from controls, Supplementary Figure S3: Diagnostic accuracy of 10-miRNA panel for identifying UC patients.

Author Contributions: Conceptualization, P.J. and O.S.; methodology, T.K. and J.B.; software, T.K.; validation, T.K.; formal analysis, T.K.; investigation, P.J., T.P. and L.K.; resources, P.J. and A.G.; data curation, P.J., J.B. and T.K.; writing—original draft preparation, P.J., T.K., J.B. and A.G.; writing—review and editing, O.S. and A.G.; visualization, T.K.; supervision, O.S. and A.G.; project administration, T.P. and L.K.; funding acquisition, A.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by CA184792, CA187956, CA227602, CA072851 and CA202797, grants from the National Cancer Institute, National Institutes of Health and by the Ministry of Health, Czech Republic—conceptual development of research organization (FNBr, 65269705) and grant no. NV21-07-00285.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Brno.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data underlying this article cannot be shared publicly due to ethical reasons. The data will be shared on reasonable request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Annex 5

frontiers in Genetics

CASE REPORT
published: 07 October 2020
doi: 10.3389/fgene.2020.561054

Novel Splicing Variant in the *PMM2* Gene in a Patient With PMM2-CDG Syndrome Presenting With Pericardial Effusion: A Case Report

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OPEN ACCESS

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Specialty section:
This article was submitted to Genetics of Common and Rare Diseases, a section of the journal Frontiers in Genetics

Received: *11 May 2020*
Accepted: *17 September 2020*
Published: *07 October 2020*

Citation:
Slaba K, Noskova H, Vesela P, Tuckova J, Jicinska H, Honzik T, Hansikova H, Kleiblova P, Stourac P, Jabandziev P, Slaby O and Prochazkova D (2020) Novel Splicing Variant in the PMM2 Gene in a Patient With PMM2-CDG Syndrome Presenting With Pericardial Effusion: A Case Report. Front. Genet. 11:561054. doi: 10.3389/fgene.2020.561054

Congenital disorders of glycosylation (CDG) are a rapidly growing family of genetic diseases with the phosphomannomutase 2 (PMM2)-CDG being the most common form of CDG. Most of these monogenic diseases are autosomal recessive and have multi-systemic manifestations, mainly psychomotor retardation, facial dysmorphisms, characteristic distribution of the fat pads, and variable coagulation abnormalities. The association of fetal hydrops with CDG has been reported, and pericardial effusion was also rarely observed in patients with PMM2-CDG. Here we describe an infant boy with PMM2-CDG. The diagnosis was suspected based on inverted nipples, fat pads, and combined coagulopathy. However, the primary symptom was progressive pericardial effusion leading to patient death at the age of 3 months. Screening for CDG performed by the use of isoelectric focusing of serum transferrin showed a typical PMM2-CDG pattern. Exome sequencing revealed one common pathogenic variant (c.691G > A/p.Val231Met) and one novel variant (c.447 + 3dupA) in the *PMM2* gene. Both *PMM2* variants were further confirmed by Sanger sequencing in both the proband and the parents' DNA. The novel variant was predicted to result in loss of donor splice site, and the analysis at mRNA level confirmed that it leads to exon five skipping (r.348_447del) and causes premature termination of translation to the protein (p.G117Kfs*4), therefore is classified as likely pathogenic. Although there is no curative therapy for the PMM2-CDG at the moment, the other supportive care options are available to be offered. The definite diagnosis of PMM2-CDG can also assist in the process of genetic counseling, family planning, and preimplantation genetic diagnosis.

Keywords: PMM2-CDG, pericardial effusion, whole exome sequencing, novel splicing variant, phosphomannomutase 2

INTRODUCTION

Congenital disorders of glycosylation (CDG) are a genetically and clinically heterogeneous group of > 130 disorders characterized by genetic defects in the synthesis and attachment of glycoprotein and glycolipid glycans (Chang et al., 2018). The most common form of CDG is phosphomannomutase 2 (PMM2)-CDG (formerly known as CDG-Ia) (OMIM 212065), a multisystem disease with a wide range of clinical presentation and the phenotype ranging from mild adulthood form to very severe neonatal form (Peanne et al., 2018). PMM2-CDG is an autosomal recessive disorder, caused by mutations in the PMM2 gene localized on chromosome 16p13 (MIM 601785), with the prevalence ranging from 1/20,000 in Dutch populations and 1/77,000 in Estonia based on isolated reports (Schollen et al., 2000; Vals et al., 2018).

From the pathophysiological point of view, PMM2-CDG is a disorder of protein N-glycosylation characterized by genetic defects leading to deficiency/dysfunction of PMM2, the enzyme responsible for the conversion of mannose-6-phosphate into mannose-1-phosphate (Matthijs et al., 2000). Mannose-1-phosphate is a precursor of guanosine diphosphate mannose (GDP-Man) and dolichol-P-mannose (Dol-Man). Deficiency of GDP-Man and Dol-P-Man causes hypoglycosylation of numerous glycoproteins, including serum glycoproteins (lysosomal enzymes and transport proteins) and membrane glycoproteins. This results in multi-organ involvement, whereas the variability of disease severity and course are not fully understood (Altassan et al., 2019).

Here, we describe a novel splicing mutation in a case of PMM2-CDG, presented with pericardial effusion, with typical dysmorphic facial features, inverted nipples, failure to thrive, and psychomotor retardation.

CASE PRESENTATION

A Czech boy was born as a first child of unrelated parents. He was diagnosed with hemodynamically insignificant pericardial effusion in the 22nd week of pregnancy. Amniocentesis was performed with no evidence of aneuploidy. There was an aberration of unknown significance Xp22.33 × 3 and three copies of the CYP21A2 gene in combination with a pathogenic variant p.Gln319* detected hypothetically associated with congenital adrenal hyperplasia; however, the determination for 17-hydroxyprogesterone in amniotic fluid provided a negative result. Routine testing of the mother for toxoplasmosis, parvovirus B19, CMV, and HSV infections was negative. Family history was unremarkable. Labor was induced in 40 weeks of pregnancy for intrauterine growth retardation and oligohydramnios. Delivery was by forceps-assisted vaginal delivery due to fetal heart rate deceleration. A birth weight of the boy was 2390 g (z-score = −1.83), length of 48 cm, and occipital frontal circumference 34 cm. His postnatal adaptation was normal, but he was noted to be highly dysmorphic. Initially, due to swallowing difficulties nasogastric tube was used for feeding.

At the age of 4 weeks pericardial effusion further progressed and the boy was referred to our department for complex evaluation and assessment. At this time, we observed a notable psychomotor retardation, significant central hypotonia, limited spontaneous movement, poor eye contact, no reaction on noise, significant failure to thrive (with only 90 g gain in 2 weeks). Arthrogenic contractures limiting the range of joins mobility were observed mainly in knee joints, ankle joints and elbow joints. His dysmorphic features included dolichocephaly, bossing forehead, dysmorphic low set ears (**Figure 1A**), enlarged fontanelle, wider philtrum, broad nasal root, prominent nares, hypertelorism, retrognathia (**Figure 1B**), inverted nipples (**Figure 1C**), abnormal fat distribution over his thighs, buttocks and suprapubic regions (**Figure 1D,E**), pilonidal sinus and hammertoes (**Figure 1F**).

Laboratory analyses at that time revealed an altered biochemical profile with findings of hypoproteinemia, hypoalbuminemia (23.1 g/l), severe combined coagulopathy with coagulation and anti-coagulation pathways alterations (aPTT 1.86R, INR 1.3, antithrombin III 20%, Factor IX 31%, fibrinogen 1.72 g/l, severe deficiency of Factor XI 5%, D-Dimer: 1.37 mg/l, Factor VII 66% and protein C bellow 5%). Other laboratory findings included mild hepatopathy with elevated levels of AST (140.96 IU/l), ALT (77.11 IU/l) and ALP (611.45 IU/l), mild elevation of lactate dehydrogenase, creatine kinase, and TSH. Infectious disease screening was negative.

Abdominal ultrasonography showed hyperechogenicity of kidneys, mild renal pelvis dilatation, ascites and small pleural effusion. There were no laboratory signs of nephropathy. The patient underwent cardiac examination. Cardiac anatomy and function were normal; however, a chronic pericardial effusion was detected by echocardiography. Initially mild to moderate pericardial effusion slowly progressed despite the administration of the diuretic (furosemide at 1mg/kg/day). The patient showed also intermittent eyelid edema and swelling of the arms and legs. Repeated infusions of albumin and plasma substitutes always led to the improvement of clinical symptoms including partial regression of pericardial effusion and peripheral edema.

Clinical symptoms and laboratory findings led to the strong suspicion of PMM2-CDG. Screening for CDG performed by the use of isoelectric focusing of serum transferrin showed pronounced increases in disialotransferrin and asialotransferin with a corresponding reduction in tetrasialotransferrin. This pattern supports the diagnosis of PMM2-CDG.

To further confirm the genetic cause of the disease exome sequencing was performed. Library for whole-exome capture and sequencing was prepared using TruSeq Exome Kit. Prepared library was loaded onto NextSeq 500/550 Mid Output Kitv2.5 (150 cycles) and sequenced on the NextSeq 500 instrument (all Illumina, CA, United States). Sequencing coverage for exomes was > 20 × at > 90% of captured regions. The variants were filtered to include those with low frequency and a predicted effect on the protein. The frequency-filter removed variants with prevalence > 1% in GnomAD or 1000 Genomes databases. The predicted effect filter excluded synonymous and non-coding variants unless they were located within 20 bp from the end of an exon. Variants annotated in ClinVar as pathogenic/likely

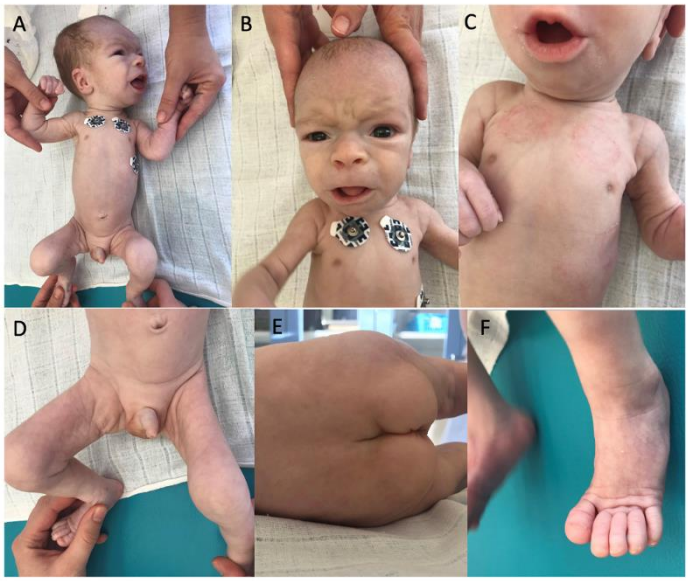

FIGURE 1 | Dysmorphic features of our patient. Dolichocephaly, bossing forehead, dysmorphic low set ears **(A)**, facial dysmorphism **(B)**, bilateral inverted nipples **(C)**, abnormal fat distribution **(D,E)**, and hammertoes **(F)**.

pathogenic were retained regardless of their frequency/function. The variants found in the proband's sample that were considered to be significant to CDG were c.691G > A/p.Val231Met in the *PMM2* gene known to be disease-causing (Matthijs et al., 1997;) and classified as class 5-pathogenic accordingly to ACMG/AMG system (Richards et al., 2015) and novel variant c.447 + 3dupA in the PMM2 gene (NM_000303.2, intron 5). We did not detect any other class 5 or 4 variants in the genes for which incidental findings are reported based on the ACMG guidelines. Confirming the results of the exome sequencing, the Sanger sequencing was done in the proband and his parents. As it was expected, results confirmed both variants in the proband and showed that c.691G > A/p.Val231Met variant was inherited from father (**Figure 2A**) and c.447 + 3dupA variant from mother (**Figure 2B**), presenting the typical autosomal recessive mode of inheritance.

The c.447 + 3dupA variant causes single-nucleotide duplication of the third intronic nucleotide after exon 5. Three different bioinformatic tools (MaxEntScan, NNSPLICE, and SpliceSiteFinder) integrated in the Alamut Visual 2.11 software predict a loss of donor splice site (Interactive Biosoftware, France) with confidence 75%. To confirm the impact of this variant to the mRNA splicing process, we have performed RNA sequencing. Total RNA from peripheral leukocytes was used to prepare the sequencing library with NEBNext Ultra II Directional Library Prep Kit (New England Biolabs, MA, United States). The library was loaded onto NextSeq 500/550 High Output Kit (75 cycles) and sequenced on the NextSeq 500 instrument (both – Illumina, CA, United States). The results of RNA sequencing were visualized by the IGV software[1] using Sashimi plots for individual exon–exon splicing events present in the transcript. We have confirmed that c. 447 + 3dupA variant causes a loss of donor splice site, leads to exon five skipping (r.348_447del100), and causes premature termination of translation and protein truncation (p.G117Kfs*4; **Figure 2C,D**), and therefore, it can be classified as a class 4—likely pathogenic.

The diagnosis of PMM2-CDG was confirmed by clinical, biochemical, and genetic findings.

At the age of 3 months, the patient was admitted to the intensive care unit for low food intake and clinical deterioration. He presented significant failure to thrive with weight being 3060 g (below 0.1 percentile, SD −5.15), length of 52 cm (below 0.1 percentile, SD −5.31), and fronto-occipital circumference 38.5 cm (below 0.1 percentile, SD −3.93). The child showed tachypnea and dyspnea becoming worse with feeding. Pericardial effusion further progressed (**Figure 3**) and finally required pericardiocentesis. Pericardial fluid was considered to be a transudate and serosanguineous. The patient was clinically deteriorating, respiratory and circulatory failure progressed. Despite all efforts, the patient died due to obstructive cardiogenic shock at the age of 3 months.

[1]http://software.broadinstitute.org/software/igv/

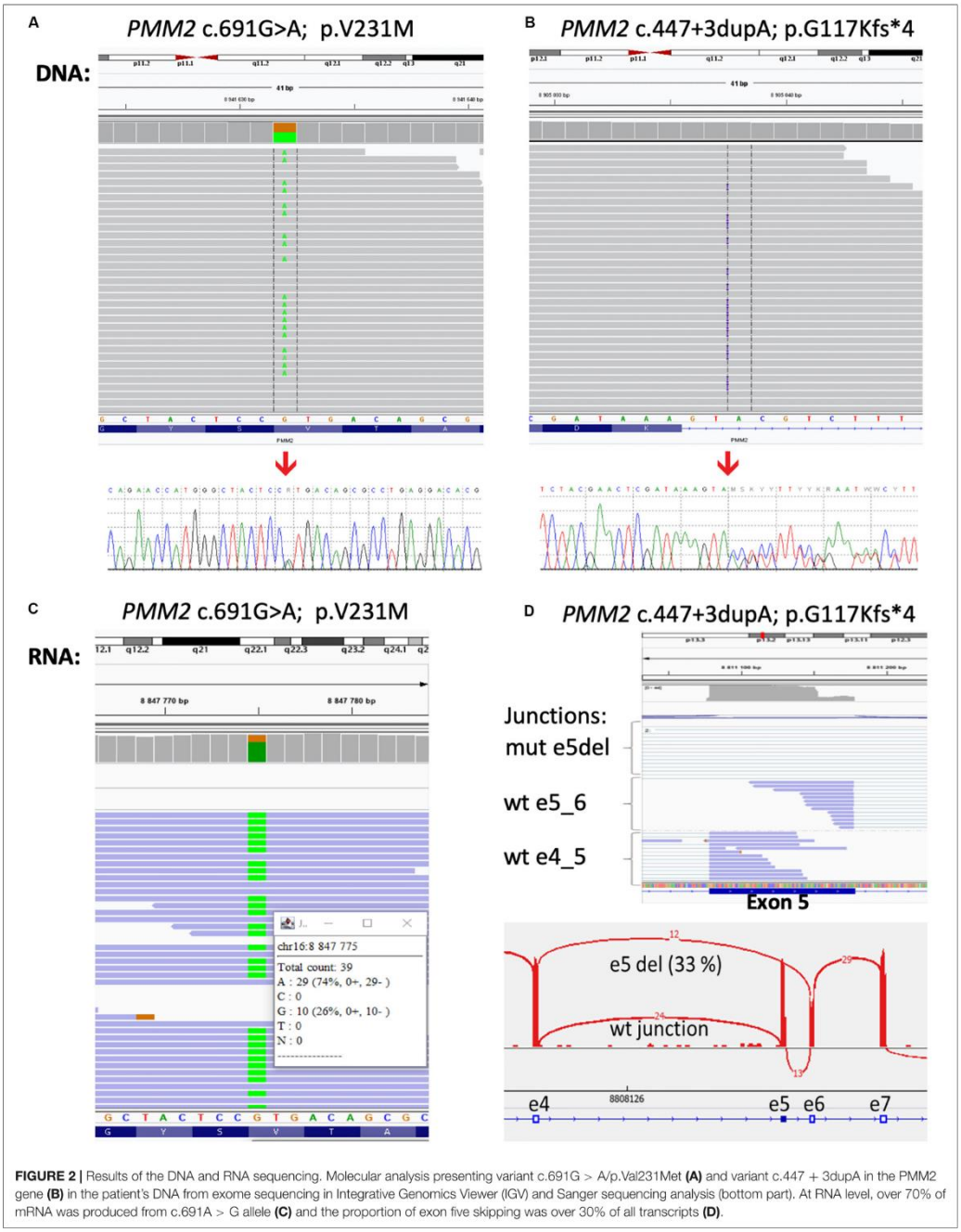

FIGURE 2 | Results of the DNA and RNA sequencing. Molecular analysis presenting variant c.691G > A/p.Val231Met **(A)** and variant c.447 + 3dupA in the PMM2 gene **(B)** in the patient's DNA from exome sequencing in Integrative Genomics Viewer (IGV) and Sanger sequencing analysis (bottom part). At RNA level, over 70% of mRNA was produced from c.691A > G allele **(C)** and the proportion of exon five skipping was over 30% of all transcripts **(D)**.

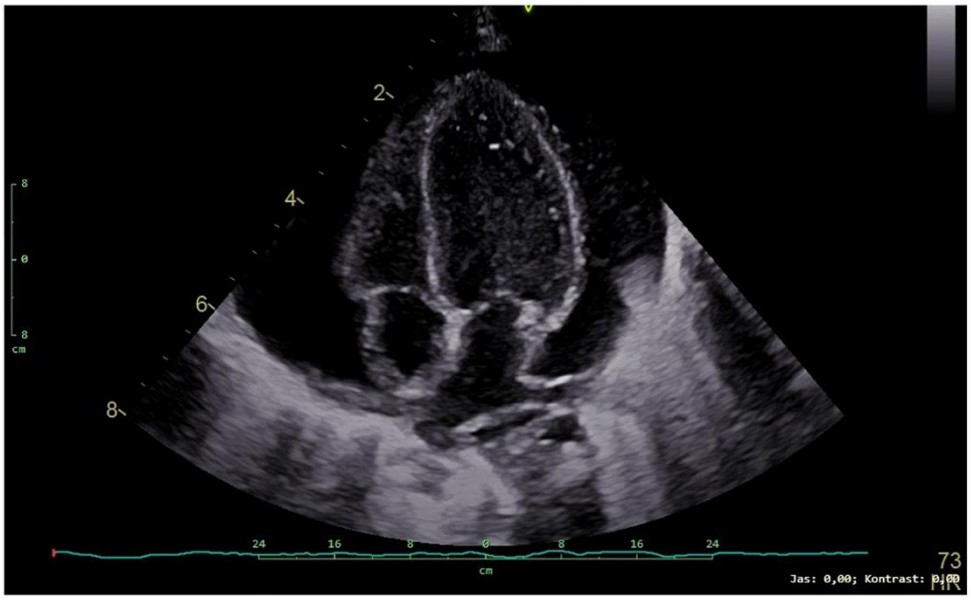

FIGURE 3 | Transthoracic echocardiogram (4-chamber view) showing a large pericardial effusion in our patient.

DISCUSSION

High clinical heterogeneity of various CDG types, and even within one particular CDG type, is considered to be the main reason behind underdiagnosis or late diagnosis of this disease (Giurgea et al., 2005). Alterations in glycosylation patterns are usually determined by isoelectric focusing of serum transferrin and apolipoprotein C III. The analytical performance of the isoelectric focusing of serum transferrin, which is a biomarker of N-glycosylation, is not satisfactory with only 60% of CDG cases being accurately found to be positive. Further, this test cannot reliably identify the subtype of CDG, and not all types can be detected by this approach (Altassan et al., 2019). A similar test to help diagnose O-linked disorders and combined N- and O-glycosylation CDGs is performed on apolipoprotein-CIII which has a single O-linked glycan which only captures abnormalities of core-1mucin type O-glycosylation. The diagnosis of PMM2-CDG can then be confirmed by measurement of PMM2 activity in either peripheral blood leukocytes or cultured skin fibroblasts (Francisco et al., 2019). The majority of PMM2-CDG patients have residual PMM2 activity below 10%, whereas in their asymptomatic parents the enzyme activity is about 50% (Francisco et al., 2019).

The variants found in the patient DNA by exome sequencing and confirmed by Sanger sequencing were a common variant c.691G > A/p.Val231Met (Altassan et al., 2019) and a novel variant c.447 + 3dupA in the *PMM2* gene. The novel mutation, inherited from mother, causes single-nucleotide duplication of the third intronic nucleotide after exon 5 and is predicted to result in loss of donor splice site indicating a novel splicing variant of the *PMM2* gene. Although three predictive algorithms predict the role of this novel variant in splicing, it has to be further experimentally confirmed to have predicted impact on the *PMM2* transcript splicing.

The variant p.Val231Met, despite being the second most frequent mutation in the *PMM2* gene with a prevalence of 10%, has only been reported so far as a compound heterozygote (Altassan et al., 2019) as in our case, where this allele was inherited from father. V231 is in the interior of the core domain, and a mutation in this residue is detrimental to its native protein structure (Silvaggi et al., 2006; Citro et al., 2018). The folding and stability defect of the Val231Met allele contributes to its reported reduced *in vitro* enzymatic activity of 38.5% (Kjaergaard et al., 1999).

Most of the PMM2-CDG patients are born with hypotonia, craniofacial dysmorphisms, and strabismus. Common are inverted nipples and a characteristic distribution of the fat pads especially in the gluteal and suprapubic regions and thighs. Clinical spectrum of PMM2-CDG further includes psychomotor retardation and mild to severe intellectual disability. PMM2-CDG patients typically show different degrees of cerebellar atrophy on MRI, mostly vermian atrophy (Serrano et al., 2015). In our patient, MRI was not feasible due to clinical deterioration. Cerebellar atrophy was not confirmed post-mortem, because

parents declined an autopsy. In the small percentage of the patients, there is a prenatal generalized edema and abnormal accumulation of fluid in two or more fetal compartments, hydrops fetalis, or accumulation of fluid in one specific compartment as in our patient with prenatal pericardial effusion.

