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TITLE

***"New insights in paediatric Inflammatory Bowel Diseases: from disease pathogenesis to clinical and therapeutic management"***


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# CHAPTER 1

# BACKGROUND AND AIMS OF THE PROJECTS

## 1.1 Background: paediatric Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD), including Crohn's disease (CD), Ulcerative colitis (UC) and IBD-unclassified (IBD-U) is a group of life-long chronic and relapsing inflammatory disorders of the gastrointestinal tract whose etiology has not been completely understood [1,2]. The most recent evidences involve a complex interaction between host genetic, environmental and microbial influences, resulting in a dysregulated mucosal immune response against the commensal intestinal microbiota [3,4]. The actual classification still distinguishes CD, characterized by the transmural, patchy inflammation with the potential involvement of all the gastrointestinal (GI) tract, from UC, mainly identified by the presence of continuous, superficial, mucosal inflammation, exclusively limited to the colon [5]. IBD-U describes all those patients with colonic disease but who otherwise have features that are not specific for CD or UC [5]. Nevertheless, it is now more evident that IBD encompasses a wide range of phenotypes with different responses to therapy and unpredictable natural history [6]. This concept is particularly pertinent to paediatric IBD, which has several specific considerations when compared with adult IBD [7]. These certainly include the relevant issues related to growth, development, pubertal maturation, bone health, and psychological impact on the patient and family, but also the unique features related to paediatric IBD phenotype, which is well-renowned to represent both a diagnostic and therapeutic challenge, being characterized by higher severity, including poor response to medical treatment, presence of extra-intestinal manifestations (EIM), and increased risk of surgery [8, 9].

### 1.2 Epidemiology: the burden of paediatric inflammatory bowel disease

Although the incidence and prevalence of IBD differ among countries, the general trend clearly highlights an overall increase over the past few decades in western countries, especially in adolescence and young adulthood [10,11]. Indeed, approximately 25% of IBD has an onset in childhood with an incidence between 8 and 11 cases/100,000 inhabitants/year and it is thought to rise more [12,13]. In a recent retrospective study conducted in Finland, during a 28-year observation period, the nationwide mean annual incidence of PIBD tripled from 7/100000 in 1987-1990 to 23/100000 in 2011-2014 [14]. Considering that the current prevalence in USA has been estimated on about 100 to 200 per 100 000 children, it is likely that most paediatricians will treat children with IBD in their practice [13, 15]. Moreover, although the major increase has been found within the age group of 10 to 12 years, it is also widely reported a higher number of cases in the sub-cohort of Very Early Onset IBD (VEO-IBD), which includes children diagnosed before 6 years of age [16]. These highly challenging cases, accounting for about 6 to 15% of all paediatric IBD, are characterized by a more aggressive phenotype and are thought to have a major genetic background [17,18].

This radical change in the epidemiology of IBD, occurring during the last decades, has certainly exerted a profound impact on healthcare systems. Indeed, a considerable proportion of IBD children will experience recurrent hospitalizations, aggressive medical treatments and complications leading to surgery, with a significant decrease of quality of life and burden of healthcare costs [19]. Already in 2008 Kappelman and colleagues assessed the direct costs of both adult and paediatric IBD [20]. The authors quantified that the mean annual costs for patients were $8265 for CD and $5066 for UC. The total annual expenditure was estimated at $3.1 billion for CD and $2.1 billion for UC [20]. The costs were significantly higher for children younger than 20 years when compared with

adults [20]. As more recently reported the widespread use of biologics in the last decade should have further increased those estimates [21].

### 1.3 Peculiarities in clinical presentation and diagnosis

Paediatric IBD is characterized by a wide spectrum of clinical presentations, ranging from striking symptoms to more elusive signs [22, 23]. Besides the classical manifestations, which may include rectal bleeding, tenesmus, diarrhoea, abdominal pain, weight loss, fever, paediatricians should be aware of the importance of extra-intestinal symptoms, which may be subtle and even cause substantial diagnostic delays [24, 25]. Among the extra-intestinal symptoms, growth deficiency certainly represents the most important and undervalued IBD sign. Indeed, a slowed height velocity may be the only manifestation for months, before the occurring of GI symptoms, particularly in children with CD [26]. Growth retardation has been reported up to 65% of CD children at diagnosis [27, 28]. It results from the combination of poor caloric intake, malabsorption and the direct effects of inflammation, causing growth hormone resistance [29]. It may be associated with the presence of significant pubertal delay, which itself represents a serious concern both for the psychological sequelae and for the follow-up of the patient [30]. In some cases, CD may manifest from the onset as a surgical emergency. This is the case of complicated forms, such as stricturing and fistulising, which may be responsible of mechanic obstruction and even perforation [31]. All otherwise previously healthy children presenting with an acute intestinal surgical emergency, with an evidence of growth deficiency or other GI complaints may raise the IBD suspicion.

In a greater percentage than adults, IBD children may present with the involvement of other organs, at the onset, before diagnosis or during the course of disease [32, 33]. EIM are usually more commonly associated with CD and the most frequently involved organs are joints, skin, liver, pancreas and eyes, with peripheral arthritis being the most prevalent

[32]. Among the others, pyoderma gangrenosum may be usually found during CD course, while sclerosing cholangitis is more often associated with UC [34, 35]. In a recent multicentre study conducted by the Swiss IBD cohort study group, EIM were detected before IBD diagnosis in 30% of the cases [36]. Within the most relevant findings, the authors confirmed that the prevalence resulted higher in paediatric than adult IBD patients and that IBD children with EIM were more likely to be treated with anti-TNF compared to those without, underlining the higher severity of the phenotype [36].

Once the IBD suspicion has been raised, the diagnosis needs to be made with specific laboratory, endoscopic, histologic and radiological criteria, which have been recently revised by the ESPGHAN Porto group [37]. A baseline extensive laboratory evaluation, including complete blood cell count with differential, systemic inflammatory indexes, such as ESR and CRP, electrolytes and organ function tests is still recommended to orientate the diagnosis and screen the subjects needing endoscopic evaluation [37]. As it will be better analysed in the chapter 4, stool culture is considered mandatory before referring the children for endoscopy in order to rule out the differential diagnosis with the main GI pathogens [37]. Finally, testing for faecal calprotectin is strongly recommended due to its high negative predictive value. Nevertheless, paediatricians should be aware of the potential false positives that are known to be higher in children than in adults, particularly in the age group<5 years [38]. The laboratory analyses need to be necessarily more extensive in the suspicion of an infantile IBD, defined as an age of onset<2 years. Indeed, in those specific cases the paediatric gastroenterologist has to rule out the differential diagnosis with a high number of primary immunodeficiencies [39].

Endoscopy remains the corner stone in the diagnosis of paediatric IBD. The ESPGHAN revised Porto criteria once more stressed the importance of performing both Upper GI endoscopy (UGIE) and ileo-colonoscopy at diagnosis, to increase the diagnostic

yield and allow a better discrimination between CD and UC [37]. The specific paediatric classification agreed in Paris in 2009 [40] and the revised Porto criteria [37] have further redefined the endoscopic pictures of paediatric IBD, well underlining the main differences with adult IBD. Nevertheless, there is still the need of specific paediatric endoscopic and histologic scores, both for the diagnosis and the monitoring of disease. In addition to the endoscopic procedures, the studies of the small bowel through the use of bowel ultrasound, magnetic resonance and, in specific cases, capsule endoscopy, have been continuously gaining importance during the last 10 years and are nowadays considered as indispensable in the first assessment of IBD in order to reduce the number of IBD-U [37].

### 1.4 Therapeutic management: current and future perspectives

The main goal of IBD therapeutic approach is to achieve and keep a long-term disease remission. However, the concept of disease remission and more generally the goals of IBD therapy have dramatically changed during the last two decades and are still in continuous evolution [41, 42]. The introduction in 1998 of therapeutic monoclonal antibodies directed against TNF$\alpha$ (Infliximab, and successively Adalimumab), a major pro-inflammatory pathogenic cytokine in CD and UC, has revolutionized the treatment of IBD, giving the possibility to switch from the non-specific conventional immunomodulatory drugs toward a pathway-based anti-inflammatory approach [43-45]. Following the same route, new biologics just came out and other will be soon available also for children, broadening the therapeutic possibilities [46].

Due to the advent of such potent medications, the paediatric gastroenterologist, who has started treating his children only aiming for symptoms control, is now able to enlarge his horizon with several, pursuable goals. The following should be nowadays

considered the main aims when deciding which therapeutic strategy starting in a child affected by IBD:

- Induce and maintain clinical remission;
- Induce mucosal healing;
- Favour linear growth, weight gain and pubertal development;
- Prevent disease complications;
- Restore quality of life;
- Minimize toxicity.

In order to reach all the above-mentioned objectives, therapeutic strategies are evolving to look for the best approach able to change the natural history of disease [47, 48]. This process is particularly crucial in the paediatric IBD population, since as already underlined, young age at onset of disease is considered at high risk for poor prognosis, reinforcing the need for a highly effective treatment approach. The initial management of most children with IBD includes the so-called conventional therapies, namely, corticosteroids (CCS), 5-aminosalicylates (5-ASA), immunomodulators, such as Azathioprine (AZA) and Methotrexate (MTX), and nutritional therapy [49, 50]. The conventional approach, also known as “step-up” strategy, is based on the gradual escalation of drugs, from those with a better safety profile but a lower efficacy (5-ASA) to those with higher efficacy but an increased risk of adverse effects (including immunomodulatory and biologic agents). The main advantage of “step-up” approach relies on reserving drugs with higher levels of toxicity only for those patients needing more intensive therapy [51, 52]. To date it has been the most used strategy in paediatrics, mainly due to its safety profile. However, the “step-up” approach is not able to alter to natural history of disease, reducing the disease

complications development and the need for surgery. Therefore, the scientific community is gradually moving toward an early aggressive approach, the so-named “top down strategy” with the aim of modifying the natural history of the disease [53-55]. The “top-down” approach is basically characterized by the early use of biologics in those patients with a higher risk of poor disease outcomes [56]. This strategy comes from the evidences that biologics are able to induce mucosal healing (MH) in a significantly higher percentage of IBD patients when compared with conventional therapies [57-58]. Although the definition is not well standardized, MH mainly refers to the healing in a various degree of the endoscopic lesions [59]. The importance of achieving MH in clinical practice is related to the recent growing data that MH contributes to lower rates of clinical relapse, hospitalization and need for surgery, with a better health-related quality of life for patients [60-62]. Next to the MH, another emerging concept has been translated from rheumatology in the last years: the idea of reaching a “deep remission” [63-64]. With deep remission, the rheumatologists mean the alteration of the biological processes underlying synovial inflammation and progressive structural destruction, thereby preventing structural joint damage and functional decline [65]. Translated to the IBD, deep remission would be the achievement of a profound remission with the absence of clinical symptoms, no biological activity, complete MH, and at most disappearance of histological signs of inflammation [66-68]. Therefore, the raising literature assessing the importance of MH and deep remission on disease outcomes is strongly pushing the adoption of “top-down strategies”, also in IBD children. This process has also been facilitated by the widespread of biologics’ drug monitoring, the so-called “trough levels”, which has helped optimize medication dosing for individual patient, increase the rate of remission, and decrease the incidence of antibody formation resulting in loss of response [69-71]. Nevertheless, the scientific debate is still opened, due to the absence of well-defined risk stratification criteria from which a clinician can decide with a high degree of confidence, which patients will benefit from early anti-

TNFα treatment [72, 73]. Indeed, the identification of patients at higher risk of poor disease outcomes represents the new paradigm of IBD research [74].

The last updated ESPGHAN guidelines on therapeutic management of paediatric CD tried to make the first steps in the risk stratification of CD children, identifying the following negative predictive factors: deep colonic ulcerations; persistent severe disease despite adequate induction therapy; pan-enteric disease; marked growth retardation; severe osteoporosis; penetrating and stricturing phenotypes; perianal disease [49]. A proper top-down strategy with anti-TNFαs has been strongly recommended only in case of active perianal disease in combination with appropriate surgical intervention and on an individualized basis in patients with severe growth retardation or other of the above-mentioned negative prognostic factors [49]. Besides these cases, biologics are recommended only in children refractory to conventional therapy. Hence, in most of the other children, it is still suggested a step-up approach, which we will briefly review.

One of the main changes respect to the past guidelines was to recognize as first-line induction therapy, the exclusive enteral nutrition (EEN), in which 100% of total calories are delivered by commercial formulas and whole table foods are excluded from the diet for 6-8 weeks [49]. Indeed, EEN, besides showing the same efficacy of CCS in inducing clinical remission, is able to achieve MH, has a better impact on nutritional issues and has no side effects [75-77]. CCS should be used only in those children not compliant or not responding to EEN [49]. For the maintenance of remission, the conventional immunosuppressants, including AZA or MTX, are still considered as valid options, due to their demonstrated efficacy [49]. Only in patients with mild colonic inflammation, 5-ASA may be used [49]. Although strongly limited by the advent of biologics, surgical intervention is still necessary in limited cases, such as active involvement of short segments, and the treatment of complications (fistulising or stricturing phenotypes) [78].

With regards to UC, based on the most recent guidelines, which are currently being updated, the therapeutic management mainly differs from CD for the absence of a nutritional therapy [50]. Indeed, the only available induction therapy is represented by CCS, which are still recommended in all moderate to severe UC children [50]. Due to the more superficial inflammation, 5-ASA are still considered very good options in many UC children and they are recommended as induction therapy in mild cases and as first maintenance therapy in mild to moderate patients. AZA is still the most used medication in UC in Europe and it is recommended at diagnosis in severe cases or otherwise in case of early relapse or intolerance to 5-ASA [50]. MTX may be used as a second-line immunosuppressant, although there is no evidence of efficacy reported in literature [50]. Anti-TNFαs have been demonstrated their efficacy also in children with UC and are recommended in all those patients refractory to conventional therapy [50]. Differently from CD, surgery is potentially curative in UC and keeps its role in those children refractory to medical treatment. The indicated intervention is the sub-total colectomy with the subsequent ileo-anal-pouch collection. This issue will be better addressed in Chapter 6.

Nevertheless, both CD and UC therapeutic algorithms will soon need to be updated due to the advent of new biologics. Particularly, two new molecules including, Vedolizumab, a humanized anti-a4b7 integrin, IgG1 monoclonal antibody, and Ustekinumab, fully human anti-p40 IgG1, have already being used off-label in IBD children refractory to anti- TNFαs, with moderate efficacy [79-82].

## 1.5 Aims

Given these premises, the aims of this thesis project are:

- To provide new insights in paediatric IBD pathogenesis, including the influence of environmental factors;
- To characterize the phenotype and the disease course of IBD in paediatric age;
- To improve the knowledge on the relationship between Gastrointestinal Infections, including *Clostridium difficile (C. difficile)* and *Yersinia enterocolitica*, and paediatric IBD, both for differential diagnosis and relapse management;
- To describe the prevalence, the natural history and the management of extra-intestinal manifestations, including IBD related anaemia and pancreatic involvement;
- To improve the current therapeutic strategies for the management of paediatric IBD.

# CHAPTER 2

# NEW INSIGHTS IN PAEDIATRIC IBD PATHOGENESIS

In the last decade, significant progress has been made in unravelling the pathogenesis of IBD. It is now quite clear that 3 major players have a role in the process of IBD development: the genetics, the intestinal microbiome and the environment [83]. During this chapter, we will review this topic, and particularly the impact of environmental factors, reporting the results of the paper "***Impact of Environmental and Familial Factors in a cohort of pediatric patients with Inflammatory Bowel Disease"*** recently published in the *Journal of Pediatric Gastroenterology and Nutrition (J Pediatr Gastroenterol Nutr. 2017; 64:569-574).*

## 2.1 The impact of environmental factors

Due to the early onset age, genetics has always been thought to play an important role in the paediatric IBD development and in some cases of VEO-IBD is certainly the preeminent factor [16, 39]. Technological advances in DNA analysis and sequencing, together with the use of huge multinational databases, allowed the achievement of numerous genome-wide association studies (GWAS) both in adults and children and the identification of more than 200 IBD risk-associated loci [84-86]. Many of them are strictly linked with immune-mediated pathways, including autophagy, cell-mediated immune responses and innate immune responses. These findings led to the hypothesis that the dysregulation of at least some of these mechanisms may interplay with intestinal microbiome, inducing and perpetuating the intestinal inflammation [4]. The importance of microbiome has been renowned since the publication in the early 90s of studies demonstrating that in animal models of colitis the absence of intestinal bacteria did not allow the development of colonic inflammation [87]. More recently, the development of next

generation sequencing technologies has deeply elucidated microbiome alterations in IBD patients. A variety of associated changes in microbiota community composition have been revealed, including: reduced diversity, decreases in the relative abundance of certain groups, such as Firmicutes and Bacteroidetes; increases in the contribution of Proteobacteria and Actinobacteria and reductions in specific species, such as Faecalibacterium prausnitzii [87, 88]. More recently, a large multicentre North-American cohort confirmed that microbiome alterations may be found already at diagnosis in CD naive-treatment children [89].

However, both the advances of genetic and microbial evidences, instead of reducing the role of environmental factors, have paradoxically reinforced their importance [90]. This is mainly due to a series of clues. First, genetics is only able to explain approximately 25% of the heritability of IBD, as confirmed by the incomplete penetrance of monozygotic twins [84]. As a matter of fact, all the identified polymorphisms, individually, only give a modest increase of the IBD risk [84]. The second fact that favours the environment influence is certainly represented by the remarkable and fast changes in IBD epidemiology. As well pointed out in Chapter 1, over the last 50 years a striking increase of the incidence has been observed both in adults and children, and it is still in progress [91]. These findings certainly cannot be explained by the genetics, considering that changes in genetic predisposition factors usually needs centuries. Finally, the geographic distribution of disease, mostly concentrated among the developed countries, has always blamed westernized lifestyle, and above all the diet [92]. This has been further confirmed by the migration studies, which demonstrated that people moving from low IBD incidence areas to high IBD incidence regions tend to acquire the risk of the host country [93, 94]. Taken together all these observations strongly suggest that environmental factors are playing a critical role in the development of these disorders and in the modulation of disease phenotypes over time.

Many researchers are now referring to the environmental factors as to the "exposome", a term that encompasses the composite of accumulated environmental exposures, starting in utero and continuing through childhood and into adulthood [95]. This aggregate "exposome" is probably able to affect the likelihood of developing IBD, interacting trough microbial and epigenetics modifications [95]. Thus, there is a growing interest in identifying the different factors within the exposome, not only to better understand the process behind IBD development, but most of all to generate dietary, lifestyle, and pharmacologic preventive interventions that will enable us to reduce the incidence and to improve the clinical course of our patients. So far, only tobacco smoking and appendectomy have been demonstrated to be strongly associated with IBD incidence [96-98]. In addition, many papers supported the so called "hygiene hypothesis", which correlates the epidemiological rise in IBD incidence over the 20th century, with the improvement in general hygienic conditions (i.e. free access to clean water, smaller family size, higher use of antibiotics, etc.) [99-100]. Many evidences suggest that dietary factors are crucial in IBD pathogenesis [101, 102]. To date we know that exposure to diets rich in the saturated fatty acids found in red meat appears to place individuals at increased risk, while an increased dietary fiber intake appears to be inversely related to the risk of developing IBD [103-105]. Moreover, some evidences in mice suggests that dietary emulsifiers, the common food additives used to facilitate the dispersion of oil into water, promote the generation of a pro-inflammatory microbiome signature and predispose to intestinal inflammation [106, 107].

Given these premises and considering this as a "hot topic" in IBD research field, we decided to perform a retrospective analysis of our paediatric IBD population with the purpose to investigate the relation between the exposure to some of the main environmental factors and the risk of developing UC and CD. In addition, we evaluated the adherence to Mediterranean diet through the administration of a validated

questionnaire, the KIDMED [108]. We hypothesized, indeed, that the Mediterranean diet, characterized by elevated intakes of fruits and vegetables, olive oil, bread, and cereals may exert a protective role in the development of IBD. We compared 264 IBD children with 203 healthy controls recruited in public schools and in the outpatients' clinic. Children and parents answered a multi-items questionnaire covering a wide range of possible environmental factors associated with IBD development from the birth to the date of diagnosis.

The results of the study confirmed the influence of environmental factors as possible explanation for the significant increase of IBD incidence. Indeed, we identified numerous risk as well as protective factors. Above all, the striking difference in Mediterranean diet adherence, between cases and controls, has to be considered the most innovative finding of our study. Indeed, the Mediterranean diet is perhaps considered one of the healthiest dietary models currently existing. Numerous epidemiological and experimental nutrition studies have demonstrated how Mediterranean countries benefit from lower rates of chronic disease morbidity and higher life expectancy [109, 110]. To the best of our knowledge this is the first paediatric study reporting the hypothesis that Mediterranean diet may have a protective effect for IBD development. If our data will be confirmed by further studies, we may have found a new possible dietary strategy for prevention and eventually treatment of our patients.

Among the other findings we confirmed many of the factors related to the Hygiene Hypothesis. Indeed, we demonstrated that owning pets, having a reduced number of toilets at home and a higher number of siblings were associated with a lower risk for both CD and UC. Family size can be used to indicate the level of overcrowding in a home, which has been associated with potential exposure to infection [111]. The same concept is related to the number of siblings. Indeed, siblings may influence the development of IBD altering exposure patterns to microorganisms in early life, affecting acute manifestation of

infections, or influencing age of transmission and severity [112]. As a further demonstration, the control group showed a higher number of familial H. Pylori infection and parasitosis. Looking at the risk factors, as previously described, we reported a higher exposure to the antibiotics in CD children. The use of antibiotics may alter the composition of the gut microbiome with the loss of potentially beneficial bacteria and the emergence of potentially pathogenic bacteria [113, 114]. Alternatively, the loss of immune interaction with potentially pathogenic bacteria at an early age fails to prime the immune system to harmful organisms that it may encounter later in life [113, 114].

We concluded that environmental factors exert a fundamental role in IBD development. Future studies will need to address the molecular mechanisms underlying the interactions between exposome, genetics and microbiome in order to increase our comprehension of pathogenetic mechanisms and design new possible preventive and therapeutic interventions.

# Impact of Environmental and Familial Factors in a Cohort of Pediatric Patients With Inflammatory Bowel Disease

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**ABSTRACT**

**Objectives:** The primary role of environment on inflammatory bowel disease (IBD) onset has been recently stressed. We aimed to investigate the effect of environmental factors in an IBD pediatric cohort.

**Methods:** A total of 467 subjects (264 IBD and 203 controls) were enrolled. All patients underwent a questionnaire including 5 different groups of environmental risk factors: family history of IBD and autoimmune diseases, perinatal period, home amenities and domestic hygiene, childhood diseases and vaccinations, and diet.

**Results:** In a multivariate model, mother's degree (odds ratio [OR]: 5.5; 2.5–11.6), duration of breast feeding >3rd month (OR: 4.3; 1.6–10.5), father's employment (OR: 3.7; 1.2–8.7), gluten introduction <6th month (OR: 2.8; 1.5–5), number of siblings <2 (OR: 2.8; 1.5–5.3), and family history of autoimmune diseases (OR: 2.7; 1.4–5.3) were significant risk factors for Crohn disease. Low adherence to Mediterranean diet (OR: 2.3; 1.2–4.5), gluten introduction <6th month (OR: 2.8; 1.6–4.9), and number of siblings <2 (OR: 2; 1.1–3.6) were significant risk factors for ulcerative colitis. Owning pets (OR: 0.3; 0.1–0.7) and bed sharing (OR: 0.2; 0.1–0.6) were protective factors for Crohn disease, whereas owning pets (OR: 0.4; 0.2–0.8) and family parasitosis (OR: 0.07; 0.01–0.4) were protective factors for ulcerative colitis.

**Conclusions:** Our study confirms that environmental factors are closely linked to IBD onset and may partly explain IBD rise in developed countries.

**Key Words:** environmental risk factors, hygiene hypothesis, inflammatory bowel disease, pediatrics

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**What Is Known**

- The increased incidence of inflammatory bowel disease over the last decades and the rise of diagnosis in pediatric age underline the importance of environmental contribution on inflammatory bowel disease onset.
- Family history, smoke, and appendectomy have been demonstrated to be strongly associated to inflammatory bowel disease incidence.

**What Is New**

- This is the first pediatric study describing the protective role of adherence to Mediterranean diet on inflammatory bowel disease development.
- Our study confirms that environmental factors are closely linked to inflammatory bowel disease onset and can explain the rise of inflammatory bowel disease in developed countries.

Inflammatory bowel diseases (IBDs) represent a group of inflammatory conditions involving the gastrointestinal tract, whose etiology remains still unknown. Ulcerative colitis (UC) and Crohn disease (CD) are the most common forms of IBD, both associated with high morbidity (1,2). In the last years, epidemiological and molecular studies stressed the importance of genetic susceptibility in causing IBD onset (3,4). Important features such as the significant geographical variation in disease incidence, the incomplete penetrance among monozygotic twins, and the striking rise of IBD over the course of the past century, however, cannot be explained with genetic predisposition alone and reinforce the environmental hypothesis for IBD etiology (5–7). So far, in addition to familial aggregation, which is the most important IBD risk factor, only tobacco smoking and appendectomy have been demonstrated to be strongly associated with IBD incidence (8–10). An emerging and interesting theory supporting the environmental influence on IBD onset, is the ''hygiene hypothesis,'' which correlates with the epidemiological rise in IBD incidence during the 20th century, both in developed and developing countries, with the improvement in general hygienic conditions (ie, free access to clean water, smaller family size, etc) (11–13). The hygiene hypothesis alone is not able to explain the increase in IBD incidence. Indeed, more recent theories emphasize the role of epigenetics, which alter cellular features through specific DNA methylation patterns (14). ''Exposomal imprinting'' means a direct or indirect influence on epigenetic patterns in specific cell types and may explain the gap in IBD heritability after genome-wide

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association studies. In contrast with genetic susceptibility that remains constant during lifetime, epigenetic changes may occur permanently, induced by microbiota components or directly by environmental triggers (14). As a matter of fact, DNA methylation changes have already been identified in IBD (15).

Moreover, several epidemiological studies suggested a role of perinatal or early life events in the etiology of IBD (5,7) such as nonspecific (gastroenteritis and other nonspecific infections) and specific (vaccines, passive smoking) exposures. A possible explanation is that a decreased prevalence of infections during childhood could lead to a major individual susceptibility in developing IBD later in life. There are, however, no definitive data demonstrating a final role of hygiene in IBD development. Understanding the role of environmental factors is important not only for the possible preventive interventions in genetically predisposed individuals, but also to offer a better disease care to those already experiencing IBD. Therefore, the purpose of our work was to investigate the relation between the exposure to some environmental factors and the risk to develop UC and CD in a cohort of pediatric patients in Southern Italy.

## MATERIALS AND METHODS

### Case Identification

A case-control study was undertaken at the Department of Translational Medical Science, Section of Pediatrics, University of Naples Federico II, in Campania, Southern Italy. Cases corresponded to pediatric patients with IBD attending our Department for clinical management from 2000 to 2014 and younger than 18 years at the time of IBD diagnosis. The diagnosis of CD and UC was based on clinical, endoscopic, radiological, and histopathological criteria (16).

### Controls

Controls were chosen both from local public schools (n = 111; 54.6%) in Campania, Italy, representatives of the general population, and from the outpatient clinic (n = 92; 45.4%) at the Department of Translational Medical Science, Section of Pediatrics, University of Naples Federico II, Campania, Italy. Only children with a diagnosis of functional gastrointestinal disorders, gastrointestinal reflux, or constipation, according to Rome III criteria and confirmed by follow-up, were enrolled (17).

### Definition of Questionnaire

Investigators personally interviewed both the study subjects and their parents. If the subject was older than 10 years, he could answer questions on his own, helped by a parent if necessary. A copy of the questionnaire (Supplemental Digital Content 1, Questionnaire, *http://links.lww.com/MPG/A715*) was given to each subject to carefully think about each answer and, if necessary, to discuss it with other family members. Patients and their parents filled the questionnaire between January and June 2014. Questions covered the period from birth to the date of diagnosis for cases and the corresponding period for control (from birth to age of the enrollment). The questionnaire was a multi-item questionnaire related to 5 different areas: family history of IBD and of autoimmune diseases (among first-degree relatives); perinatal period (gestational age at birth, mother diseases during pregnancy, infections, or hospitalization during the first month of life); home amenities (presence of bathrooms, hot water), water consumption (tap or bottle water), type of housing (flat, house or other), personal hygiene, presence of animals (pets, other animals), number of siblings, sharing the bed or the room with other family members, active and passive smoking, mother's and father's degree and employment, and stressing events susceptibility (generalized anxiety, social anxiety, panic disorders, bereavement, divorce); childhood infections (measles, mumps, rubella, chicken pox, whooping cough, croup, gastroenteritis, respiratory infections during the first years of life), other childhood diseases (atopy), use of antibiotics, appendectomy, and vaccination history (both compulsory such as measles-mumps-rubella vaccine, diphtheria, tetanus, poliomyelitis, and pertussis and optional such as *pneumococcus* and meningococcal vaccines); in the latter category positive history for other infective diseases, both in the patients or in the family components, (such as *Helicobacter Pylori*, parasites) were also included; and infants and children diet (breastfeeding, age of introduction of gluten). For each question, all answers were also compared with patients' medical records. In addition, a dietician performed a separate validated questionnaire designed to assess the adherence to Mediterranean diet, as previously described (18) (Supplemental Digital Content 2, Test, *http://links.lww.com/MPG/A716*). The total duration of the interview was approximately 1 hour.

### Ethical Considerations

The institutional review board of the University of Naples "Federico II" approved the study protocol and questionnaire, and all participants gave informed written consent.

### Statistics

The association between potential risk factors and IBD, CD, or UC was explored by Pearson chi-square test for categorical variables. A $P$ value of $<0.05$ ($P^*$) was considered to denote a statistically significant difference. A Bonferroni correction was applied to correct for multiple comparisons. A multivariate model was performed to consider the effect (odds ratio [OR]) of each single factor, after having considered the contribution of all other factors. We selected a stepwise forward conditional procedure to identify the strongest risk factor and then we added the factors that contribute independently to further risk. We included in the analysis all the significant factors at the univariate analysis. We compared CD and UC cases with controls to estimate the odds of being a control (not affected) by a Logistic Regression Model. Because environmental risk factors may be related to the socioeconomic level of the family, we included in the first step of the multivariate model, mother's education and father's occupation, considered as the best markers of the socioeconomic status and positive IBD family history and autoimmune diseases, the 2 strongest IBD-related risk factors. To test the goodness of fit, Hosmer-Lemeshow (H-L) test was performed. The presence of collinearity among predictors was assessed by computing the variance inflation factor (VIF) for each of the variables included in the multivariable model. In addition, we also performed Cramer V index to exclude possible associations among the independent variables included in the model. For a case-control study, a $P$ value $<0.05$, a power of 80%, a mean risk factor prevalence of 30%, and expected relative risk of 2.5 (selected for biological significance), the required sample size for cases and controls separately was estimated to be around 80 (19). Statistical analysis was performed using SPSS statistical software package for Windows (version 19.0; SPSS, Chicago, IL).

## RESULTS

### Description of Inflammatory Bowel Disease Cohort and Controls

All IBD cases and controls agreed to participate in the study. A total of 467 subjects ages between 1 and 18 years were enrolled.

TABLE 1. Baseline characteristics of the enrolled patients

| | IBD | | Controls | | |
|---|---|---|---|---|---|
| Variables (n, %) | CD (n = 102) | UC (n = 162) | OP (n = 92) | SC (n = 111) | *P* |
| Median age (y; range) | 11 (4–17) | 10 (2–17) | 10.9 (5–17) | 14.4 (5–17) | 0.3 |
| Median age at diagnosis (y; range) | 9 (2–16) | 10 (2–17) | – | – | NA |
| Male sex (n, %) | 58 (56.8) | 80 (49.3) | 56 (60.8) | 62 (55.8) | 0.22 |
| Living area (n, %) | | | | | 0.08 |
| Urban | 73 (71%) | 106 (65%) | 64 (69%) | 89 (80%) | |
| Rural | 29 (28%) | 56 (34%) | 28 (30%) | 22 (20%) | |

CD = Crohn disease; IBD = inflammatory bowel disease; OP = outpatients; SC = school children; UC = ulcerative colitis.

Among these 102 were affected by CD (median age: 11 years; range: 4–17 years; M/F: 58/44) and 162 by UC (median age: 10 years; range: 2–17 years; M/F: 80/82). In the IBD groups no case of IBD-unclassified (IBD-U) was present. In addition, 203 controls (median age: 12 years; range: 5–17; M/F: 118/85) were also enrolled. Baseline characteristics of enrolled patients were not significantly different among cases and controls, as reported in Table 1.

## Univariate Analysis

### Family History of Inflammatory Bowel Disease and Autoimmune Diseases

A family history of CD was significantly associated with the risk of being affected by CD (CD: 3% vs controls: 0%; $\chi^2$: 6.1; $P = 0.01$), and a family history of UC was significantly higher in UC children when compared with controls (UC: 3.1% vs controls: 0%; $\chi^2$: 6.4; $P = 0.01$). The presence of concomitant autoimmune diseases was associated to the risk of developing IBD, with a significant difference compared with controls both for CD (CD: 37% vs controls: 19.4%; $\chi^2$: 10.85; $P = 0.002$) and UC (UC: 40%; controls: 19.4%; $\chi^2$: 19.7; $P < 0.001$) (Tables 2 and 3).

### Perinatal Period

Gestational age, mothers' diseases during pregnancy, and infections or hospitalization during the first month of life were not significantly different between cases and controls.

### Home Amenities, Domestic, and Personal Hygiene

A higher percentage of stressing events in the family was found in both group of cases compared with controls (CD: 46.1% vs controls: 34.5%; $\chi^2$: 3.9; $P = 0.05$; UC: 54.7% vs controls: 34.5%; $\chi^2$: 14.9; $P < 0.001$). Bed sharing was significantly more frequent in controls patients when compared with patients with CD (controls: 37.1% vs CD: 17.6%; $\chi^2$: 12.1; $P < 0.001$); this finding was not confirmed in patients with UC (UC: 30.8% vs controls: 37.1%; $\chi^2$: 1.6; $P = 0.1$). The number of siblings resulted to be lower (<2 siblings) in the CD group (CD: 69.3% vs controls: 50.7%; $\chi^2$: 9.5; $P = 0.002$) and UC group (UC: 67.7% vs controls: 50.7%; $\chi^2$: 10.6; $P = 0.001$) when compared with controls. A higher (>1) number of toilets was found in subjects' houses compared with the controls both for CD (CD: 73.5% vs controls: 61.6%; $\chi^2$: 4.3; $P = 0.04$) and UC (UC: 77.6% vs controls: 61.6%; $\chi^2$:10.8; $P = 0.001$). Furthermore, owning a pet was more frequent in controls compared with

TABLE 2. Association of Crohn disease cases with childhood factors

| Variables (n, %) | CD (n = 102) | Controls (n = 203) | OR | CI (95%) | $P^*$ | $P^\dagger$ |
|---|---|---|---|---|---|---|
| CD family history | 3 (3) | 0 (0) | 3.0 | 2.6–3.6 | 0.03 | – |
| Autoimmune diseases | 37 (37.0) | 38 (19.4) | 2.4 | 1.4–4.1 | 0.002 | + |
| Mother degree | 90 (88.2) | 121 (60.5) | 4.8 | 2.5–9.5 | <0.001 | + |
| Father employment | 87 (85.3) | 150 (74.6) | 1.9 | 1.0–3.7 | 0.02 | – |
| Family stress | 47 (46.1) | 70 (34.5) | 1.6 | 1.0–2.6 | 0.05 | – |
| Bed sharing | 18 (17.6) | 75 (37.1) | 0.3 | 0.2–0.6 | <0.001 | + |
| No. siblings <2 | 70 (69.3) | 102 (50.7) | 2.1 | 1.3–3.6 | 0.002 | + |
| No.° toilets >1 | 75 (73.5) | 125 (61.6) | 1.7 | 1.0–2.9 | 0.04 | – |
| Pets | 18 (17.6) | 70 (34.7) | 0.4 | 0.2–0.7 | 0.001 | + |
| Family smoke | 53 (52.5) | 85 (41.9) | 1.5 | 0.9–2.4 | 0.05 | – |
| Family parasitosis | 3 (3.1) | 32 (16.0) | 0.1 | 0.04–0.5 | 0.001 | + |
| Family *H pylori* | 9 (9.2) | 35 (17.9) | 0.4 | 0.2–1 | 0.03 | – |
| Antibiotic therapy | 31 (30.4) | 43 (21.2) | 1.6 | 0.9–2.7 | 0.05 | – |
| Low adherence to Mediterranean diet | 30 (29.4) | 34 (17.3) | 1.9 | 1.1–3.5 | 0.01 | – |
| Breast feeding >3 months | 61 (78.2) | 93 (57.1) | 2.7 | 1.4–5 | 0.001 | + |
| Gluten <6th month | 54 (54.0) | 58 (29.3) | 2.8 | 1.7–4.6 | <0.001 | + |

CD = Crohn disease; CI = confidence interval; HC = healthy controls; OR = odds ratio.
*$\chi^2$ test.
†Significance after Bonferroni correction.

TABLE 3. Association of ulcerative colitis cases with childhood factors

| Variables (n, %) | UC (n = 162) | Controls (n = 203) | OR | CI (95%) | $P^*$ | $P^\dagger$ |
|---|---|---|---|---|---|---|
| UC family history | 5 (3.1) | 0 (0) | 2.3 | 2.0–2.5 | 0.016 | – |
| Autoimmune diseases | 65 (40.0) | 38 (19.4) | 2.8 | 1.7–4.6 | <0.001 | + |
| Father employment | 140 (87.5) | 150 (74.6) | 2.3 | 1.3–4.1 | 0.002 | + |
| Family stress | 88 (54.7) | 70 (34.5) | 2.2 | 1.4–3.5 | <0.001 | + |
| No. siblings <2 | 109 (67.7) | 102 (50.7) | 2.0 | 1.3–3.1 | 0.001 | + |
| No. toilets >1 | 125 (77.6) | 125 (61.6) | 2.1 | 1.3–3.4 | 0.001 | + |
| Pets | 38 (23.5) | 70 (34.7) | 0.5 | 0.3–0.9 | 0.02 | – |
| Family parasitosis | 2 (1.3) | 32 (16.0) | 0.06 | 0.01–0.2 | <0.001 | + |
| Family *H pylori* | 14 (8.9) | 35 (17.9) | 0.4 | 0.2–0.8 | 0.02 | – |
| Low Mediterranean diet adherence | 61 (37.7) | 34 (17.3) | 2.8 | 1.7–4.7 | <0.001 | + |
| Gluten <6 mo | 88 (55.3) | 58 (29.3) | 2.9 | 1.9–4.6 | <0.001 | + |

CI = confidence interval; HC = healthy controls; OR = odds ratio; UC = ulcerative colitis.
*$\chi^2$ test.
†Significance after Bonferroni correction.

both group of cases (CD: 17.6% vs controls: 34.7%; $\chi^2$: 9.5; $P = 0.001$; UC: 23.5% vs controls: 34.7%; $\chi^2$: 5.4; $P = 0.02$). In CD families tobacco smoking was statistically more frequent than in control's families (CD: 52.5% vs controls: 41.9%; $\chi^2$: 3.1; $P = 0.05$).

### Childhood Diseases, Vaccinations

No statistical significant difference was found in the exposure to vaccination or incidence of childhood exanthema (measles, mumps, and rubella) between cases and controls. A positive family history for intestinal parasitosis and *H pylori* infection resulted more frequently in controls than in CD and UC subjects (parasitosis: CD: 3.1% vs controls: 16%; $\chi^2$: 10.6; $P = 0.001$; UC: 1.3% vs controls: 16%; $\chi^2$: 22.136; $P < 0.001$; *H pylori*: CD: 9.2% vs controls: 17.9%; $\chi^2$: 3.8; $P = 0.03$; UC: 8.9% vs controls: 17.9%; $\chi^2$: 5.8; $P = 0.02$). A higher use of antibiotic therapy was found in the CD group compared with controls (CD: 30.4% vs controls: 21.2%; $\chi^2$: 3.1; $P = 0.05$). Recurrence of intestinal parasitosis and *H pylori* infection was not significantly different in cases (both CD and UC) compared with the control group. No difference was found regarding atopy (eczema, asthma), appendectomy, and consumption of nonsteroidal anti-inflammatory drugs.

### Infants and Children Diet

The number of subjects who showed a low adherence to Mediterranean diet was higher for CD (CD: 29.4% vs controls: 17.3%; $\chi^2$: 5.9; $P = 0.01$) and for UC (UC: 37.7% vs controls: 17.3%; $\chi^2$: 19; $P < 0.001$) when compared with controls. Patients with CD (CD: 78.2% vs controls: 57.1%; $\chi^2$: 10.2; $P = 0.001$) showed a higher frequency of prolonged breastfeeding (>3 months) compared with controls. Gluten was introduced in child's diet more frequently before the 6th month of age in both CD (CD: 54% vs controls: 29.3%; $\chi^2$: 17.3; $P < 0.001$) and UC (UC: 55.3% vs controls: 29.3%; $\chi^2$: 24.77; $P < 0.001$) compared with controls.

## Multivariate Logistic Regression Analysis

### Inflammatory Bowel Disease Versus Controls

In a multivariate logistic regression model being affected by CD or UC were used as dependent variables. Mother's degree was among the most significant variables associated with CD (OR: 5.5; 95% CI 2.5–11.6; $P = 0.01$), whereas a low adherence to Mediterranean diet resulted significantly associated with UC (OR: 2.3; 95% CI 1.2–4.5; $P = 0.01$). Owning pets and bed sharing were independent protective factors for CD development (OR: 0.3; 95% CI 0.1–0.7; $P = 0.007$; OR: 0.2; 95% CI 0.1–0.6; $P < 0.001$). Owning pets and family parasitosis were significant protective factors for UC (OR: 0.4; 95% CI 0.2–0.8; $P = 0.004$; OR: 0.07; 95% CI 0.01–0.4; $P = 0.01$). All variables independently associated with the risk of being affected by CD and UC after multivariate analysis are shown in Table 4. Model fit was assessed with the H-L test, to determine whether there was a statistically significant difference between the rates of predicted and observed cases. H-L test was not significantly different for both CD and UC multivariate analysis, attesting the goodness of fit ($P = 0.3$; $P = 0.6$, respectively). To exclude the presence of collinearity we performed VIF analysis. The greatest VIF, associated to the factor mothers' degree, was 1.13; all the others were <1.1. These results allowed us to exclude the presence of collinearity among predictors. As a further confirm, we also computed the Cramér V index among the 3 variables that could be associated with each other (mother's degree, breastfeeding, and gluten introduction). The values of

TABLE 4. Multivariate logistic regression analysis for inflammatory bowel disease children

| Variables | Adjusted OR | CI 95% | *P* |
|---|---|---|---|
| CD | | | |
| Mother's degree | 5.5 | 2.5–11.6 | 0.01 |
| Breast feeding >3rd mo | 4.3 | 1.6–10.5 | 0.002 |
| Father's employment | 3.7 | 1.2–8.7 | 0.008 |
| Gluten introduction <6th mo | 2.8 | 1.5–5.0 | 0.001 |
| No. siblings <2 | 2.8 | 1.5–5.3 | 0.01 |
| Autoimmune diseases | 2.7 | 1.4–5.3 | 0.003 |
| Pets | 0.3 | 0.1–0.7 | 0.007 |
| Bed sharing | 0.2 | 0.1–0.6 | 0.001 |
| UC | | | |
| Low adherence to Mediterranean diet | 2.3 | 1.2–4.5 | 0.01 |
| Gluten introduction <6th mo | 2.8 | 1.6–4.9 | <0.001 |
| No. siblings <2 | 2.0 | 1.1–3.6 | 0.01 |
| Pets | 0.4 | 0.2–0.8 | 0.004 |
| Family parasitosis | 0.07 | 0.01–0.4 | 0.01 |

CD = Crohn disease; CI = confidence interval; IBD = inflammatory bowel disease; OR = odds ratio; UC = ulcerative colitis.

the V statistics were 0.01 between mother's degree and breastfeeding, 0.10 between breastfeeding and gluten introduction, and 0.09 between gluten introduction and mother's degree. None of these associations showed a significant chi-square statistics ($P > 0.05$).