Up to 15% of non-immune hydrops fetalis cases may be due to inborn errors of metabolism, and a large proportion of cases linked to metabolic disorders remains undiagnosed. The pathophysiology of fetal hydrops, edema, and pericardial effusions in inherited metabolic diseases is not fully understood. The fluid accumulation is due to an unbalanced interstitial fluid production and lymphatic return. The etiology of these symptoms in CDG appears to be multifactorial, frequently occurring due to a decreased plasma albumin concentration secondary to enteral and renal protein loss and a decreased synthetic function of the liver (Truin et al., 2008). There are additional factors leading to fluid leakage to pericardial and peritoneal spaces. Focal mixed inflammatory changes with mesothelial proliferation and a damaged pericardial protein barrier have been suggested in PMM2-CDG patients previously (Kristiansson et al., 1998). Truin et al. (2008) described three patients with severe *PMM2* mutations, who developed life-threatening accumulation of pericardial and abdominal fluids. In two cases this severe extravascular fluid accumulation progressed to decompensation and death.

Abnormal glycosylation of cell surface proteins in PMM2-CDG patients may result in disequilibrium of normal fluid balance and protein transport through the pericardial and peritoneal membranes and may cause life-threatening complications (Isikay et al., 2014).

CONCLUSION

In this case report, the patient's non-specific but uncommon findings of pericardial effusion, inverted nipples, fat pads, and combined coagulopathy pointed us toward an early clinical suspicion of a PMM2-CDG disease. Consequent targeted biochemical analysis and genetic findings led to the confirmation of the PMM2-CDG. We identified one common pathogenic variant and one novel variant predicted to result in loss of donor splice site, leading to exon five skipping and causing premature termination of translation to the protein. There is no curative therapy for the PMM2-CDG at the moment, but other supportive care options were available to be offered. The definite diagnosis of PMM2-CDG can also assist in the process of genetic counseling, family planning, and preimplantation genetic diagnosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was reviewed and approved by the Ethics Committee of University Hospital Brno. Written informed consent to participate in this study was provided by the participants' legal guardian. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KS, OS, and DP designed the study and drafted the manuscript. HN, PV, PK, and OS performed the genetic analysis and evaluation of variants. KS, JT, DP, TH, PS, HJ, and PJ conducted the clinical evaluations. HH and TH performed the biochemical analysis. All authors approved the final manuscript.

FUNDING

This work was supported by the Ministry of Health, Czech Republic, Conceptual Development of Research Organization (FNBr, 65269705). TH and HH were supported by the Ministry of Health of the Czech Republic (MZ CR-RVO VFN64165). This study was supported by the Ministry of Health of the Czech Republic by the Czech Health Research Council (Project No. NV18-03-00024).

ACKNOWLEDGMENTS

We acknowledge the CF Genomics of CEITEC supported by the NCMG research infrastructure (LM2015091 funded by MEYS CR) for their support with obtaining scientific data presented in this article.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annex 6

frontiers in Immunology

CASE REPORT
published: 18 September 2019
doi: 10.3389/fimmu.2019.02194

Novel SAMD9 Mutation in a Patient With Immunodeficiency, Neutropenia, Impaired Anti-CMV Response, and Severe Gastrointestinal Involvement

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OPEN ACCESS

Edited by:
Yenan Bryceson, Karolinska Institute (KI), Sweden

Reviewed by:
Kimberly Gilmour, Great Ormond Street Hospital, United Kingdom
Silvia Clara Giliani, University of Brescia, Italy

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Specialty section:
This article was submitted to Primary Immunodeficiencies, a section of the journal Frontiers in Immunology

Received: *23 January 2019*
Accepted: *30 August 2019*
Published: *18 September 2019*

Citation:
Formankova R, Kanderova V, Rackova M, Svaton M, Brdicka T, Riha P, Keslova P, Mejstrikova E, Zaliova M, Freiberger T, Grombirikova H, Zemanova Z, Vlkova M, Fencl F, Copova I, Bronsky J, Jabandziev P, Sedlacek P, Soukalova J, Zapletal O, Stary J, Trka J, Kalina T, Skvarova Kramarzova K, Hlavackova E, Litzman J and Fronkova E (2019) Novel SAMD9 Mutation in a Patient With Immunodeficiency, Neutropenia, Impaired Anti-CMV Response, and Severe Gastrointestinal Involvement. Front. Immunol. 10:2194. doi: 10.3389/fimmu.2019.02194

Mutations in the Sterile alpha motif domain containing 9 (*SAMD9*) gene have been described in patients with severe multisystem disorder, MIRAGE syndrome, but also in patients with bone marrow (BM) failure in the absence of other systemic symptoms. The role of hematopoietic stem cell transplantation (HSCT) in the management of the disease is still unclear. Here, we present a patient with a novel mutation in *SAMD9* (c.2471 G>A, p.R824Q), manifesting with prominent gastrointestinal tract involvement and immunodeficiency, but without any sign of adrenal insufficiency typical for MIRAGE syndrome. He suffered from severe CMV (cytomegalovirus) infection at 3 months of age, with a delayed development of T lymphocyte functional response against CMV, profound T cell activation, significantly reduced B lymphocyte counts and impaired lymphocyte proliferative response. Cultured T cells displayed slightly lower calcium flux and decreased survival. At the age of 6 months, he developed severe neutropenia requiring G-CSF administration, and despite only mild morphological and immunophenotypical disturbances in the BM, 78% of the BM cells showed monosomy 7 at the age of 18 months. Surprisingly, T cell proliferation after CD3 stimulation and apoptosis of the cells normalized during the follow-up, possibly reflecting the gradual development of monosomy 7. Among other prominent symptoms, he had difficulty swallowing, requiring percutaneous endoscopic gastrostomy (PEG), frequent gastrointestinal infections, and perianal erosions. He suffered from repeated infections and periodic recurring fevers with the elevation of inflammatory markers. At 26 months

of age, he underwent HSCT that significantly improved hematological and immunological laboratory parameters. Nevertheless, he continued to suffer from other conditions, and subsequently, he died at day 440 post-transplant due to sepsis. Pathogenicity of this novel *SAMD9* mutation was confirmed experimentally. Expression of mutant *SAMD9* caused a significant decrease in proliferation and increase in cell death of the transfected cells.

Conclusion: We describe a novel *SAMD9* mutation in a patient with prominent gastrointestinal and immunological symptoms but without adrenal hypoplasia. Thus, SAMD9 mutations should be considered as cause of enteropathy in pediatric patients. The insufficient therapeutic outcome of transplantation further questions the role of HSCT in the management of patients with *SAMD9* mutations and multisystem involvement.

Keywords: SAMD9, MIRAGE, immunodeficiency, neutropenia, cytomegalovirus infection, dysphagia, hematopoietic stem cell transplantation, gastrointestinal disorder

BACKGROUND

In 2016, Narumi et al. (1) reported mutations in Sterile alpha motif domain-containing protein 9 (SAMD9) in 11 patients examined primarily for adrenal hypoplasia. Most of the patients shared strikingly similar phenotypes, and thus, a novel multisystem disorder, MIRAGE (myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, and enteropathy) syndrome, was defined. Two patients from the cohort developed myelodysplastic syndrome (MDS) accompanied by loss of the chromosome 7 carrying the SAMD9 mutation. In 2017, Buonocore et al. (2) found similar *de-novo*, heterozygous *SAMD9* mutations in 8 children with a complex multisystem growth restriction phenotype. Adrenal insufficiency was frequently but not constantly present.

The appropriate treatment of the patients with SAMD9 mutations is not currently known. Fourteen of 19 patients from the first two studies died, mostly due to severe infections, in first 2 years of age. Two patients from the surviving group developed MDS with monosomy 7 and received hematopoietic stem cell transplantation (HSCT). Monosomy 7, deletions of 7q or secondary somatic loss of function mutation in SAMD9 frequently developed as a compensatory mechanism for the mutated allele, which rescued the growth-restricting effect of the *SAMD9* mutation, but it could lead to MDS in some of the patients. Schwarz reported a germline *SAMD9* mutation in three siblings with MDS and monosomy 7. Interestingly, the patients had an otherwise mild phenotype with no signs of MIRAGE syndrome except for hypospadia and bifid scrotum in one boy, and even had an asymptomatic mother carrying the same mutation (3). Bluteau et al. found 6 patients with mutated *SAMD9* and 10 patients with a mutation in SAMD9 counterpart *SAMD9L* (4) in a cohort of 86 patients with BM failure of suspected inherited origin (5). The patients presented with mild BM failure and monosomy 7, and only one presented typical signs of MIRAGE syndrome.

CASE PRESENTATION

We describe the case of a Caucasian boy from the 4th gravidity of healthy, non-consanguineous parents. In the first month after a preterm birth (32 weeks and 3 days of pregnancy, weight 1,450 g), he manifested with bilateral bronchopneumonia and hepatopathy that progressed to septicemia with bradycardia and respiratory failure requiring ventilation support. Generalized primary cytomegalus virus (CMV) infection was confirmed at the age of 3 months. His health status was complicated by bilateral pneumonia followed by respiratory distress that demanded ventilation support complicated by disseminated intravascular coagulation and septic shock. A 6-week treatment with ganciclovir was introduced. Antimycotic treatment was introduced for suspected aspergillus infection. A huge persisting cutaneous defect in the gluteal region with uretroscrotal fistula was present from the second month of age complicated by scrotal abscess at the age of 5 months.

He suffered from recurrent upper respiratory tract infections but also sepsis of unknown origin with high fever, and high C-reactive protein (CRP) responding to antibiotic treatment. From the age of 14 months, he had recurring pneumonia with respiratory distress and septicemia at the age of 18 months. Recurrent oral, nasal and urethral candidiasis were confirmed.

Gastrointestinal Involvement

Because of hypoproteinic malnutrition, failure to thrive and inability to swallow presumably caused by frequent vomiting, percutaneous endoscopic gastrostomy (PEG) was introduced at the age of 5 months. PEG tube management was complicated by extensive leakage. He suffered from sublingual erosions, diarrhea, recurrent proctocolitis with intestinal bleeding, and chronic perianal erosions. Hemorrhagic proctocolitis caused by *Pseudomonas aeuruginosa* with septicemia manifested at the age of 13 months. Severe *Clostridium difficile* gastroenteritis demanding intensive care manifested at the age of 23 months. Gastroscopy and colonoscopy at 18 months of age did not reveal any significant disturbances. Histologic evaluation of the

duodenal mucosa showed a mild deficit of disaccharides and other enzymes of the brush-border and mild chronic non-active enteritis.

Hematopoietic System Involvement

Immediately after birth, the patient presented with transient thrombocytopenia and anemia (**Figure 1A**). From 6 months of age, he had significant neutropenia with absolute neutrophil count (ANC) $< 0.5 \times 10^9$/l. Granulocyte colony stimulating factor G-CSF was introduced at the age of 19 months. BM evaluation at 18 months of age revealed normocellularity with reduced myeloid lineage (31%) with gradual maturation and increased erythroid lineage (48%); megakaryocytes were in the normal range, and atypical cells were not documented.

Other Symptoms

Hypospadia, micropenis, central hypotonic syndrome, pseudobulbar syndrome, psychomotor retardation, and mild orofacial stigmatization with macroglossia and hypomimia were documented.

HSCT

The patient was indicated for HSCT for unspecified primary immunodeficiency with severe infections, neutropenia and lack of B-cells at the age of 26 months. Conditioning regimen included busulfan targeted to plasma concentrations of 500–700 ng/mL from days−5 to−2, fludarabine 40 mg/m^2/day from days−6 to−3 and alemtuzumab in total dose 1 mg/kg from days−8 to−6. Graft-vs.-host (GVHD) prophylaxis administered from day−1 consisted of cyclosporine A (CsA) and mycophenolate mofetil (MMF). Plasmapheresis was performed for high titers of anti-A antibodies in the situation of ABO incompatibility on days −10, −9, −8, and on day 0. He received a BM graft from his HLA identical older brother (2.6×10^8 nucleated cells/kg, 5.6×10^6 of CD34pos cells). Neutrophil engraftment defined as the first of 3 days with ANC above 0.5×10^9/l was achieved on day +18, thrombocyte engraftment (the thrombocytes count above 20×10^6/l without transfusion in previous 7 days) on day +33, respectively. Complete donor chimerism (>98% donor cells) in non-separated peripheral blood (PB) evaluated by PCR amplification of the microsatellite markers was documented from day +21. The early post-transplant period was complicated by mucositis grade III, febrile neutropenia and CMV reactivation on day +20 with good response to ganciclovir therapy. He was discharged on day +42, without signs of acute GVHD, with diarrhea, vomiting and inability to swallow, persisting from the pre-transplant period.

MMF was stopped on day +60, CsA on day +164. Recurrent febrile states with elevation of inflammatory markers, vomiting and abdominal pain started again from day +270. Gastroduodenoscopy and colonoscopy performed for suspicion of pseudo obstruction showed no pathology. In contrast with an unsatisfactory clinical condition, absolute numbers of CD3+ T cells, CD19+ B cells and CD3+CD16+56+ NK cells and proliferative response to phytohemagglutinin were comparable to controls 1 year after SCT. Serum concentrations of IgG, IgA, IgM were in the normal ranges on continuous treatment with IVIG, and BM evaluation showed normocellular trilineage hematopoiesis. On day +440, he developed sepsis with hemoculture positive for *Streptococcus salivarius* and rapid progression to septic shock and despite antibacterial treatment and intensive care he died from multiple organ failure.

CLINICAL AND LABORATORY INVESTIGATIONS

Genetic Analysis

The cytogenetic evaluation performed from PB at 5 months of age did not reveal any structural or numerical abnormality, and retrospective FISH evaluation using CEP7 probe did not reveal monosomy 7. Whole-genome SNP array and FISH analysis from BM sample taken at 18 months of age found monosomy 7 in 78.5% of interphase nuclei.

Whole-exome sequencing analysis was performed at 18 months of age. Mutations in the genes causing congenital neutropenia were excluded, as well as variants in genes causing dyskeratosis congenita, because Hoyeraal Hreidarsson syndrome was considered. No other potentially causative variants were found using the virtual panel of genes associated with bone marrow failure or immunodeficiency. After publication of the SAMD9 patient cohort in 2016, the data were reanalyzed, and a novel, previously unreported *de-novo* heterozygous mutation in the *SAMD9* gene (c.2471 G>A, p.R824Q) was reported. Although this change is predicted as tolerated by SIFT (6) and benign by PolyPhen2 (7), with a CADD (8) score of 15.2, the residue is located near previously reported mutations p.K821M (9) and p.N834Y (1), and the mutation was not found in the ExAC or gnomAD population databases (10).

Immunological Evaluation and Functional Testing

The results of immunological testing during the course of the disease are summarized in **Figure 1B**. Flow cytometry (FC) determination (first performed at the age of 5 months) of major lymphocyte subsets did not provide conclusive results. CD3 cells were overrepresented; their percentages fluctuated from 87 to 96% (ref. range 39–77%), absolute CD3 cell numbers from 2.99 to 9.88 10^9/l (ref. range 2.4–6.9 10^9/l). CD4 cell numbers were normal: 21–42% (ref. range 25–50%), absolute number 1.34–2.28 10^9/l (ref. range 1.04–5.10 10^9/l). CD8+ cells were abundant: 47–69% (ref. range 13–26%), absolute number 1.62–7.49 10^9/l (ref. range 0.6–2.2 10^9/l). CD19+ cells were markedly decreased with repeatedly estimated representation of 2% (ref. range 13–35%), absolute number 0.07–0.22 10^9/l (ref. range 0.7–2.5 10^9/l). CD4/C8 ratio varied between 0.30 to 0.83 (ref 0.7–3.08) NK cell (CD16/56+CD3-) numbers were normal: from 7 to 11% (ref. range 2–13%), abs number 0.76–0.38 (ref range 0.7–1.0 10^9/l).

Levels of immunoglobulins were elevated (IgG 10.6 g/l, IgM 7.1 g/l, IgA level was within ref. range: 0.204 g/l). Thereafter, IgG and IgA levels remained within normal levels, IgM decreased to 3.25 g/l. IgE: was repeatedly <17 UI/ml. Total hemolytic

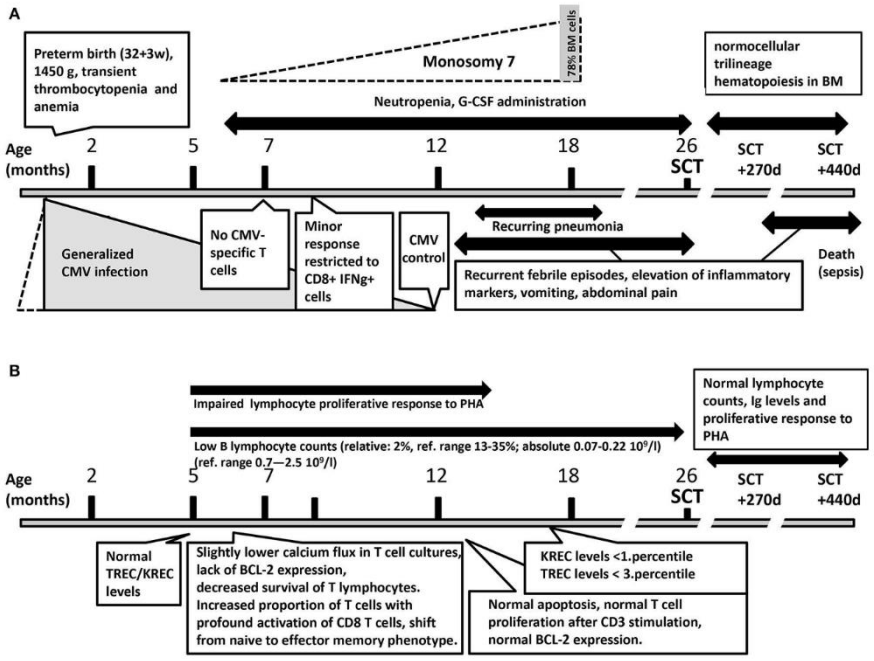

FIGURE 1 | (A) The results of hematological testing and infectious diseases during the course of the disease. **(B)** The results of immunological testing during the course of the disease. BM, bone marrow; CMV, cytomegalovirus; IFNg, interferon gamma; SCT, hematopoietic stem cell transplantation.

complement (CH 50) and granulocyte function test ("burst test) results were normal.

Detailed FC evaluation at 7 months of age revealed increased proportion of T cells, with profound activation of CD8 T cells (HLA-DR+ 72%) and shift from naive (6%) to effector memory phenotype (77%). This was presumably in response to persistently present CMV viremia. However, no functionally responding CMV specific T cells were detected. Minor response restricted to CD8+ IFNg+ producing cell was detected at 8.5 months of age that did not lead to CMV control. CMV reactivation control was restored only at one year of age (PCR CMV negativity). Retrospective analysis of neonatal dry blood spot did not reveal any presence of CMV.

Lymphocyte proliferation in response to phytohemagglutinin (PHA) using 3H-Thymidine incorporation was repeatedly found reduced between 5 and 14 months of age. At 7 months of age, activated T-cell cultures were established by stimulating PBMCs with immobilized anti-CD3ε antibody followed by propagation in the presence of IL-2. These cultures displayed reduced viability, accompanied by the lack of anti-apoptotic Bcl-2 protein expression (**Figures 2A,B**). Upon CD3 re-stimulation, they showed slightly decreased calcium flux (**Figure 2C**), while no difference in overall tyrosine phosphorylation after TCR stimulation was observed (data not shown). However, examination of activated T-cell cultures newly established 6 months later (coincident with CMV control) revealed normal level of apoptosis, normal cell proliferation and Bcl-2 expression comparable to controls (data not shown).

Neither T-cell receptor excision (TREC) nor kappa-deleting element excision (KREC) circle levels were decreased at 5 months of age in PB, but at 17 months of age, the KREC numbers were decreased to the levels observed in SCID patients, and TREC levels were reduced below the 3rd percentile of age-matched controls.

FUNCTIONAL EVALUATION OF *SAMD9* MUTATION

Transient ectopic expression of wt SAMD9 resulted in a significant decrease in proliferation of the transfected cells. The impact of mutant SAMD9 was even more profound resulting in a dramatic drop in the number of proliferating cells (**Figure 3A**). Expression of SAMD9 (both wt and mutant) also caused an increase in apoptosis. Interestingly, mutant SAMD9 induced primary necrosis of the cultured cells further demonstrating the gain-of-function impact of p.R824Q on SAMD9 (**Figure 3B**).

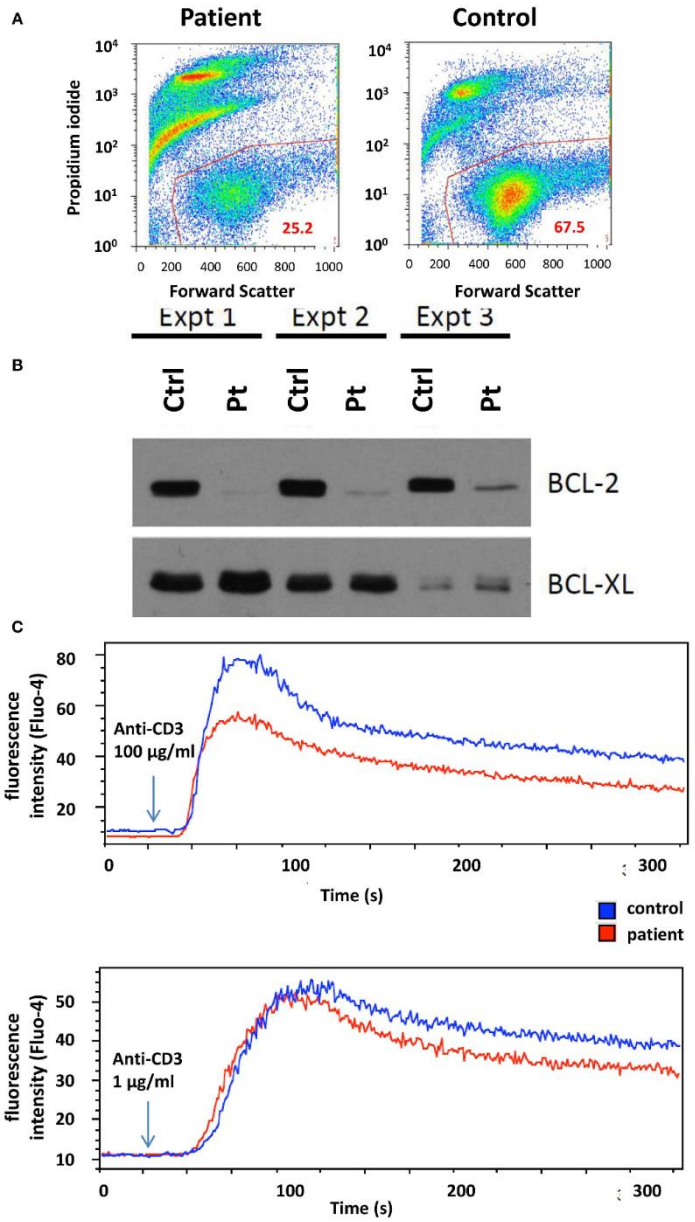

FIGURE 2 | (A) T-cell culture viability determined by flow cytometry. Live cells were gated based on propidium iodide and forward scatter signals. **(B)** Western blot analysis of Bcl-2 and Bcl-xL expression in T cells after 2, 3, and 5 weeks of culture. Ctrl, control, Pt, patient. **(C)** Flow cytometry analysis of T-cell calcium response to activation with 1 and 100 µg/ml anti-CD3ε antibody. The arrow indicates the time point when antibody was added to the sample.