## DISCUSSION

Our study suggests numerous evidences supporting the influence of environmental factors as possible explanation for the significant increase of IBD incidence in the last decades (20). To the best of our knowledge this is the first pediatric study addressing the hypothesis that the development of IBD may be related to a lower adherence to Mediterranean diet, assessed with a validated questionnaire.

As previously reported, having a first-degree relative with IBD represents an important factor determining an individual's risk for developing the disease (21), confirming the importance of genetic background. The fact that environmental factors, however, play a major role in IBD development is corroborated by many of our results. We confirmed that antibiotics are associated with increased risk of CD (22,23). The use of antibiotics may alter the composition of the gut microbiome with the loss of potentially beneficial bacteria and the emergence of potentially pathogenic bacteria (22,23). Alternatively, the loss of immune interaction with potentially pathogenic bacteria at an early age fails to prime the immune system to harmful organisms that it may encounter later in life (22,23). Moreover, our study confirms an association between crowded housing and protection against the development of both IBD forms: owning pets, reduced number of toilets at home, and higher number of siblings were associated with a lower risk for both CD and UC. Family size can be used to indicate the level of overcrowding in a home, which has been associated with potential exposure to infection. A small family size and thus a less propensity for exposure to infections have been associated with a higher risk for IBD (24). Siblings may influence the development of IBD altering exposure patterns to microorganisms in early life, affecting acute manifestation of infections, or influencing age of transmission and severity (25). Therefore, it is not surprising that *H pylori* infection and parasitosis were significantly more frequent in the families of the control group. In a recent meta-analysis, *H pylori* infection was inversely associated with IBD (26,27). Also helminthes are thought to have an immunoregulatory role within the intestinal microbiome, and may have potentially protective effect in IBD development (27,28), as demonstrated in a recent case-control study conducted in South Africa (29). We have to underline that the history of *H pylori* infection and parasitosis was, however, investigated only through a questionnaire and this represents a limitation of the study. Moreover, also a higher educational level and a better social class according to mother's degree and father's occupation were significant risk factors for CD development, as previously reported by Lopez-Serrano et al (30).

Interestingly, in our pediatric population we found a more frequent incidence of stressing events in life compared with controls. The exact mechanism behind the effect of stress on intestinal inflammation is unclear; however, the evidence that stress can modulate the course of IBD is provided by clinical observations and by animal models of colitis and neuroimmune studies (31). Depression, anxiety, and stress have also been associated with increased rates of relapse and surgery for patients with IBD (32). Nevertheless, we also have to consider the possibility that this result may have been influenced by disease itself. Indeed, children with chronic diseases may highlight stressful events and relate these to the disease onset more than controls and this may have biased the result.

Above all, the striking difference in Mediterranean diet adherence, between cases and controls, has to be considered the most innovative finding of our study. Indeed, the Mediterranean diet is perhaps considered one of the healthiest dietary models currently existing. Numerous epidemiological and experimental nutrition studies have demonstrated how Mediterranean countries benefit from lower rates of chronic disease morbidity and higher life expectancy (18). There are several proposed mechanisms of action to explain the association between IBD and dietary choices. These proposed mechanisms involve a direct effect of dietary antigens, alteration of gut permeability, and the autoinflammatory response of the mucosa due to changes in the microbiota (33). In a case-control study of children published in 2008, a positive association with CD was found in girls with a diet rich in meats, fatty foods, and desserts, whereas, a diet of vegetables, fruits, olive oil, fish, grains, and nuts was inversely associated with CD in both sex. As a matter of fact, we found that traditional Mediterranean dietary patterns characterized by elevated intakes of fruits and vegetables, olive oil, bread, and cereals may exert a protective role in the development of IBD. Diet is, however, difficult to study as it is a multifactorial exposure, and patients may alter dietary habits based on symptom onset before diagnoses or as a result of increased disease activity (34). Unfortunately, there is still a large gap in knowledge due to limitations of retrospective data collection and recall bias for dietary histories (35). As reported from Baron et al (36), we found that breastfeeding was associated with an increased risk for CD. It is known that breastfeeding provides immunological protection to the newborn; therefore, Baron et al (36) speculated that when weaning occurs, delayed infections may lead to an inappropriate immune response and persistence of intestinal inflammation. The authors, however, conclude that short- and long-term benefits of breastfeeding overrule by far the increased risk of IBD that was observed in the study. We also explored the age of gluten introduction and we confirmed the results from Lopez-Serrano et al (30) who found that the first exposure to wheat before 6 months of age was a significant risk factor for IBD in the univariate and multivariate analysis. Lammers et al (37) demonstrated that gliadin is able to induce a direct increase in small intestinal permeability. It is, therefore, hypothesized that a longer exposure to gluten may be responsible of a detrimental effect on the gut barrier, participating in the chronic inflammatory process that leads to IBD onset. Moreover the duration of exposure to gluten has been related to an increase in the risk of other autoimmune disorders in patients with celiac disease (38). As previously reported (39), we found that passive tobacco smoking contributes to an increased risk for CD, but we did not observe a protective effect on UC. In contrast, we did not find any association with appendectomy, which is considered an important and strong protective factor for UC and a risk factor for CD (40). One of the reasons may be the relatively small sample size of our patients.

The main limitations of the present study are certainly related to the retrospective nature and, therefore, the possibility of recall biases has to be taken into account. Moreover, due to a lack of a pediatric validated questionnaire, we referred to the main risk factors previous analyzed. In addition, although reasonably excluded by VIF analysis and Cramer V index, it is acknowledged that some of the socioeconomic factors included in the multivariate analysis may have affected the model through the phenomenon of collinearity.

## CONCLUSIONS

Our study strongly supports the role of environmental factors in IBD pathogenesis. In addition, we reported for the first time the low adherence to Mediterranean diet as a possible risk factor for

developing IBD. Considering the retrospective nature, there is a strong need for future prospective studies. Indeed, it is impossible to rule out the possibility, that the relation between IBD and environment-related factors, is not an indirect effect that may serve as a mechanism for other yet unknown lifestyle factors. Future research should also be targeted for the identification of potential mechanisms underlying associations with hygiene-related factors to provide new clues for a better comprehension of IBD etiopathogenesis.

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# CHAPTER 3

# CHARACTERIZATION OF PAEDIATRIC IBD PHENOTYPE

As partially underlined during Chapter 1, childhood-onset IBD is considered a different phenotype from adult-onset or elderly-onset disease with many peculiarities in its phenotypic expressions and generally a more aggressive disease course [115]. Within this chapter we will describe both paediatric CD and UC phenotypes reporting the results of 2 different papers. The first **"*Autophagy genes variants and paediatric Crohn's disease phenotype: a single-centre experience*"** has been published *in Digestive and Liver Disease (Dig Liver Dis. 2014; 46:512-7)*; the second ***"The changing face of paediatric ulcerative colitis: a population-based cohort study"*** has been recently submitted and it is currently under review.

## 3.1 Impaired autophagy and paediatric Crohn's disease (CD) phenotype

The severity of paediatric CD is known since the first population-based studies published in the early 2000 [31, 115] and it has been confirmed by further papers [116, 117]. Compared to adults, the main characteristics of paediatric CD are: a more extensive anatomic involvement, including a higher frequency of pan-enteric disease and a major involvement of colon; an inflammatory behaviour at diagnosis with an early disease-progression toward complicated forms, including both stricturing and fistulising disease; an early need for immunomodulation and a higher risk for surgery [31, 115-118]. Therefore, in the last 10 years an effort has been made in order to characterize possible clinical, laboratory, microbial and genetics biomarkers able to differentiate children with a higher risk of poor disease outcomes [74, 119, 120]. In this paragraph, we will analyse the role of genetic factors in predicting CD disease phenotype.

The understanding of the genetic basis of CD started in 1996, with the discovery of NOD2/CARD15, the first identified gene, and probably one of the first successes in the genetics of complex polygenic diseases [121]. As underlined in chapter 2, GWAS have given evidence for several determinants, including genes encoding ATG16L1 (autophagy-related 16-like 1) and IRGM1 (immunity-related GTPase family M) [122]. All these genes are involved in a biological process known as autophagy, which plays a role in protein degradation, antigen processing, regulation of cell signalling, and many other pathways essential to the regulation of inflammation [123]. Some studies tried to correlate the presence of the above-mentioned polymorphisms with CD phenotype, both in adults and children. In details, 2 different papers found a strong association between NOD/CARD15 SNPs and major ileal or ileo-colonic localization of CD in children [124-125]. With regards to ATG16L1, a correlation between ATG16L1 risk allele and a more frequent ileal involvement of CD has been described in adults, but there are no evidences in children [126]. In addition, only one paediatric study analysed the recurrence of IRGM1 SPNs and CD risk, but no associations were found between these SPNs and the clinical course of the disease [127].

Considering this background literature, the aims of our study, carried out in our cohort population in 2014, was to evaluate the relationship between the main risk alleles of NOD2/CARD15 (rs2066844; rs2066845; rs2066847), ATG16L1 (rs2241880) and IRGM1 (rs13361189; rs4958847), and the corresponding clinical phenotype. In particular, we wanted to ascertain if patients carrying the above-mentioned alleles showed a more aggressive phenotype. Hence, we retrospectively analysed the clinical course of our cohort of CD children diagnosed from 2001 to 2013. All the children were genotyped for all the above-reported risk alleles.

Our data clearly underlined that patients carrying the homozygous risk allele rs2241880 of ATG16L1 had a significant trend to switch from an inflammatory to a

stricturing behaviour during the course of disease. In line with this finding, ATG16L1 risk allele was also associated with the absence of perianal disease, confirming that the expression of this genetic determinant is specifically linked with the development of stricturing and fibrosis rather than fistulising phenotype. To our knowledge, this is the first paediatric study reporting an association between the presence of rs2241880 risk polymorphism of ATG16L1 in children with CD and a more severe phenotype of disease. To support the hypothesis of a worst phenotype in homozygous patients, we found that rs2241880 risk polymorphism was related to a major incidence of clinical relapses and with the earlier introduction of immunosuppressants. In addition, children carrying rs2241880 risk allele show higher values of faecal calprotectin and CRP at diagnosis compared with patients carrying ATG16L1 heterozygous or wild type variants. Indeed, although the role of faecal and serologic markers in predicting IBD disease course is still controversial, the hypothesis that higher values at diagnosis could predict a more severe disease course has been claimed [128].

Differently from ATG16L1, several studies tried to identify the role of NOD2/CARD15 variant alleles in determining clinical phenotype in both adults and children. The most recurrent finding of this literature is that children and adolescents carrying NOD2/CARD15 have a more frequent small bowel involvement, and more precisely, the disease tends to be localized in terminal ileum [129, 130]. In agreement with these evidences, our analyses of genotype-phenotype correlation showed that patients carrying the NOD2/CARD15 rs2066847 (1007fs) heterozygous allele had a more frequent ileal involvement than children showing the wild type variant for the same SNP.

We also investigated whether IRGM1 gene, recently identified to play a role in the development of CD and also shown to be involved in autophagy, may have a role in predicting CD phenotype. In contrast, to the extensive amount of work that has been accomplished in identifying and characterizing the complex network of ATG molecular

mechanisms, there is much less known about the role of the IRGM1 related autophagic pathways. At the univariate analysis, we found that the presence of IRGM1 rs13361189 variant allele was associated with a lower use of immunosuppressant therapy, highlighting a possible role on the development of a milder phenotype. However, this association was not confirmed at the multivariate logistic regression analysis. Therefore, we weren't able to find a genotype-phenotype correlation for IRGM1 and other studies are needed to elucidate its role in CD pathogenesis.

In conclusion, the findings of this work confirmed that genetic susceptibility might have an important role in the etiology of paediatric-onset IBD and in the determination of CD phenotype. In our paediatric cohort, ATG16L homozygous risk allele seemed to be a key player in determining stricturing complications. In addition, we confirmed that NOD2-CARD15 is associated with ileal involvement. These data stress once more the importance of genetic susceptibility research in larger paediatric onset IBD cohorts in order to find new genes and possibly allow an early treatment stratification of these patients.

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Alimentary Tract

# Autophagy genes variants and paediatric Crohn's disease phenotype: A single-centre experience

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ABSTRACT

*Background and aims:* Little evidence demonstrating the correlation between several single nucleotide polymorphisms and a specific phenotype of Crohn's disease has been reported in children. We investigated the relationship between autophagy genes variants and clinical features in our children with Crohn's disease.

*Methods:* Genotyping for *ATG16L1*, *NOD2/CARD15*, and *IRGM1* was performed in 80 consecutive patients with Crohn's disease (median age: 11 years; range: 0.7–17.9 years). Crohn's disease location and behaviour were classified using the Paris classification. Additional data were collected from clinical records on patients' demographics, age at symptom onset and diagnosis, extraintestinal manifestations, therapy, clinical relapses, and need of surgical intervention.

*Results:* Patients homozygous for the risk allele *ATG16L1* (T300A) showed a trend towards switching to a stricturing phenotype during the course of disease compared to children either homozygous for the wild-type allele or heterozygous for the *ATG16L1* single nucleotide polymorphism ($p = 0.01$). Homozygosity for the *ATG16L1* risk allele was associated with a major recurrence of clinical relapses and earlier introduction of immunosuppressants ($p = 0.006$ and $p = 0.04$, respectively). Heterozygosity for the *NOD2* rs2066847 allele was associated with major ileal involvement ($p = 0.01$).

*Conclusion:* In patients carrying the T300A variant, Crohn's disease follows a more aggressive clinical course.



## 1. Introduction

The molecular basis of the pathogenesis of Crohn's disease (CD) is not completely understood. Strong epidemiological evidence for a genetic predisposition has stimulated recent efforts to identify the susceptibility genes [1,2]. In 1996, the discovery of *NOD2/CARD15*, the first gene identified as being associated with CD [3], represented one of the first success stories in the genetics of a complex polygenic disease. Subsequently, in 2001, the characterization of the gene at the IBD1 locus as *NOD2/CARD15* was a landmark observation [4]; since then, a large number of replication studies confirmed the association of the *NOD2/CARD15* variant alleles with an increased susceptibility to CD in adults [5]. Genome-wide association studies (GWAS) provided evidence for several determinants, including genes encoding *ATG16L1* (autophagy-related 16-like 1) and *IRGM1* (immunity-related GTPase family M), providing further insights into disease pathogenesis [6]. These genes are involved in a biological process known as autophagy, which plays a role in protein degradation, antigen processing, regulation of cell signalling, and many other pathways essential to the regulation of inflammation [7]. The group of Hampe et al. was the first to implicate the autophagy pathway in CD pathogenesis [8]. An association of *NOD2/CARD15*, *ATG16L1*, and *IRGM1* risk alleles with CD susceptibility has already been confirmed both in adults and children; however, there is little evidence describing a possible correlation between these single nucleotide polymorphisms (SNPs) and a spe-

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**Table 1**
Clinical features of 80 paediatric patients with Crohn's disease.

| Clinical features | Total *n* (%) |
|---|---|
| Median age at diagnosis (years; range) | 11 (0.7–17.9) |
| Gender | |
| Male | 53 (66.2) |
| Median disease duration (months; range) | 36.4 (12–154) |
| Positive family history | 2 (2.5) |
| Location of disease | |
| Ileum only (L1) | 17 (21.2) |
| Colon only (L2) | 27 (33.8) |
| Ileum and colon (L3) | 35 (43.8) |
| Upper gastrointestinal tract (L4a) | 1 (1.2) |
| Perianal disease | 20(25) |
| Extraintestinal manifestation | 15 (18.7) |
| Disease behaviour | |
| Inflammatory (B1) | 68 (85) |
| Stricturing (B2) | 8 (10) |
| Fistulizing (B3) | 4 (5) |
| Change in clinical behaviour | 12 (15) |
| Need for surgery | 10 (12.5) |

cific CD phenotype in children. Various studies found a strong association, in paediatric patients, between the *NOD/CARD15* SNPs and early-onset disease, as well as a major ileal or ileo-colonic localization of CD [9,10]. However, as regards ATG16L1, a correlation between the *ATG16L1* risk allele and a more frequent ileal involvement of CD has been described in adults [11], but not in children. In addition, only one paediatric study [12] analyzed the recurrence of *IRGM1* SPNs and CD risk, and no associations were found between these SPNs and the clinical course of the disease. The purpose of our study is to evaluate the relationship between the main risk alleles of *NOD2/CARD15* (rs2066844; rs2066845; rs2066847), *ATG16L1* (rs2241880), and *IRGM1* (rs13361189; rs4958847) and the clinical features in our cohort of children affected by CD.

## 2. Materials and methods

### 2.1. Study population

The study population included 80 children diagnosed with CD between January 2001 and August 2013. Demographic and clinical data of each patient were retrospectively collected from medical records (Table 1). Only children with at least 1 year of follow-up were included in this study. Diagnosis of CD was based on clinical, endoscopic, radiological, and histopathological criteria [13]. For the diagnosis, all children underwent ileo-colonoscopy, upper GI endoscopy, and imaging analyses, including abdominal ultrasound and entero-MRI or small bowel follow-through.

### 2.2. Phenotype analysis

CD disease location was categorized using the Paris classification: L1 for cecal and distal ileum involvement, L2 for colonic disease, L3 for ileocolonic disease, L4a for upper disease proximal to the ligament of Treitz, and L4b for upper disease distal to the ligament of Treitz. Perianal disease was defined by the presence of fissures, perianal ulcers, abscesses or fistulae, and skin (tags). Disease behaviour based on clinical history, was categorized as fistulising, stricturing, or inflammatory using the Paris classification guidelines criteria [14]. Stricturing disease referred to the presence of a constant luminal narrowing diagnosed radiologically, endoscopically, or surgically. Penetrating disease referred to radiographic, endoscopic, surgical, or clinical evidence of an abscess or fistula in any location. Patients who had neither stricturing nor fistulising disease at diagnosis and throughout follow-up were classified as having inflammatory disease behaviour. Moreover, information regarding changes from the inflammatory to the fistulising or stricturing patterns, as well as any clinical relapses, was collected.

For each patient, clinical activity of the disease was evaluated at the time of diagnosis using the paediatric Crohn's disease activity index (PCDAI) [15]. Clinical remission was defined as a PCDAI score of <10, while clinical response to the induction treatment was identified by a change in the PCDAI score of at least 15 points from baseline. Clinical relapse was defined as the occurrence or worsening of symptoms accompanied by a PCDAI score of >10 points, sufficient to require rescue treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery [16]. Family history was defined as positive if at least one first or second-degree relative was diagnosed with Inflammatory Bowel Disease (IBD). We also considered clinical therapy at disease onset and during follow-up. Two expert paediatric gastroenterologists (AS and EM) made all decisions regarding therapeutic interventions, in line with the validated international guidelines [17]. Exclusive enteral nutrition for 6–8 weeks or oral steroid treatment (oral methylprednisolone: 1 mg/kg/day, max 40 mg/day per 4 weeks) was used as induction therapy in all patients. Aminosalicylates (mesalazine 50 mg/kg/day) were used as maintenance therapy in patients with mild disease. Patients in whom standard induction therapy had failed or patients with early relapse (<6 months) were treated with azathioprine (2–2.5 mg/kg/day). Methotrexate was used as second-line immunosuppressant in those patients intolerant or refractory to azathioprine. infliximab (5 mg/kg/dose at weeks 0, 2 and 6, and then 8-weekly) was given as a first biological agent in patients refractory or intolerant to steroids and immunomodulators. Patients refractory or intolerant to infliximab therapy were treated with adalimumab (loading dose: 160/80 mg or 80/40 mg at weeks 0 and 2, respectively in patients weighing ≥40 kg or <40 kg; maintenance dose: 80 mg and 40 mg every 2 weeks, respectively in patients weighing ≥40 kg or <40 kg). Extraintestinal manifestations included eye, joint, skin, or liver involvement and persistent fever, defined as temperature ≥38 °C for 3 days during the week before the treatment. The laboratory tests used for inflammation parameters included: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), haemoglobin concentration, and faecal calprotectin. Written, informed consent was obtained from the participants' parents, and assent was obtained for all patients older than 10 years of age. The study was approved by the Institutional Review Board of the University of Naples "Federico II".

### 2.3. Genotype analysis

Genomic DNA was isolated from 10 ml of peripheral venous blood anticoagulated with EDTA and extracted using the modified salting out technique [18]. DNA samples of the patients were analyzed for the following gene variants: *NOD2/CARD15* rs2066844 and rs2066845 (gene accession number: NC_000016.9, region: 50721584.50772911), *IRGM1* rs13361189 and rs4958847 (gene accession number: NC_000005, region: 150225762.150228552), and *ATG16L1* rs2241880 (gene accession number: NC_000002.11, region: 234153601.234210934). The PCR allelic discrimination assay was performed using a predesigned TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) performed in 20-μL reactions consisting of 5 ng of genomic DNA and including primer, probes, and Universal PCR Master Mix. Cycling conditions were: 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. The implemented software (Eppendorf) automatically called and plotted genotypes based on a two-parameter plot using fluorescence intensities of carboxyfluorescein (FAM) and 2-chloro-7-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Indeterminate calls were excluded from the analysis. A combination of PCR, SSCP, and direct sequencing of amplified fragments was used to analyze NOD2/CARD15 rs2066847. A fragment of

**Table 2**
Genotype frequencies based on Hardy–Weinberg equilibrium.

| Polymorphism | Wild-type | Heterozygous | Risk | $\chi^2$ test | $p$ |
|---|---|---|---|---|---|
| | AA | AG | GG | | |
| ATG16L1rs2241880 | 8 (10%) | 46 (57.5%) | 26 (32.5%) | 3.5 | 0.06 |
| | TT | TC | CC | | |
| IRGM rs13361189 | 45 (56.3%) | 22 (27.5%) | 13 (16.3%) | 9.5 | 0.002 |
| | GG | GC | CC | | |
| IRGM rs4958847 | 50 (62.5%) | 27 (33.8%) | 3 (3.8%) | 0.07 | 0.7 |
| | CC | CG | | | |
| NOD2/CARD15 rs2066844 | 70 (87.5%) | 10 (12.5%) | | 0.5 | 0.35 |
| | GG | GC | CC | | |
| NOD2/CARD15 rs2066845 | 67 (32.5%) | 10 (47.5%) | 3 (10%) | 7.4 | 0.006 |
| | CC | CG | | | |
| NOD2/CARD15 rs2066847 | 58 (78.4%) | 16 (21.6%) | | 0.08 | 0.2 |

202 bp in molecular weight was amplified using the following primer pairs: NOD2-rs2066847-FPGGACAGGTGGGCTTCAGTAG; NOD2-rs2066847RPGCCTTACCAGACTTCCAGGAT. The oligonucleotides used were designed with the primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR-SSCP analysis was performed as previously described [19]. Sequencing analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of the NOD mRNA sequence (GenBank accession number: NM_022162.1).

### 2.4. Statistical analysis

Statistical analysis was performed using the SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). The patients' allele frequencies were tested for Hardy-Weinberg equilibrium (HWE) by comparing expected and observed genotypes with the Fisher's exact test. All markers showed no statistically significant deviation from the HWE, except for *IRGM1* rs13361189 ($p = 0.002$) and *NOD2/CARD15* rs2066845 ($p = 0.006$) (Table 2). The Student's *t*-test and the Mann–Whitney test were used for comparison of continuous variables, and the $\chi^2$ and Fisher's exact tests were used for categorical variables, as appropriate. In order to explore the weight of clinical and laboratory factors, discriminating homozygous patients from wild-type and heterozygous patients, a stepwise discriminant analysis was performed. Multivariate conditional logistic regression analysis was used to explore the odds associated with each polymorphism status. *ATG16L1*, *IRGM1*, and *NOD2/CARD15* were used as dependent variables, while the effect of all the above mentioned parameters for phenotype/expression was analyzed by a stepwise procedure.

## 3. Results

### 3.1. Clinical features

A total of 80 consecutive CD patients were included in the study [males: 53 (66.2%); median age: 11 years, range 0.7–17.9 years]. Clinical features of the study population are summarized in Table 1. Eighteen out of 80 patients (22.5%) underwent induction treatment with steroids at diagnosis, while 58 (72.5%) received nutritional therapy, and 4 (5%) had both steroid and nutritional therapy. Thirty-one out of 80 patients (38.7%) relapsed during the first year of disease. The median time of relapse was 6 months (range, 15 days to 12 months). At diagnosis, 68 out of 80 patients (85%) showed inflammatory disease behaviour, while 8 presented with a stricturing phenotype, and 4 with a fistulising pattern. During the course of disease, 12 out of 68 patients (17.6%) with an initial inflammatory disease changed their clinical phenotype: 8 (66.6%) switched to a stricturing CD, while 4 (33.4%) developed a penetrating phenotype. The median period in which patients switched their phenotype was 29 months (range 9–132 months). Twenty-nine patients (36.2%) needed to start immunosuppressant therapy for relapse of disease during the first year of follow-up. Sixteen patients (57%) were treated with azathioprine (AZT), 4 (14%) with methotrexate (MTX), and 8 (28%) firstly with AZT and then, due to lack of response, with MTX. Ten out of 80 patients (12.5%) needed to start biological therapy with infliximab for refractoriness to conventional therapy, and in 3 of them (30%) infliximab therapy was later replaced with adalimumab due to a lack of efficacy. In 10 out of 80 patients (12.5%) surgical intervention was needed for complications of disease. Fifteen out of 80 patients (18.7%) presented extraintestinal manifestations, 8 (53.3%) were affected by arthropathy, 3 (20%) by erythema nodosum, and 2 (13.3%) by persistent fever. Moreover, 1 of these 15 patients (6.6%) suffered from pancreatitis, while another presented with vaginal ulcers.

### 3.2. Genotype/phenotype correlation

#### 3.2.1. ATG16L1

The evaluation of the *ATG16L1* polymorphism, rs2241880, was performed in 80 CD patients. The overall frequency of the G allele was 61%. Homozygosity for the risk allele G was found in 26 out of 80 patients (32.5%), while the number of subjects carrying the wild-type and the heterozygous alleles was 8 (10%) and 46 (57.5%), respectively. The *ATG16L1* genotype/phenotype correlations are summarized in Table 3. The presence of the rs2241880 risk polymorphism was significantly associated with the development of stricturing behaviour. Six out of the 8 patients (75%) who developed stricturing behaviour were homozygous for the *ATG16L1* risk allele ($p = 0.01$). This mutation was more greatly associated with the occurrence of relapses during the first year of disease and with the use of immunosuppressant therapies than were the wild-type and heterozygous variants ($p = 0.006$ and $p = 0.04$, respectively). Moreover, homozygosity for the risk allele was significantly associated with a higher value of CRP and faecal calprotectin at diagnosis ($p = 0.05$, $p = 0.007$, respectively). Conversely, homozygous risk allele carriers showed a trend towards a lower risk of developing perianal disease than heterozygous and wild-type patients ($p = 0.06$ for both). Homozygosity for the risk allele occurred more frequently in males than in females, with a trend towards statistical significance ($p = 0.07$). Furthermore, the rs2241880 risk polymorphism of *ATGL16L1* was associated with earlier age at diagnosis, specific clinical behaviour, change in fistulising phenotype, family history of IBD, disease localization, PCDAI and ESR scores at diagnosis, surgery, and the start of biological therapy (Table 3).

#### 3.2.2. IRGM1

All enrolled patients were genotyped for the *IRGM1* risk alleles rs13361189 and rs4958847. Thirteen out of 80 patients (16.3%) were homozygous for the rs13361189 risk allele of *IRGM1*, while 22 (15%) were heterozygous, and 45 (68.7%) carried only the wild-type

**Table 3**
Characteristics of patients with Crohn's disease according to ATG16L1 s2241880 gene status.

| Variables (n, %) | Risk | Het + WT | p |
|---|---|---|---|
| Sex | | | 0.07[c] |
| Male | 21/26 (80.8) | 32/54 (59.3) | |
| Median age at diagnosis (years, range) | 11 (2.7–17.9) | 11 (0.7–16.8) | 0.4[a] |
| Family history | 1/26 (4) | 1/54 (2) | 1[b] |
| Disease behaviour at diagnosis | | | 0.7[b] |
| Inflammatory (B1) | 23/26 (88.4) | 45/54 (83.3) | |
| Stricturing (B2) | 3/26 (11.5) | 5/54 (9.3) | |
| Fistulizing (B3) | 0/26 (0) | 4/54 (7.4) | |
| Development of stricturing phenotype | 6/26 (23.1) | 2/54 (3.7) | 0.01[b] |
| Development of fistulising phenotype | 2/26 (7.7) | 2/54 (3.7) | 0.5[b] |
| Localization of disease at diagnosis | | | 0.6[b] |
| Ileum only (L1) | 7/26 (26.9) | 10/54 (18.5) | |
| Colon only (L2) | 7/26 (26.9) | 20/54 (37) | |
| Ileum and colon (L3) | 12/26 (46.2) | 23/54 (42.6) | |
| Upper gastrointestinal tract (L4a) | 0 (0) | 1/54 (1.9) | |
| Perianal disease | 3/26 (11.5) | 17/54 (31.5) | 0.06[b] |
| Extraintestinal manifestations | 5/26 (19.2) | 10/54 (18.5) | 1[b] |
| Relapse during the first year of disease | 16/26 (61.5) | 15/54 (27.8) | 0.006[b] |
| Immunosuppressants | 13/26 (50) | 13/54 (24.5) | 0.04[b] |
| Biologic therapy | 4/26 (15.4) | 5/54 (9.3) | 0.4[b] |
| Surgery | 3/26 (11.5) | 7/54 (13) | 1[b] |
| PCDAI score (median, range) | 28.7 (10–48) | 27.7 (5–60) | 0.6[a] |
| CRP (median, range) | 9.7 (0–174) | 4.8 (0–165) | 0.05[a] |
| ESR (median, range) | 33 (2–90) | 25 (2–102) | 0.1[a] |
| Calprotectin (median, range) | 500 (150–1470) | 375 (15–790) | 0.007[a] |

Het: heterozygous; WT: wild type; PCDAI: paediatric Crohn disease activity Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.
[a] Mann–Whitney test.
[b] Fisher's test.
[c] $\chi^2$ test.

**Table 4**
Characteristics of Crohn's disease patients according to NOD2/CARD15 1007 fs gene status.

| Variables (n, %) | Risk | WT | p |
|---|---|---|---|
| Sex | | | 0.7[c] |
| Male | 12/16 (75) | 39/58 (67.2) | |
| Median age at diagnosis (years, range) | 10.3 (6.4–15.7) | 11.2 (2–17.9) | 0.2[a] |
| Family history | 0/16 (0) | 2/58 (3.4) | 1[b] |
| Disease behaviour at diagnosis | | | 0.4[b] |
| Inflammatory (B1) | 15/16 (93.8) | 47/58 (81) | |
| Stricturing (B2) | 1/16 (6.2) | 7/58 (12.5) | |
| Fistulizing (B3) | 0/16 (0) | 4/58 (6.9) | |
| Development of stricturing phenotype | 5/16 (8.6) | 3/58 (18.8) | 0.1[b] |
| Development of fistulising phenotype | 4/16 (6.9) | 0/58 (0) | 0.5[b] |
| Localization of disease at diagnosis | | | 0.008 [b] |
| Ileum only (L1) | 8/16 (50) | 8/58 (13.8) | |
| Colon only (L2) | 1/16 (6.2) | 22/58 (37.9) | |
| Ileum and colon (L3) | 7/16 (43.8) | 27/58 (46.6) | |
| Upper gastrointestinal tract (L4a) | 0/16 (0) | 1/58 (1.7) | |
| Perianal disease | 4/16 (25) | 16/58 (27.6) | 1[a] |
| Extraintestinal manifestations | 1/16 (6.2) | 13/58 (22.4) | 0.2[a] |
| Relapse during the first year of disease | 5/16 (31.2) | 25/58 (43.1) | 0.5[b] |
| Immunosuppressants | 5/16(31.2) | 21/58 (36.2) | 0.7[b] |
| Biologic therapy | 2/16 (12.5) | 7/58 (12.1) | 1[b] |
| Surgery | 2/16 (12.5) | 8/58 (13.8) | 1[b] |
| PCDAI score (median, range) | 25 (18–50) | 27.5 (5–60) | 0.6[a] |
| CRP (median, range) | 1.8 (0–61) | 5.8 (0–174) | 0.2[a] |
| ESR (median, range) | 30 (2–84) | 25 (3–102) | 0.9[a] |
| Calprotectin (median, range) | 418 (15–500) | 450 (60–1470) | 0.5[a] |

WT: wild type; PCDAI: paediatric Crohn disease activity index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.
[a] Mann–Whitney test.
[b] Fisher's test.
[c] $\chi^2$ test.

allele. The overall frequency of the risk allele was 30%. Homozygosity for the risk allele was significantly associated with less frequent relapse during the first year of the disease and with a lower use of immunosuppressant therapy ($p = 0.001$ and $p = 0.05$, respectively). Among the 80 patients, 3 (3.8%) were homozygous for the *IRGM1* rs4958847 risk polymorphism, 27 (33.8%) were heterozygous, and 50 (62.5%) carried only the wild-type allele. *IRGM1* rs4958847 was not associated with any of the clinical features analyzed.

#### 3.2.3. *NOD2/CARD15*

The evaluation of the *NOD2/CARD15* polymorphism, rs2066847, was possible in 74 of the CD patients. The overall frequency of the G allele was 10.8%. Fifty-eight (78.4%) out of 74 patients carried only the wild-type allele, while 16 (21.6%) were found to be heterozygous for the rs2066847 polymorphism. The heterozygous variant was significantly associated with an ileal localization of the disease ($p = 0.01$, Table 4). Allelic variants of rs2066844 and rs2066845 were not significantly associated with any of the clinical features analyzed.

### 3.3. Discriminant analysis

In order to explore the weight of the clinical and laboratory parameters discriminating homozygous patients from wild-type and heterozygous patients, we attempted a discriminant analysis to progressively select the factors that can best distinguish patients, having considered all the other factors in a multivariate fashion. Wilks-Lambda is an estimate of the discriminant capacity of each factor, while the variance rate, *F*, assigns a weight to each discriminant factor. Once the discriminant function is developed, a discriminant score is assigned to each subject, regardless of his phenotype. The classification analysis shows the efficiency of the discrimination based on the selected variables. Development of stricturing behaviour, relapse during the first year of life, faecal calprotectin, and male gender correctly classified 77.5% of the ATG16L1 rs2241880 allele-carrying patients. In order to validate the analysis, a jack-knifing procedure was applied (Table 5). Considering the few variables involved in discriminating *NOD2/CARD15* rs2066847 and *IRGM1* rs13361189 and rs4958847 it was not feasible to outline a discriminant function.

### 3.4. Multivariate logistic regression analysis

In a multivariate logistic regression model, the *ATG16L1* rs2241880, *NOD2/CARD15* rs2066847, and *IRGM1* rs13361189 polymorphisms were used as dependent variables. The variables

**Table 5**
Discriminant analysis – Wilk's Lambda for ATG16L1 polymorphism.

| Variables | WL | *F* | p |
|---|---|---|---|
| *ATG16L1* | | | |
| Development of stricturing phenotype | 0.87 | 12.5 | 0.001 |
| Faecal calprotectin | 0.76 | 11.6 | 0.0001 |
| Relapse during the first year of disease | 0.71 | 10.3 | 0.0001 |
| Gender | 0.6 | 9.4 | 0.0001 |

WL: Wilk's-Lambda; *F*: variance rate *F*.

**Table 6**
Multivariate logistic regression analysis.

| | OR | CI 95% | *p* |
|---|---|---|---|
| *ATG16L1* | | | |
| Development of stricturing behaviour | 18.4 | 2.5–135 | 0.001 |
| Relapse during the 1st year of disease | 1.2 | 1–1.4 | 0.002 |
| Perianal disease | 0.2 | 0.05–1 | 0.05 |
| *NOD2/CARD15 1007fs* | | | |
| Ileal disease | 5.4 | 1.6–18 | 0.01 |

OR: odds ratio; CI: confidence interval.

that were still significantly associated with the presence of the *ATG16L1* risk allele in homozygosity were the development of stricturing behaviour (OR = 18.4; $p = 0.004$) and the relapse during the first year of disease (OR = 1.2; $p = 0.002$) (Table 6). The presence of perianal disease was independently associated with homozygosity of the *ATG16L1* risk allele as a protective factor (OR 0.2; $p = 0.05$) (Table 6). The sole variable found to be associated with heterozygosity of the *NOD2/CARD15* allele was the presence of ileal disease (OR = 5.4; $p = 0.001$) (Table 6). None of the variables were independently correlated with the presence of the *IRGM1* rs13361189 and rs4958847 risk alleles in homozygosity.

## 4. Discussion

To our knowledge, this is the first paediatric study reporting an association between the presence of the rs2241880 risk polymorphism of *ATG16L1* in children with CD and a more severe phenotype of the disease. It was not the aim of our paper to reassess the role of ATG16L1 in susceptibility to paediatric Crohn's disease, since it has already been widely described in paediatric literature [20–23]. Nonetheless, in our cohort of paediatric CD patients, the frequency of the G allele (61%) was comparable with that reported by the single Italian multicentre study published by Latiano and colleagues (59%) [20]. Instead, we here tried to look for possible phenotypic associations, and we clearly demonstrated that homozygous risk allele carriers show a significant trend towards changing from inflammatory to stricturing behaviour, suggesting that the ATG16L1 risk allele may be somehow linked with the early development of fibrosis. This finding is in agreement with those of Fowler and colleagues [24]. These authors genotyped a large population of Australian adults with CD and found that the GG variant was independently associated with some more complicated disease courses, such as the development of stricturing disease and intra-abdominal penetrating disease. Consistent with Fowler's data [24], we here demonstrated an association between the rs2241880 risk polymorphism and the absence of perianal disease, suggesting that the *ATG16L1* risk allele may exert a protective effect at least on this manifestation in CD children. To support the hypothesis of a more severe phenotype in homozygous patients, we found that the rs2241880 risk allele was related to a higher incidence of clinical relapses and also to the introduction of immunosuppressants. In addition, children carrying the rs2241880 risk allele showed higher values of faecal calprotectin and CRP at diagnosis compared to heterozygous and wild-type *ATG16L1* patients. However, the role of faecal and serologic markers in predicting IBD disease course is still controversial, and the hypothesis that higher values of these markers at diagnosis could predict a more severe course of disease has been claimed [25]. Our data were strengthened by both a discriminant function and a multivariate logistic regression analysis. Indeed, our multivariate logistic regression analysis demonstrated that development of stricturing behaviour, followed by relapse during the first year of disease and absence of perianal disease, were the most significant variables associated with the ATG16L1 risk allele. In accordance with these genotype-/phenotype correlations, our data from functional analyses show that the monocyte-derived dendritic cells (DC) from paediatric patients with CD carrying the *ATG16L1* risk polymorphism are antigen-sampling and processing impaired. We found a marked reduction of bacteria particle localization in DC from CD children with the rs2241880 risk variant of ATG16L1 compared to DC of children with the wild-type variant. Furthermore, we found that DC from the risk group almost completely failed to upregulate HLA-DR and CD86, the 2 key molecules for the activation of the T-cell-mediated immune response [26]. Since intestinal microorganisms have been suggested to be one of the causes responsible for bowel inflammation [27], it is likely that an alteration of the autophagy process might lead to uncontrolled microorganism growth in the intestine of IBD patients [26].

In agreement with Lauriola et al., we found that the *ATG16L1* risk allele is not associated with a positive family history or with the presence of extraintestinal manifestations [28]. The association between the rs2241880 risk polymorphism of *ATG16L1* and CD localization is controversial. The rs2241880 variant was described as being associated with an ileal CD phenotype in children [11,22,29], while, according to Lakatos et al., ATG16L1 homozygosity for rs2241880 was associated with colon disease only [30]. In our study population we did not find any specific disease localization according to the stratification of CD with the Paris classification.

The *IRGM1* gene has been recently identified as playing a role in the development of CD and has also been shown to be involved in autophagy. In contrast to the extensive amount of work that has been accomplished in identifying and characterizing the complex network of *ATG* molecular mechanisms, little is known about the role of *IRGM1*-related autophagic pathways [31]. No Italian study has ever assessed the IRGM1 frequency in a CD population. We found that the presence of the *IRGM1* rs13361189 variant allele was associated with a lower use of immunosuppressant therapy, highlighting a possible role in the development of a milder phenotype. However, none of the associations were confirmed by the multivariate logistic regression analysis; thus, further studies are needed to provide definitive conclusions regarding *IRGM1* rs13361189.

Several studies supported a significant association between ileal disease and the carriage of one or more *NOD2/CARD15* variant alleles [32–34]. In our cohort of paediatric CD patients, the frequency of the G allele for NOD2/CARD15 rs2066847 (10.6%) was comparable to that reported by an Italian multicentre study by Annese and colleagues (9.3%) [35]. According to the current evidence, our analyses of genotype/phenotype correlation showed that patients heterozygous for the *NOD2/CARD15* rs2066847 (1007 fs) allele had more frequent ileal involvement than children showing the wild-type variant for the same SNP. In contrast with previous studies [36,37], we did not find any significant relationship between the three *NOD2/CARD15* risk genes studied and parameters such as a specific pattern of CD, positive family history, extraintestinal manifestations, perianal disease, or surgery. Our study has some limitations, including the small cohort of patients; despite this, the associations were confirmed by powerful statistical tests as to avoid potential bias.

In conclusion, we believe that genetic susceptibility may have a very important role in the aetiology of paediatric-onset IBD. Certainly, IBD has a multifactorial origin, and environmental factors are crucial in the development of the disease. Nevertheless, if genetic susceptibility is greater, paediatric IBD patients can be expected to have a more severe clinical course of the disease. Within paediatric-onset CD, specific genotype/phenotype associations can be found. In our paediatric cohort, homozygosity for the ATG16L risk allele was associated with a more aggressive disease course, including development of stricturing behaviour, early relapse, and premature use of immunosuppressants. As previously demonstrated, heterozygosity for the NOD2/CARD15 was significantly correlated with major ileal disease. These data highlight the

importance of genetic susceptibility research in larger paediatric-onset IBD cohorts in order to find new genes and allow an early stratification for the treatment of these patients.

## Conflict of interest

None declared.