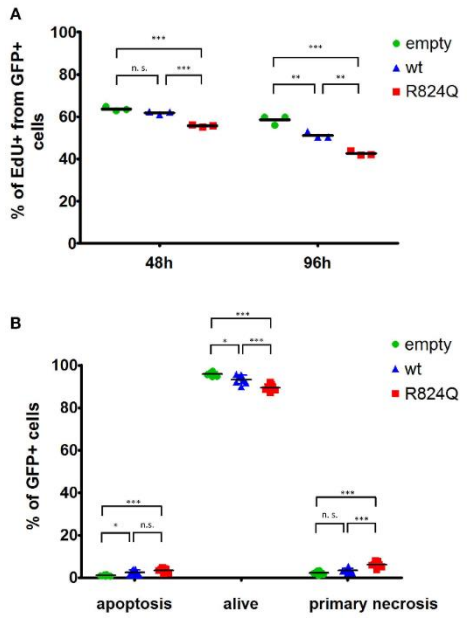

FIGURE 3 | Analysis of proliferation **(A)**, cell death **(B)** of HEK293T cells transfected with wt SAMD9-GFP, mutant SAMD9-GFP or empty GFP-positive control expression vector. Representative data are shown with results depicted as a fraction of GFP+ cells. Statistical significance: (*$P \leq 0.05$; **$P \leq 0.001$; ***$P \leq 0.0001$).

DISCUSSION

In concordance with previously published cases (1, 2), our patient was delivered preterm and seriously ill in the neonatal period and needed intensive care. He showed genital anomalies, and he suffered from recurrent infections and chronic diarrhea. Thus, his phenotype was consistent with MIRAGE syndrome apart from the adrenal insufficiency, the sign that defined the original published cohort. Buonocore et al. reported severe adrenal insufficiency in 6 patients, while mild and no adrenal involvement were reported in the remaining two patients (2). Our patient repeatedly suffered from hyponatremia, but hyperpigmentation of the skin was not observed, and cortisol levels were only found decreased in one of three evaluations.

One of the two dominant clinical features in our patient were the inability to swallow, requiring PEG feeding, and chronic vomiting, symptoms which have not been highlighted as diagnostic signs so far. However, case presentations in the cohort of Narumi et al. reveal that at least four of 11 patients required feeding tubes, and one had a gastrostomy tube due to aspiration pneumonias, esophageal stricture, or achalasia. Also the two patients reported recently by Sarthy et al. had enteral feeding intolerance (9). Thus, SAMD9 mutations should be considered in cases of unexplained disturbances of the upper gastrointestinal tract. His other gastrointestinal symptoms included chronic diarrhea, which is a common symptom in MIRAGE syndrome [reported in 9 of 11 patients in the original cohort (1)].

The second dominant clinical feature was severe neutropenia requiring the administration of G-CSF. One of the three siblings reported by Schwartz et al. manifested with severe neutropenia, but it was associated with macrocytosis, thrombocytopenia and hypocellularity and trilineage dysplasia with 1% blasts in the BM. In our patient, BM aspiration performed at 18 months was without signs of myelodysplasia, only with reduction in the myeloid lineage. Thus, congenital neutropenia was primarily considered at that time. Monosomy 7 was revealed incidentally by SNP array, and retrospective evaluation of BM by FISH revealed monosomy 7 in the 78.5% of nuclei.

The complex immunodeficiency complications in our patient are in good accordance with manifestation of patients described by Narumi et al. where all 7 described patients were prone to complicated or recurrent infections, including episodes of pneumonia; one described patient suffered from severe CMV infection as well. In another patient, recurrent fever with high CRP was reported. Bluteau et al. reported severe recurrent infections in 4/6 patients with SAMD9 mutation (5). Despite clinically manifested immunodeficiency since birth, the laboratory immunological investigation did not show any gross abnormality. Examination of the lymphocyte subsets revealed decreased B lymphocytes, and the lymphocyte proliferation test showed decreased response after PHA stimulation. This finding is consistent with the previous observation by Narumi et al. that showed decreased numbers of B lymphocytes and decreased NK activity in several patients. Bluteau et al. reported immunoglobulin deficiency in 2/6 patients (5). The most prominent sign of immunodeficiency in our patient was non-responsiveness of T lymphocytes to CMV despite severe CMV infection in the early infancy period.

SAMD9 acts as a growth repressor, and SAMD9 mutations are considered gain-of function mutations, thus, further intensifying the growth suppression. The p.R824Q mutation present in our patient was predicted as benign by two prediction tools. However, neither PolyPhen2 (7) nor SIFT (6) tools are adjusted for gain-of-function predictions and, thus, should be used with caution (11). Growth of HEK293 cells transfected with SAMD9 mutants was profoundly restricted in several studies (1, 9, 12). The cells transfected with the p.R824Q mutant showed a significant growth restriction as well. Interestingly, we also observed an increase in cell death of the cultured cells. The involvement of SAMD9 in cell death has already been predicted (13). During the initial evaluation of our patient, we observed a reduced growth, higher rate of apoptosis of cultured T lymphocytes together with the lack of Bcl-2 expression. However, this observation was not confirmed during repeated evaluation after 6 months. We can speculate that the renewal of the proliferation capacity could have been caused by the gradual emergence of cells with compensatory loss of chromosome 7, as observed in other studies (2).

The role of HSCT in the management of patients with SAMD9 mutations is not entirely clear. To our knowledge, 14 transplanted patients with SAMD9 mutation have been reported so far, including our patient. One patient from the cohort of Narumi et al. (1) was transplanted due to MDS, but died due

to Epstein-Barr virus-related post-transplant lymphoproliferative disorder. The only 2 surviving patients from the cohort described by Buonocore et al. (2) were also transplanted due to MDS. However, these patients had the mildest phenotype, with only fewer syndromic features, compared to the rest of the patients. This was also the case of three other reported patients (3, 14) who survived after HSCT. Wilson et al. retrospectively identified a SAMD9 mutation in a patient with MIRAGE phenotype after HSCT due to MDS. The patient survived more than 10 years after transplant but suffered from multiple other medical issues related to syndromic features (12). Bluteau et al. reported four transplanted patients, of whom three survived without major complication, while the only one patient with MIRAGE phenotype died. Interestingly, 11 of 13 patients with SAMD9 or SAMD9L mutations, who were not transplanted immediately, showed spontaneous improvement in blood cell counts, and HSCT was even canceled in 5 of them with no impact on survival (5). Recently, Sarthy et al. reported two patients with SAMD9 mutations and severe MIRAGE phenotype transplanted due to BM failure, who both died after HSCT due to multiorgan complications connected with the syndrome (9). This was also the case of our patient, who tolerated relatively well the transplantation procedure and successfully restored hematopoiesis, but died 14 months after HSCT due to worsening of his other symptoms. Taken together, 5 of 6 reported patients with the MIRAGE phenotype died after SCT, while all 9 reported patients without severe syndromic features survived. These results show that in patients with the MIRAGE phenotype, the transplantation management is rarely successful due to accompanying multiorgan issues. BM disturbances can show spontaneous improvement, including the disappearance of monosomy 7. Thus, a watch and wait strategy should be an option for both syndromic and non-syndromic patients. However, their treatment must be managed by primary immunodeficiency centers with access to intensive care units with multidisciplinary teams.

In conclusion, we report the case of a patient with novel mutation in *SAMD9* with severe gastrointestinal involvement, neutropenia and immunodeficiency, underscoring the role of *SAMD9* in the differential diagnosis of patients with these symptoms. We further question the role of HSCT in the management of the disease.

METHODS

The detailed descriptions of CMV response detection (15), activated T-cell culture (16), cell viability, BCL-2 expression, calcium response (16), TREC/KREC analysis, and of the functional assessment of SAMD9 mutation *in-vitro* are available in the **Supplementary File** (17–19).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the 2nd Medical Faculty Ethics Committee Guidelines. The parents of the patient gave written informed consent with the study in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the 2nd Medical Faculty, Charles University Prague. Written informed consent was obtained from the parents of the participant for the publication of this case report.

AUTHOR CONTRIBUTIONS

RF, EF, TK, VK, MS, and TB analysis and interpretation of data for the study and drafting the manuscript. MR, KS, VK, MS, TB, EH, and HG functional experiments. PR, MZ, EM, TF, ZZ, EH, JB, PJ, PS, JSo, JSt, MV, JL, JT, IC, FF, PK, and OZ analysis and interpretation of data.

FUNDING

This project was supported by NV18-07-00430 (to EM and JSt), NV19-05-00332 (to EF and VK) projects of the Czech Ministry of Health and by 17-04941Y from the Czech Science Foundation. The research facilities were supported by the project for the conceptual development of research organization 00064203 and LO1604, the infrastructure was supported by CZ.2.16/3.1.00/24505. EF and MS were supported by PRIMUS/17/MED/11. KS and MR were supported by PRIMUS/19/MED/04. ZZ was supported by the project for the conceptual development of research organization RVO-VFN64165.

ACKNOWLEDGMENTS

We would like to thank the family of the patient for their kind cooperation and permission to publish this paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02194/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annex 7

doi: 10.48095/ccgh2020469

Eosinophilic esophagitis – 10 years of experience in five Czech pediatric endoscopy centers

Eozinofilní ezofagitida – 10 let zkušeností pěti českých pediatrických endoskopických center

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Summary: **Background:** Eosinophilic esophagitis (EoE) is a chronic, progressive inflammatory disease of the esophagus characterized by local eosinophilic infiltration accompanied by symptoms of esophageal dysfunction. The aim of this study was to characterize features of EoE diagnosed regionally and to describe local strategies for its treatment. **Methods:** The observational survey retrospectively analyzed a data set of child patients with histologically proven EoE from five pediatric endoscopy centers. This analysis focused on describing their general situation (age, gender, symptoms) and also aimed to investigate any possible linkage between age and symptoms or length of diagnosis period. Demographic features; clinical symptoms; laboratory, endoscopic, and histopathological findings; and chosen treatment of patients were recorded and analyzed. **Results:** From January 2010 to September 2020, 33 new cases of EoE were reported. Strong association of EoE with male sex is consistent with the results from formerly published studies (81.8%). The median age of symptom onset was 7 years, while the median age for diagnosis was almost 13 years. The most common symptoms were reflux symptoms in general (39.4%), followed by vomiting (36.4%), and dysphagia (33.3%). Examination for sensitization to food allergens was performed on 23 (69.7%) patients with diagnosed EoE. Out of these, 17 (51.5% of all cases) were found to be sensitive to some allergens. Most of this subgroup (and 48.5% of all cases) were examined by specific IgE testing, and just 2 (6.1% of all cases) patients were tested only by skin prick tests. Another allergic comorbidity was present in 75.8% of patients, and the most common of these were bronchial asthma and allergic rhinoconjunctivitis (45.5% and 42.2%, respectively). More than two-thirds of patients (69.7%) had abnormal macroscopic findings during diagnostic endoscopy, and the most common were longitudinal furrows and white exudates. The most common initial modality of treatment was to use proton-pump inhibitors (PPI; 93.9%), followed by food elimination (75.8%) and then by corticosteroid administration (63.6%). The vast majority of patients treated with corticosteroids received a topical preparation (90.9%). **Conclusion:** This is the first retrospective study on pediatric patients with EoE in the Czech Republic. We found similar features of EoE as reported in formerly published works elsewhere. Collecting long-term prospective observational data in a national EoE register of patients in the Czech Republic would significantly improve our knowledge of this disease.

Key words: eosinophilic esophagitis – children – pediatrics – endoscopy

Souhrn: **Úvod:** Eozinofilní ezofagitida (EoE) je chronické progresivní zánětlivé onemocnění jícnu charakterizované lokální eozinofilní infiltrací doprovázenou příznaky dysfunkce jícnu. Cílem této studie bylo charakterizovat rysy regionálně diagnostikovaných případů EoE a popsat lokální strategie léčby. **Metody:** V observační studii byla retrospektivně analyzována data dětských pacientů s histologicky prokázanou EoE v některém z pěti dětských endoskopických center. Tato analýza se zaměřila na obecné charakteristiky pacientů (věk, pohlaví, příznaky) a jejich možnou souvislost s trváním symptomatického onemocnění do stanovení diagnózy. U pacientů byly dále analyzovány demografické parametry, klinické příznaky, laboratorní, endoskopické a histopatologické nálezy a použití různých modalit léčby. **Výsledky:** Od ledna 2010 do září 2020 bylo zaznamenáno 33 nových případů EoE. Silná vazba EoE na mužské pohlaví byla v souladu s výsledky dříve publikovaných studií (81,8 %). Ačkoliv medián věku pacientů v době prvních příznaků byl 7 let, medián věku v době stanovení diagnózy byl téměř 13 let. Nejčastěji referovaným symptomem byly refluxní příznaky obecně (39,4 %), následované zvracením (36,4 %) a dysfagií (33,3 %). Stanovení senzibilizace proti různým potravinovým alergenům bylo provedeno u 23 (69,7 %) pacientů s EoE. Z těchto pacientů byla u 17 (51,5 % vyšetřovaných) prokázána senzibilizace na některý z potravinových alergenů. Téměř polovina pacientů (48,5 %) byla vyšetřována stanovením specifických IgE a pouze u 2 pacientů (6,1 %) bylo provedeno vyšetření kožními prick testy. Alergická komorbidita byla zjištěna u 75,8 % pacientů, nejčastěji to bylo bronchiální astma a alergická rinokonjunktivitida (45,5 %; resp. 42,2 %). U více než dvou třetin pacientů prokázala diagnostická endoskopie abnormální makroskopické nálezy (69,7 %), nejčastěji podélné rýhy a bílé exsudáty. Nejčastěji zvoleným způsobem léčby byly inhibitory protonové pumpy (93,9 %), následované eliminační dietou (75,8 %) a podáváním kortikosteroidů (63,6 %). Naprostá většina pacientů léčených kortikosteroidy byla léčena topickými formami (90,9 %). **Závěr:** Jedná se o první retrospektivní studii u pediatrických pacientů s EoE v České republice. U pacientů s EoE byly prokázány velmi podobné charakteristiky jako v dříve publikovaných zahraničních pracích. Dlouhodobě sbíraná prospektivní data v národním registru EoE pacientů České republiky by významně napomohla v dalším získávání znalostí o tomto onemocnění.

Klíčová slova: eozinofilní ezofagitida – děti – pediatrie – endoskopie

Introduction

Eosinophilic esophagitis (EoE) is a chronic progressive inflammatory disease characterized by local eosinophilic infiltration of the esophageal mucosa and symptoms resulting from its dysfunction. EoE and gastroesophageal reflux disease (GERD) are today the most commonly identified esophageal causes of difficulty in swallowing among children and adolescents [1]. EoE is currently a relatively common finding in esophagoscopy (up to 7%) and can no longer be considered rare (overall prevalence 0.5–1 case per 1,000 persons) [2]. Despite the number of publications analyzing various attributes of EoE, the cause of the exponential increase in its prevalence within Western Europe over the past three decades remains unclear. This is the first retrospective analysis of pediatric EoE in the Czech Republic. The aims of this study were to analyze the demographic, clinical, laboratory, and endoscopic features of pediatric EoE from a large geographic region, to better understand EoE across a wide range of variable manifestations, and to provide feedback to improve the diagnostic-therapeutic process.

Tab. 1. Presenting symptoms leading up to diagnosis of EoE in general and in age-related groups.
Tab. 1. Hlavní příznaky vedoucí k diagnóze EoE celkově a v různých věkových skupinách.

Symptom	All age groups	6 years and less	7–14 years	15–19 years
Reflux symptoms	8 (24.24%)	2 (28.57%)	3 (23.08%)	3 (23.08%)
Impaction	8 (24.24%)	0 (0.00%)	4 (30.77%)	4 (30.77%)
Stomach ache	4 (12.12%)	1 (14.29%)	2 (15.38%)	1 (7.69%)
Dysphagia	4 (12.12%)	0 (0.00%)	0 (0.00%)	4 (30.77%)
Refusing food	3 (9.09%)	2 (28.57%)	1 (7.69%)	0 (0.00%)
Vomiting	3 (9.09%)	1 (14.29%)	2 (15.38%)	0 (0.00%)
Odynophagia	2 (6.06%)	0 (0.00%)	1 (7.69%)	1 (7.69%)
Failure to thrive	1 (3.03%)	1 (14.29%)	0 (0.00%)	0 (0.00%)
Total	**33 (100%)**	**7 (100%)**	**13 (100%)**	**13 (100%)**

Material and methods

The data for patients with the EoE diagnosis were collected from three university hospitals in Moravia and two regional pediatric departments that are capable of endoscopic examinations and further to look after patients with EoE. All participating pediatricians, pediatric gastroenterologists, and allergologists were sent an e-mail asking them to report all diagnoses of histologically proven pediatric EoE (≥ 15 eosinophils per high-power field [HPF] in a sample of esophageal mucosa with the highest number of eosinophils) made in the previous 10 years. The identities of all cases remained anonymous. Data collected via a standardized questionnaire included patient demographics, symptoms, macroscopic and histopathological findings at diagnosis and control endoscopy, therapeutic management, allergic comorbidity, and examination of sensitization. The collected data are presented as means or medians, with categorical variables summarized as percentages. The data were analyzed using the SAS and SPSS programs. The threshold for statistical significance of tests used in the analysis was 5%.

Results

Demographics

We have collected reports from 33 cases of pediatric EoE, 27 of which were for male patients (81.8%) and 6 (18.2%) for female patients. The male-to-female ratio is thus approximately 4 : 1.

Clinical symptoms

Mean age for the occurrence of EoE symptoms was 8.05 years (SD 5, median 7.0) and the mean age for diagnosis was almost 11.4 years (SD 4.60, median 12.75). Various presenting symptoms leading up to the diagnosis irrespective of and according to age are described in Tab. 1.

The age of manifestation for each individual presenting symptom of EoE and symptomatic time to diagnosis were established, and these data are summarized in Tab. 2. A median of fewer than 3 years was needed to diagnose EoE in patients with food impaction, vomiting, odynophagia, dysphagia, and abdominal pain. In cases of reflux symptoms, failure to thrive, and food refusal, however, it took a longer time to be referred and diagnosed. The wide range of symptomatic periods makes it difficult to interpret this data and subsequent collection of data would be appropriate.

Irrespective of patient age, reflux symptoms were the most common and affected more than 39% of all patients with EoE. This was closely followed by vomiting (36%) and dysphagia (33%), then by food impaction and stomach ache. The occurrence of clinical symptoms significantly varied depending on children's ages at the time of diagnosis (Tab. 3).

The youngest patients (≤ 6 years) most frequently showed a failure to thrive (71%), followed by food refusal (57%), and vomiting (43%). Patients from 7 to 14 years of age most frequently complained about dysphagia (55%), followed by vomiting and food impaction (both around 40%), then by reflux symptoms (just less than one-third). Symptoms of patients above 15 years of age were pretty similar to those of the previous age-related group. Reflux symptoms in general, including cough and pyrosis, were typical (almost 54%) and were followed by dysphagia and impaction (both 38%). Adolescents relatively often also referred to vomiting (31%), stomach ache (30%), and odynophagia (23%).

The overall numbers of symptoms manifested by patients with EoE generally and within age-related groups are shown in Tab. 4.

We also looked at the symptomatic time length in relation to the age of the patients at the time of the first symptoms. The data are depicted graphically in Fig. 1.

Patients were divided into three groups according to their age at occurrence of the first symptoms, and the results are summarized in Tab. 5.

Tab. 2. Age of first EoE signs manifestation and symptomatic time distribution according to the main symptom (in years).
Tab. 2. Věk při manifestaci prvních příznaků EoE a délka jejich trvání před stanovením diagnózy (roky).

Symptom	Age of manifestation		Symptomatic time to diagnosis	
	mean	median (min–max)	mean	median (min–max)
Failure to thrive	0.67	0.67 (0.67–0.67)	3.00	3.00 (3.00–3.00)
Refusing food	2.58	0.75 (0.50–6.50)	4.83	4.50 (0.00–10.00)
Reflux symptoms	5.96	6.00 (0.67–12.00)	4.01	3.17 (0.08–12.50)
Vomiting	6.50	7.00 (2.50–10.00)	2.11	2.00 (1.00–3.33)
Stomach pain	10.25	12.00 (3.00–14.00)	1.06	0.92 (0.17–2.25)
Impaction	10.00	10.25 (5.00–15.00)	3.50	2.67 (0.00–9.00)
Odynophagia	11.25	11.25 (7.50–15.00)	1.25	1.25 (0.00–2.50)
Dysphagia	11.60	14.96 (1.00–15.50)	5.02	1.54 (0.50–16.50)

Tab. 3. Occurrence of clinical symptoms according to the children's ages.
Tab. 3. Výskyt klinických příznaku podle věku pacientů.

Symptom	All age groups	6 years and less	7–14 years	15–19 years
Reflux symptoms	13 (39.39%)	2 (28.57%)	4 (30.77%)	7 (53.85%)
Vomiting	12 (36.36%)	3 (42.86%)	5 (41.67%)	4 (30.77%)
Dysphagia	11 (33.33%)	0 (0.00%)	6 (54.55%)	5 (38.46%)
Impaction	10 (30.30%)	0 (0.00%)	5 (38.46%)	5 (38.46%)
Stomach ache	8 (24.24%)	2 (28.57%)	2 (15.38%)	4 (30.77%)
Failure to thrive	6 (18.18%)	5 (71.43%)	1 (7.69%)	0 (0.00%)
Refusing food	5 (15.15%)	4 (57.14%)	1 (7.69%)	0 (0.00%)
Odynophagia	5 (15.15%)	0 (0.00%)	2 (15.38%)	3 (23.08%)
Regurgitation	3 (9.09%)	0 (0.00%)	2 (15.38%)	1 (7.69%)
Diarrhea	2 (6.06%)	0 (0.00%)	0 (0.00%)	2 (15.38%)

Tab. 4. Numbers of symptoms generally and by age group.
Tab. 4. Celkový počet příznaků a jejich počet v různých věkových skupinách.

Numbers of symptoms	All age groups	6 years and less	7–14 years	15–19 years
1	11 (33.33%)	1 (14.29%)	5 (38.46%)	5 (38.46%)
2	7 (21.21%)	3 (42.86%)	3 (23.08%)	1 (7.69%)
3	9 (27.27%)	2 (28.57%)	3 (23.08%)	4 (30.77%)
4	5 (15.15%)	1 (14.29%)	1 (7.69%)	3 (23.08%)
5	1 (3.03%)	0 (0.00%)	1 (7.69%)	0 (0.00%)

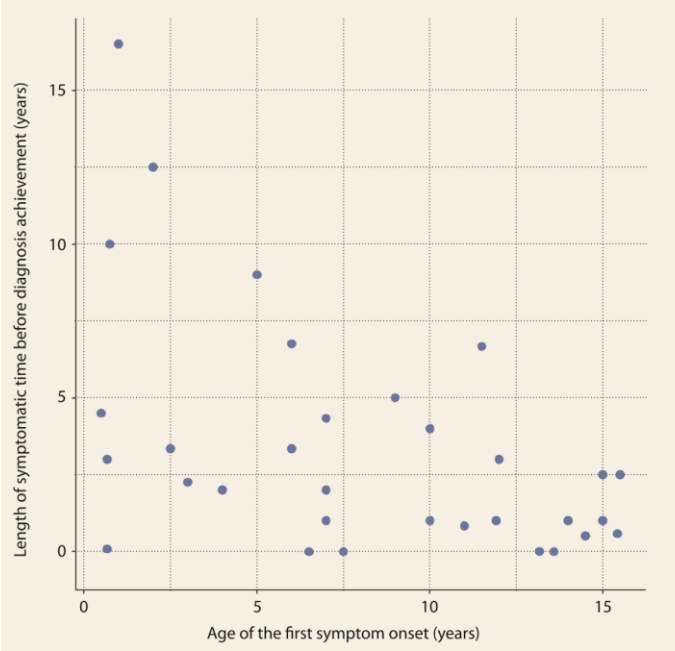


Fig. 1. Length of symptomatic time before diagnosis achievement in relation to the age of first symptom onset (years).
Obr. 1. Délka trvání příznaků před stanovením diagnózy ve vztahu k věku při jejich manifestaci (roky).

Tab. 5. The length of symptomatic period before diagnosis achievement according to the age o patients (years).
Tab. 5. Délka symptomatického období před stanovením diagnózy podle věku pacientů (roky).

Age group	N	Mean (SD)	Median (min–max)
≤ 3 years	8	6.52 (5.78)	3.92 (0.08–16.50)
4–10 years	13	2.99 (2.76)	2.00 (0.00–9.00)
11–16 years	11	1.72 (1.92)	1.00 (0.00–6.67)

The longest symptomatic period prior to establishing the diagnosis was reported in a boy nearly 18 years old who had intermittent dysphagia, vomiting, and reflux symptoms already from his second year of life. The longest-waiting girl was at first diagnosed as having an eating disorder. From infancy she showed food refusal and failure to thrive, followed later by esophageal reflux symptoms, regurgitation, and nausea. At age 5 years, she refused to swallow solid parts of food and suffered an aspiration episode in attempting to swallow a berry. At 7 years of age, she underwent esophagogastroduodenoscopy, unfortunately without performance of esophageal biopsy. She was referred to a neurologist for suspicion of autistic spectrum disorder. Finally, at 10 years of age, due to persisting mild problems like dysphagia and regurgitation, she underwent a control endoscopy which, despite normal macroscopic features of esophageal mucosa, showed histopathological features typical of EoE.

Endoscopic and histopathological findings

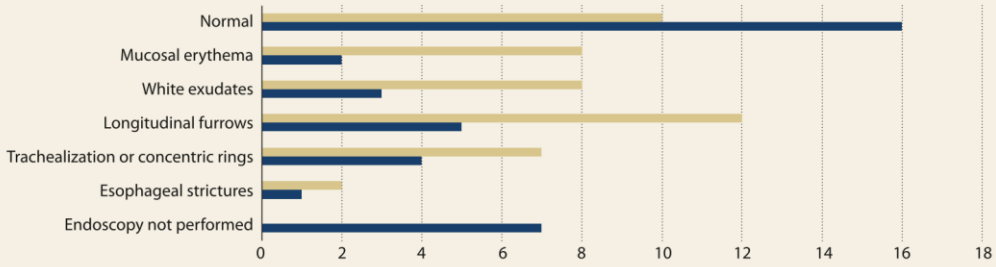
Almost one-third of the 33 patients with EoE had normal macroscopic findings during the first endoscopy (30%). Otherwise longitudinal furrows (sometimes referred to as "crepe paper-like appearance"), white exudates, and mucosal erythema were the most common findings (30%, 8%, and 7%, respectively). Much less frequently, features of chronic inflammation like trachealization or concentric rings (21%) and esophageal strictures (6%) were apparent upon first biopsy. In comparison with the features more frequently seen, however, these had a stronger tendency to persist even after the administration of treatment. Fig. 2 summarizes the presence of various endoscopic findings apparent during diagnostic and control endoscopy in the 33 patients with EoE.

The typical age of patients and symptomatic time to achieving diagnosis according to the particular endoscopic findings are summarized in Tab. 6. A hypothetical association between the age of patients or symptomatic time to diagnosis with certain endoscopic findings was examined using Mann-Whitney U-test, but no significant relationship was proven. On the other hand, due to the low number of specific endoscopic findings, the hypothetical association of specific endoscopic findings with the length of a symptomatic period is not conclusively debunked, and this possibility is indicated in the present data set.

The median intraepithelial eosinophil count (IEE) per high-power field (HPF) in the diagnostic biopsy samples obtained from the esophagus in EoE patients was 51.16/HPF, while in the control samples it was 35.48/HPF. Decrease in local eosinophilic inflammation during treatment was significant, and this was also confirmed by Wilcoxon signed-rank test (p-value = 0.0002). Nevertheless, this median value is still more than twice the cutoff.

Allergological evaluation

The median relative eosinophil count in peripheral blood samples of patients



	Endoscopy not performed	Esophageal strictures	Trachealization or concentric rings	Longitudinal furrows	White exudates	Mucosal erythema	Normal
First endoscopy	0	2	7	12	8	8	10
Control endoscopy	7	1	4	5	3	2	16

Fig. 2. Occurrence of various endoscopic findings at first and later endoscopy.
Obr. 2. Výskyt různých endoskopických nálezů při diagnostické a kontrolní endoskopii.

with EoE was 5.6% (1.70–30.70) and the median immunoglobulin E (IgE) level was 300 kU/l (24–1,960). Relative eosinophil counts and especially IgE levels were significantly higher in younger patients and there was a significant decreasing trend with higher age. The correlation of both variables with age at diagnosis was significant (IgE: Spearman's Rho = 0.41, p-value = 0.034; Eosinophils: Spearman's Rho = –0.37, p-value = 0.037). Sensitization to food allergens was examined in 23 (69.70%) patients overall. Of these, 17 (51.52% of all cases) were found to be sensitive to some of the food allergens. Most of this subgroup of patients – 16 (48.48% of all cases) – were examined by laboratory-specific IgE assessment and only 2 (6.06% of all cases) by skin prick-to-prick testing with native foods. Five (15.15%) of the patients were tested by both methods. Three out of 7 patients (42.86%) from the group of the youngest children (≤ 6 years of age) were sensitized to some food. In 2 (28.57%) cases sensitivity was to cow's milk, in 2 cases to eggs, and in 2 cases to peanuts. One patient for each (14.29%) had sensitivity to soy, gliadin, and wheat. In the patient group aged 7–14 years, 7 (53.85%) were sensitized. The most common sensitization was to proteins of cow's milk, with 4 cases (30.77%), followed by hazelnuts with 3 cases (23.08%). Wheat, walnuts, peanuts, and eggs sensitized 2 (15.38%) patients each, and apples, potatoes, gliadin, and soy 1 (7.69%) patient each. The oldest group of adolescent patients aged 15 years or older had the highest sensitization rate to food allergens: 8 (61.54%) out of 13 overall. These patients were the most often polysensitized to food allergens. The most common food allergens which sensitized this older group were hazelnuts in 7 (53.85%) patients, closely followed by wheat, peanuts, and apples in 6 (46.15%) patients each. Other allergens sensitized adolescents in much lower numbers. Three (23.08%) adolescent patients were allergic to peppers (in 1 case explicitly specified as green peppers), 2 (15.38%) patients reported allergy to either egg, soy, oats, cow's milk, walnuts, almonds, or rice. Allergy to corn, lupine, lentils, peas, beans, peaches, carrots, rye, barley, and gluten, in general, were reported only in 1 (7.69%) older patient each. An evaluation of patient histories revealed bronchial asthma in 45.5% (n = 15), allergic rhinoconjunctivitis in 42.4% (n = 14), food allergy in 27.3% (n = 9), and atopic dermatitis in 24.2% (n = 8). No additional

Tab. 6. Age at diagnosis and symptomatic time before diagnosis in relation to endoscopic findings (in years).
Obr. 6. Věk při stanovení diagnózy a trvání příznaků před stanovením diagnózy u různých endoskopických nálezů (roky).

Endoscopic finding	Age		Symptomatic time	
	mean	median (min–max)	mean	median (min–max)
Normal	9.41	9.17 (0.75–17.50)	3.44	2.00 (0.00–16.50)
Longitudinal furrows	12.9	13.38 (5.83–16.00)	2.94	1.00 (0.00–10.00)
White exudates	9.80	7.75 (3.67–18.17)	2.47	2.63 (0.00–6.67)
Mucosal erythema	14.66	14.25 (10.75–18.17)	5.57	5.83 (0.17–12.50)
Trachealization or concentric rings	14.12	15.00 (7.42–18.17)	1.79	0.83 (0.42–6.67)
Stricture formation	15.00	15.00 (14.00–16.00)	4.79	4.79 (0.58–9.00)

Tab. 7. Rates of administering various treatment combinations.
Tab. 7. Relativní výskyt různých kombinací léčby.

Treatment type	N (%)
PPI + CS + diet	14 (42.4%)
PPI + diet	9 (27.3%)
PPI + CS	5 (15.2%)
PPI	3 (9.1%)
CS + diet	2 (6.1%)

allergic comorbidity was found in 24.4% of patients with EoE (n = 8).

Management

In the 33 patients with EoE analyzed, the most commonly used therapeutic option consisted of proton-pump inhibitors (PPI), which were used in 31 (93.9%) patients. PPI were followed by some type of elimination diet in 25 (75.8%) patients and then by corticosteroids (CS) administration in 21 (63.6%) patients (Tab. 7). Patients usually received a combination of treatments and the most common was all three of the aforementioned modalities, which was seen in 14 (42.4%) patients. PPI was the only therapeutic option used without any of the others only in 3 (9.1%) patients. The rates of administering the various treatments are summarized in Tab. 7.

PPI were administered in 93.9% of EoE cases in this data set, and we may state that the vast majority of patients used PPI in an appropriate dosage (average dose 0.9 mg/kg/day, median 1 mg/kg/day, min. 0.2, max 1.5).

In the group of patients who were treated with topical CS more than half of patients (57.1%) got budesonide suspension with an appropriate median dose of 2 mg/day. A minority of patients was treated with swallowed fluticasone MDI with a median dose 200 µg/day (min. 100.0, max. 375.0). Only 3 patients were treated with systemic corticosteroids.

Food elimination was a very common treatment among our patients, with 25 (75.8%) eliminating at least one. The most commonly eliminated food consisted of nuts (by 17 patients).

Although the strict six-food diet (SFD) was observed by only 1 (4.00%) patient, another 4 (16.0%) patients adhered to the diet with the exception of one food from SFD (fish, wheat, or soy). The numbers of patients within our data set eliminating various foods are summarized in Fig. 3.

No esophageal dilation was needed, and only 2 of all patients manifested complications from treatment. A 13-year-old boy manifested mycotic esophagitis after budesonide suspension administration and an 8-year-old boy complained about itching throat and intermittent dysphagia after fluticasone treatment, which was impossible to differentiate from the manifestation of disease activity.

The rates of clinical, endoscopic, and histopathological remission achievement by the time of the control endoscopy were investigated, and those results are summarized in Tab. 8.

Discussion

In the presented study we found the basic demographic data to be consistent with that from similar published case series. We demonstrated the vast majority of patients to be male (81%) and the median age at onset to be 7 years. This only strengthens the reliability and reproducibility of our subsequent findings for the data set of patients representing the typical demography of an EoE population as reported in large cross-sectional surveys elsewhere [3].

The complex EoE immunopathogenesis constituting the background for histopathological changes resulting in

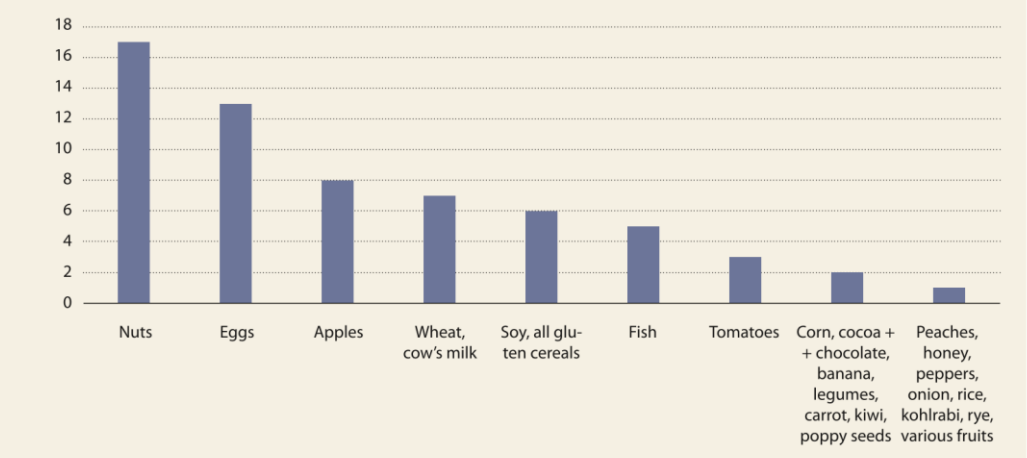


Fig. 3. Number of patients eliminating specific foods.
Obr. 3. Počet pacientů eliminujících určité potraviny.

various endoscopic features and clinical symptoms is summarized in Fig. 4.

Basically, immunopathological mechanisms involved in the pathogenesis of EoE are IgE-mediated and non-IgE-mediated reactions, the latter of which are more delayed at the onset. Immediate allergic reactivity is in connection with an immunoglobulin isotype switch to allergen-specific IgE formation, which is set as a result of a predominant allergenic type 2 T-helpers signalization (at the expense of tolerogenic signaling of type 1 T-helpers). This results in loss of immune tolerance and leads to a process of sensitization to food- and aeroallergens that occurs at various sites (e. g., respiratory tract, skin) [10].

EoE is often associated with atopy and food allergy, so nonspecific allergy markers were analyzed in this study (total serum IgE and relative eosinophilia) and these were just slightly higher in patients with EoE than are the reference intervals for pediatric patients. This is in concordance with recently published data showing an absence of clinical effect of anti-IgE antibody (omalizumab) in patients with EoE and demonstrating that the non-IgE mechanism is the dominant immunopathological mechanism of EoE development [11–13]. Almost three-quarters of patients in the present study had been examined and more than half of those had proven sensitization to food allergens. These findings confirm a strong association between food allergy and EoE reported previously [14]. In the present study, we found that laboratory assessment of specific IgE levels is the predominant method for detecting sensitization to food allergens. The severity of symptoms cannot be predicted by either the specific IgE level or the degree of skin testing positivity, but it was proven previously that the probability of symptom onset is directly related. Considering this fact, the authors consider it important to mention that skin prick-to-prick testing with native food triggers (i.e., cow's milk, eggs, peanuts, and fish) has been shown in studies to have very high negative predictive value and thus it provides reliable, noninvasive, and relatively low-cost exclusion of IgE-mediated mechanisms in the background of EoE pathogenesis [15]. There were significant differences among proven food allergens in the different age groups. Cow's milk, eggs, and peanuts were dominant food allergens of children up to 7 years of age. Above this age, tree nuts, wheat, apples, and peanuts rise in significance. These results are quite similar in comparison to those from other, previously published Western studies [16]. These observations are in agreement with the known phenomenon of oral tolerance and disappearance of allergic reactivity in relationship to some food allergens that is common at an early age and with rising relevance of other food allergens that often are homologous with some of the aeroallergens (i.e., Bet v1) [17]. More than one-quarter (27.3%) of the 33 analyzed EoE patients suffered from oral allergy syndrome (sometimes reported as a food-pollen syndrome), which is caused by cross-reactivity of an IgE-mediated immune reaction to specific aeroallergens having proteins similar to those in some fruits, vegetables, or tree-nuts. Large retrospective surveys have proven a strong association of EoE with commonly seen atopic diseases (most frequently with allergic rhinoconjunctivitis) [3,18]. Furthermore, three-quarters of all patients in the present study showed some allergic comorbidity, with bronchial asthma and/or allergic rhinoconjunctivitis diagnosed in almost half of them. It is noteworthy that statistical analysis of the remission achievement rate in relation to other allergic comorbidities in our 33 patients showed no impact of these comorbidities on the results of the treatment.

Allergic inflammation results in esophageal dysfunction that is accompanied by clinical symptoms related to the age of the patients. Younger children usually manifest only nonspecific symptoms that have a considerable degree of overlap with symptoms of gastroesophageal reflux. Vomiting, food refusal, and failure to thrive are the most common symptoms of EoE in preschool children. Controversially, abdominal pain in this age group is particularly tricky to interpret. Parents of young children are usually referring about so-called "colics", and preschool children may also complain of epigastric pain despite having only an esophageal disease with chest pain (odynophagia), dysphagia, or pyrosis (heartburn) [19]. When EoE is chronic, without adequate therapy, and usually progressing, the disease shows many and variable combinations, with symptoms changing through time during childhood. Earlier publications mention these clinical features of EoE as a pitfall of EoE diagnostics, and especially in younger children

The youngest patients in this survey were two boys who both had manifested difficulty feeding, food refusal in combination with various symptoms of gastroesophageal reflux, and failure to thrive at 6 and 9 months of age. It took more than 5 years – and even almost 13 years in the second case – to achieve an appropriate endoscopic diagnosis of EoE. Our results point to a trend of longer symptomatic time in children having their first symptoms at a younger age,

Tab. 8. Remission achievement.
Tab. 8. Dosažení remise.

Clinical remission	**N/33 (%)**
yes	21/33 (63.6%)
no	7/33 (21.2%)
unknown	5/33 (15.2%)
Endoscopic remission	**N/33 (%)**
yes	16/33 (48.5%)
no	7/33 (21.2%)
unknown	10/33 (30.3%)
Histopathological remission	
yes	10/33 (30.3%)
no	16/33 (48.5%)
unknown	7/33 (21.2%)
Any remission	
yes	26/33 (78.8%)
no	7/33 (21.2%)

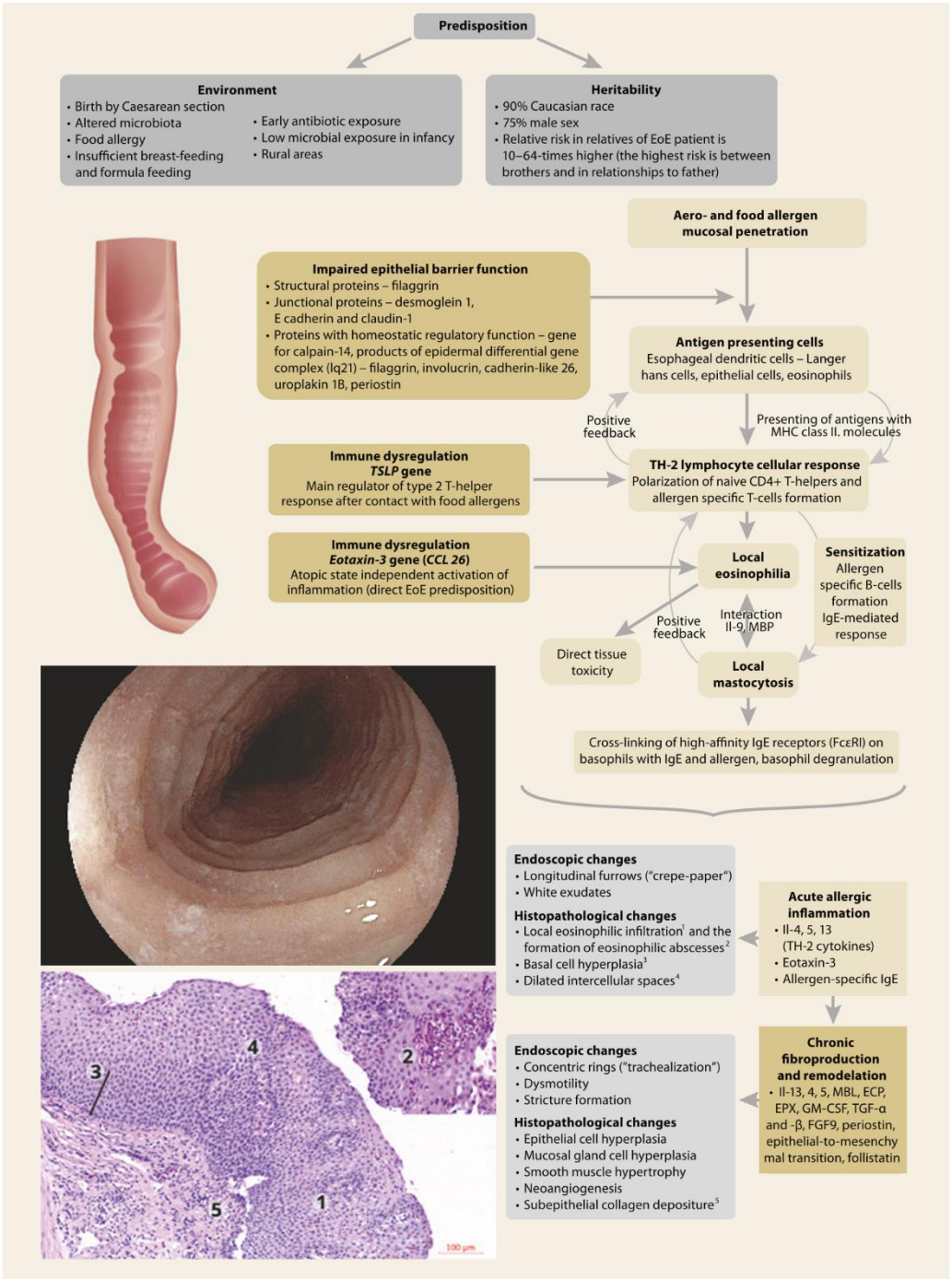

Predisposition
Environment
• Birth by Caesarean section
• Altered microbiota
• Food allergy
• Insufficient breast-feeding and formula feeding
• Early antibiotic exposure
• Low microbial exposure in infancy
• Rural areas
Heritability
• 90% Caucasian race
• 75% male sex
• Relative risk in relatives of EoE patient is 10–64-times higher (the highest risk is between brothers and in relationships to father)
Aero- and food allergen mucosal penetration
Impaired epithelial barrier function
• Structural proteins – filaggrin
• Junctional proteins – desmoglein 1, E cadherin and claudin-1
• Proteins with homeostatic regulatory function – gene for calpain-14, products of epidermal differential gene complex (Iq21) – filaggrin, involucrin, cadherin-like 26, uroplakin 1B, periostin
Antigen presenting cells
Esophageal dendritic cells – Langer hans cells, epithelial cells, eosinophils
Positive feedback
Presenting of antigens with MHC class II. molecules
Immune dysregulation
TSLP gene
Main regulator of type 2 T-helper response after contact with food allergens
TH-2 lymphocyte cellular response
Polarization of naive CD4+ T-helpers and allergen specific T-cells formation
Immune dysregulation
Eotaxin-3 gene (CCL 26)
Atopic state independent activation of inflammation (direct EoE predisposition)
Local eosinophilia
Sensitization
Allergen specific B-cells formation IgE-mediated response
Positive feedback
Interaction Il-9, MBP
Direct tissue toxicity
Local mastocytosis
Cross-linking of high-affinity IgE receptors (FcεRI) on basophils with IgE and allergen, basophil degranulation
Endoscopic changes
• Longitudinal furrows ("crepe-paper")
• White exudates
Histopathological changes
• Local eosinophilic infiltration[1] and the formation of eosinophilic abscesses[2]
• Basal cell hyperplasia[3]
• Dilated intercellular spaces[4]
Acute allergic inflammation
• Il-4, 5, 13 (TH-2 cytokines)
• Eotaxin-3
• Allergen-specific IgE
Endoscopic changes
• Concentric rings ("trachealization")
• Dysmotility
• Stricture formation
Histopathological changes
• Epithelial cell hyperplasia
• Mucosal gland cell hyperplasia
• Smooth muscle hypertrophy
• Neoangiogenesis
• Subepithelial collagen depositure[5]
Chronic fibroproduction and remodelation
• Il-13, 4, 5, MBL, ECP, EPX, GM-CSF, TGF-α and -β, FGF9, periostin, epithelial-to-mesenchymal transition, follistatin
1
2
3
4
5
100 µm

Fig. 4. Pathophysiology of EoE.
Environment, and especially during early childhood, has the dominant impact on the multifactorial development of EoE. The relevant factors are listed in the scheme above. Hereditary factors are generally considered to have a minor effect [4,5]. Nevertheless, genetic predispositions for EoE include mutations in the *calpain-14* gene and down-regulation of some additional genes whose products are involved in epithelial barrier function (*desmoglein-1, filaggrin, involucrin*, and others) against allergens from an external environment, and that barrier's disruption is one route for sensitization [6.] Whole exome sequencing has identified numerous variants of the *TSLP* (thymic stromal lymphopoietin) gene, which is currently considered to be a major regulator of the TH-2 cellular response in terms of allergenic polarization after contact with allergens [7]. Moreover, the genetic predisposition is associated also with genes for eotaxin-3 in esophageal epithelial cells, which is probably a major atopy-independent genetic factor for EoE development [8]. After an allergen's penetration of the esophageal mucosa, antigen--presenting cells present the processed antigens to T helpers type 2. Activation of the so-called TH-2 response is accompanied by release of numerous cytokines, of which interleukin-13 (IL), IL-4, and IL-5 are considered to be most important in development of the acute inflammatory phase of EoE, which results in increased eotaxin-3 expression followed by local eosinophilic hyperfiltration. Esophageal tissue damage leads to additional disruptions of the superficial epithelium followed by lingering release of proinflammatory chemokines by activated eosinophils maintaining chronic inflammatory infiltration and fibrous tissue production that results in irreversible esophageal strictures formation [9].