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### 3.2 Phenotype and clinical course of paediatric Ulcerative Colitis

The onset of UC in paediatric age is today considered both a diagnostic and a therapeutic challenge, when compared with adults [8, 115, 131]. From the diagnostic perspective, adults' UC has a quite clear and recognizable pattern, mainly characterized by a typical superficial, chronic mucosal inflammation, which involves the colon starting from the rectum and proceeding continuously towards proximal segments. Differently, paediatric UC may be characterized by the presence of atypical phenotypes, which are responsible of many IBD-U and even wrong CD diagnosis. The revised Porto criteria, published in 2014, tried to identify and classify all of them, with the aim of improving the quality of diagnosis in children [37]. Six different atypical UC phenotypes have been identified:

1) Rectal sparing: absence of macroscopic disease in rectum or recto-sigmoid;
2) Short duration of disease variant: patchy disease in biopsies or lack of typical architectural distortion in pathological specimens;
3) Cecal patch: left-side colitis plus an area of inflammation in cecal region;
4) Upper gastrointestinal involvement (UGI): presence of gastric erosions and non-serpiginous small ulcers;
5) Acute severe colitis (ASC): disease contiguous from the rectum and trans-mural inflammation with deep ulcers;
6) Backwash ileitis: macroscopic, short segment, non-stricturing terminal ileitis, without granulomata.

To date the incidence of atypical features has been poorly described. In addition, all the papers, addressing this issue, have been published before the Porto classification, therefore identifying some, but not all the phenotypes. This is the case of Eurokids data

registry, published in 2013, which reported only the incidence of UGI, rectal sparing and backwash ileitis [132].

From a therapeutic perspective, it is well known that childhood-onset UC is characterized by a more extensive colonic involvement and a more rapid severe course when compared to adult UC, including a higher risk of corticosteroids dependency, an earlier immunosuppressive therapy introduction and surgery occurrence [115, 131]. Already in 2008, Van Limbergen and colleagues comparing a cohort of children with UC with an adult population demonstrated that 74.5% of paediatric patients were diagnosed with an extensive colitis based on the Montreal classification [115]. These findings were confirmed by the data from EPIMAD registry. As a matter of fact, Gower Rosseau et al found that paediatric UC is characterized by widespread localization, a high rate of disease extension and a colectomy rate of 20% after 5 years of follow-up [131].

Although this could seem a huge amount of data, as well underlined in a recent systematic review, most of the studies have been conducted in the pre-biologics era, before the widespread use of immunosuppressants and biological therapies [133]. Considering the need to identify therapeutic strategies able to modify the course of disease, it is worth to re-evaluate the natural history of paediatric UC in the last decade to understand if the earlier introduction these more effective drug exerted an effect on the natural history of paediatric UC.

Therefore, the aims, of our paper were to describe UC phenotype at diagnosis, also looking at the prevalence of atypical features, and to characterize the natural history of disease in our large cohort of UC patients. We performed a paper chart review of children and adolescents diagnosed with UC between 2 and 18 years from January 2007 to January 2016 and we finally included in the analysis 111 children.

Among the most innovative findings of our paper, we firstly reported the prevalence of all the atypical phenotypes at diagnosis, based on the recent Porto classification. We

demonstrated that the overall prevalence of atypical phenotypes at diagnosis in paediatric UC is rather high, reaching the 49.5% of our cohort, thus confirming the risk of misdiagnosis. The most recurrent phenotype resulted UGI, with a frequency of 20.5%, while patchy histologic inflammation, or short disease duration, was the second most frequent atypical feature. Not surprisingly, we found a higher prevalence of ASC among the early-onset IBD group (age<6 years), once more underlining severe clinical course of this specific group-age. More generally, the whole paediatric UC confirmed its major severity respect to the adult UC. Indeed, the phenotype of UC in our cohort is well synthetized by the high percentage of pancolitis/extensive colitis at diagnosis (60%), the disease extension during the course of disease (30%) and the early relapse rate (47.7%). We were also able to provide useful insights regarding possible early predictors of a worst outcome of disease. Many recent papers assessed the value of PUCAI at diagnosis and at 3 months, as the best predictor of sustained steroid free remission, ASC and early need for rescue therapy [134-136]. In our cohort, children with an early relapse within the 6th month showed higher values of PUCAI, CRP and faecal calprotectin at diagnosis, which may therefore be used as potential markers to orientate the initial treatment strategies.

Looking at therapeutic strategies, up to 54% of our patients needed to start AZA and very early during the course of disease, with a median time of 7 months. At the maximal follow-up about 8.1% started infliximab. These data confirm that we tend to use more immunosuppressive and biologic drugs and that we use them earlier during the course of disease. Although this aggressive strategy is certainly reflective of the phenotype severity, our findings suggest at least a partial efficacy on the clinical course. Indeed, we reported a surgery rate at 5 and 10 years of 8 and 16%, respectively, which is significantly lower when compared with the early publications and it is in line with the more recent papers. As a matter of fact, looking at the literature we can observe a progressive

decrease of surgery rate, starting from the 26.1% at 5 years reported from Van Limbergen and colleagues in 2008 [115] and arriving to the more recent 8% reported by Malmborg et al. and confirmed by our results [137]. Although it is still unclear whether surgery is merely postponed, immunosuppressive and biologics may have modified the disease course.

In conclusion, we confirmed that paediatric UC represent both a diagnostic and a therapeutic challenge, for the high percentage of atypical features and for the severe clinical course. Nevertheless, the decreased surgery rate observed in the last decade may suggest that an early aggressive approach may be able to modify the natural history of disease.

**The changing face of paediatric ulcerative colitis: a population-based cohort study.**

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**Contributors' Statement**

*Massimo Martinelli* and *Francesca Paola Giugliano* participated equally in this study;

*Massimo Martinelli* contributed to conception and design of the study, collection, analysis and interpretation of data, drafting the article and final approval of the version to be published;

*Francesca Paola Giugliano* contributed to conception and design of the study, collection, analysis and interpretation of data, drafting the article and final approval of the version to be published;

*Marina Russo* contributed to the collection, analysis and interpretation of data, drafting the article and final approval of the version to be published;

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*Laura Perrone* contributed to conception and design of the study, drafting the article and final approval of the version to be published;

*Annamaria Staiano* contributed to conception and design of the study, drafting the article and final approval of the version to be published.

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*Erasmo Miele* contributed to conception and design of the study, drafting the article and final approval of the version to be published.

*Pierluigi Marzuillo* contributed to conception and design of the study, drafting the article and final approval of the version to be published.

*Caterina Strisciuglio* contributed to conception and design of the study, collection, analysis and interpretation of data, drafting the article and final approval of the version to be published.

**Abstract**

**Objectives:** Few paediatric studies described the disease course of Ulcerative Colitis (UC) in the era of immunosuppressive and biologic therapies. The aims of this retrospective study were to describe UC phenotype at diagnosis and to characterize the natural history of disease in a large cohort of children.

**Methods:** This was a retrospective, single-centre study. We reviewed the charts of UC patients diagnosed between 2 and 18 years at our referral centre from January 2007 to January 2016. Laboratory and clinical features at diagnosis, such as disease location, atypical phenotypes, extra-intestinal manifestations (EIMs) and therapies, as well as pattern changes during the follow-up, including relapse rate, disease extension and the cumulative risk for colectomy were collected.

**Results:** One-hundred-eleven patients were identified. Disease location at diagnosis was pancolitis in 36.9% and extensive colitis in 22.5% of patients. Atypical phenotypes were identified at diagnosis in 55 out of 111 patients (49.5%). EIMs were detected in 16 out of 111 (14.4%) at the diagnosis. First relapse rate was 47.7% (53/111) at 6 months and 68.5% (76/111) at 24 months. Disease extension was reported in 21 out of 70 patients (30%). At the last follow-up 60 out of 111 (54%) patients needed to start Azathioprine and 10 out of 111 patients underwent surgery, resulting in a cumulative risk of 8% at 5 years and 16% at 10 years.

**Conclusion:** Paediatric UC is associated with a severe phenotype and a high percentage of atypical features. Surgery rate seems to be decreased from early reports.

**Key words:** Atypical phenotypes, Paediatric, Ulcerative Colitis, Inflammatory Bowel disease.

**What is known**

- The diagnosis of paediatric onset Ulcerative Colitis (UC) may be more challenging due to the existence of atypical phenotypes;
- UC onset during childhood has a different disease pattern and a more aggressive development compared to adult onset.

**What is new**

- This is the largest study assessing the prevalence of UC atypical features at diagnosis, after the publication of the revised Porto criteria;
- Although associated with a higher percentage of atypical features and with a more severe phenotype, we observed a decrease in the surgery rate during the last decade in comparison with the early studies.

## Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and Ulcerative colitis (UC), is a group of life-long chronic and relapsing inflammatory disorders of the gastrointestinal tract whose pathogenesis is still unknown (1,2). Although the incidence and prevalence of IBD in paediatric population differ among countries, the general trend shows an overall increase over the past few decades especially in adolescence and young adulthood (3,4). Differently from most studies reporting a predominance of CD incidence over UC (5), in Italy it has been described an opposite trend, with UC exceeding CD diagnoses (52% versus 40%) (6). UC is typically characterized by a chronic mucosal inflammation, which involves the intestinal tract starting from the rectum and proceeding continuously towards proximal segments. Nevertheless, the diagnosis of paediatric onset UC may be more challenging due to the existence of atypical phenotypes (7). In particular, 6 different atypical UC phenotypes have been recently identified in the revised Porto criteria for the diagnosis of paediatric IBD: rectal sparing, short duration, cecal patch, upper gastrointestinal involvement (UGI), acute severe colitis (ASC) and backwash ileitis (8). The incidence of atypical features has been poorly described. It is well known that childhood-onset UC is characterized by a more extensive intestinal involvement and a more rapid progression when compared to adult UC, including a higher risk of corticosteroids dependency, an earlier immunosuppressive therapy introduction and surgery occurrence (9,10). In 2009 Gower-Rosseau et al. reported the data from EPIMAD registry and found that paediatric UC is characterized by widespread localization, a high rate of disease extension and a colectomy rate of 20% after 5 years of follow-up (11). However, as well stated in a recent systematic review (12), limited studies are available in the era of immunosuppressants and biological therapies. More recent data are needed in order to evaluate the impact of new treatment strategies on the natural history of paediatric UC. Therefore, the aims of this retrospective study were to describe UC phenotype at

diagnosis and to characterize the natural history of disease in a large cohort of paediatric patients.

## Materials and Methods

This was a retrospective, single-centre study. We performed a paper chart review of children and adolescents diagnosed with UC between 2 and 18 years at the paediatric IBD referral centre of the Department of Translational Medical Science, University of Naples "Federico II" from January 2007 to January 2016. The inclusion criteria were: a confirmed diagnosis of UC; age at diagnosis≤18 years; a clinical follow-up of at least 12 months. We excluded from the analysis children affected by CD and IBD-U and patients with an initial diagnosis of UC, switching to CD during the follow-up. The diagnosis of UC was established on the basis of clinical, endoscopic, radiological and histological criteria according to the Porto criteria until 2014 (13). Revised Porto criteria were used after their publication in June 2014 (8). At diagnosis, all patients underwent colonoscopy with mucosal biopsies and small bowel imaging, including abdominal ultrasound and MRI or small bowel follow through. Not all, but the majority of the patients was also investigated with upper gastrointestinal endoscopy. The period between the first onset of IBD symptoms and diagnosis date was defined as diagnostic delay. Patients with a diagnosis at an age ≤6 years were defined as early-onset (EO) IBD, as previously reported (14). For the purpose of this manuscript, disease location was characterized on the basis of Paris classification (15). Disease extension was defined as the endoscopic and histologic involvement of at least one additional segment during the follow-up. In addition, we retrospectively identified at diagnosis the following atypical phenotypes on the basis of the revised Porto criteria (8): rectal sparing, defined as the absence of macroscopic disease in rectum or recto-sigmoid; short duration disease, characterized by patchy disease in

biopsies or lack of typical architectural distortion in pathological specimens; cecal patch, represented by the presence of left-sided colitis with an area of cecal inflammation; UGI, characterized by erosions or small ulcers in stomach, neither serpiginous nor linear; ASC, defined as disease contiguous from the rectum and trans-mural inflammation with deep ulcers; backwash ileitis, represented by macroscopic, short segment, non stenosing terminal ileitis, without granulomata (8). Clinical activity of the disease was evaluated at time of diagnosis using the Paediatric Ulcerative Colitis Activity Index (PUCAI) with the previously validated cut-off values for remission (<10 points), mild disease (<35), moderate disease (<65) and severe disease (≥65)(16). In addition, timing of first clinical relapse, defined as the occurrence or worsening of symptoms accompanied by an increase of PUCAI >10 points, was also recorded. A relapse occurring within 6 months from the diagnosis was defined as an early relapse. Family history was defined as positive if at least one first-degree relative was diagnosed with IBD. Symptoms and laboratory parameters at diagnosis including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), haemoglobin concentration, platelets count and faecal calprotectin were also collected. Extra-intestinal manifestations (EIMs) at diagnosis included skin, joints, ocular manifestations, pancreatic involvement and primary sclerosing cholangitis. Two expert paediatric gastroenterologists (AS and EM) made all decisions regarding therapeutic interventions, in line with the validated international guidelines (17). The followed strategy was a classical step-up approach. Oral steroid treatment *(oral methylprednisolone: 1mg/kg/day, max 40 mg/day per 4 weeks)* was generally used as induction therapy in patients with moderate to severe disease. Steroid dependency was defined as remission with corticosteroids but recurrence of symptoms when the dose was lowered or within 3 months following complete taper, or if steroids couldn't be stopped within 14 to 16 weeks; steroid refractoriness was defined as a non-response at oral steroids within 7-14 days (17). Aminosalicylates (5-ASA) *(mesalazine 50 mg/kg/day)* were used as induction

therapy in patients with mild to moderate disease and as a standard maintenance therapy. Patients in whom induction therapy had failed or patients with early relapse were treated with azathioprine (AZA) *(2 to 2.5mg /kg/day)*. Methotrexate (MTX) was used as second-line immunosuppressant in those patients intolerant or refractory to AZA. Infliximab (*5mg/kg/dose at weeks 0, 2, and 6 and then 8 weekly*) was given as first biologic agent in patients refractory or intolerant to steroids and immunomodulators. Patients refractory or intolerant to Infliximab therapy were treated with Adalimumab (*Loading dose: 160/80 or 80/40 mg at weeks 0 and 2 respectively in patients weighing ≥ 40 kg or <40 Kg; Maintenance dose: 80 and 40 mg every two weeks respectively in patients weighing ≥ 40 kg or <40 Kg*). Surgery was taken into considerations in patients refractory to medical therapy. In line with UC guidelines, subtotal colectomy with ileo-anal pouch anastomosis in 2-3 stages, was considered the preferred option (17).

***Statistical Analysis***

Variables were screened for their distribution, and appropriate parametric or non-parametric tests were adopted as necessary. The Student's t-test, the ANOVA test and the Mann-Whitney test for continuous variables and the $\chi^2$ and Fisher's exact tests for categorical variables were used where appropriate. The Kaplan-Meier method was used to estimate the cumulative risk of surgery at 1, 2 and 5 and 10 years from the diagnosis. Multivariate conditional logistic regression analysis was used to explore the odds associated with the risk of surgery. Surgery was used as dependent variable, while the effect of all the parameters was analyzed by a stepwise procedure. Statistical significance was predetermined as $p<0.05$. Percentages were rounded to the nearest whole numbers. SPSS version 15 was used for all statistical analyses.

### *Ethical Considerations*

The Institutional Review Board of the University of Naples "Federico II" approved the study protocol with the registration number 176/16. Written, informed consent was obtained from all parents and also from children, where appropriate.

## Results

### *Demographic and clinical data at diagnosis*

One-hundred-eleven patients with a confirmed diagnosis of UC were identified between January 2007 and January 2016 (median age: 16 yrs; range 6-27.9; M/F: 50/61). Median age at diagnosis was 11.9 years (range 2.1-17.5 yrs) and 23 out of 111 (20.7%) UC patients had an EO-disease (age<6 yrs) (Table 1). Median time between onset of symptoms and diagnosis was 3 months (range: 0-80), and 34.2% (n= 38) of children had a diagnostic delay exceeding 6 months. Family history was identified in 17 out of 111 (15.3%). The main symptoms at diagnosis are showed in Table 1. EIMs were detected in 16 out of 111 (14.4%) at the diagnosis: skin involvement (n=2; 1.8%), axial arthropathies and peripheral arthritis/arthralgia (n=3; 2.7%), pancreatic involvement (n=6; 5.4%) and primary sclerosing cholangitis (n=5; 4.5%). Median PUCAI score at diagnosis was 40 (range: 15-75). On the basis of PUCAI score, 34.2% (n=38) of patients presented with mild disease, 62.2% (n=69) with moderate and 3.6% (n=4) with severe disease. Disease location at diagnosis was pancolitis (E4) in 41 out of 111 patients (36.9%), extensive colitis (E3) in 22.5% (n=25), left-sided colitis (E2) in 18.9% (n=21) and proctosigmoiditis (E1) in 21.6% of patients (n=24) (Table 1) (Figure 1). The presence of pancolitis was significantly associated with a younger age of the patients at the diagnosis (9.9 yrs vs 11.7 yrs; p=0.01). Atypical phenotypes were identified at diagnosis in 55 out of 111 patients

(49.5%). A patchy colonic inflammation, defined as short duration disease, was found in 22 out of 111 patients (19.8%). At diagnosis only 68 out of 111 (61.2) underwent upper gastrointestinal endoscopy and among them 20.5% (14/68) presented with UGI. Cecal patch was identified in 7/111 (6.3%) patients and rectal sparing in 5/111 (4.5%). Four out of 111 (3.6%) patients presented with ASC and 3/111 (2.7%) with backwash ileitis (Table 1). Regarding induction therapy at the diagnosis, 54.1% (n=60) received oral or intravenous corticosteroids, while 92 patients (82.9%) started 5-ASA or sulfasalazine. Twenty-five out 111 children (22.5%) needed a treatment with topical steroids and 24/111 (21.6%) with topic mesalazine. Seventeen out of 111 patients (15.3%) started AZA as maintenance therapy from the diagnosis (Table 1).

***Data at the follow-up***

Median follow-up was 51 months (range 12-185 months). The rate of 1$^{st}$ relapse from the diagnosis was: 24.3% (27/111) at 3 months, 47.7% (53/111) at 6 months, 61.3% (68/111) at 12 months and 68.5% (76/111) at 24 months. The median time from the 1$^{st}$ relapse was 6 months (range 1-110). When compared with the remaining patients, children with an early relapse within 6 months showed higher values of PUCAI (Mean ± SD: 42±13.3 vs 36±12.7; p=0.02), CRP (Mean ± SD: 107.1±294 vs 55.8±222.7; p=0.001) and faecal calprotectin (Mean ± SD: 662±760 vs 432±451; p=0.01) at diagnosis.

Disease extension at maximal follow-up was reported in 21 out of 70 patients (30%). Among the patients with E1 at diagnosis 4.1% (n=1), 8.3% (n=2) and 20% (n=5) progressed to E2, E3 and E4, respectively; among children with an initial E2 location 14.2% (n=3) advanced to E3 and 23.8% (n=5) to E4; among UC children with E3 at diagnosis 20% (n=5) progressed to E4. At maximal follow up, disease location was E4 in 45 out of 111 patients (40%), E3 in 12.6% (n=14), left-sided colitis (E2) in 8.1% (n=9) and E1 in 18.9% of patients (n=21); only 5 patients were in remission of disease (Figure 1).

During the follow-up steroid-dependency was 23.4% and steroid-refractoriness 7.2%. At the last follow-up 60 out of 111 (54%) patients needed to start AZA therapy; 13.3% (n=8) of these patients needed to switch to MTX. The median time to start immunosuppressive drugs was 7 months (range 0-110 months). Nine out of 111 patients (8.1%) started Infliximab. Two of these 9 children (22.2%) switched to Adalimumab. At the maximal follow-up, 10 out of 111 patients underwent surgery, resulting in a crude colectomy rate of 9%. The cumulative probability of surgery was 0.03 (95% CI: 0.02–0.28) at 1 year, 0.07 (95% CI: 0.06–0.211) at 2 years, 0.08 (95% CI: 0.07–0.2) at 5 years and 0.16 (95% CI: 0.1–0.3) at 10 years from time of diagnosis (Figure 2).

***Early onset versus late-onset disease***

The presence of EIMs at diagnosis was significantly higher in the EO group when compared with late-onset UC [8/23 (34.7%) vs 8/88 (9%); p=0.003]. A higher number of patients with EO-IBD needed to have induction of remission with oral or intravenous steroids respect to children with late-onset IBD [9/23 (78.2%) vs 47/88 (56.6%); p=0.03]. ASC resulted to be significantly higher in EO group than in late-onset UC children [3/23 (13%) vs 1/88 (0.9%); p=0.02]. With regards to laboratory parameters at diagnosis, EO-IBD children showed significant decreased values of haemoglobin and a significant higher platelets' count when compared with late-onset group (Mean ± SD: 10.9±2.2 vs 11.9±1.7, p=0.03; 525±179 vs 354±116, p=0.002, respectively). None of other variables including gender, PUCAI at diagnosis, family history for IBD, diagnostic delay>6 moths, disease location, CRP, ESR, fecal calprotectin, disease extension and therapy was significantly different between the two groups.

***Predictive factors for surgery***

Patients undergoing surgery had significantly higher values of PUCAI score and decreased haemoglobin values at diagnosis when compared to the remaining children (Mean±SD: 48.7±12.1 vs 37.6±12.5, p=0.01; 10.1±1.8 vs 11.9±1.8, p=0.001, respectively). At univariate analysis, PUCAI≥35 at diagnosis [(Odds Ratio (OR)=1.6], need to start AZA at diagnosis (OR=4.5), first relapse within the first 6 months (OR=4.9), induction therapy with steroids at diagnosis (OR= 7.7), steroid refractoriness (OR=23.7), need to start MTX (OR=94) and biologic therapy (OR=164) during the follow-up were associated with an increased risk of colectomy (Table 2). At the multivariate analysis, the only variables that resulted independently associated with an increased risk of surgery were steroid refractoriness (OR=47.9) and the need of biological therapy during the follow-up (OR=236.5) (Table 2).

**Discussion**

The present retrospective study describes the clinical features at diagnosis and the disease course of paediatric UC over the last 10 years at a referral centre in Southern Italy. To the best of our knowledge this is the largest study assessing the prevalence of atypical features at diagnosis, after the publication of the revised Porto criteria. Although confirming that paediatric UC is associated with a more severe phenotype, we demonstrated a significant reduction of surgery rate at 5 and 10 years when compared with early reports.

As finally stated in the revised Porto criteria (8), atypical phenotypes represent a peculiarity of paediatric UC and are associated with relevant diagnostic challenges, being often responsible of IBD-U diagnosis (18). Nevertheless, few studies have assessed their actual prevalence. Earlier in 2013, the Eurokids registry study tried to give an answer to this question (7). However, these data were published before the proper classification of Porto criteria and the authors only investigated some of these phenotypes, including UGI, rectal sparing and backwash ileitis (7). In our study population, we demonstrated that the overall prevalence of atypical phenotypes at diagnosis in paediatric UC is rather high, reaching the 49.5% of our cohort. The most recurrent phenotype resulted UGI, with a frequency of 20.5%. Levine et al. found that 4.2% of UC children presented UGI (7). Previously, Tobin and colleagues described histological gastritis in up to 50% of UC children (19). Not all our population was screened at diagnosis with an Upper GI endoscopy and in addition we do not routinely repeat it during the follow-up in UC patients, unless symptomatic. Therefore, larger prospective studies are needed to establish the effective incidence of UGI in paediatric UC and clarify the need for further upper gastrointestinal control endoscopies. Patchy histologic inflammation, or short disease duration, was the second most frequent atypical feature. Short disease duration seems to

be the result of an early diagnosis, immediately after the starting of symptoms (8, 20). Our prevalence of 19.8% is in agreement with the previous data from Aloi et al. (21). Regarding the association of UC atypical features with other clinical or laboratory variables, not surprisingly we found a higher frequency of ASC among EO-IBD children. Differently from Eurokids data, although rectal sparing was higher in EO-IBD group, the significance was not reached (7).

It is well known that UC onset during childhood has a different disease pattern and a more aggressive development compared to adult onset (22, 23). Already in 2008, Van Limbergen and colleagues comparing a cohort of children with UC with an adult population demonstrated that 74.5% of paediatric patients were diagnosed with an extensive colitis based on the Montreal classification (10). These data were even worst at the maximal follow-up (82.2%) and significantly higher than adult UC patients (47%) (10). The largest paediatric cohort data using Paris classification derives again from Eurokids registry (7). The authors reported that almost 78% of the enrolled children were affected by extensive colitis or pancolitis (7). The severity of UC paediatric phenotype was confirmed in our cohort, as demonstrated by the high percentage of pancolitis/extensive colitis at diagnosis (60%), the disease extension during the course of disease (30%) and the early relapse rate (47.7%). Furthermore, our study provides useful insights regarding possible early predictors of a worst outcome of disease. The identification of markers able to predict the disease course is strongly needed in order to stratify patients and orientate the treatment strategies. Many recent papers assessed the value of PUCAI at diagnosis and at 3 months as the best predictor of sustained steroid free remission, ASC and early need for rescue therapy (24-26). In our cohort, children with an early relapse within the 6th month showed higher values of PUCAI, CRP and fecal calprotectin at diagnosis. In addition, as previously reported the presence of pancolitis was significantly associated with a younger age of the patients and it was even more common among the 23 UC children with EO-UC

(age<6 years) with a frequency at diagnosis >40% (21, 27). Another marker of phenotype severity is usually represented by EIMs, which have been reported over a broad range from 3 to 30% (28, 29). Aloi and colleagues more recently demonstrated in another Italian paediatric UC cohort that EIMs might be detected in at least the 16% of patients at diagnosis (27). In our population EIMs reach the percentage of around 14.4%, confirming the previous data. In addition, the majority of children with EIMs at diagnosis were detected within the EO-IBD group. This finding is in line with the consolidated hypothesis that EO-IBD is usually associated with a more severe phenotype, including the possible involvement of other organs and systems (21, 30).

In accordance with the current therapeutic management of paediatric UC (17), the majority of our children received 5-ASA both as first-line therapy for induction and maintenance of remission in children with mild to moderate UC, while 54.1% of children needed to receive a cycle of steroids for the induction of remission. As reported in other cohorts (31), up to 54% of children needed to start AZA and very early during the course of disease, with a median time of 7 months. These data are strikingly different from the initial percentages reported two decades ago from Hyams et al. (9), and it is reflective of what it was anticipated from the Scottish cohort and from the EPIMAD registry (10, 11). We use more immunosuppressive and biologics drugs and we use them earlier. This tendency to introduce earlier immunosuppressive drugs may be partly responsible of the decreased risk of surgery, more recently reported (32). In 2008 and in 2009, Van Limbergen et al. and Gower-Rosseau and colleagues described a cumulative risk of surgery of 26.1% and 20% at 5 years, respectively (10, 11). In 2013 Aloi et al reported a cumulative risk at 5 years of 14% (27), while more recently in a Swedish cohort, Malmborg and colleagues found that the risk of surgery was only 8% at 5 years (33). Data of our study-population are perfectly in line with this more recent literature with a crude colectomy rate of 9%. The Kaplan-Meyer analysis confirmed that the cumulative risk of surgery at 5 and 10 years

were only 8% and 16%, respectively. These data clearly confirm a decreased surgery rates when comparing with the earlier studies. One could question whether surgery is merely postponed or if immunosuppressive drugs may actually modify the course of disease. With regards to the factors associated with the risk of surgery, previous studies identified EIMs and disease extension as the main risk factors (11, 12). At the multivariate analysis, the need to start biologic therapy and the steroid-refractoriness, resulted the most important predictive factors for surgery in our cohort. These data are strongly reflective of our therapeutic strategy. Indeed, although introducing earlier immunosuppressive drugs, we tend to use biologics only in those patients with non-response to steroids and conventional immunosuppressive drugs. Therefore, these associations may probably represent the selection of more aggressive phenotypes.

It is acknowledged that this study is not without limitations. The main limitation is of course related to the retrospective nature, and therefore the concrete possibility of recall biases needs to be taken into account. In addition, this was a single-center study. The main strength lies in the availability of detailed medical records of a well-defined large cohort of paediatric patients with UC with a median follow-up duration of 4 years.

**Conclusions**

In conclusion this retrospective, single-centre study in Southern Italy confirmed that Paediatric UC represent both a diagnostic and a therapeutic challenge. Although associated with a higher percentage of atypical features and with a more severe phenotype, we observed a decrease in the surgery rate at 5 and 10 years during the last decade. Further prospective, multicentre, longitudinal studies, are needed to clarify whether this more aggressive medical strategy is able to modify the natural history of paediatric UC.

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**Table 1. Baseline characteristics of 111 UC children at the time of diagnosis**

| | |
|---|---|
| Median age at enrolment (yrs, range) | 16.5 (6-27.9) |
| Median age at diagnosis (yrs, range) | 12 (2.1-17.5) |
| Gender (n, %) | |
| *Male* | 51 (45) |
| *Female* | 60 (55) |
| Median diagnostic delay (mths, range) | 3 (0-80) |
| Diagnostic delay>6 months (n, %) | 38 (34.2) |
| Early onset (<6 yrs) (n, %) | 23 (20.7) |
| Family History (n, %) | 17 (15.3) |
| Symptoms (n, %) | |
| *Abdominal Pain* | 80 (72.1) |
| *Bloody diarrhea* | 70 (63.1) |
| *Rectal bleeding* | 32 (28.8) |
| *Tenesmus* | 22 (19.8) |
| *Weight loss* | 32 (28.8) |
| Extraintestinal Manifestations (n, %) | 16 (14.4) |
| Median PUCAI (range) | 40 (15-75) |
| Paris Classification at diagnosis (n,%) | |
| *Proctosigmoiditis (E1)* | 24 (21.6) |
| *Left-sided colitis (E2)* | 21 (18.9) |
| *Extensive colitis (E3)* | 25 (22.5) |
| *Pancolitis (E4)* | 41 (36.9) |
| Atypical phenotypes (n, %) | |
| *Acute severe colitis* | 4 (3.6) |
| *Backwash Ileitis* | 3 (2.7) |
| *Cecal patch* | 7 (6.3) |
| *Rectal Sparing* | 5 (4.5) |
| *Short duration disease* | 22 (19.8) |
| *UGI* | 14/68 (20.5) |
| Induction therapy at diagnosis (n, %) | |
| *Steroids* | 60 (54.1) |
| *Mesalazine* | 92 (82.9) |
| *Immunosuppressants* | 17 (15.3) |
| *Topic steroids* | 25 (22.5) |
| *Topic mesalazine* | 24 (21.6) |

UC: Ulcerative Colitis; UGI: Upper Gastrointestinal Involvment

**Table 2. Risk factors associated with colectomy in 111 children with a diagnosis of Ulcerative Colitis at univariate and multivariate analysis.**

| Variables | OR | 95% CI | p |
|---|---|---|---|
| ***Univariate analysis*** | | | |
| PUCAI≥35 at diagnosis | 1.6 | 1.3-1.9 | 0.01 |
| Azathioprine therapy at diagnosis | 4.5 | 1.1-18.1 | 0.03 |
| Early relapse<6 months | 4.9 | 1-24.6 | 0.04 |
| Steroids therapy at diagnosis | 7.7 | 0.9-62 | 0.02 |
| Steroid refractoriness | 23.7 | 3.3-170 | 0.004 |
| Need to start Methotrexate therapy | 94 | 13.1-674 | <0.001 |
| Need to start biologic therapy | 164 | 20-1350 | <0.001 |
| ***Multivariate analysis*** | | | |
| Steroid refractoriness | 47.9 | 2.2-1009 | 0.01 |
| Need to start biologic therapy | 236.5 | 20-3011 | <0.001 |

95%CI: 95% Confidence Intervals

**Figures legend**

**Figure 1.** Disease location according to the Paris classification at diagnosis and at maximal follow-up in 111 children affected by Ulcerative Colitis.

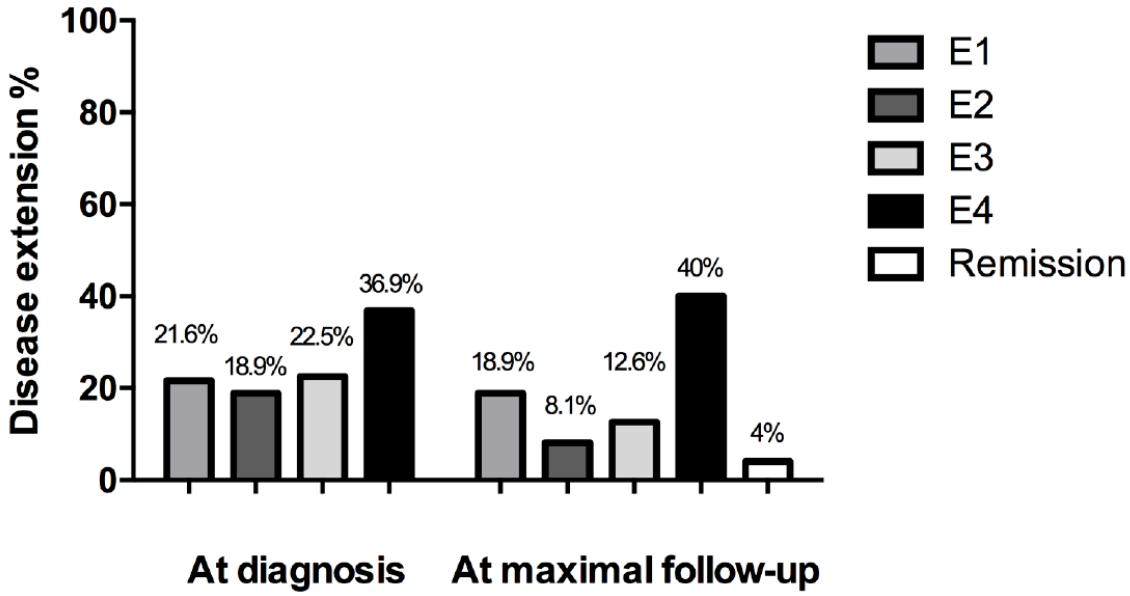
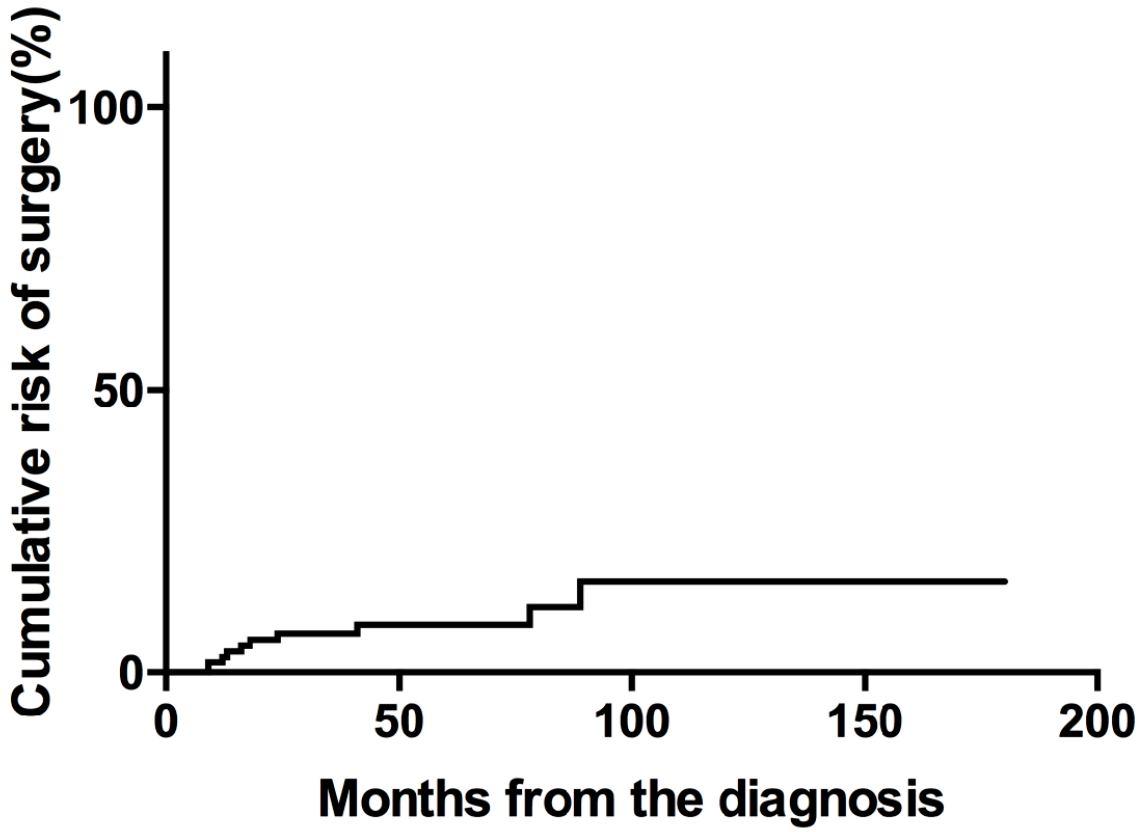


**Figure 2.** Cumulative risk of surgery in 111 children affected by Ulcerative Colitis. The cumulative probability of surgery was 0.03 (95% CI: 0.02–0.28) at 1 year, 0.07 (95% CI: 0.06–0.211) at 2 years, 0.08 (95% CI: 0.07–0.2) at 5 years and 0.16 (95% CI: 0.1–0.3) at 10 years from time of diagnosis.



## CHAPTER 4

## GASTROINTESTINAL INFECTIONS AND PAEDIATRIC IBD

The management of GI infections in paediatric IBD is still considered a hot topic, being crucial both at the disease onset and during the follow up for differential diagnosis and therapeutic issues [138]. Furthermore, it is still unclear whether or not GI infection may trigger the disease and act as a player in IBD pathogenesis [139].

As a matter fact within the revised Porto criteria for IBD diagnosis [37], the following concepts are mainly stressed:

- In children with suspected IBD, enteric infections should be excluded as cause of the symptoms preferentially before endoscopy is performed;
- The search for bacterial infections should include a stool culture to exclude *Salmonella, Shigella, Yersinia, Campylobacter, and C. difficile* toxins in all of the children. Screening for enteric viruses is rarely helpful, while testing for *Giardia lamblia* is recommended in high-risk populations or endemic areas;
- The identification of a pathogen does not necessarily exclude a diagnosis of IBD, since a first episode or flare of IBD may be triggered by a documented enteric infection.

The last point is mainly correlated to the concrete risk of a delayed diagnosis [37].

We will revise this topic within this chapter through the description of the paper ***"Clostridium difficile and pediatric Inflammatory Bowel Disease: a prospective, comparative, multicenter, ESPGHAN study"*** published in *Inflammatory Bowel Disease (Inflamm Bowel Dis. 2014; 20:2219-25)* and the letter to the editor ***"Yersinia Enterocolitica Ileitis Mimicking Pediatric Crohn's Disease"*** also recently published in *Inflammatory Bowel Disease (Inflamm Bowel Dis. 2017; 23: E15-E16).*

### 4.1 The relationship between *Clostridium difficile* infection and paediatric IBD

*C difficile* is a gram-positive, spore-forming obligate anaerobe that causes a spectrum of clinical presentations. Symptoms of *C difficile* infection can range from mild, self-limited diarrhoea to severe colitis with cramping, haematochezia, pseudo-membrane formation, and intestinal perforation [140]. Despite considerable advances in understanding the epidemiology, immunology, and pathogenesis, *C. difficile* is still the most frequent cause of nosocomial bacterial infectious diarrhoea in developed countries and it has grown in frequency and severity over the three decades. In the last 10 years the epidemiology of *C. difficile* infection has profoundly changed. Next to the classical risk groups, including patients treated with broad-spectrum antibiotics, hospitalization, oncologic and severely immunocompromised children, new factors favouring the infection came out [140]. Among those, being affected by IBD has been widely reported to increase the risk of *C. difficile* infection both in adults and children [139, 141-147]. However, the prevalence of *C. difficile* infection among children with IBD is still unclear due to the variable data reported, ranging from 3.5% to 69% [139, 146-150]. The increased risk of infection is not believed to be due to the frequent use of immunosuppressive agents and hospital based-services, but also to IBD itself [151].

The management of *C. difficile* during the IBD natural history encompasses different phases, being associated with IBD in several ways, including triggering disease flares, sustaining activity, and in some cases, acting as an “innocent” bystander. This usually makes things complicated for the clinician. Indeed, when a flaring IBD patient is found to be infected with *C. difficile*, the therapeutic dilemma is whether to withhold immunosuppression or alternatively to administer antibiotics along with intensified immunosuppressant therapy to treat a possible concurrent IBD exacerbation [152]. Furthermore, some reports also underlined that IBD course may substantially deteriorate, after *C. difficile* infection [153, 154]. In order to answer some of these questions and to collect

a higher number of cases, we performed a prospective, multicentre study, involving 8 different ESPGHAN centres.

The primary aim of this study was to investigate the occurrence of *C. difficile* infection in paediatric patients with IBD and to compare with a group of children affected by another GI pathology, such as celiac disease; the secondary aim of the study was to evaluate the natural history and the disease course of *C. difficile* infected IBD children. Between October 2010 and October 2011, the study group enrolled 211 children affected by IBD and 112 celiac children. All the children were tested for the presence of *C. difficile* toxins A and B in their stools. IBD group had a further stool collection after 6 and 12 months from the enrolment.

Examining the results, we were able to observe a whole epidemiologic picture of *C. difficile* infection in IBD children in Europe and Israel. The occurrence of *C. difficile* at the enrolment in the IBD group was 7.5%, while the overall 1-year occurrence, considering patients completing the follow-up, was 20.1%. As expected the occurrence in IBD was significantly higher than celiac children (0.8%). We decided to test patients with celiac disease as control group, to verify whether another gastrointestinal pathology, with described microbiota alterations [155], may confer a higher susceptibility to *C. difficile* infection. This finding confirms that the increased risk is specifically related to the IBD. As a further validation of this hypothesis, the classical *C. difficile* risk factors, including antibiotic therapy, immunomodulators and hospitalization did not result to be associated with the infection. On the contrary, the infection seemed to be more community acquired. Among the risk factors IBD related, in agreement with the literature, we found that the colonic involvement was highly prevalent in children with *C. difficile* and that there is no specific type of IBD predisposing to the infection [156].

Not surprisingly we found a huge variation of *C. difficile* infected patients among the different countries, ranging from 0 to 24.1%. Despite these variations, we assisted to all

the possible *C. difficile* clinical manifestations. *C. difficile* was diagnosed in a high percentage of cases at disease onset, as previously described by Kelsen et al [147]. This result underlines the possibility of a diagnostic delay and once more addresses the question of a possible role of *C. difficile* as disease trigger. During the follow up, *C. difficile* was significantly associated with an active disease, confirming the relevance of testing it during IBD flares. On the other side of the coin, we also reported a non-negligible number of cases showing an asymptomatic carriage of *C. difficile*. The significance of asymptomatic finding of *C. difficile* toxins in the stools and the risk of a subsequent relapse is still unclear in literature [157]. Nevertheless, our study may provide useful insights. Indeed, to our knowledge this represents the first prospective multicentre study, addressing the follow-up of *C. difficile* infected IBD children. We found that *C. difficile*–positive patients at the enrolment, including those with an asymptomatic carriage, showed a substantial risk for exacerbated disease course, as demonstrated by the need for immunosuppressant escalation, the higher frequency of active disease, the increased colectomy rate, and the higher number of hospitalization at 6 months. Differently from previous studies [146], we found a high success rate of *C. difficile* eradication (86.6%) after treatment. Nevertheless, as reported above, in most of the cases, the eradication of the infection did not prevent a subsequent complicated IBD course, suggesting that *C. difficile* may act more as a relapse trigger rather than a “leading actor” of the symptoms. Another possibility is that *C. difficile* detection may be an expression of significant dysbiosis, and therefore, it may be interpreted as an indicator of severe disease course.