CCL 26 – chemokine (C-C motif) ligand 26, ECP – eosinophil cationic protein, EPX – eosinophil peroxidase, FGF9 – fibroblast growth factor 9, IL – interleukin, MBP – major basic protein, TGF-α and β – transforming growth factor α and β, TH-2 – T helper type 2, TSLP – thymic stromal lymphopoietin.

Figure 4 was prepared in accordance with Davis et al [9] and created in collaboration with the Service Center for E-Learning at Masaryk University, Faculty of Informatics (part of user support at IS MU, Faculty of Informatics, Masaryk University, Brno).

Obr. 4. Patofyziologie EoE.
Environmentální vlivy, zejména pak v raném dětství, mají dominantní dopad na multifaktoriální vývoj EoE. Relevantní faktory jsou uvedeny v předkládaném schématu. Obecně se má za to, že dědičné faktory mají pouze malý vliv [4,5]. Mezi genetické predispozice pro EoE však patří mutace genu *calpain-14* a down-regulace některých dalších genů, jejichž produkty se podílejí na funkci epiteliální bariéry (*desmoglein-1, filaggrin, involucrin* a další) proti alergenům z vnějšího prostředí a narušení této bariéry je jednou z cest senzibilizace [6.] Metodou celoexomového sekvenování byla identifikována řada variant genu *TSLP* (thymic stromal lymphopoietin), který je v současné době považován za hlavní regulátor buněčné odpovědi TH-2 z hlediska alergenní polarizace po kontaktu s alergeny [7]. Genetická predispozice je navíc spojena také s geny pro eotaxin-3 v epiteliálních buňkách jícnu, což je pravděpodobně hlavní genetický faktor nezávislý na atopii pro vývoj EoE [8]. Po průniku alergenu do sliznice jícnu antigen prezentující buňky předkládají zpracované antigeny pomocným T lymfocytům typu 2. Aktivace takzvané TH-2 odpovědi je doprovázena uvolňováním mnoha cytokinů, z nichž jsou interleukin-13 (IL), IL-4 a IL-5 považovány za nejdůležitější ve vývoji akutní zánětlivé fáze EoE, která vede ke zvýšené expresi eotaxinu-3 následované lokální eozinofilní infiltrací. Poškození tkáně jícnu vede k dalšímu narušení povrchového epitelu, po kterém následuje přetrvávající uvolňování prozánětlivých chemokinů aktivovanými eozinofily, kdy dochází k chronické zánětlivé infiltraci a fibrotizaci tkáně, což vede k tvorbě ireverzibilních striktur jícnu [9].

CCL 26 – chemokinový ligand 26 (C-C motiv), ECP – eozinofilní kationický protein, EPX – eozinofilní peroxidáza, FGF9 – fibroblastový růstový faktor 9, IL – interleukin, MBP – hlavní bazický protein, TGF-α a β – transformující růstový faktor α a β, TH-2 – pomocný T lymfocyt typu 2, TSLP – thymický stromální lymfopoetin.

Obr. 4 byl převzat a upraven dle Davis et al [9] a vytvořen ve spolupráci se Střediskem služeb pro e-learning Masarykovy univerzity (součást uživatelské podpory na IS MU, Fakulta informatiky Masarykovy univerzity, Brno).

as expected. The correlation between age of first symptoms and length of diagnosis is statistically significant (Spearman's rho = –0.42, p-value = 0.014), and it is obvious that EoE in young children is usually underdiagnosed.

Moreover, one-third of all of the patients experienced only one of the EoE symptoms, which then led to the decision to perform endoscopic evaluation and histopathological diagnosis. This was unusual in the youngest group of patients, and among those children this occurred only once (in the case of a patient refusing food). The appearance of just one symptom before proceeding to biopsy was observed more often among older patients, and most frequently it was abdominal pain or food impaction that led directly to the diagnosis without demonstrating any additional symptoms. This phenomenon also supports the hypothesis that EoE could be underdiagnosed in young children, as they usually must manifest multiple symptoms to get an appropriate diagnosis. Furthermore, symptoms, such as food impaction, that seem able to lead to diagnosis on their own occur not so frequently in young children, further complicating their reaching a diagnosis of EoE. Nevertheless, the statistical test did not confirm any relationship at the 5% significance level between age at diagnosis and number of symptoms. Older children with dysmotility of the eso-

phagus usually complain about concrete difficulties like dysphagia, odynophagia, or chest pain, as well as various gastroesophageal reflux symptoms. The relative manifestation rates of various symptoms among patients in the present study quite closely correspond to previously published data [20,21]. If these symptoms of esophageal dysmotility in older children are not diagnosed precisely and on time and then adequately treated they may lead to irreversible remodeling of the esophageal wall and irreversible stricture formation with recurrent food impaction [19,22,23].

The mean duration of symptoms in patients involved in this study was 3.36 (SD 3.87) years and the median 2.25 years with a very wide range of symptomatic time to diagnosis of 0–16.5 years.

Interestingly, the cases of girls with EoE in our study showed an even longer symptomatic period (mean 4.9 years with SD 3.8 and median 4.2 years with range 0–10). Unfortunately, in comparison with data published elsewhere, such long symptomatic intervals before appropriate diagnosis in children are not unusual [22,24].

Endoscopic findings in the present pediatric EoE data set included both inflammatory (erythematous mucosa, linear furrows, and patchy white exudates) and fibrostenotic changes (crepe paper-like appearance of the mucosa, concentric ring-like esophageal changes or strictures). The most common endoscopic findings in the present pediatric patient data set were longitudinal furrows and white exudates, which is in concordance with previously published pediatric data from Western countries [22].

If EoE is not treated adequately and in a timely manner, the chronic inflammation in the esophageal wall develops complications associated with the remodeling process. The concentric rings and esophageal strictures can be seen among adult patients with EoE dominant macroscopic findings [2]. Also in our data set these macroscopic signs were seen with the highest median patient's age among all findings. The results of retrospective studies have shown that the most important risk factor for esophageal remodeling and stricture formation is late establishment of the diagnosis [25].

Detailed guidelines for the management of EoE in pediatric patients are regularly published by the Joint Task Force on Allergy-Immunology Practice Parameters, and the respected professional authorities the European Academy of Allergy and Clinical Immunology (EAACI) and European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) publish a joint communication covering current knowledge on EoE respecting the rules of evidence-based medicine [1,26]. Management of EoE in children aims for the resolution of clinical symptoms as well as endoscopic and histological remission. Based on individual evaluation as to the severity of clinical manifestations, the results of allergological examinations, and macroscopic findings, a decision among the various treatment modalities is made. Treatment options include PPI, elimination diets, and topical or systemic CS administration, as well as their combinations [27]. Systemic CS are reserved for severe cases where rapid relief of symptoms is required and other treatment has not proven successful. This conservative attitude regarding their use was apparent also in our data set [28]. Magistraliter prescription of budesonide suspension and swallowing of fluticasone aerosol forms are off-label administrations of these drugs. Jorveza® is a new preparation of topical CS budesonide in an orodispersible tablet that is registered in the Czech Republic to treat adults with eosinophilic esophagitis. In a double-blind, placebo-controlled phase III clinical trial (EOS-1), Jorveza® showed both high safety and efficacy for induction and long-term treatment of EoE in adults. To date, there are no available data concerning Jorveza® administration in the pediatric population, but clinical trials are currently being conducted [29].

An elimination diet is considered as a first-line therapeutic modality for children and adults with EoE and, together with food challenges, these are irreplaceable in the diagnostic approach to food allergies. EoE is a chronic disease by its nature, and gradually progressive esophageal wall remodeling is a long-term process that allows too many patients to adapt to and thus underestimate the symptoms. From these clinical features of EoE there follows a major limitation upon the use of elimination diets, because to objectively measure the complex therapeutic effect of a diagnostic diet necessitates repeated endoscopy assessment and esophageal biopsies after food challenges. The invasivity of these procedures constitutes the main limitation upon administering individual therapeutic diets in childhood. Unfortunately, strong data suggest that the highest efficiency involves elemental diets (90.8%) in comparison with SFD (72.1%), the four-food diet (53.4%), and allergy skin test result-directed food elimination (45.5%) [30]. All these approaches are recommended for the treatment of EoE under the current guidelines [31]. Nevertheless, in order to achieve the highest possible adherence and maintain the best quality of life, it is appropriate to strive for as much targeted elimination in the diet as is possible, and this must be chosen individually for each patient. Time- and cost-effective procedures may point to recommending a diet eliminating a few of the most allergenic foods (i.e., the four-food diet). Then, in order to increase the effectiveness of the diagnostic diet, the standard approach in the health care facilities of the present authors consists in conducting skin prick-to-prick testing with a huge set of the most allergenic foods, including certain oral allergy syndrome triggers and individually referred food suspects. Their positivity is then quantified by detection of IgE to specific components of allergens in order to increase reproducibility and clinical relevance of these results [7,32].

In comparison with a recent meta-analysis, our study showed a slightly lower rate of remission achievement. This may be due to a relatively high proportion of incomplete evaluations, occurring, among other reasons, because some patients did not return for ambulatory controls, a significant number of patients did not undergo control endoscopy and/or biopsy, and the availability of nutritional consulting varied by different health care facility. Moreover, our survey had quite strictly set terms for remission achievement (absence of all symptoms, disappearance of all macroscopic findings, and decrease of IEE strictly below the diagnostic cutoff). Statistical tests were performed to investigate the relationships between certain treatments and rate of remission (clinical, endoscopic, and histopathological) achievement. Categorical variables and remission achievement were tested using Fisher's exact test and continuous variables using Mann-Whitney U-test. None of the tested variables (PPI use and its dosage, CS use and its dosage, food elimination, range of food elimination, combination of various treatments) proved to be statistically significant. Whereas median IEE count in the control biopsies was still above the diagnostic cutoff, the authors consider it appropriate to mention that local mucosal eosinophilia is not fully pathognomonic and so it may accompany various gastrointestinal diseases (i.e., inflammatory bowel disease) for which differential diagnosis must be borne in mind.

The results of the present analysis are to a great extent consistent with the results of pediatric EoE experience previously published elsewhere, and we claim that the analyzed set of patients truly constitute a representative demographic sample of the regional EoE population.

Conclusion

This study's comprehensive overview of the present regional management of pediatric EoE may increase pediatricians' awareness about the characteristics of EoE and may lead to an increase in the frequency of endoscopic verifications and biopsies, especially in younger children often presenting with nonspecific symptoms for a long period. Early detection of EoE and initiation of effective treatment during the inflammatory stage are very important. The therapeutic elimination diet is a safe and reliable approach to the long-term effective management of pediatric patients with EoE. The results of this study are limited by its retrospective design, the relatively small number of patients, and the lack of standardization of the treatment protocols as ensues from the multicenter collection of the analyzed data. The authors would appreciate if this survey would contribute to the founding of a national register of patients with EoE in the Czech Republic.

Acknowledgement

Figure 4 was created in cooperation with Service Center for e-learning at MU (part of user support at IS MU, Faculty of Informatics, Masaryk University, Brno).

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Submitted/Doručeno: 2. 10. 2020
Accepted/Přijato: 24. 11. 2020

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Conflict of Interest: The authors declare that the article/manuscript complies with ethical standards, patient anonymity has been respected, and they state that they have no financial, advisory or other commercial interests in relation to the subject matter.

Publication Ethics: This article/manuscript has not been published or is currently being submitted for another review. The authors agree to publish their name and e-mail in the published article/ manuscript.

Dedication: This work was supported by Ministry of Health, Czech Republic – conceptual development of research organization (FNBr, 65269705).

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

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Dedikace: Podpořeno MZ ČR - RVO (FNBr, 65269705).

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Annex 8

doi: 10.14735/amgh2020233

Herpetic esophagitis in a 7-year-old immunocompetent patient

Herpetická ezofagitida u imunokompetentního sedmiletého pacienta

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Summary: Herpetic esophagitis is a disease diagnosed especially in immunocompromised patients. Although the disease is rare in immunocompetent individuals, the diagnosis should be considered in the presence of its acute triad of clinical symptoms – odynophagia, chest pain, and fever of unknown origin. Herpes simplex virus type 1 (HSV1) is the most common causative agent. In the majority of cases, the disease develops by re-activation of latent HSV1 infection or, rather rarely, by primo-infection. The basis of diagnosis is endoscopic examination of the esophagus with biopsy and direct detection of the virus in the bioptic sample. In immunocompromised patients, treatment with acyclovir, which is the first-line virostatic in this indication, is always indicated. In immunocompetent patients, this is a self-limiting disease, where in most cases merely symptomatic treatment is sufficient. This case report describes an immunocompetent patient with a suddenly occurring typical triad of symptoms caused by herpetic esophagitis. The diagnosis was confirmed by the presence of viral DNA as determined by polymerase chain reaction from a sample taken during endoscopic examination. Due to the more severe course of the disease, the patient was treated with acyclovir and the general condition and local endoscopic findings then quickly improved.

Key words: herpetic esophagitis – children – acyclovir – endoscopy

Souhrn: Herpetická ezofagitida je onemocnění diagnostikované zejména u imunokompromitovaných pacientů. U imunokompetentních jedinců se jedná o vzácné onemocnění, na které je ale třeba pomýšlet při akutně vzniklé triádě potíží – odynofagie, bolesti na hrudi a horečka nejasného původu. Nejčastějším původcem je herpes simplex virus typu 1 (HSV1). Ve většině případů vzniká onemocnění reaktivací latentní infekce HSV1, vzácněji při primoinfekci. Základem diagnostiky je endoskopické vyšetření jícnu s provedením biopsie a přímým průkazem přítomnosti viru v bioptickém vzorku. U imunokompromitovaných pacientů je vždy indikována léčba acyklovirem, který je v této indikaci virostatikem první volby. U imunokompetentních pacientů se jedná o tzv. self-limiting onemocnění, kdy v naprosté většině případů postačuje symptomatická léčba. Kazuistika popisuje imunokompetentního pacienta s náhle vzniklou typickou triádou potíží způsobených herpetickou ezofagitidou. Diagnóza byla potvrzena průkazem přítomnosti virové DNA metodou PCR ze vzorku odebraného při endoskopickém vyšetření. Vzhledem k těžšímu průběhu onemocnění byl pacient přeléčen acyklovirem a došlo k rychlé úpravě celkového stavu i lokálního endoskopického nálezu.

Klíčová slova: herpetická ezofagitida – děti – acyklovir – endoskopie

Introduction

Along with cytomegalovirus and candida, herpes simplex virus (HSV) is one of the possible causative agents of ulcerative esophagitis in immunocompromised patients (e. g., HIV patients, immunosuppressed patients, patients with severe systemic disease or severe burns, patients with long-term corticosteroid therapy) [1,2]. Despite the high prevalence of primary and recurrent HSV infection in the population [3], clinically manifested herpetic esophagitis (HE) is rare in immunocompetent patients [4]. Isolated cases of HE in immunocompetent children are nevertheless described in the literature [5–11].

Case report

A 7-year-old patient who had previously been healthy was transferred to the De-

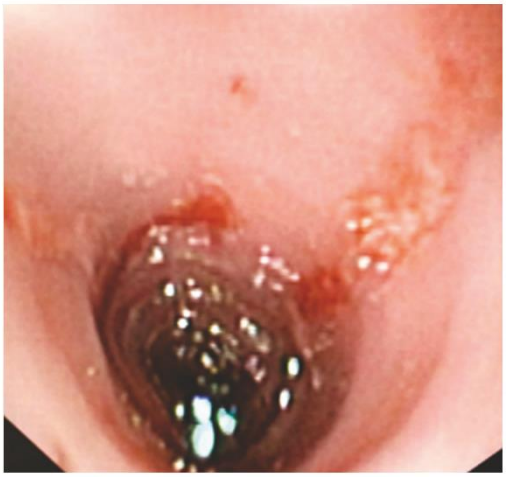

Fig. 1A. Endoscopic imaging – view of distal esophagus. Ulceration covered by mucus, mucosa fragile to the touch, contact bleeding.
Obr. 1A. Endoskopický obraz – pohled na distální jícen. Ulcerace kryté hlenem, sliznice křehká na dotek, kontaktně krvácející.

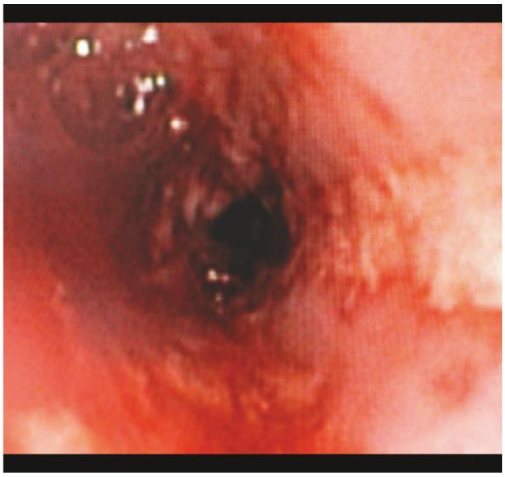

Fig. 1B. Endoscopic imaging – ulceration of the distal esophagus.
Obr. 1B. Endoskopický obraz – ulcerace distálního jícnu.

partment of Pediatrics of the University Hospital Brno from the pediatric department of a district hospital. He was a normally thriving boy with adequate psychomotor development who had been properly vaccinated with no adverse manifestations. He had had no physiological morbidities up to that time and no allergic disease. He had not taken any medication for a long time. Before the occurrence of the clinical symptoms, the boy had spent a month of summer holidays in Montenegro. Otherwise, the epidemiological history obtained on admission was negative. Four days before the admission to the hospital, the boy had had a fever that reached a maximum of 38.5 °C. The fever had responded well to antipyretic therapy. The boy had no other problems. On the second day of the fever, dysphagia and odynophagia had occurred. After examination by a GP for children and adolescents, proton pump inhibitor therapy in the usual dosage had been recommended. Despite the established treatment, the swallowing problems worsened over subsequent days and the boy had to be hospitalized in the children's ward of the district hospital. The possibility of having ingested a foreign body was excluded by the patient and parents. There had been no previous signs of primary immunodeficiency or other signs of gastrointestinal disease in the patient.

There was significant elevation of CRP (110 mg/l) in the baseline blood samples, mild leukocytosis in the blood count, and significant ketonuria without glycosuria in the urine. During hospitalization, a chest CT scan was performed but did not show any specific pathology. Parenteral nutrition was initiated and symptomatic therapy (proton pump inhibitor, nonsteroidal anti-inflammatory drugs) continued. For persisting difficulties without significant improvement, the boy was transferred for further examination to the university hospital.

The initial clinical examination was without noteworthy changes. In a detailed supplement to the epidemiological history, it was found that 4 days prior to the onset of the patient's complaints, his mother had had a significant eruption of herpes labialis. The patient himself had never had such symptoms. As a further step to the diagnosis, an esophagogastroduodenoscopic examination was performed. Significant inflammatory changes transitioning to longitudinal ulcerations in the distal third of the esophagus were revealed (Fig. 1A, B). In addition to the standard sampling of esophageal tissue, biopsy material was also sent for bacteriological and mycological examination and also for the detection of HSV and cytomegalovirus by polymerase chain reaction (PCR).

Given that this was a relatively unusual finding in a pediatric patient, empirical therapy with antibiotics (cefuroxime), antifungal agent (fluconazole), and antiviral drug (acyclovir) were administered intravenously. Over the next two days, there was a significant subjective improvement in symptoms. The patient began to tolerate fluids and subsequently a semi-solid diet, and the fever disappeared.

The result of HSV DNA detection by PCR from the esophageal mucosa was positive. Histological examination of the tissue taken from the affected area

of the esophagus confirmed the presence of nonspecific ulceration (necrosis and granulation tissue) (Fig. 2), but the pathognomonic features of herpesvirus infection (inclusion, multinucleated keratinocytes) were not found in the sample. Immunohistochemistry (anti-CMV antibody) and special staining (PAS, Grocott silvering) excluded the presence of cytomegalovirus and fungal infection.

Due to the relatively unusual diagnosis, an immunological examination was performed. This did not indicate any impairment of immunity. After completing the results of the microbiological examination, the antimicrobial treatment was revised. Antiviral therapy was maintained until the result of the control esophagogastroduodenoscopy, which was performed 3 weeks after the diagnosis. Endoscopic and histopathological examination confirmed the complete disappearance of inflammatory changes in the esophagus.

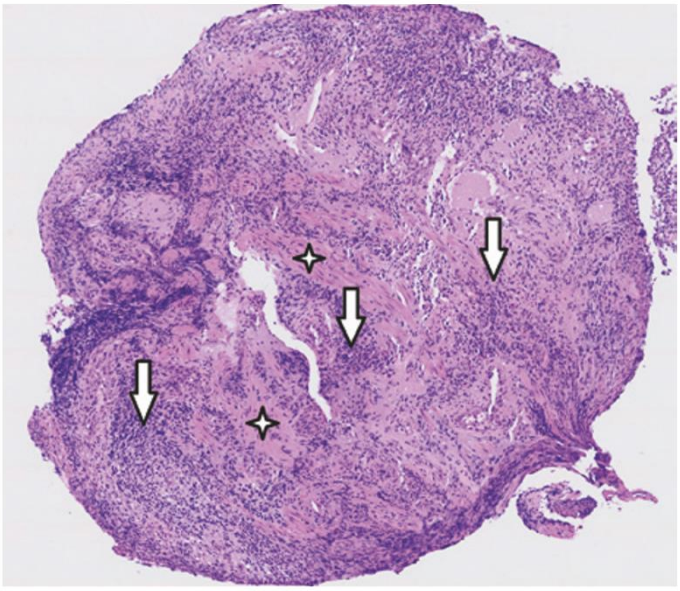

Fig. 2. Histological examination – base of ulceration consisting of granulation tissue with mixed inflammatory infiltrate.
The presence of smooth muscle bundles verifies the depth of ulceration to the muscularis propria. Hematoxylin-eosin staining, magnification 30-times.
Stars indicate smooth muscle bundles, vertical arrows indicate inflammatory infiltrate.
Obr. 2. Histologické vyšetření – spodina ulcerace tvořená smíšeně zánětlivě celulizovanou granulační tkání.
Přítomnost snopců hladké svaloviny dokumentuje hloubku ulcerace až ke svalovině muscularis propria. Barvení hematoxylin-eozinem, zvětšení 30krát. Hvězdičky – snopce hladké svaloviny, svislé šipky – zánětlivý infiltrát.

Discussion

HE was first described in 1940 by Johnson [12] and confirmed histopathologically by the Pearce team 3 years later [13]. HE is regarded today as an opportunistic infection that usually affects patients with immune disorders [1]. The first larger set of endoscopic findings of immunocompromised patients in which the diagnosis was based on virus culture was described by McBan in 1991 [14]. In 2000, Ramathan et al published an overview of HE described in immunocompetent patients, including several children [4].

HE can be caused by human HSV types 1 and 2. Type 2 is, however, rarely the causative agent [6]. Nevertheless, 20% of adult HE patients have been reported to have concomitant oropharynx and genital involvement [4]. The HSV rarely attacks internal organs. In such cases as it does so, the esophagus is often affected but the lungs and kidneys very rarely. Cases with stomach involvement also have been described [15,16]. In a series of 1,307 autopsies performed in adults, a study from Japan found an incidence of HE in 1.8% patients [17]. HE can develop as a result of direct expansion in the primary infections of the orofacial region or as a result of reactivation of latent infection when invasion of the esophagus mucosa by anterograde neuronal migration had occurred via the vagus nerve [1].

One of the predisposing factors of HE in immunocompetent patients may be esophageal mucosal trauma. This predisposition may be gastroesophageal reflux disease, previous esophageal instrumental examination, long-term insertion of a nasogastric tube, or swallowing of a foreign body [5]. HE is typically manifested by a triad of symptoms: odynophagia, chest pain, and fever of unknown origin. These symptoms are usually accompanied by weight loss [4,14].

The diagnosis of HE consists of an endoscopic examination, exclusion of other causes (from infection, especially cytomegalovirus and candida), and confirmation of HSV's presence. Bioptic examination almost always shows inflammatory changes of the mucosa, but only in some cases can the typical diagnostic signs of herpesvirus infection (i.e., intranuclear inclusion in the epithelium of the esophageal mucosa, either eosinophilic [Cowdry A type] or ground-glass [Cowdry B type]) be found [18]. Distinguishing of HSV and varicella-zos-

ter virus is not possible with the use of light microscopy. In the past, the virus has been proven by a combination of immunohistochemical methods and cultivation [4,14]. Presently, due to its indisputable advantages, the detection of viral DNA by PCR is used [19].

The diagnosis is therefore usually based on clinical signs, upper gastrointestinal endoscopy, histology, microbiological examination, and then PCR, respectively. Serological examination may be another marker for diagnosis [20].

Other esophageal infections (cytomegalovirus, varicella-zoster virus, candida, or various bacterial agents), trauma, esophageal burns, and inflammatory diseases such as Crohn's disease or Behcet's disease may be considered in the differential diagnosis of ulcerative esophagitis [21].

Acyclovir, famciclovir, and valacyclovir are used to treat HE in immunocompromised patients [22].

HE in immunocompetent patients appears generally to be self-limiting [23]. The reasons for administering antiviral therapy may include expectation to reduce the length of the period of infection or to treat or prevent complications.

Primary HSV infection is a common disease in childhood. Positive antibodies to HSV 1 occur in as many as 90% of all adolescents. The highest incidence of the disease with clinical manifestation is around 2 years of age, and gingivostomatitis is the most common clinical form of the disease [5]. During primo-infection, HE can occur rather frequently and thus be underdiagnosed. It is very likely that undiagnosed esophagitis may also be suffered by patients with severe primo-infection (i.e., herpetic gingivostomatitis or pharyngitis), as well as patients with gastroesophageal reflux disease treated with proton pump inhibitors. HE in immunocompetent children is mostly represented by primo-infection, but it may also follow reactivation of latent infection. Although HSV infections of the visceral organs are the result of viremia, esophagitis as such is rather the result of a direct spread of oropharyngeal involvement. Boys are more affected than girls in a ratio of 3.4/1.

The described patient had been in contact with his mother who had symptoms of labial herpes 4 days before the onset of his symptoms. It can be assumed, therefore, that this was a primary HSV infection that then spread to the esophagus. This would correspond to the initial negative serological examination of HSV antibodies in the patient. Also, our patient showed no other clinical signs of immunodeficiency and the results of the immunological examination were normal.

The clinical manifestations described in the literature (which means the triad of odynophagia, chest pain, and fever) were also present in this case.

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Conflict of Interest: The authors declare that the article/manuscript complies with ethical standards, patient anonymity has been respected, and they state that they have no financial, advisory or other commercial interests in relation to the subject matter.

Publication Ethics: This article/manuscript has not been published or is currently being submitted for another review. The authors agree to publish their name and e-mail in the published article/manuscript.

Dedication: Supported by Ministry of Health of the Czech Republic – conceptual development of research organization (FNBr, 65269705) and by Ministry of Health of the Czech Republic, grant nr. NU20-03-00126.

The Editorial Board declares that the manuscript met the ICMJE „uniform requirements" for biomedical papers.

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Publikační etika: Příspěvek nebyl dosud publikován ani není v současnosti zaslán do jiného časopisu pro posouzení. Autoři souhlasí s uveřejněním svého jména a e-mailového kontaktu v publikovaném textu.

Dedikace: Podpořilo Ministerstvo zdravotnictví České republiky – konceptuální rozvoj výzkumné organizace (FNBr, 65269705) a Ministerstvo zdravotnictví České republiky, grant č. NU20-03-00126.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Submitted/ Doručeno: 27. 3. 2020
Accepted/ Přijato: 29. 3. 2020

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Annex 9

Journal of Crohn's and Colitis, 2020, 361–368
doi:10.1093/ecco-jcc/jjz153
Advance Access publication September 10, 2019
Original Article

Original Article

The Influence of Microscopic Inflammation at Resection Margins on Early Postoperative Endoscopic Recurrence After Ileocaecal Resection for Crohn's Disease

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Abstract

Background and Aims: The pathogenesis and risk factors for early postoperative endoscopic recurrence of Crohn's disease [CD] remain unclear. Thus, this study aimed to identify whether histological inflammation at the resection margins after an ileocaecal resection influences endoscopic recurrence.

Methods: We have prospectively followed up patients with CD who underwent ileocaecal resection at our hospital between January 2012 and January 2018. The specimens were histologically analysed for inflammation at both of the resection margins [ileal and colonic]. We evaluated whether histological results of the resection margins are correlated with endoscopic recurrence of CD based on colonoscopy 6 months after ileocaecal resection. Second, we assessed the influence of known risk factors and preoperative therapy on endoscopic recurrence of CD.

Results: A total of 107 patients were included in our study. Six months after ileocaecal resection, 23 patients [21.5%] had an endoscopic recurrence of CD. The histological signs of CD at the resection margins were associated with a higher endoscopic recurrence [56.5% versus 4.8%, $p < 0.001$]. Disease duration from diagnosis to surgery [$p = 0.006$] and the length of the resected bowel [$p = 0.019$] were significantly longer in patients with endoscopic recurrence. Smoking was also proved to be a risk factor for endoscopic recurrence [$p = 0.028$].

Conclusions: Histological inflammation at the resection margins was significantly associated with a higher risk of early postoperative endoscopic recurrence after an ileocaecal resection for CD.

Key Words: Crohn's disease; ileocaecal resection; early postoperative endoscopic recurrence

1. Introduction

Despite the era of biologics, surgical treatment of Crohn's disease [CD] still plays an important role. Up to 80% of patients need surgery during their lifetime.[1,2] Moreover, early postoperative endoscopic recurrence [EPER] of the disease occurs in approximately 70% of patients within the first year after the surgery.[2,3] As EPER precedes clinical symptoms, its detection is essential in the further management of postoperative CD.[2,4] The exact pathogenesis of EPER is unknown. However, according to the literature, the risk factors include smoking, previous intestinal surgery, absence of prophylactic treatment, penetrating disease at index surgery, perianal location, granulomas in the resection specimen, and myenteric plexitis.[5] Although some recent data have suggested that histopathology results of the resection margins could predict EPER,[1,6,7] such relationship remains to be further established. Recurrence prevention concerning patient selection as well as postoperative treatment and its timing remains a clinical challenge, as no standard recommended algorithm exists.[1,8–10] Hence, this study aimed to identify whether histological inflammation at the resection margins influences EPER in patients with CD after an ileocaecal resection [ICR], and to assess the influence of the aforementioned risk factors and preoperative therapy on EPER.

2. Materials and Methods

2.1. Patient selection

In this prospective study, we have included patients with CD who underwent ICR between January 2012 and January 2018 at the University Hospital in Brno. ICR was performed by either the open or the laparoscopic approach. The resection extended to macroscopically uninvolved tissue, and a primary anastomosis was constructed. All the surgeries were performed by three surgeons who are experienced in colorectal and IBD surgery. Ileocolonoscopy was performed 6 months after ICR. Patients who underwent other resection procedures, such as a hemicolectomy, multiple ileal resections, and stricturoplasties, or had either a permanent or a diverting loop ileostomy, were excluded. Moreover, patients with any postoperative prophylactic treatment before the postoperative ileocolonoscopy check-up and those with missing data were also excluded.

All patients provided written informed consent and procedures were performed in accordance with the ethical standards according to the Declaration of Helsinki.

2.2. Variables analysed

We analysed the general characteristics of the patients, including age, gender, the Montreal classification of CD [including perianal disease], and the disease duration from diagnosis to ICR. In regard to the surgery, we evaluated the type of approach [open or laparoscopic], length of the resected bowel, type of anastomosis [end-to-end or side-to-side], and postoperative complications [according to the Clavien-Dindo classification].[11]

Histopathological analysis of the resected tissue was performed by an experienced IBD pathologist. As there is no histopathology score validated for CD,[12] the pathologist marked the resection margin as positive if the following signs of CD were noted: architectural distortion, inflammation activity [mild: cryptitis in <25% crypts; moderate: cryptitis in >25% of crypts; severe: with ulcerations], granulomas, ulcerations, erosions, fibrosis, neuronal hyperplasia, Paneth cells metaplasia, or signs of chronic inflammation [transmural inflammation with lymphoid aggregates and basal plasmacytosis]. When assessing the resection margins, the pathologist always evaluated both the ileal and the colonic margins and assessed for the aforementioned signs of CD. Specimens with inflammation in the ileal, colonic, or both resection margins were counted as positive.

Regarding EPER, ileocolonoscopy 6 months after the ICR was performed and endoscopic recurrence was evaluated using Rutgeert's score [positive EPER was defined as Ri ≥2]. The use of preoperative medication [≤12 weeks before ICR] as well as smoking and any family history of CD as possible risk factors were also analysed.

2.3. Statistical analysis

EPER was the primary endpoint of this study. The difference in the primary endpoint between the two study groups according to the histological inflammation at the resection margins was evaluated using a univariate logistic regression model.

No sample size calculations were performed before the study started. However, the number of patients in both the study arms [90 patients with negative and 17 patients with positive resection margins] as well as the observed difference in the primary endpoint ensured a statistical power of the univariate logistic regression model of >99%.

Standard frequency tables and summary statistics, i.e. means, standard deviation, median, minimum, and maximum, were used to describe the baseline demographic and clinical characteristics. Statistical significance of the differences for categorical variables was assessed using Fisher's exact test. Comparisons of continuous variables were performed using the Mann–Whitney U test. The secondary outcomes were evaluated using univariate and multivariate logistic regression models. A p <0.05 was considered statistically significant for all analyses. All statistical analyses were performed with SPSS Statistics for Windows, version 24.0 [IBM Corp., Armonk, NY, USA].

3. Results

A total of 107 patients, who provided written informed consent, were included in this study. Thirty-six patients were excluded because of a different intestinal resection or prophylactic treatment administered before postoperative ileocolonoscopy. The baseline characteristics of the study group are listed in Table 1.

For EPER, ileocolonoscopy was performed 6 months after ICR, and the endoscopic results were evaluated using Rutgeert's score as shown in Table 2. With reference to the primary outcome of the study, histological results showed that inflammation at the resection margins has a significant statistical influence on EPER [56.5% versus 4.8%, $p < 0.001$; Figure 1]. The histological signs of inflammation in the resection margins were as follows: ileal [I]-positive and colonic [C]-negative in 21.7%; I-negative and C-positive in 13.0%; and I-positive and C-positive in 21.7%. In particular, the risk of EPER was 26-fold higher in patients with positive than in those with negative histology (odds ratio [OR] 26.00, 95% confidence interval [CI] 7.09–95.33) [Tables 3 and 4].

A significant difference in the time from diagnosis of the disease to the ICR between patients with endoscopic recurrence (median 8 years [0–14 years]) and those with endoscopic remission (median 3 years [0–18 years]) [p = 0.006] was found. Thus, the longer the disease duration, the higher the risk of EPER [every year, the risk increases by 14%; OR 1.14, 95% CI 1.03–1.26; p = 0.015]. Moreover, a significant difference in terms of smoking, which was identified as a risk factor for endoscopic recurrence, between patients with recurrence and those with remission was found [p = 0.028] [Tables 3 and 4].

Table 1. Baseline characteristics of the patients, subdivided in the two groups according to the histological signs of inflammation at the resection margins, expressed as median [minimum-maximum] or frequency [percent].

Characteristics		Patients [n = 107]	Negative resection margins [*n* = 90]	Positive resection margins [*n* = 17]
Age at diagnosis	[Years]	Median [min–max]		
		26 [8–75]	26 [8–75]	28 [8–62]
Time from diagnosis to ICR	[Years]	Median [min–max]		
		3 [0–18]	3 [0–15]	7 [0–18]
Gender	Male	48 [44.9%]	39 [43.3%]	9 [52.9%]
	Female	59 [55.1%]	51 [56.7%]	8 [47.1%]
Montreal classification				
A [age]	A1 [<16 years]	13 [12.1%]	10 [11.1%]	3 [17.6%]
	A2 [17–40 years]	74 [69.2%]	64 [71.1%]	10 [58.8%]
	A3 [>40 years]	20 [18.7%]	16 [17.8%]	4 [23.5%]
L [location]	L1 [ileum]	74 [69.2%]	62 [68.9%]	12 [70.6%]
	L1 + L4 [ileum + upper GI]	2 [1.9%]	1 [1.1%]	1 [5.9%]
	L3 [ileum + colon]	28 [26.2%]	24 [26.7%]	4 [23.5%]
	L3 + L4 [ileum + colon + upper GI]	3 [2.8%]	3 [3.3%]	0 [0.0%]
B [behaviour]	B1 [inflammatory]	13 [12.1%]	12 [13.3%]	1 [5.9%]
	B2 [stenosing]	58 [54.2%]	46 [51.1%]	12 [70.6%]
	B3 [perforating]	36 [33.6%]	32 [35.6%]	4 [23.5%]
Perianal disease	No	82 [76.6%]	68 [75.6%]	14 [82.4%]
	Yes	25 [23.4%]	22 [24.4%]	3 [17.6%]
Ileocaecal resection				
Surgical approach	Open	66 [61.7%]	58 [64.4%]	8 [47.1%]
	Laparoscopic	41 [38.3%]	32 [35.6%]	9 [52.9%]
Length of resected bowel	[cm]	Median [min–max]		
		25 [5–80]	25 [6–80]	25 [5–50]
Anastomosis	Side to side	91 [85.0%]	76 [84.4%]	15 [88.2%]
	End to end	16 [15.0%]	14 [15.6%]	2 [11.8%]
Postoperative complications	No	71 [66.4%]	61 [67.8%]	10 [58.8%]
	Yes	36 [33.6%]	29 [32.2%]	7 [41.2%]
Clavien-Dindo classification of postoperative complications	Grade 1	12 [11.2%]	8 [8.9%]	4 [23.5%]
	Grade 2	7 [6.5%]	4 [4.4%]	3 [17.6%]
	Grade 3a	2 [1.9%]	2 [2.2%]	0 [0.0%]
	Grade 3b	15 [14.0%]	15 [16.7%]	0 [0.0%]
	Grade 4	0 [0.0%]	0 [0.0%]	0 [0.0%]
	Grade 5	0 [0.0%]	0 [0.0%]	0 [0.0%]
Histological results				
Presence of granulomas	Negative	71 [66.4%]	64 [71.1%]	7 [41.2%]
	Positive	36 [33.6%]	26 [28.9%]	10 [58.8%]
Preoperative therapy				
Medication ≤12 weeks before ICR	Antibiotics	43 [40.2%]	39 [43.3%]	4 [23.5%]
	5-ASA	58 [54.2%]	49 [54.4%]	9 [52.9%]
	Local GCS	26 [24.3%]	21 [23.3%]	5 [29.4%]
	Systemic GCS	23 [21.5%]	21 [23.3%]	2 [11.8%]
	AZA	31 [29.0%]	26 [28.9%]	5 [29.4%]
	MTX	0 [0.0%]	0 [0.0%]	0 [0.0%]
	Biologic treatment	13 [12.1%]	13 [14.4%]	0 [0.0%]
Smoking	Non-smoker	49 [45.8%]	44 [48.9%]	5 [29.4%]
	Ex-smoker	26 [24.3%]	23 [25.6%]	3 [17.6%]
	Smoker	32 [29.9%]	23 [25.6%]	9 [52.9%]
Family history of IBD	No	94 [87.9%]	80 [88.9%]	14 [82.4%]
	Yes	13 [12.1%]	10 [11.1%]	3 [17.6%]

ICR, ileocaecal resection; GI, gastrointestinal tract; 5-ASA, 5-aminosalicylates; GCS, glucocorticoids; AZA, azathioprine; MTX, methotrexate; IBD, inflammatory bowel disease.

With respect to disease phenotype, the number of patients who developed EPER was significantly higher in those with stenosing behaviour [73.9%] than in those with perforating behaviour [13%] [p = 0.045]. Perianal involvement was present only in four patients [17.4%] with EPER; however, the results were not significant [p = 0.582] [Tables 3 and 4].

Another significant difference was found in terms of the length of the resected bowel; the longer the resection needed, the more likely EPER should occurr [median 30 cm [5–80 cm] in patients with endoscopic recurrence versus 25 cm [6–70 cm] in patients with endoscopic remission; p = 0.019]. On average, patients with EPER required a 7 cm longer resection. With reference to the surgical

Table 2. Assessment of EPER using Rutgeerts score [Ri] 6 months after ICR.

Endoscopic recurrence	Ri score	*n*	%
Negative	Ri 0	39	36.4
	Ri 1	45	42.1
		84	**78.5**
Positive	Ri 2	14	13.1
	Ri 2–3	3	2.8
	Ri 3	4	3.7
	Ri 4	2	1.9
		23	**21.5**

EPER, early postoperative endoscopic recurrence; ICR, ileocaecal resection.

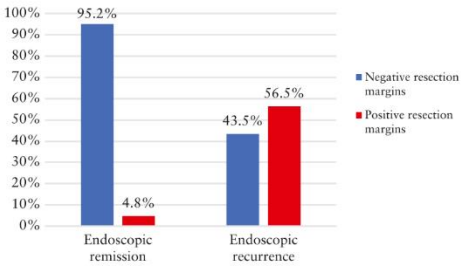

Figure 1. Influence of histologically inflamed resection margins on endoscopic recurrence.

approach, 66 of 107 [61.7%] procedures were open surgeries and 41 [38.3%] were performed laparoscopically. Of 43 surgeries that were initially started laparoscopically, two had to be converted to open approach [the conversion rate was 4.6%]. Postoperative complications showed no statistical influence on EPER [$p = 0.333$] [Table 3].

Furthermore, out of 107 specimens, 36 specimens were positive for granulomas [33.6%]. In the group with positive resection margins [$n = 17$], 10 specimens [58.8%] had granulomas. Moreover, a significant correlation between the presence of granulomas and EPER was found [60.9% versus 26.2%; $p = 0.003$] [Tables 1 and 3]. Regarding preoperative therapy, the use of antibiotics at <12 weeks before the surgery decreased EPER by up to 66% [OR 0.34, 95% CI 0.11–0.99; $p = 0.048$] [Tables 3 and 4].

According to the multivariate analysis results, positive resection margins have the greatest influence on EPER [OR 26.46, 95% CI 6.42–109.06; $p < 0.001$]. A statistically significant effect of disease duration [OR 1.20, 95% CI 1.02–1.41; $p = 0.031$] and stenosing disease behaviour [OR 0.08, 95% CI 0.01–0.87; $p = 0.038$] was also found [Table 5]. The groups were comparable in the other analysed variables [demographic characteristics, other surgical characteristics, and preoperative therapy, with the exception of use of antibiotics and family history of IBD].

4. Discussion

Disease recurrence after surgical resection in CD has been studied since the end of the 20th century.[13] The incidence of postoperative recurrence differs considerably in the literature and ranges from 10% to 90%, depending on the used definition of recurrence [i.e. clinical, endoscopic, radiological, or surgical recurrence], study designs, and postoperative therapy.[2,3,14,15] In our cohort, we have investigated EPER in patients without any postoperative therapy. EPER occurred in 21.5% of patients in our study.

It has been proven that endoscopic recurrence both precedes and predicts clinical relapse.[3,4] Currently, postoperative ileocolonoscopy within the first year following surgery is recommended.[4,5] We performed ileocolonoscopies 6 months after ICR, and the findings were assessed according to Rutgeert's score.

A diagnosis of EPER seems to be crucial for further postoperative management to prevent any flare-up of the disease.[16] According to the current guidelines, the risk factors used as predictors of postoperative recurrence include smoking, previous intestinal surgery, absence of prophylactic treatment, penetrating disease at index surgery, perianal location, granulomas in the resection specimen, and myenteric plexitis.[4] Smoking appears to be the only consistently reported risk factor that increases the risk of postoperative recurrence by 2.5-fold.[4,16,17] In our study, we have also confirmed that smoking is a risk factor [among non-smoking patients, 52.4% had endoscopic remission and 21.7% had endoscopic recurrence; $p = 0.028$]. Nevertheless, a clear identification of patients at a higher risk of EPER, who would benefit from more aggressive and financially demanding therapy, remains a clinical challenge, as no standard for postoperative treatment exists.[1,8–10]

The primary aim of our study was to identify whether thistological inflammation at the resection margins after ICR could be considered a significant risk factor for EPER. In the literature, data concerning this assumption are inconsistent.[4] The relevant studies, mainly published in the late 1990s, reported no relationship between the microscopic involvement of the resection margins and postoperative recurrence.[18–21] However, the frequently cited study of Fazio *et al.*,[19] who conducted a randomised controlled trial of 152 patients, showed that microscopic CD at the resection margins is correlated with surgical recurrence in the long-term follow-up [up to 96 months]. The types of operation in their study population included not only primary ileocolic resection [49%] but also secondary resection of ileocolic anastomosis [46%], as well as other types. The patients did not receive any postoperative immunoprophylaxis.[19] In our cohort, we have specifically assessed endoscopic recurrence after 6 months only in patients who had primary ICR. Consistent with the study of Fazio *et al.*, our patients did not receive any postoperative therapy before ileocolonoscopy. Furthermore, recent studies have confirmed that histological inflammation at the resection margin is associated with a higher risk of postoperative recurrence.[1,6,7] The retrospective study of Bobanga *et al.*, including 142 patients, investigated the different aspects of postoperative recurrence after ileocaecal resection between adolescents and adults; multivariate analysis showed that positive resection margins are predictive of endoscopic recurrence in the adult group [$p = 0.007$].[1] Concerning the histological assessment of the resection margins, we have always evaluated both the ileal and the colonic margins. On the other hand, previous studies have either assessed only the proximal margin or the margins have not been further specified.[1,6,19] Disease duration as a risk factor is not uniformly accepted.[5,8,9] However, our results imply that the longer the disease duration, the higher the risk of EPER. The longer duration could be explained by the more conservative therapeutic approach at our hospital, which would in turn lead to more complicated cases with a higher EPER incidence.