In conclusion, our results show the whole picture of the vexed relationship between *C. difficile* and IBD. Paediatric IBD confers a specific risk of *C. difficile* infection, probably linked to its dysbiosis. *C. difficile* positive toxins may be encountered at onset of disease, during the IBD course, in both symptomatic and asymptomatic patients. It is therefore worth testing in any case, since it seems that *C. difficile* detection may complicate the

natural history of disease or it may simply represent the microbial signature of a worst outcome.

# *Clostridium difficile* and Pediatric Inflammatory Bowel Disease: A Prospective, Comparative, Multicenter, ESPGHAN Study

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**Background:** *Clostridium difficile* infection is associated with pediatric inflammatory bowel disease (IBD) in several ways. We sought to investigate *C. difficile* infection in pediatric patients with IBD in comparison with a group of children with celiac disease and to evaluate IBD disease course of *C. difficile* infected patients.

**Methods:** In this prospective, comparative, multicenter study, 211 pediatric patients with IBD were enrolled from October 2010 to October 2011 and tested for the presence of *C. difficile* toxins A and B in their stools at 0, 6, and 12 months. During the same study period, stool specimens for *C. difficile* toxins analysis were collected from 112 children with celiac disease as controls.

**Results:** *Clostridium difficile* occurrence was significantly higher in patients with IBD compared with patients with celiac disease (7.5% versus 0.8%; $P = 0.008$). *Clostridium difficile* was associated with active disease in 71.4% of patients with IBD ($P = 0.01$). Colonic involvement was found in 85.7% of patients with *C. difficile*. Antibiotics, proton pump inhibitors, hospitalization, and IBD therapies were not associated with increased *C. difficile* detection. At 12 months, a higher number of *C. difficile*–positive patients at the enrollment started immunosuppressant/biological therapy compared with patients without *C. difficile* ($P = 0.01$). At 6 and 12 months, patients with *C. difficile* were more frequently in active disease than patients without *C. difficile* ($P = 0.04$; $P = 0.08$, respectively). Hospitalizations were higher at 6 months in *C. difficile* group ($P = 0.05$).

**Conclusions:** In conclusion, this study demonstrates that pediatric IBD is associated with increased *C. difficile* detection. Patients with *C. difficile* tend to have active colonic disease and a more severe disease course.

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**Key Words:** *Clostridium difficile*, Crohn's disease, ulcerative colitis

Despite considerable advances in understanding the epidemiology, immunology, and pathogenesis, *Clostridium difficile* (*C. difficile*) infection is still the most frequent cause of nosocomial bacterial infectious diarrhea in developed countries, and it has grown in frequency and severity over the 3 decades that has passed since the identification of the organism as a pathogen.[1] Recent changes in the epidemiology of *C. difficile* infection include the identification of adult and pediatric patients with inflammatory bowel disease (IBD) as a group at risk in comparison with the general population.[2–5] The prevalence of *C. difficile* infection in pediatric patients with IBD is reported over a broad range from 3.5% to 69%.[6–11] It also seems that antibiotic exposure, a uniformly identified risk factor in the general population, is frequently absent in patients with IBD infected with *C. difficile*. The increased risk of infection is believed to be due not only to the frequent use of immunosuppressive agents and hospital based-services but also

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to IBD itself. However, whether the underlying inflammatory disease process per se confers an additional risk of infection in patients with IBD is still unclear.[12] An additional concern is that *C. difficile* infection in patients with IBD seems to be mostly community-acquired.[6–9] *Clostridium difficile* may associate with the course of IBD in several ways, including triggering disease flares, sustaining activity, and in some cases, acting as an "innocent" bystander.[13] Therefore, when a flaring patient with IBD is found to be infected with *C. difficile*, the clinician usually faces a therapeutic dilemma whether to withhold immunosuppression or alternatively to administer antibiotics along with intensified immunosuppressant therapy to treat a possible concurrent IBD exacerbation.[14] The complexity of this situation has increased with recent reports showing that IBD may deteriorate after *C. difficile* infection.[8,10,15,16] Most of our current knowledge stems from studies in adults with numerous confounding factors. Despite the association with pediatric IBD, data regarding the health care burden related to *C. difficile* infection in children with IBD are limited to few studies. The primary aim of this study was to investigate the occurrence of *C. difficile* infection in pediatric patients with IBD and to compare with a group of children affected by celiac disease; secondary aim of this study was to evaluate natural history and disease course of *C. difficile* infection in pediatric patients with IBD.

## METHODS

### Patient Population

We conducted a prospective, comparative, multicenter study in pediatric inpatients and outpatients with a diagnosis of IBD. Patients affected by Crohn's disease (CD) or ulcerative colitis (UC) were consecutively enrolled between October 2010 and October 2011 and tested for the presence of *C. difficile* toxins A and B in their stools. During the same study period, stool samples for testing *C. difficile* toxins A and B were also obtained from a control group of children, admitted to the outpatient clinic of the enrolling centers, with a diagnosis of celiac disease. Patients with IBD were followed as part of normal follow-up necessary to control the underlying disease and stool samples for detection of *C. difficile* toxins A and B were further examined after 6 and 12 months. The control patients enrolled delivered stool samples for detection of toxins A and B to *C. difficile* only at the time of the enrollment and if negative no more. Eight tertiary care sites from 5 countries participated in this study: the Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Italy; Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, Rome, Italy; Department of Pediatric Gastroenterology, Hepatology and Nutrition, Hospital San Joan De Deu, Barcelona, Spain; Pediatric Gastroenterology and Nutrition Unit, Wolfson Medical Center, Tel Aviv University, Tel Aviv, Israel; Shaare Zedek Medical Center, The Hebrew University of Jerusalem, Jerusalem, Israel; Children's Hospital, Zagreb, Croatia; Department of Pediatrics, Hvidovre University Hospital, Hvidovre, Denmark; and Semmelweis University, Budapest, Hungary. For all children data on age and sex, duration of bowel disorder, consistency of feces, presence of abdominal pain or fever (>38°C), and antibiotic treatments 30 days before the stool collection were recorded. Data were also obtained about use of proton pump inhibitors (PPIs) and hospitalization within the preceding 2 months before *C. difficile* toxin collection. For the IBD group, type of IBD, anatomical distribution of disease, symptoms, disease activity, and treatments, including surgery, were recorded at each time point. The diagnosis of IBD was established on the basis of clinical, endoscopic, radiological, and histological criteria according to the Porto criteria.[17] For the purpose of this article, disease location was described according to Paris classification.[18] Disease activity was scored by the Pediatric Crohn's Disease Activity Index or the Pediatric Ulcerative Colitis Activity Index for CD and UC, respectively.[19,20] Exclusion criteria from the study were age: ≤2 years or >18 years; patients with inflammatory bowel disease unclassified; inability or unwillingness to give informed consent. Children with *C. difficile* toxins in the stools and symptoms suggestive of *C. difficile* infection were treated according to the physicians' discretion. The treatment for each patient was recorded. Asymptomatic patients with a positive test were not treated. *Clostridium difficile* recurrence was defined as a stool study positive within 60 days of the previous infection, with at least 1 stool test negative for *C. difficile* between the 2 infections. All parents or guardians signed a consent form indicating their awareness of the investigational nature and possible risks of this study. Where appropriate, we also obtained children's assent. The study was approved by the institutional review board of each involved center.

### Microbiological Methods

Each laboratory of the units involved in the study, performed the immunoenzymatic test, routinely used. *Clostridium difficile* toxins A and B enzyme immunocard (Meridian Bioscience, Cincinnati, OH) was used in Hvidovre, Naples, Rome, and Zagreb. This qualitative, horizontal-flow enzyme immunoassay has a sensitivity of 83 ± 6.7% and a specificity of 95 ± 1.6%. *C. diff* Quik Chek Complete (TechLab, Blacksburg, VA) was performed in Barcelona, Budapest, Jerusalem, and Tel Aviv. This assay, comprising a dual rapid membrane enzyme immunoassay for toxins A and B and for glutamate dehydrogenase antigen, has a sensitivity of 87.8% and a specificity of 99.4%. The assays were performed according to the manufacturer's instructions.

### Statistical Analysis

Variables were screened for their distribution, and appropriate parametric or nonparametric tests were adopted as necessary. The Student's $t$ test and the Mann–Whitney test for continuous variables and the $\chi^2$ and Fisher's exact tests for categorical variables were used where appropriate. Statistical significance was predetermined as $P < 0.05$. Percentages were rounded to the nearest whole numbers. To evaluate the association between *C. difficile* detection and the primary and secondary factors, we conducted 2 tests for matched-pair data along with multivariate conditional

**TABLE 1.** Number of Enrolled Children for Participating Centers

| Centers | Patients with IBD (n = 211) | Patients with Celiac Disease (n = 112) |
|---|---|---|
| Barcelona | 11 | 8 |
| Budapest | 29 | 26 |
| Hvidovre | 28 | 17 |
| Jerusalem | 10 | 1 |
| Naples | 76 | 41 |
| Rome | 24 | — |
| Tel Aviv | 14 | — |
| Zagreb | 19 | 19 |

logistic regression analysis. SPSS version 15 was used for all statistical analyses. The sample size of 100 children in each group was estimated with a 90% power to detect a difference of at least 20%, between the 2 groups with an alpha of 0.05.

## RESULTS

Three hundred twenty-three patients met the inclusion criteria and were enrolled between October 2010 and October 2011, of whom 211 were affected by IBD (UC: 93; CD: 118; median age: 13.1 yr; range, 2–18 yr; M/F: 121/90) and 112 by celiac disease (median age: 11.1 yr; range, 2–18 yr; M/F: 53/59). Number of enrolled patients for participating center and baseline characteristics are shown in Table 1 and Table 2, respectively. At the enrollment, *C. difficile* was detected significantly more often in patients affected by IBD compared with patients with celiac disease (16/211 [7.5%] versus 1/112 [0.8%]; $P = 0.008$; odds ratio = 9; 95% confidence interval, 1.8–68). One hundred ninety-six patients completed follow-up at 6 months. Six of 196 patients with IBD (3%) were positive to *C. difficile* toxins test at 6 months of follow-up. One hundred seventy-three patients completed the follow-up at 12 months. Two patients (1.1%) were positive at 12 months. Twenty of 21 *C. difficile*–positive patients (95.2%) completed the study. Considering patients completing the follow-up at 12 months, the overall 1-year occurrence was 11.5% (20/173 total patients). *Clostridium difficile* occurrence varied among different enrolling centers. Geographical distribution of *C. difficile* infection is shown in Figure 1. Recurrence rate among *C. difficile*–positive patients completing the follow-up was 10% (2/20 patients). One patient had 2 recurrences. Twelve of 224 patients (5.3%) tested with Meridian Immunocard resulted to be *C. difficile* positive compared with 10 of 99 patients (10.1%) undergoing *C. diff* Quik Chek Complete, without any statistical difference ($P = 0.1$).

### Clinical Associations with *Clostridium difficile* Detection in Patients with IBD

We did not identify a specific type of IBD predisposing to *C. difficile* infection ($P = 0.3$). *Clostridium difficile* infection was not associated with age of patients at the time of infection and duration of IBD ($P = 0.9$ and $P = 0.8$, respectively). Three of 21 patients with *C. difficile* (14.2%) detection had new-onset IBD compared with 24 of 195 patients without *C. difficile* detection (12.3%) ($P = 0.4$). The presence of *C. difficile* toxins was associated with active disease in 15 of 21 patients with IBD (71.4%) ($P = 0.01$). In particular, *C. difficile* infection was found in 15.4% of all patients with IBD in relapse (15/82). Mean Pediatric Ulcerative Colitis Activity Index score was significantly increased in *C. difficile* infected patients with UC compared with noninfected patients, whereas mean Pediatric Crohn's Disease Activity Index score was higher in *C. difficile*–positive patients with CD, but statistical significance was not reached ($P = 0.01$ and $P = 0.5$, respectively) (Table 3). Six patients with IBD (28.5%) with positive *C. difficile* toxins were totally asymptomatic. Eighteen of 21 patients with IBD with *C. difficile* infection (85.7%) showed a colonic disease. However, disease location did not result to be significant different between *C. difficile*–positive and negative

**TABLE 2.** Baseline Characteristics

| Characteristics (n, %) | Patients with IBD | Patients with Celiac Disease | *P* |
|---|---|---|---|
| Median age (range), yr | 13.1 (2–18) | 11.1 (2–18) | 0.01 |
| Gender | | | 0.1 |
| Male | 121 (57.3) | 53 (47.3) | |
| Female | 90 (42.7) | 59 (52.7) | |
| *C. difficile* detection | 16 (7.5) | 1 (0.8) | 0.008 |
| IBD characteristics | | | |
| Type | | | NA |
| CD | 118 (55.9) | | |
| UC | 93 (44.1) | | |
| Disease location | | | NA |
| CD | | | |
| Ileum only (L1) | 27 (22.8) | | |
| Colon only (L2) | 30 (25.4) | | |
| Ileum and colon (L3) | 59 (50) | | |
| Upper gastrointestinal tract (L4a) | 10 (8.5) | | |
| Upper gastrointestinal tract (L4b) | 14 (11.8) | | |
| UC | | | |
| Proctosigmoiditis (E1) | 19 (20.4) | | |
| Left-sided colitis (E2) | 22 (23.6) | | |
| Extensive colitis (E3) | 12 (12.9) | | |
| Pancolitis (E4) | 40 (43) | | |
| Treatment | | | NA |
| Steroids | 34 (16.1) | | |
| Mesalazine | 103 (48.8) | | |
| Immunosuppressants | 85 (40.2) | | |
| Enteral nutrition | 4 (1.8) | | |
| Biologics | 29 (13.7) | | |

**TABLE 3.** Clinical Associations with *Clostridium difficile* Detection in Patients with IBD

| Characteristics (n, %) | *C. difficile* Positive (n = 21) | *C. difficile* Negative (n = 190) | *P* |
|---|---|---|---|
| Gender | | | |
| Male | 15 (71.4) | 106 (55.8) | 0.2 |
| Female | 6 (28.6) | 84 (44.2) | |
| Mean age (range), yr | 13.4 (2–18) | 12.8 (2–18) | 0.9 |
| Mean disease duration (range), mo | 28.2 (0–118) | 30.3 (0–132) | 0.8 |
| Disease onset | 3 (14.2) | 24 (12.3) | 0.4 |
| IBD type | | | 0.3 |
| CD | 14 (66.7) | 104 (54.7) | |
| UC | 7 (33.3) | 86 (45.3) | |
| Active disease | 15 (71.4) | 82 (43.1) | 0.01 |
| Mean PCDAI (range) | 22.7 (5–35) | 20.4 (0–45) | 0.3 |
| Mean PUCAI (range) | 26.2 (0–35) | 20 (0–50) | 0.01 |
| Disease location | | | |
| Ileum only (L1) | 3 (14.2) | 24 (12.6) | 0.5 |
| Colon only (L2) | 4 (19) | 26 (13.7) | 0.7 |
| Ileum and colon (L3) | 7 (33.3) | 52 (26.8) | 0.2 |
| Upper gastrointestinal tract (L4a) | 2 (9.5) | 8 (4.2) | 0.3 |
| Upper gastrointestinal tract (L4b) | 2 (9.5) | 12 (6.3) | 0.6 |
| Proctosigmoiditis (E1) | 0 (0) | 19 (10) | 0.2 |
| Left-sided colitis (E2) | 2 (9.5) | 20 (10.5) | 0.2 |
| Extensive colitis (E3) | 1 (4.7) | 11 (5.7) | 1 |
| Pancolitis (E4) | 4 (19) | 36 (18.9) | 1 |
| Colonic involvement | 18 (85.7) | 166 (87.4) | 0.7 |
| Treatment | | | |
| Antibiotics | 3 (14.2) | 21 (11) | 0.1 |
| PPIs | 3 (14.2) | 23 (12.1) | 1 |
| Immunosuppressants | 12 (57.1) | 88 (46.3) | 0.3 |
| Recent Hospitalization | 2 (9.5) | 39 (20.1) | 0.05 |

PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index.

groups (Table 3). Antibiotics, PPIs, and immunosuppressive therapies did not predispose patients to *C. difficile* infection ($P = 0.1$, $P = 1$, and $P = 0.3$, respectively). Previous hospitalizations were registered significantly more frequently in patients with IBD without *C. difficile* infection than in patients with IBD with *C. difficile* infection ($P = 0.05$). Multivariate logistic regression analysis with the risk of *C. difficile* detection as the dependent variable, confirmed that having IBD was the only factor that significantly contributed to the development of the infection (odds ratio = 2.6; 95% confidence interval, 1.8–7.3; $P = 0.03$). None of the other factors resulted to be independently associated to the infection.

## Natural History of *Clostridium difficile* Infected Patients

To evaluate the effect of *C. difficile* detection on IBD course, we examined the escalation of immunosuppressant and/or biological therapy, disease activity, rate of surgery, and number of hospitalizations during the study follow-up in *C. difficile*–positive patients at the enrollment. Fifteen of 16 patients with IBD (93%) *C. difficile* positive at the enrollment completed the follow-up at 6 and 12 months. Natural history of *C. difficile*–positive patients at the enrollment performing all the follow-up is shown in Table 4. Within 12 months, there was a higher number of *C. difficile*–positive patients who started immunosuppressant or biological therapy compared with patients without *C. difficile* (7/15 [46.6%] versus 30/158 [18.9%]; $P = 0.01$; odds ratio = 3.7; 95% confidence interval, 1.2–11). In details, among *C. difficile*–positive patients, 5 switched from mesalamine to an immunomodulator, and 2 patients, already under immunosuppressants, started biological therapy. At 6 and 12 months, patients with *C. difficile*–positive stools at the enrollment were significantly more often in active disease compared with patients without *C. difficile* (7/15 [46.6%] versus 38/181 [20.9%], $P = 0.04$; 6/15 [40%] versus 28/158 [17.7%], $P = 0.08$, respectively). One of 15 patients with *C. difficile* detection (6.6%) underwent total colectomy within 12 months compared with 1 of 158 (0.6%), but the difference was not statistically significant ($P = 0.1$). Number of hospitalizations was significantly higher at 6 months in *C. difficile*–positive group (4/15 [25%] versus 19/196 [9.6%], $P < 0.05$) but not different at 12 months ($P = 0.4$).

## Treatment of *Clostridium difficile* Cases

Fifteen of 21 total patients with IBD with *C. difficile*–positive stools (71.4%) showed signs and symptoms compatible with the infection. Eight of them (53.3%) were treated with oral vancomycin, whereas 7 patients (46.6%) were treated with oral metronidazole for 14 days. None of the patients withdrew immunosuppressive therapy. In 2 of 15 patients (13.3%), both treated with oral vancomycin, *C. difficile* was not eradicated, and another cycle of oral vancomycin was needed. The only patient with positive celiac was totally asymptomatic and therefore was not treated.

# DISCUSSION

To the best of our knowledge, this is the first prospective, multicenter pediatric study characterizing the occurrence and natural history of *C. difficile* infection/colonization in patients with IBD. We found that although infection is common in certain countries and rarer in others, it seems to be associated with approximately 15% of relapses. A number of adult studies[2–5,12,21] have examined the incidence of *C. difficile* infection, whereas only few

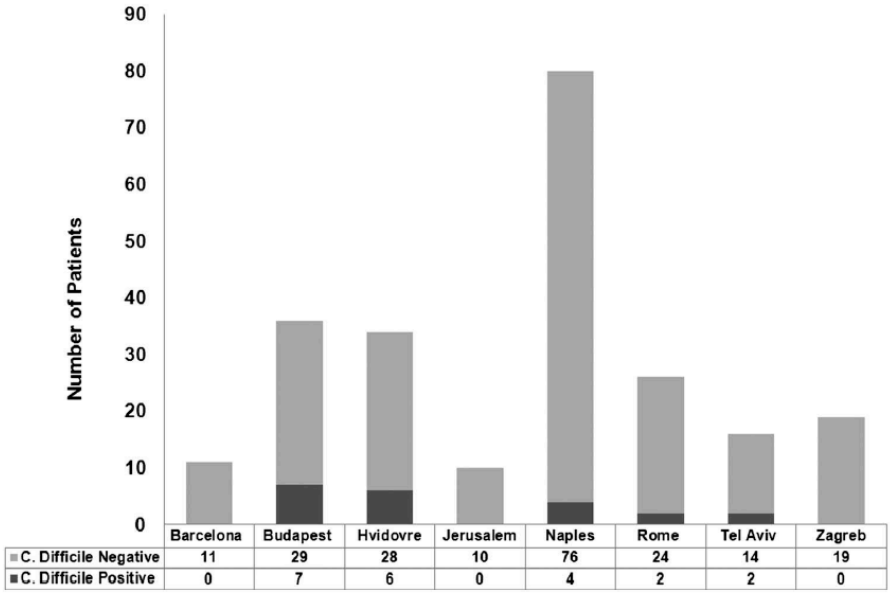


FIGURE 1. *Clostridium difficile* occurrence among different enrolling centers.

retrospective pediatric studies[6–11] tried to assess the prevalence, with different values. Pascarella et al[6] first reported a prevalence of 24.7% in pediatric IBD population, whereas more recently, Pant et al[11] assessed a rate of 3.5%. In our study population, *C. difficile* detection varied hugely among the enrolling centers, ranging from 0% to 24.1%. *Clostridium difficile* infection was significantly higher in patients with IBD than patients with celiac disease. We decided to test patients with celiac as control group, to verify whether another gastrointestinal pathology with described microbiota alterations,[22] such as celiac disease, may confer a higher

**TABLE 4.** Natural History of *Clostridium difficile* Infected Patients at the Enrollment Completing the Follow-up

| | | Baseline | | T6 | | T12 | |
|---|---|---|---|---|---|---|---|
| Patients | Diagnosis | *C. difficile* | Act | *C. difficile* | Act | *C. difficile* | Act |
| 1 | CD | Positive | Moderate | Negative | Moderate | Negative | Remission |
| 2 | CD | Positive | Severe | Negative | Moderate | Negative | Remission |
| 3 | CD | Positive | Moderate | Negative | Remission | Negative | Remission |
| 4 | CD | Positive | Moderate | Negative | Severe | Negative | Moderate |
| 5 | UC | Positive | Moderate | Negative | Remission | Negative | Remission |
| 6 | UC | Positive | Severe | Negative | Remission | Negative | Remission |
| 7 | CD | Positive | Mild | Positive | Remission | Negative | Remission |
| 8 | CD | Positive | Moderate | Negative | Remission | Negative | Moderate |
| 9 | CD | Positive | Remission | Negative | Moderate | Negative | Remission |
| 10 | UC | Positive | Severe | Positive | Moderate | Positive | Severe (colectomy) |
| 11 | CD | Positive | Remission | Negative | Remission | Negative | Remission |
| 12 | UC | Positive | Remission | Negative | Remission | Negative | Remission |
| 13 | CD | Positive | Mild | Negative | Moderate | Negative | Moderate |
| 14 | UC | Positive | Remission | Negative | Remission | Negative | Moderate |
| 15 | CD | Positive | Mild | Negative | Mild | Negative | Severe |

Act, disease activity; T6, 6 months; T12, 12 months.

susceptibility to *C. difficile* infection. Our data confirm that *C. difficile* increased risk is related to IBD itself. Although adult studies[3,4,12] documented an increased incidence of *C. difficile* in patients with UC, we could not confirm a similar association, and there was no specific IBD type predisposing to *C. difficile* colonization. This is consistent with published pediatric literature.[8,9] Our data seem to indicate that colonic involvement rather than disease type is the explanation for the association with UC. Most patients with IBD with *C. difficile* in our cohort (85.7%) showed colonic involvement, independent of the type of disease, as previously reported.[23] In agreement with previous articles, *C. difficile* was mainly community-acquired.[6–11] This is a further warning that *C. difficile* epidemiology is changing, and classical risk factors are often not involved.