In relation to the length of the resected bowel, current data are also equivocal. Our results showed that the patients with EPER needed, on average, a 7 cm longer resection, which could

Table 3. Influence of the variables on EPER. Results expressed as median [minimum-maximum] or frequency [percent].

Characteristics		Normal endoscopy [*n* = 84]	Endoscopic recurrence [*n* = 23]	*p*-value
Age at diagnosis	[Years]	Median [min – max] 25 [8–75]	28 [8–62]	0.660
Time from diagnosis to ICR	[Years]	Median [min – max] 3 [0–18]	8 [0–14]	**0.006**
Gender	Male	39 [46.4%]	9 [39.1%]	0.638
	Female	45 [53.6%]	14 [60.9%]	
Montreal classification				
A [age]	A1 [<16 years]	10 [11.9%]	3 [13.0%]	0.817
	A2 [17–40 years]	59 [70.2%]	15 [65.2%]	
	A3 [>40 years]	15 [17.9%]	5 [21.7%]	
L [location]	L1 [ileum]	58 [69.0%]	16 [69.6%]	0.649
	L1 + L4 [ileum + upper GI]	1 [1.2%]	1 [4.3%]	
	L3 [ileum + colon]	22 [26.2%]	6 [26.1%]	
	L3 + L4 [ileum + colon + upper GI]	3 [3.6%]	0 [0.0%]	
B [behaviour]	B1 [inflammatory]	10 [11.9%]	3 [13.0%]	**0.045**
	B2 [stenosing]	41 [48.8%]	17 [73,9%]	
	B3 [perforating]	33 [39.3%]	3 [13.0%]	
Perianal disease	No	63 [75.0%]	19 [82.6%]	0.582
	Yes	21 [25.0%]	4 [17.4%]	
Ileocaecal resection				
Surgical approach	Open	53 [63.1%]	13 [56.5%]	0.631
	Laparoscopic	31 [36.9%]	10 [43.5%]	
Length of resected bowel [cm]		25 [6–70]	30 [5–80]	**0.019**
Anastomosis	Side to side	74 [88.1%]	17 [73.9%]	0.106
	End to end	10 [11.9%]	6 [26.1%]	
Postoperative complications	No	55 [65,5%]	16 [69.6%]	0.807
	Yes	29 [34.5%]	7 [30.4%]	
Clavien - Dindo classification of postoperative complications [grade]	1	9 [10.7%]	3 [13.0%]	0.333
	2	4 [4.8%]	3 [13.0%]	
	3a	2 [2.4%]	0 [0.0%]	
	3b	14 [16.7%]	1 [4.3%]	
	4	0 [0.0%]	0 [0.0%]	
	5	0 [0.0%]	0 [0.0%]	
Histological results				
Inflammation at resection margins	Negative	80 [95.2%]	10 [43.5%]	**<0.001**
	Positive	4 [4.8%]	13 [56.5%]	
	Ileal negative, colonic negative	80 [95.2%]	10 [43.5%]	
	Ileal positive, colonic negative	3 [3.6%]	5 [21.7%]	**<0.001**
	Ileal negative, colonic positive	1 [1.2%]	3 [13.0%]	
	Ileal positive, colonic positive	0 [0.0%]	5 [21.7%]	
Presence of granulomas	Negative	62 [73.8%]	9 [39.1%]	**0.003**
	Positive	22 [26.2%]	14 [60.9%]	
Preoperative therapy				
Medication ≤12 weeks before ICR	Antibiotics	38 [45.2%]	5 [21.7%]	0.055
	5-ASA	45 [53.6%]	13 [56.5%]	0.818
	Local GCS	19 [22.6%]	7 [30.4%]	0.425
	Systemic GCS	20 [23.8%]	3 [13.0%]	0.392
	AZA	23 [27.4%]	8 [34.8%]	0.604
	MTX	0 [0.0%]	0 [0.0%]	–
	Biologic treatment	11 [13.1%]	2 [8.7%]	0.730
Smoking	Non-smoker	44 [52.4%]	5 [21.7%]	**0.028**
	Ex-smoker	18 [21.4%]	8 [34.8%]	
	Smoker	22 [26.2%]	10 [43.5%]	
Family history of IBD	No	73 [86.9%]	21 [91.3%]	0.730
	Yes	11 [13.1%]	2 [8.7%]	

EPER, early postoperative endoscopic recurrence; ICR, ileocaecal resection; GI, gastrointestinal tract; 5-ASA, 5-aminosalicylates; GCS, glucocorticoids; AZA, azathioprine; MTX, methotrexate; IBD, inflammatory bowel disease.

be attributed to disease severity. Nonetheless, several studies reported that the length of the resected bowel does not significantly affect EPER.[1,9,22] Moreover, other variables associated with surgery [surgical approach, type of anastomosis, postoperative complications] did not show any significant effect on EPER. The guidelines state that perforating disease is an independent risk factor [level 2

Table 4. Associations between endoscopic recurrence of CD and categorical variables [standard logistic regression models, $n = 107$].

Characteristics		*n*	OR [95% CI]	*p*-value
Inflammation of resection margins	Negative	90	1.00 [-]	–
	Positive	17	**26.00 [7.09–95.33]**	**<0.001**
Age at diagnosis	Increase of 10 years	107	1.09 [0.78–1.54]	0.618
Disease duration	Increase of 1 year	107	**1.14 [1.03–1.26]**	**0.015**
Montreal classification				
A [age]	A1 [<16 years]	13	1.00 [-]	–
	A2 [17–40 years]	74	0.85 [0.21–3.47]	0.818
	A3 [>40 years]	20	1.11 [0.22–5.73]	0.900
B [behaviour]	B1 [inflammatory]	13	1.00 [-]	–
	B2 [stenosing]	58	1.38 [0.34–5.65]	0.653
	B3 [perforating]	36	0.30 [0.05–1.74]	0.181
Perianal disease	No	82	1.00 [-]	–
	Yes	25	0.63 [0.19–2.07]	0.448
Preoperative therapy				
Medication ≤12 weeksbefore ICR	Antibiotics	43	**0.34 [0.11–0.99]**	**0.048**
	5-ASA	58	1.13 [0.45–2.85]	0.801
	Local GCS	26	1.50 [0.54–4.17]	0.441
	Systemic GCS	23	0.48 [0.13–1.79]	0.273
	AZA	31	1.41 [0.53–3.78]	0.489
	Biologic therapy	13	0.63 [0.13–3.08]	0.570

CD, Crohn's disease; ICR, ileocaecal resection; 5-ASA, 5-aminosalicylates; GCS, glucocorticoids; AZA, azathioprine; MTX, methotrexate; OR, odds ratio; CI, confidence interval.

Table 5. Multidimensional logistic regression model [$n = 107$] of the relationship between selected variables and recurrence of Crohn's disease.

Characteristics		*n*	OR [95% CI]	*p*-value
Inflammation at resection margins	Negative	90	1.00 [-]	–
	Positive	17	**26.46 [6.42–109.06]**	**<0.001**
Disease duration	Increase of 1 year	107	**1.20 [1.02–1.41]**	**0.031**
Montreal classification				
A [age]	A1 [<16 years]	13	1.00 [-]	–
	A2 [17–40 years]	74	3.24 [0.31–33.52]	0.323
	A3 [>40 years]	20	5.16 [0.35–76.62]	0.233
B [behaviour]	B1 [inflammatory]	13	1.00 [-]	–
	B2 [stenosing]	58	0.66 [0.12–3.65]	0.629
	B3 [perforating]	36	**0.08 [0.01–0.87]**	**0.038**
Perianal disease	No	82	1.00 [-]	–
	Yes	25	1.15 [0.23–5.69]	0.863
Preoperative therapy				
Medication ≤12 weeks before ICR	Antibiotics	43	0.45 [0.12–1.71]	0.242
	5-ASA	58	1.11 [0.28–4.43]	0.885
	Local GCS	26	1.43 [0.33–6.25]	0.635
	Systemic GCS	23	0.68 [0.13–3.54]	0.652
	AZA	31	1.66 [0.46–5.92]	0.436
	Biologic therapy	13	1.54 [0.22–10.80]	0.664

ICR, ileocaecal resection; 5-ASA, 5-aminosalicylates; GCS, glucocorticoids; AZA, azathioprine; MTX, methotrexate; OR, odds ratio; CI, confidence interval.

evidence].[5] However, conflicting data with regard to the early recurrence of perforating disease exist based on the meta-analysis by Similis *et al.*[23] Similarly, our data showed that the number of patients who developed EPER was fewer among those with a perforating behaviour of the disease than in those with a stenosing disease [$p = 0.045$].

Perianal disease is also a risk factor according to the guidelines;[5] nevertheless, the cited studies in the guidelines included patients who had different types of surgeries [not only ICR], presumably because of a more extensive type of the disease. Moreover, patient follow-up after surgery for perianal disease is much longer [up to years], which could be attributed to a cumulative frequency of perianal disease.[4] This could explain why our data did not confirm perianal disease as a risk factor for EPER [$p = 0.582$] as our cohort included only EPER assessed 6 months after the ICR.

The current guidelines also reported on histological features, such as presence of granulomas and myenteric plexitis, as risk factors for EPER.[5] In our study, we have confirmed that granulomas in the specimens are significantly correlated with EPER [$p = 0.003$]. Myenteric plexitis has been reported as a risk factor only recently in the guidelines [level 3 evidence].[5,24] During the

initiation of our study in 2012, myenteric plexitis was not widely accepted as a risk factor; thus, this histological sign was not evaluated by our pathologist. We have included this as a limitation of our study.

As previously mentioned, preoperative therapy with antibiotics [e.g. metronidazole] administered <12 weeks before the surgery decreases the risk of EPER. However, in a multidimensional analysis, the results were not statistically significant and have to be further verified. In the literature, only the possible protective effect of the postoperative antibiotic therapy was discussed.[25,26]

According to the multivariate analysis, positive resection margins have the greatest influence on EPER. A statistically significant effect of disease duration and stenosing disease behaviour [as discussed previously] was also found. However, with regard to the small number of patients and presentation of characteristics in our cohort, such a multivariate analysis is quite unstable, which could be seen, for example, in the 95% CI for OR of the resection margins.

Our study has some limitations. The group of patients in our cohort was specifically chosen [patients on prophylactic therapy or who had different types of surgery were excluded], which could in turn decrease the generalisability of the study. Moreover, the patients were followed up 6 months after ICR only, and we have not included myenteric plexitis as a risk factor in this study.

In conclusion, in our prospective study of 107 patients, we have shown that microscopic inflammation at the resection margins after ICR is associated with EPER. Although validation of this finding by larger studies is needed, our results may contribute to the identification of patients with a high risk of EPER, who could benefit from a more aggressive and targeted postoperative therapy. Consensus on a clear and definite recommendation remains to be reached, to identify the best practice for these patients.

Funding

This work was supported by the Ministry of Health, Czech Republic – conceptual development of research organisation [FNBr 65269705 - Sup 16/19].

Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

KP: study concept and design, analysis and interpretation of data, writing, literature review; LK: concept and design of the study, review of data and the manuscript, consultant; FM: consultant, concept and design of the study, literature review; ZK: consultant, review of the manuscript; VP: consultant, review of data, concept and design of the study, literature review; JD: consultant, review of the manuscript; VZ: consultant, review of the data and the manuscript; PK: statistical analysis; TP: statistical analysis; PJ: consultant, review of the manuscript; ZP: histological analysis; JV: histological analysis; LM: consultant, literature review. All authors have made scientific contribution to the study design and discussion and have read and approved the final version of the manuscript. Moreover, all authors had full access to all the data in this study and had final responsibility for the decision to submit for publication.

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Annex 10

Jabandziev *et al. Critical Care* 2014, **18**:R1
http://ccforum.com/content/18/1/R1

CRITICAL CARE

RESEARCH Open Access

Multiple gene-to-gene interactions in children with sepsis: a combination of five gene variants predicts outcome of life-threatening sepsis

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Abstract

Introduction: The aim of the study was to identify the dependency structure of genetic variants that can influence the outcome for paediatric patients with sepsis.

Methods: We evaluated the role of single nucleotide polymorphisms for five genes: bactericidal permeability increasing protein (*BPI*; rs5743507), lipopolysaccharide-binding protein (*LBP*; rs2232618), toll-like receptor 4 (*TLR4*; rs4986790), heat shock protein 70 (*HSP 70*; rs2227956), and interleukin 6 (*IL-6*; rs1800795) in 598 children aged 0 to 19 years that were admitted to a paediatric intensive care unit with fever, systemic inflammatory response syndrome, sepsis, severe sepsis, septic shock, or multiple organ dysfunction syndrome. A control group of 529 healthy individuals was included. Multi-way contingency tables were constructed and statistically evaluated using log-linear models. Typical gene combinations were found for both study groups.

Results: Detailed analyses of the five studied gene profiles revealed significant differences in sepsis survival. Stratification into high-risk, intermediate-risk, and low-risk groups of paediatric patients can predict the severity of sepsis.

Conclusions: Analysis of single nucleotide polymorphisms for five genes can be used as a predictor of sepsis outcome in children.

Introduction

Sepsis remains one of the most threatening conditions in intensive care units [1,2]. It is defined as the systemic inflammatory response of the human host that is triggered by an invading pathogen (bacterial, viral, fungal, parasitic or combined). Despite outstanding achievements in research and clinical practice, sepsis remains among the major causes of morbidity and mortality worldwide [3,4]. Hitherto, only limited data on this condition have been available from children. A population-based epidemiologic study estimated that in 2003 a total of about 300,000 infectious disease hospitalizations occurred among infants (less than one year of age) in the United States alone and accounted for 42.8% of all infant hospitalizations [5]. Septic states remain one of the most common causes of neonatal morbidity and mortality, especially in the preterm population [6]. The high incidence, associated costs and mortality rate of patients with sepsis has in recent decades led the critical care scientific community to develop specific strategies aimed at improving the outcome of septic states [7,8]. Nevertheless, sepsis mortality has not decreased dramatically during the past decade [4].

Patients admitted to intensive care units with general conditions that seemingly correspond to the severity of infection may nevertheless present fundamentally different survival rates. We hypothesize that at least part of this variability in the sepsis outcome may be due to variation in genes coding components of the innate immune response. Individual differences in disease manifestation influenced by the genetic predisposition have been recognized by the PIRO concept that stratifies patients with sepsis on the basis of their Predisposing conditions, the nature and extent of the Insult (infection or trauma), the nature and magnitude of the host Response, and the degree of concomitant Organ dysfunction [9]. The P, R

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and O components of the PIRO concept depend largely on the genetic predisposition of the individual patient.

Gene variants (mainly single nucleotide polymorphisms (SNPs)) in genes related to inflammatory and immune system regulations may explain, at least to some extent, the variability of clinical course observed in sepsis and infections. Most previous studies related to SNPs in sepsis were performed on adults [10]. Our group focuses on the paediatric population and previously demonstrated that interleukin 6 (IL-6) and bactericidal permeability increasing protein (BPI) polymorphisms are associated with different outcomes of sepsis [11,12]. We recently have focused on multiparametric analyses of five polymorphisms in five genes that play critical roles in or are associated with inflammatory response, sepsis severity and mortality in order to identify possible predictive mechanisms for sepsis risk stratification. The following genes and their SNPs were investigated: 1) toll-like receptor 4 (*TLR*, OMIM acc. No. 603030) (rs4986790), which is a part of the lipopolysaccharides (LPS) recognition/response unit [13]; 2) lipopolysaccharide-binding protein (*LBP*, OMIM acc. No. 151990) (rs2232618), which is a soluble acute phase protein that binds to LPS of Gram-negative bacteria and facilitates the transfer of bacterial LPS to the specific receptor CD14 [14]; 3) bactericidal permeability increasing protein (*BPI*, OMIM acc. No. 109195) (rs5743507), which displays activity against a wide range of Gram-negative bacteria, reflecting high affinity to lipid A of the LPS regions and potent endotoxin-neutralizing activity [12,15]; and 4) interleukin 6 (*IL-6*, OMIM acc. No. 147620) (rs1800795), which is a key proinflammatory cytokine and plays an important role in the development, pathogenesis and outcome of systemic inflammatory response syndrome, sepsis and septic shock [11]. Plasma levels of IL-6 are elevated in patients with sepsis and high IL-6 concentrations are associated with increased mortality [16,17]. Genetic variation within the regulatory part of the *IL-6* gene may affect the incidence and outcome of sepsis [10,11]. Finally, the role of heat shock protein A1L (*HSP 70*, OMIM acc. No. 140560) (rs2227956), which helps to protect cells from thermal or oxidative stress [18,19], was investigated. While several studies have revealed the importance of genetic polymorphisms in the course and outcome of sepsis [10,20], only a few [21] have demonstrated the influence of combinations of genetic polymorphisms, even though the genetic predisposition to sepsis is polygenic and with many variants in multiple gene loci playing different roles. Only limited data are available from the paediatric sepsis population regarding genetic polymorphism studies and their role in sepsis severity prediction. To our knowledge, no multiple gene SNPs analysis has been performed to demonstrate predictability in paediatric patients with sepsis. This study presents data based on multiparametric analyses of five SNPs of immune-related and inflammation-related genes that are involved in the immune response in sepsis.

Materials and methods

A total of 598 paediatric patients aged 0 to 19 years (325 males, 273 females) were enrolled if they met the following inclusion criteria: 1) admission to the Paediatric Intensive Care Unit (PICU) at University Hospital Brno for at least 24 h; 2) fever (defined as body temperature above 39°C or above 38.5°C measured consecutively at two occasions at least 6 h apart), systemic inflammatory response syndrome, sepsis, severe sepsis, septic shock or multiple organ dysfunction syndrome (MODS) based on generally accepted consensus criteria published and modified for paediatric patients by the American College of Chest Physicians and the Society of Critical Care Medicine [22,23]; 3) signed informed consent by their parents or legal guardians; and 4) successful genotyping of all studied gene variants.

Patients were enrolled from September 2003 to December 2009, their clinical status was monitored on a daily basis, and the outcome of the stay at the PICU was carefully evaluated. Patients hereinafter referred to as non-survivors died as a direct consequence of the septic event.

As a control group, 529 healthy individuals (269 male and 260 female) aged 26 to 67 years were analysed after signing a written informed consent. This group represents a random sample of the population of two districts of the Czech Republic selected according to the World Health Organization protocol (Multinational monitoring of trends and determinants in cardiovascular diseases (the MONICA Project)). This study hereinafter refers to patients generally as the patient group (PG), patients with severe sepsis, septic shock, or MODS as the patient group with severe condition (PGS), and healthy controls as the control group (CG).

This study was approved by the Institutional Review Board of the University Hospital Brno in accordance with the 1964 Declaration of Helsinki.

Genetic analysis

DNA was isolated according to the standard protocol from EDTA blood as previously described [24]. DNA variants of the genes studied (that is, *BPI*, *LBP*, *TLR*, *HSP 70* and *IL-6*) were analysed using PCR and restriction analyses. For more details about the oligonucleotide sequences, restriction enzyme used and detailed PCR conditions, see the authors' previous work [11,12] or contact the corresponding author.

Statistical methods

The statistical theory of log-linear models and logit analysis for the evaluation of multi-way contingency tables was used [25], including the theory of graphical models [26]. The adequate log-linear graphical model was chosen by stepwise

procedure [27] using STATISTICA software manufacturer: (StatSoft Inc., Tulsa, OK, USA) and likelihood ratio statistics G^2. The U statistics based on arcsine transformation [28] and Fisher's exact test with mid-*P*-value [25] were used to compare the equality of two independent binomial populations (frequencies) for small sample sizes. The theory of generalized linear models [25] was used to classify the combinations of risk SNP variants into groups according to the level of risk and to calculate probabilities for the risk groups. The software STATISTICA (version 10.0.1011.0) and MATLAB software manufacturer: (MathWorks Inc., Natick, MA, USA) (version 7.11.0.584) were used for computing.

Statistical analysis

Descriptive statistics were used for basic characterization of both PG and CG. Individual genotypes were distinguished and labelled as follows: major/common homozygote (aa), heterozygote (ab) and minor allele homozygote (bb). Some genotype frequencies in group (bb) were very small, including only three or fewer subjects. For this reason, it was necessary to re-code the gene variants to meet the requirements of the statistical tests and enable reliable evaluation of information from the data sets. Thus, genotypes of all studied genes were coded and labelled according to the following key: common, major homozygotes (aa) were labelled (A) and those remaining (that is, heterozygotes (ab) and minor homozygotes (bb)) were collapsed and labelled (B). Absolute and relative frequencies were determined for the occurrence of variant A of the respective gene in PG, PGS and CG.

Further analyses were based on comparisons of relative frequencies between PG and CG or between PGS and CG. These comparisons were performed not only for individual SNPs of each gene, but also for the combinations of two, three, four and five genes. Initially, two five-way contingency tables (for each PG and CG) were created. Each table was formatted as $2 \times 2 \times 2 \times 2 \times 2$ for the five SNPs studied. These contingency tables were then analysed using log-linear models for five dimensions, the optimal graphical model for both groups was found, and comparisons of groups were made using Fisher's exact test and mid-*P*-value as well as by the test using U statistics for comparing two independent binomial frequencies. Two different statistics were used to compare the adequacy of the chosen tests in cases of small frequencies. When both tests yielded the same results, only results based on Fisher's exact test are reported. An identical approach was then applied for comparisons between PGS and CG.

Based on results of previous comparisons, the theory of generalized linear models was used and the SNP combinations were classified according to the value of the probability that the given SNP combination would appear in PG or CG. The SNP combination risk groups could be determined using this method. If the results of all methods are consistent, typical SNP variants associated with high risk and low risk of sepsis outcome could be identified and described.

Results

Patient clinical characteristics

Most of the patients were admitted to the PICU due to infection (181 patients with respiratory tract infection (30.3%), 60 with urogenital infection (10%), 73 with central nervous system infection (12.2%), 56 with abdominal infection (9.4%) and 49 with other infections (8.2%)). The remaining 179 (29.9%) were admitted due to trauma or some other surgical condition. Overall survival was 575 (96.2%) out of 598 patients enrolled. All patients experiencing only a febrile episode (131 patients) or systemic inflammatory response syndrome (314 patients) survived. As expected, the mortality rate was low in the sepsis subgroup (2.6%, that is, 1 out of 39 patients), higher in the severe sepsis subgroup (5.5%, that is, 4 out of 73 patients), and highest in the septic shock and MODS subgroup (43.9%, that is, 18 out of 41 patients). Detailed characteristics of non-survivors are summarized in Table 1. The presence of an infection either upon admission or that developed during the stay at the PICU was confirmed in 297 (49.7%) of 598 paediatric patients. The cause of infection was Gram-positive bacteria in 123 cases (41.4%), Gram-negative bacteria in 112 cases (37.7%), viruses in 37 cases (12.5%) and fungi or other infectious agents in 25 cases (8.4%).

Single nucleotide polymorphisms

The complete genotyping was successful in 598 patients and in 529 control individuals. No age or gender differences have been demonstrated in the distribution of gene variants in either PG or CG. The distributions of individual genotypes of both polymorphisms are in Hardy-Weinberg equilibrium in both groups. The genotype relative frequencies in PG and CG are shown in Figure 1 and Table 2.

To describe interactions among the five genes studied, the adequate statistical association structures of SNPs in both PG and CG were determined using optimal association graphs. Associations are demonstrated in Figure 2. Both graphs were constructed to reveal the most typical statistical associations among the SNPs studied. The differences between PG and CG are demonstrated at Figure 1, Figure 2 and Table 2. For PG, the association among *BPI*, *TLR* and *LBP* is typical and there is a three-factor interaction. This means that each pair of these three variables may be conditionally dependent and an odds ratio for any pair of these three variables may vary across levels of the third variable. In addition, *IL-6* is conditionally independent from *BPI* and *LBP* provided that the presence of the *TLR* SNP is fixed, whereas the occurrence of the *HSP 70* SNP is independent of the preceding four

Table 1 Clinical and SNP characteristics of non-survivors

Original diagnosis	Cause of death	Causative pathogen	*BPI*	*LBP*	*TLR*	*HSP 70*	*IL-6*	Risk variants of five genes
Crohn's disease	Septic shock	CMV, Candida	A	A	A	B	A	H
Pneumonia	Septic shock	Not identified	A	A	A	B	A	H
Peritonitis	Septic shock	Not identified	A	A	A	A	B	H
Pneumonia	Severe sepsis	G + bacteria	A	B	A	A	B	I
Pneumonia	Septic shock	Not identified	A	A	A	A	B	H
Pneumonia	Septic shock	Not identified	A	B	A	A	A	H
Pneumonia	Septic shock	G- bacteria	A	A	A	A	B	H
Pneumonia	Severe sepsis	G + bacteria	A	B	A	B	B	I
Multiple injury	Septic shock	G + bacteria	A	A	A	A	B	H
Pneumonia	Septic shock	Not identified	A	A	A	B	A	H
Pneumonia	Severe sepsis	G- bacteria	A	A	A	B	A	H
Pneumonia	Septic shock	Not identified	A	A	A	A	A	I
Ileus	Septic shock	G + bacteria	A	A	A	A	B	H
Multiple injury	Sepsis	Not identified	A	A	A	A	B	H
Pneumonia	Severe sepsis	Not identified	B	A	A	B	B	I
Cranial injury	Septic shock	G + bacteria	A	A	A	A	B	H
Pneumonia	Septic shock	Aspergillus	A	B	A	B	B	I
Meningitis	Septic shock	Not identified	B	A	B	A	A	I
Pulm. embolism	Septic shock	Actinomyces	A	A	A	A	B	H
Pneumonia	Septic shock	G + bacteria	A	A	B	A	A	I
Pneumonia	Septic shock	Candida	A	B	B	A	B	I
Ileus	Septic shock	G- bacteria	A	B	A	A	B	I
Gastroenteritis	MODS	G- bacteria	A	A	A	A	B	H

A, common (wild-type) homozygotes (aa); B, heterozygotes (ab) and minor homozygotes (bb); H, high-risk sepsis combination of five single nucleotide polymorphism (SNP) variants; I, intermediate risk sepsis combination of five SNP variants.

genes. In CG, associations are described between *BPI* and *HSP 70*, *HSP 70* and *LBP*, and *LBP* and *IL-6*. The *TLR* SNP is independent from the other four SNPs. The graphs in Figure 2 were used in further searching for high-risk and low-risk variants of SNP combinations.

In view of Figure 2, detailed comparison was made between the frequencies of the following three SNP combinations: 1) *BPI*, *LBP* and *TLR*; 2) *BPI*, TLR and *IL-6*; and 3) *LBP*, *TLR* and *IL-6*. Binomial testing based on U statistics and Fisher's exact test was used. The

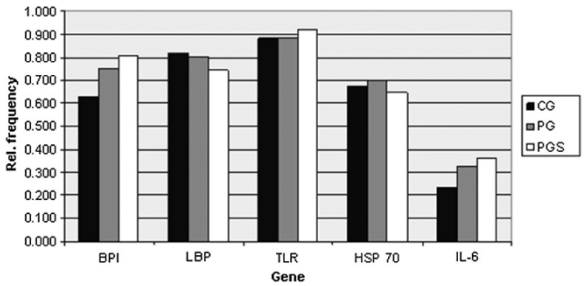

Figure 1 Relative frequencies of wild-type homozygote variants. Relative frequencies of the occurrence of major homozygotes for the given genes in the control group (CG), group of patients (PG) and group of patients with highly serious sepsis - severe sepsis, septic shock and multiple organ dysfunction syndrome (MODS) (patient group with severe condition, PGS).

Table 2 Wild type homozygote frequencies

Gene	CG (n = 529)		PG (n = 598)		PGS (n = 114)	
	abs.	rel.	abs.	rel.	abs.	rel.
BPI	333	0.629	450	0.753	92	0.807
LBP	432	0.817	480	0.803	85	0.746
TLR	466	0.881	531	0.888	105	0.921
HSP 70	355	0.671	420	0.702	74	0.649
IL-6	124	0.234	194	0.324	41	0.360

Absolute (abs.) and relative (rel.) frequencies of the occurrence of major homozygotes for the given genes in the control group (CG), group of patients (PG), and group of patients with highly serious sepsis - severe sepsis, septic shock and multiple organ dysfunction syndrome (MODS) (patient group with severe condition, PGS).

results comparing PG and CG as well as PGS and CG demonstrated significant differences, and the findings of the two statistical tests were in good agreement. Therefore, only the results of Fisher's exact test are reported in Table 2. Table 2 also demonstrates risk prediction in unrelated *BPI* and *HSP 70* SNPs as well as non-associated combination of *BPI* and *HSP 70* and combination of all five examined SNPs. Based on these results, we can clearly identify high, intermediate and low sepsis risk populations in paediatric patients.

Detailed statistical analysis of a unique *BPI*, *LBP* and *TLR* gene triplet detected significant differences in genetic structure between the studied groups. The high-risk combination associated with sepsis development was BPI A + LBP A + TLR A. The proportion of combined major homozygotes was significantly higher in PG compared to CG ($P = 0.005$). The comparison between the PGS and the CG revealed BPI A + LBP B + TLR A as a high-risk combination ($P = 0.034$). On the contrary, a low-risk sepsis development combination was BPI B + LBP A + TLR A for both PG and PGS in comparison with CG ($P < 0.001$; $P = 0.003$, respectively). Similar to the previous triplet, the combination of major homozygosity for *BPI*, *TLR* and *IL-6* SNPs was associated with high risk of sepsis development ($P < 0.001$). Furthermore, this association was even more expressed in PGS ($P = 0.003$). In contrast, the combination of BPI B + TLR A + IL-6 B represents low risk for PG and PGS ($P < 0.001$; $P = 0.001$, respectively).

Analyses of the specific triplet *LBP*, *TLR* and *IL-6* showed LBP A + TLR B + IL-6 A and LBP B + TLR A + IL-6 A combinations ($P = 0.006$; $P = 0.012$, respectively) to be at high risk for sepsis development, but occurrence of the proposed variants was low (4.2 and 4.8% of patients). A low-risk variant for PGS was the LBP A + TLR A + IL-6 B combination ($P = 0.027$), which was the most common variant in this group (41.2% of patients).

A non-associated combination of wild-type homozygote variants of *BPI* and *HSP 70* genes together represented high risk for sepsis development ($P < 0.001$), and this was statistically significant for PGS ($P = 0.004$). The combination BPI B + HSP 70 A for the sepsis group ($P < 0.001$) and for PGS ($P < 0.001$) represented low risk.

This highly complex analysis of the five studied genes' distribution showed significant differences between patients and control groups. A high-risk combination for sepsis development and typical for the patients group was the quintet of wild-type homozygotes ($P = 0.005$), but the most common specific combination in the three studied groups was BPI A + LBP A + TLR A + HSP 70 A + IL-6 B. This combination also represented high risk for sepsis development ($P = 0.016$). A low-risk variant was BPI B + LBP A + TLR A + HSP 70 A + IL-6 B both in patients ($P = 0.006$) and PGS ($P = 0.001$). Frequencies of other low-risk variants BPI B + LBP A + TLR B + HSP 70 A + IL-6 B and BPI B + LBP B + TLR A + HSP 70 A + IL-6 B differ significantly between the groups (for details, see Table 3).

These data are in agreement with results of the other statistical method, the generalized linear model (optimal logit model). These models calculated the probabilities

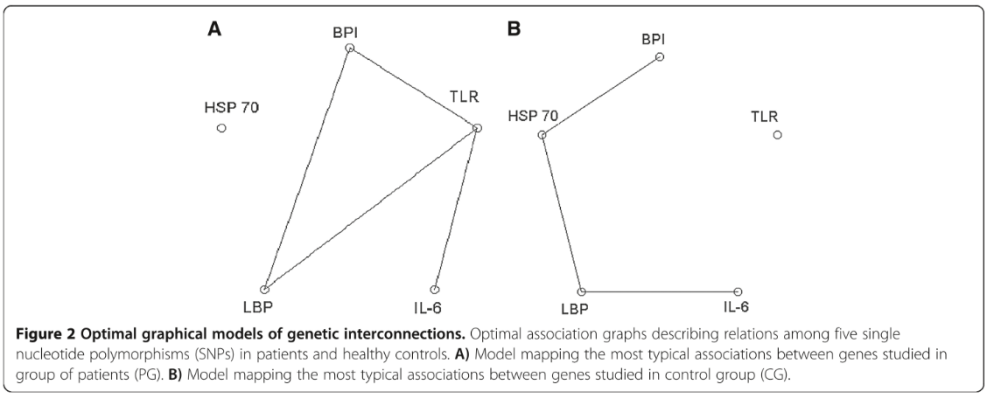

Figure 2 Optimal graphical models of genetic interconnections. Optimal association graphs describing relations among five single nucleotide polymorphisms (SNPs) in patients and healthy controls. **A)** Model mapping the most typical associations between genes studied in group of patients (PG). **B)** Model mapping the most typical associations between genes studied in control group (CG).

that individuals with defined combinations of SNPs belong to CG or PG. The logit model then enables setting 95% confidence intervals for these probabilities. The results of these analyses confirmed that all combinations for high-risk and low-risk cases shown in Table 3 are adequate (see the Additional file 1 for further details).

Finally, in the group of patients with severe condition, a comparison was made between survivors and non-

Table 3 Evaluation of sepsis risk based on five SNPs in paediatric patients

			Control group (n = 529)		Patient group (n = 598)				Patient group with severe condition (n = 114)			
	Genes	**SNP* variant**	**No.**	**(%)**	**No.**	**(%)**	**Fisher Mid-*P***	**Risk**	**No.**	**(%)**	**Fisher Mid-*P***	**Risk**
Associated SNP combinations	*BPI + LBP + TLR*	AAA	237	43.5	319	53.3	0.005	H	62	54.4	0.071	I
		AAB	31	5.9	34	5.7	0.950	I	4	3.5	0.236	I
		ABA	58	11.0	87	14.5	0.068	I	23	20.2	0.034	H
		ABB	7	1.3	10	1.7	0.721	I	3	2.6	0.308	I
		BAA	144	37.8	110	18.4	<0.001	L	17	14.9	0.003	L
		BAB	20	3.8	17	2.8	0.361	I	2	1.8	0.224	I
		BBA	27	5.1	15	2.5	0.022	L	3	2.6	0.198	I
		BBB	5	0.9	6	1.0	0.882	I	0	0	0.188	I
	BPI + TLR + IL-6	AAA	69	13.0	127	21.2	<0.001	H	31	27.2	0.003	H
		AAB	226	42.7	279	46.7	0.177	I	54	47.4	0.378	I
		ABA	7	1.3	21	3.5	0.016	H	1	0.9	0.818	I
		ABB	31	5.9	23	3.8	0.109	I	6	5.3	0.745	I
		BAA	44	8.3	36	6.0	0.148	I	7	6.1	0.398	I
		BAB	127	24.0	89	14.9	<0.001	L	13	11.4	0.001	L
		BBA	4	0.8	10	1.7	0.146	I	2	1.8	0.489	I
		BBB	21	4.0	13	2.8	0.100	I	0	0	0.008	L
	LBP + TLR + IL-6	AAA	102	19.3	134	22.4	0.200	I	32	28.1	0.050	I
		AAB	279	52.7	295	49.3	0.245	I	47	41.2	0.027	L
		ABA	8	1.5	25	4.2	0.006	H	3	2.6	0.602	I
		ABB	43	8.1	26	4.3	0.011	L	3	2.6	0.015	L
		BAA	11	2.1	29	4.8	0.012	H	6	5.3	0.206	I
		BAB	74	14.0	73	12.2	0.401	I	20	17.5	0.345	I
		BBA	3	0.6	6	1.0	0.416	I	0	0	0.278	I
		BBB	9	1.7	10	1.7	0.909	I	3	2.6	0.596	I
Non-associated SNP combination	*BPI + HSP 70*	AA	212	40.1	323	54.0	<0.001	H	63	55.3	0.004	H
		AB	121	22.9	127	21.2	0.494	I	29	25.4	0.585	I
		BA	143	27.0	97	16.2	<0.001	L	11	9.6	<0.001	L
		BB	53	10.0	51	8.5	0.382	I	11	9.6	0.932	I
All SNPs in combination	*BPI + LBP + TLR + HSP 70 + IL-6*	AAAAA	36	6.8	70	11.7	0.005	H	13	11.4	0.157	I
		AAAAB	111	21.0	162	27.1	0.016	H	29	25.4	0.354	I
		AABBB	14	3.6	3	0.5	0.004	L	1	0.9	0.172	I
		ABAAA	6	1.1	22	3.7	0.005	H	5	4.4	0.133	I
		BAAAB	70	13.2	48	8.0	0.006	L	4	3.5	0.001	L
		BABAB	15	2.8	7	1.2	0.041	L	0	0	0.026	L
		BBAAB	20	3.8	8	1.3	0.009	L	2	1.8	0.224	I
		others	257	48.6	278	46.5	0.492	I	60	52.6	0.440	I

Single nucleotide polymorphisms' (SNPs') frequencies and their sepsis risk evaluation for three sets of associated genes, non-associated genes and five gene SNPs. SNP variants indicate the wild-type (most frequent) homozygote with label "A", while heterozygote or minor homozygotes are shown as "B". SNP variants are stated in the same order as genes.

survivors using the Fisher's exact test and mid-*P*-values. Due to limited numbers in each subgroup, the power of the tests used is low and the data demonstrate only trends or statistical significance at the 10% level (Table 4). The one-side and two-side alternatives were considered.

The distribution showed no differences in any patient group when compared to the control group. However, specific combinations indicated a tendency to be over-represented in the non-survivors group, such as *BPI* major homozygotes ($P = 0.077$), combination of TLR B and IL-6 A ($P = 0.055$), combination of LBP A + TLR B + IL-6 A ($P = 0.055$) and BPI A + LBP A + TLR A + HSP 70 A + IL-6 B ($P = 0.055$).

Discussion

The immune response in sepsis is an extremely multifaceted cascade of events involving inflammatory and anti-inflammatory processes, humoral and cellular reactions, and circulatory abnormalities [29]. Several risk factors for sepsis development have been identified [30], but the cause of basic differences in susceptibility between individuals and populations remains unclear.

Host genetic variability in the regulatory and coding regions of genes for components of the innate immune system, inflammatory cytokines and coagulation cascade may influence the susceptibility to and/or outcome from sepsis. SNPs can result in absolute deficiency of a protein, an altered protein, a change in the level of normal protein expression, or no discernible change in protein function or expression, and they are thought to explain at least in part the interindividual differences in susceptibility [30].

In recent years, many investigators have observed potential associations between immune-related gene polymorphisms and the development, course and outcome of septic episodes - and often with apparently conflicting results [10,20]. Differences in study design, ethnicity, as well as gene-to-gene and gene-to-environmental interactions could be limiting factors. The outcome of a septic condition is influenced by multiple host and pathogen factors, including the patient's age, gender and race, as well as the presence of comorbid conditions, the patient's underlying immune status, and the specific pathogen involved [31]. It is clear that only a part of the genetic contribution to sepsis development may be explained by the identified gene polymorphisms.

Differences in environmental exposures and genetic heterogeneity between ethnic groups may have complicated the search for genetic and gene-environmental determinants. The contributions of gene-gene interaction to the risk of diseases have been documented (for example, in the case of breast cancer) [32,33]. Data from populations with sepsis are poor and, moreover, developmental differences that affect the haemodynamic, inflammatory, coagulation and immune responses make it difficult to extrapolate data from adult studies to paediatric populations [31].

This study evaluates combined genetic polymorphisms for their possible association with susceptibility to septic conditions and outcome of all septic episodes. To the best of our knowledge, we have evaluated for the first time in a Central European population of critically ill children the influence of genetic polymorphism interactions of five genes related to immune response. The results demonstrate that the specific combinations of genetic polymorphisms seem to be associated with sepsis development. We believe that we can conclude this, despite the fact that we have used healthy adults as controls. There is no evidence that allelic frequencies in some genes are significantly different in children and in adults.

The study points out the importance of interactions among the *BPI*, *LBP*, *TLR*, *HSP 70* and *IL-6* polymorphisms and also highlights the relevance of the combination of gene polymorphisms to sepsis outcome. Specific

Table 4 Risk of death in patients with severe condition

Genes	SNP variant	Survivors (n = 91)		Non-survivors (n = 23)		*P* (one)	*P* (both)	Risk of death SNP variant
		No.	(%)	No.	(%)			
BPI	A	71	78.0	21	91.3	0.077	0.088	Risk
BPI	B	20	22.0	2	8.7	0.077	0.088	Non-risk
BPI; TLR	BA	19	20.9	1	4.3	0.029	0.029	Non-risk
TLR; IL-6	BA	1	1.1	2	8.7	0.055	0.56	Risk
BPI; LBP; TLR	BAA	16	17.6	1	4.3	0.056	0.062	Non-risk
BPI; TLR; IL-6	BAA	7	7.7	0	0	0.098	0.098	Non-risk
LBP; TLR; IL-6	ABA	1	1.1	2	8.7	0.055	0.56	Risk
BPI; LBP; TLR; HSP 70; IL-6	AAAAB	20	22.0	9	39.1	0.055	0.155	Risk
	AAABB	8	8.8	0	0	0.077	0.077	Non-risk

All single nucleotide polymorphism (SNP) combinations revealing at least 10% level of statistical significance between survivors and non-survivors of severe sepsis, septic shock or multiple organ dysfunction syndromes (MODS) (based on two-sided Fisher's exact test) are shown. Other SNP combinations did not demonstrate statistically significant differences and are not presented.

combinations of common homozygosity for *BPI*, *LBP*, *TLR*, *HSP 70* and *IL-6* variants were typical within the septic group and were associated with a high risk of sepsis development. Generally, in the group of children with sepsis studied, individuals carrying wild-type alleles of the proposed genes in various combinations had increased risk for sepsis development compared to those with the minor alleles. Similar to our findings, a study by Flores *et al.* [34] had concluded that a common SNP risk haplotype of *LBP* was strongly associated with susceptibility to severe sepsis and homozygous carriers of the risk haplotype had increased risk for severe sepsis.

Benermo *et al.* revealed that the G allele of 174 G>C SNP in the promoter region of the *IL-6* gene is functional *in vivo* with increased inflammatory response [35]. This result could be consistent with the fact that early increased and uncontrolled release of cytokines (known as a cytokine storm) and proinflammatory mediators are peculiar for sepsis development and associated with increased mortality [16,17].

Our data indicate that specific combinations of gene polymorphisms - most frequently wild-type homozygote variants - were significantly associated with sepsis development in a large cohort of paediatric patients. In addition, we revealed significant associations between genetic structure in patients with severe septic conditions (severe sepsis, septic shock and MODS). Moreover, low-risk variants, typical for the control group and representing low risk for sepsis development were also described. Our hypothesis arising from our previous genetic observations states that mutated variants of gene polymorphisms seem to be protective against sepsis development. The exact protective mechanism is unknown due to an incomplete understanding of the complex pathophysiologic nature of sepsis development. Compared to non-carriers, however, volunteers with the mutated *TLR 299* allele have been shown to have lower concentrations of some of the inflammatory cytokines, acute-phase reactants and other mediators of inflammation relatively late after the onset of experimental endotoxemia [36].

Conclusions

Future genome-wide expression profiling studies will allow researchers to reveal specific interactions between polymorphisms in genes involved in innate immunity and to stratify patients according to their risk for certain outcomes. The first reported genome-wide comparison of expression patterns among healthy children and children with septic shock revealed more than 2,000 genes that were differentially expressed or repressed in patients experiencing septic shock relative to healthy controls [37]. It is only a question of time before a reasonable number of risky combinations for sepsis development and for poor outcomes can be determined [30]. High-risk patients could benefit from being so identified through early introduction of specific preventive or therapeutic interventions.

These data demonstrate that such an approach can clearly identify SNP variants that are associated with favourable and unfavourable sepsis outcomes.

Key messages

- Five single nucleotide polymorphisms of genes involved in inflammation can stratify paediatric patients for risk of sepsis survival.
- Stratification into high-, intermediate- and low-risk groups of paediatric patients can predict the severity of sepsis.

Additional file

Additional file 1: Table S1. Results based on the optimal graph model.

Abbreviations

BPI: Bactericidal permeability increasing protein; CG: Control group; HSP 70: Heat shock protein A1L; IL-6: Interleukin 6; LBP: Lipopolysaccharide-binding protein; LPS: Lipopolysaccharide; MODS: Multiple organ dysfunction syndrome; PG: Patient group; PGS: Patient group with severe condition - severe sepsis septic shock or multiple organ dysfunction syndrome; PICU: Paediatric intensive care unit; SNP: Single nucleotide polymorphisms; TLR: Toll-like receptor 4.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PJ collected data, managed the study database and composed the manuscript. MS and JM Sr performed the statistical analyses and helped with designing the study. MF interpreted the clinical characteristics of patients. LK collected data and interpreted the clinical characteristics of patients. JAH carried out the molecular genetic studies, analyzed the data, interpreted the results and proofread the manuscript. JM Jr designed and supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgement

This work was supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic NR 9894-4 and the Ministry of Education, Youth and Sports of the Czech Republic, the National Research Programme II 2B08066, the MoH (the Ministry of Health of the Czech Republic) Institutional project for the Development of Research Organization 00023001 (IKEM, Prague, Czech Republic), and by the FEM (Faculty of Economics and Management) Institutional support Development Project Economic Laboratory and Development of Methods for Solving Unstructured Decision Making Problems at the MoD (the Ministry of Defence of the Czech Republic).

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Received: 8 July 2013 Accepted: 31 October 2013
Published: 2 January 2014

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doi:10.1186/cc13174
Cite this article as: Jabandziev *et al.*: Multiple gene-to-gene interactions in children with sepsis: a combination of five gene variants predicts outcome of life-threatening sepsis. *Critical Care* 2014 **18**:R1.