Aside from disease location, other traditional risk factors seem to be less important or have no association with pediatric IBD. We could not demonstrate that medications such as PPIs or immunomodulators were associated, and hospitalization did not increase the risk for acquiring *C. difficile*. Freedberg et al[24] have recently questioned the role of PPIs. As described by Kelsen et al,[9] we found a high percentage of patients with *C. difficile* at onset of disease. Although not significant, this result underlines the possibility of a diagnostic delay and once more raises the question of a possible role of *C. difficile* in IBD pathogenesis. Furthermore, the presence of *C. difficile* toxins in the stools significantly correlated with an active disease, confirming the relevance of testing *C. difficile* during IBD flares.[25] As recently described, we found a considerable number of patients with IBD showing an asymptomatic carriage of *C. difficile*. Clayton et al[13] in a prospective evaluation reported that *C. difficile* was detected in stool cultures from 8% of patients with IBD in remission compared with 1% of healthy controls. More recently, Hourigan et al[26] found that asymptomatic *C. difficile* carriage was significantly more frequent in IBD (17%) versus controls (3%). Although the role of *C. difficile* carriage in subsequent *C. difficile*–associated disease or in IBD relapse needs to be further elucidated, our study provides important insight. Indeed, *C. difficile*–positive patients at the enrollment, including those with an asymptomatic carriage, seem to show a substantial risk for subsequent IBD exacerbation. This hypothesis is supported by the need for immunosuppressant escalation, the higher frequency of active disease, the increased colectomy rate, and the higher number of hospitalization at 6 months. Kelsen et al[9] in a retrospective analysis demonstrated that *C. difficile* infection worsened IBD severity, in terms of longer hospital admission, escalation of therapy, and colectomy rate. More recently, Pant et al[11] reported that *C. difficile* infected patients with IBD tended to have lengthier hospital stays, higher charges, and greater need for parenteral nutrition and blood transfusions. Differently from previous studies,[8] we found a high success rate of *C. difficile* eradication (86.6%) after treatment. However, this finding should be cautiously interpreted. The low treatment failure may partly reflect that European *C. difficile* strains are different from North-American B1/NAP1/027[27,28]; nevertheless, in most of the cases, eradication of the infection did not prevent a subsequent complicated IBD course, suggesting that *C. difficile* may act more as a relapse trigger rather than a "leading actor" of the symptoms. Another possibility is that *C. difficile* detection may be a expression of significant dysbiosis,[29] and therefore, it should be considered as an indicator of severe disease course. This study has some limitations. First, we limited our diagnosis to the use of immunoenzymatic assays. However in a recent report by Wang et al,[30] the outcomes of *C. difficile* diagnosed by polymerase chain reaction or enzyme-linked immunosorbent assay, seemed comparable despite the greater percentage of patients tested positive by polymerase chain reaction compared with enzyme-linked immunosorbent assay. There seemed to be a selection bias such that over 80% of patients had colonic involvement, which might reflect differences in phenotypes of disease, or enrollment of more severe colonic disease because of hospitalizations. It is well-known that Northern latitudes, such as Scandinavian and Scottish patients with IBD, as well as younger pediatric patients present with more colonic involvement in CD.[18] In addition, use of PPIs in our cohort was very limited, and we may have been underpowered to detect a difference. Furthermore, since the use of PPIs in children is low, this is less likely a priori to be a major risk factor in children. Finally, we did not perform *C. difficile* strains characterization, which could differ along the different countries.

## CONCLUSION

In conclusion, this prospective, multicenter study confirms that pediatric IBD is associated with increased *C. difficile* detection. *Clostridium difficile* is associated with a severe disease course, as demonstrated by the escalation of immunosuppressive therapy, the higher frequency of active disease, the colectomy rate, and the higher number of hospitalizations at 6 months. A consistent number of patients show an asymptomatic carriage, which should be carefully evaluated, considering the possibility of a quick worsening of disease. Future studies will clarify whether *C. difficile* has a causative role on IBD course exacerbation or if may simply colonize those patients with a more severe phenotype. In addition, the relationship between *C. difficile* and IBD pathogenesis should be further investigated.

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### 4.2 Differential diagnosis between *Yersinia enterocolitica* ileitis and CD

Yersiniosis is the third most commonly reported bacterial zoonoses in humans and it is a frequently reported gastrointestinal disease in Europe, especially in infants and young children [158]. The consumption of contaminated food, milk or water is the route of transmission of yersiniosis. *Yersinia Enterocolitica* is the most frequent *Yersinia* species accountable for human cases [158]. *Yersinia* infection is most commonly detected in 0-4-year-old children. Clinical presentation is variable from mild enteric symptoms to severe case with fever and bloody diarrhoea and complicated by extra-intestinal involvement [159]. An age-dependent symptomatology has been described. Indeed, in younger children the infection leads to an enterocolitis characterized by fever, vomiting and diarrhoea, while infants <3 months have an increased risk of extra-intestinal manifestations, including bacteraemia and metastatic infections, such as liver and spleen abscesses, pneumonia, septic arthritis, meningitis and endocarditis [160, 161]. In older children, abdominal pain, mainly localized in the lower right quadrant, is the most common symptom and usually represents the clinical expression of terminal ileitis and mesenteric lymphadenitis [160, 161]. A small percentage of patients may develop a chronic active ileitis and/or colitis, with persistent abdominal pain and/or diarrhoea, clinically mimicking IBD. Indeed, *Yersinia Enterocolitica* enteritis strongly resembles the major aspects of CD [162, 163]. Abdominal ultrasound examination is indistinguishable, showing thickening of terminal ileum wall and enlarged mesenteric lymph nodes, while colonoscopy is usually characterized by round or oval elevation and ulcers at the terminal ileum. Finally, *Yersinia* species may also histologically mimic Crohn's disease causing epithelioid granulomas with lymphoid cuffing, transmural inflammation with lymphoid aggregates, mucosal ulceration and cryptitis [164].

Here, we reported the case of an 11-year-old boy referred to our unit for a 4-month history of recurrent abdominal pain (RAP). At the onset of symptoms, the patient presented abdominal pain associated with diarrhoea, which disappeared few days later.

Successively, RAP was invariably associated with nausea and loss of appetite. Our laboratory investigations, including a complete and a differential blood count, organs function markers and acute phase reactants were normal. Faecal calprotectin was 185 ug/g. Celiac disease antibodies, anti-tissue transglutaminase and anti-endomysium, resulted negative. Our orientation was for a functional GI disorder, but an ultrasound exam of the abdomen revealed thickness (3.8 mm), loss of stratification and increased vascularization of terminal ileum wall, associated with enlarged mesenteric lymph nodes, with a suspicion of CD. Thus, we decided to perform an ileo-colonoscopy. The exam revealed the presence of aphthous ulcers and granularities at the terminal ileum. These findings were consistent with a diagnosis of CD, A1b-L1-B1-$G_0$ on the basis of Paris Classification [40]. Histology was confirmative, revealing active terminal ileitis with chronic inflammation at the lamina propria, cryptitis, and superficial erosion. Therefore, EEN was started, with poor results on the abdominal pain. Eight days after EEN's beginning, stool cultures revealed positivity for *Yersinia enterocolitica*. EEN was discontinued and trimethoprim/sulfamethoxazole was started for a 10-day course with a prompt clinical improvement. At the 1-month follow-up, the patient referred no episode of abdominal pain. A magnetic resonance enterography demonstrated the absence of small bowel mural thickening, no increased vascularization and disappearance of the previous lymphadenopathy.

This case once more suggests the importance of an extensive infectious diagnostic evaluation in the suspicion of paediatric IBD. As specified within this chapter, the ESPGHAN revised Porto criteria clearly state that in all children with suspected IBD, enteric infections should be excluded as cause of the symptoms, preferentially before endoscopy [37]. Yersiniosis is included within the stool culture panel to be performed [37]. Unfortunately, this is not always feasible taking into account the time necessary to achieve all microbiological cultures. In addition, most clinical microbiology laboratories do not

routinely use selective techniques to isolate *Yersinia* on stool cultures unless specified. To complicate this scenario, the identification of a pathogen does not necessarily exclude a diagnosis of IBD because a first episode of IBD may be triggered by a documented enteric infection. As a matter of fact, it has been reported that paediatric patients with a diagnosis of CD showed a significant higher presence of *Yersinia* DNA in colonic specimens when compared with controls [165]. Indeed, as indicated, a clinical longitudinal follow-up of these patients may be recommended [37]. In our case, the lack of response to EEN and the immediate resolution of both symptoms and radiologic signs of inflammation, after antibiotic therapy, should have definitely rule out the diagnosis of IBD.

In conclusion, this case demonstrates the clinical, sonographic and endoscopic similarities between *Yersinia enterocolitica* ileitis and CD. Thus, in the diagnostic work-up of suspected paediatric IBD, it is mandatory to exclude yersiniosis, requesting specific stool cultures. In any case, these patients may benefit of a clinical follow-up to definitely exclude an IBD diagnosis.

# *Yersinia Enterocolitica* Ileitis Mimicking Pediatric Crohn's Disease

***To the Editor:***

*Yersinia enterocolitica* is a Gram-negative bacillus, causing a variety of clinical manifestations ranging from self-limited enteritis to life-threatening systemic infection.[1] In older children and adolescents, *Y. enterocolitica* infection is characterized by abdominal pain associated with terminal ileitis and mesenteric adenitis, mimicking other diseases such as appendicitis and inflammatory bowel disease (IBD), often delaying diagnosis and treatment.[2,3] Hereby, we describe the case of an 11-year-old boy referred to our department with a 4-month history of recurrent abdominal pain. At the onset of symptoms, the patient presented abdominal pain associated with diarrhea, which disappeared few days later. Successively, recurrent abdominal pain was invariably associated with nausea and loss of appetite. The pain was mainly localized at mesogastrium and hypogastrium, and it was characterized by a daily occurrence and by nocturnal episodes, frequently awaking the patient. His past medical and family history were unremarkable. At presentation, his physical examination revealed normal growth. A mild abdominal tenderness was elicited by deep palpation in the right lower quadrant. Laboratory investigations resulted normal, including complete blood count, inflammatory markers, and celiac disease antibodies. Fecal calprotectin was 185 μg/g. An abdomen ultrasound examination revealed thickness (3.8 mm), loss of stratification, and increased vascularization of terminal ileum wall, associated with enlarged mesenteric lymph nodes. In the suspicion of Crohn's disease (CD), we decided to perform an ileocolonoscopy. The examination revealed the presence of aphthous ulcers and granularities at the terminal ileum (Fig. 1). These findings were consistent with a diagnosis of CD, A1b-L1-B1-$G_0$ on the basis of Paris Classification.[4] Histology revealed active terminal ileitis with chronic inflammation at the lamina propria, cryptitis, and superficial erosion, consistent with a diagnosis of CD (Fig. 2). Exclusive enteral nutrition (EEN) with a polymeric formula (Alicalm, Nutricia) was started. After one week of EEN, the patient presented unchanged abdominal pain. Eight days after EEN's beginning, stool cultures revealed positivity for *Y. enterocolitica* serotype O:8. EEN was discontinued and trimethoprim/sulfamethoxazole was started for a 10-day course with a prompt clinical improvement. At the 1-month follow-up, the patient referred no abdominal pain. A magnetic resonance enterography demonstrated the absence of small bowel mural thickening, no increased

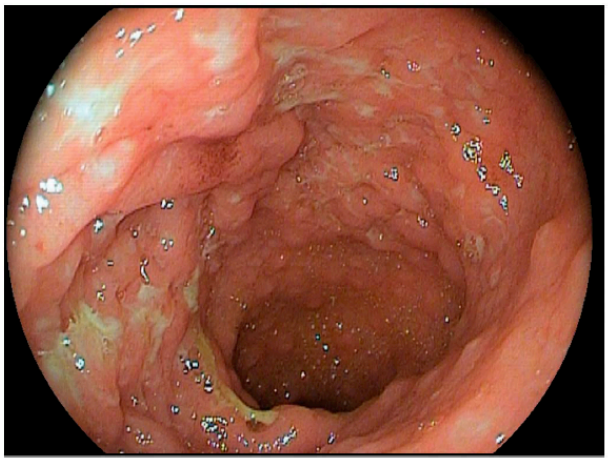

FIGURE 1. Ileocolonoscopic photograph reveals aphthous ulcers and granularities at the terminal ileum.

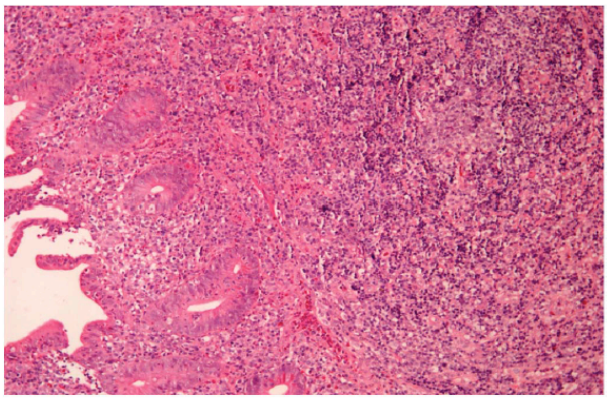

FIGURE 2. Ileal mucosa enlarged by hyperemic and active inflammation with necrosis characterized by hyperplastic-Peyer's patches with overlying ulceration.

Annamaria Staiano, MD, served as investigator and member of advisory board for the following companies: D.M.G, Valeas, Angelini, Miltè, Danone, Nestlé, Sucampo, Menarini. Erasmo Miele, MD, PhD, served as speaker, as investigator, and member of advisory board for the following companies: Abbvie, Angelini, Bioprojet, Ferring, Menarini, Milte, Valeas. The remaining authors have no conflict of interest to declare.



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vascularization, and disappearance of the previous lymphoadenopathy.

This case demonstrates that clinical manifestations, sonographic, endoscopic, and histologic findings of *Yersinia Enterocolitica* enteritis can resemble Crohn's disease hallmarks, confirming the importance of considering this pathogen in the differential diagnosis of inflammatory bowel disease.[5]

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# Patients with Inflammatory Bowel Disease May Use Electronic Communication with Their Physicians More Frequently Than Those Without Inflammatory Bowel Disease

***To the Editor:***

It is with interest that we read the article by Reich et al, entitled "A Survey of Social Media Use and Preferences in Patients with Inflammatory Bowel Disease" which revealed that social media can be a useful avenue for information delivery and that there may be a desire for patients with inflammatory bowel disease (IBD) to receive information from their gastroenterologist through electronic media. We would like to further support the conclusions of this article with significant findings from our own study regarding individuals with IBD and electronic communications. We hypothesized that a younger patient demographic population is more likely to use electronic communications with providers. We evaluated the differences in utilization of electronic physician–patient communication in individuals with IBD as compared with non-IBD patients, as well as, the differences in these communications by patient age, sex, and ethnicity.

In our retrospective chart review, which was at an urban academic medical center over 6 months, we recorded patient demographics, methods of provider communication, and IBD diagnosis. We identified a control group of patients seen at the gastroenterology clinic for chronic diagnoses other than IBD, such as GERD and IBS. All patients were under the care of faculty gastroenterologists. Controls were matched by age, sex, and treating gastroenterologist. Statistical analyses were conducted using a 2-tailed Fisher's exact test with significance set at $P < 0.05$.

There were 242 (29%) patients with IBD who used electronic communications compared with 21 (6%) non-IBD patients ($P = 0.0001$). There were 161 (34%) patients younger than 40 years of age who used electronic communications compared with 81 (23%) patients older than 40 years of age ($P = 0.0003$). There were 163 (48%) females who used electronic communications compared with 79 (32%) males ($P = 0.0001$). No statistically significant differences were observed among ethnicities.

Access to health care providers is important, particularly for those with chronic illnesses requiring complex management. The increased utilization of electronic communications by patients with IBD highlights the need to focus on faster, easier patient–physician communication modalities among this population. Patients with IBD may have a greater need for urgent delivery of care. Our data indicate that younger patients with IBD, as well as female patients, electronically communicated with their physicians more frequently. In a world that increasingly values faster communication, it is important that we consider making electronic methods, such as email and social media, accessible to patients.

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# CHAPTER 5

# MANAGEMENT OF EXTRA-INTESTINAL MANIFESTATIONS

As previously underlined in chapter 1, one of the peculiarities of paediatric IBD is the higher percentage of EIM [32, 33]. Their management is usually problematic due to the absence of specific treatments and the need to find a common therapeutic strategy both for the intestinal and extra-intestinal disease [34]. In some cases, particularly when it is involved the muscle-skeletal system, patients will need a quick escalation to biologics [36]. Within this chapter we will only face two of the most frequent and challenging EIM associated to paediatric IBD: 1) anaemia and 2) pancreatic involvement.

We will discuss these issues reporting the results of 2 different papers: ***"Serum Hepcidin and Iron Absorption in Pediatric Inflammatory Bowel Disease"*** published in *Journal of Crohns and Colitis (J Crohns Colitis. 2016; 10:566-74)* and ***"Natural history of pancreatic involvement in paediatric inflammatory bowel disease"*** published in *Digestive and Liver Disease (Dig Liver Dis. 2015; 47:384-9).*

## 5.1 The role of hepcidin in IBD related anaemia

IBD related anaemia is probably the most frequent EIM both in children and adults, with a prevalence ranging from 15 to 75%, on the basis of the different reports [166-168]. However, despite its relative high prevalence, costs and impact on patient's quality of life, it is rarely considered and adequately treated [169, 170]. One of the main difficulties in IBD related anaemia is understanding which is the mechanism underneath. Indeed, at least 3 types of anaemia are identifiable during the management of patients with IBD: 1) iron deficiency anaemia (IDA), which represents the result of the chronic blood loss from the GI tract; 2) anaemia of inflammatory etiology, or anaemia of chronic disease (ACD), which is mainly the result of systemic inflammation, impairing both iron absorption and iron release into the blood; 3) combined IDA+ACD, determined in a variable measure by both the

reported mechanisms [171, 172]. Determining which type of anaemia may be important to better orientate the therapeutic management. Indeed, in case of IDA, with negative inflammatory indexes, a cycle of oral iron may be recommended. On the contrary, in case of clear ACD oral iron is strongly discouraged being completely useless and possibly harmful [173]. However, children with a mixed component represent the most challenging cases. In those patients, it is often difficult to discriminate which is the prevalent mechanism with the traditional biomarkers (serum iron, ferritin, transferrin, CRP, etc.) and the clinician may face the dilemma whether or not to use oral iron [174].

The research of new possible biomarkers may certainly improve the management of IBD related anaemia. Recent elucidations revealed hepcidin, as a possible candidate. As well known, hepcidin is one of the most important iron regulator. Its expression is, indeed, transcriptionally regulated in response to changing serum iron levels. Elevated serum iron promotes hepcidin expression, leading to downregulation of ferroportin and decreased entry of iron into the circulation. Conversely, low serum iron leads to reduced hepcidin expression, elevated ferroportin, and increased movement of iron into the circulation [175, 176]. Hepcidin is thought to be the key markers in the pathogenesis of ACD. Hence, in addition to iron status, inflammatory cytokines, such as interleukin 6, can also influence transcription of the hepcidin gene [177, 178]. Some papers tried to elucidate the role of hepcidin in the mechanisms of anaemia in pediatric IBD. However, the results are limited and conflicting [179-183]. This may suggest that hepcidin in human IBD is likely to be influenced by various factors such as age, type of disease, and disease activity.

Given these premises, with the objective of clarifying some of these unanswered questions, we decided to conduct a prospective, observational study in our IBD cohort. The primary aim was to correlate hepcidin serum levels in IBD children with disease activity, inflammatory markers and iron absorption; the secondary aims were to compare

serum hepcidin levels of IBD patients with a group of celiac and healthy patients, and to establish which iron parameter better correlates with hepcidin.

To realize these goals, we prospectively enrolled between December 2012 and June 2013 50 IBD children, 45 celiac children and 50 healthy controls. All the children underwent an extensive blood panel including the traditional iron markers and hepcidin measurement. In addition, in order to evaluate the efficacy of iron absorption in IBD children, an iron load test (ILT) was performed.

The results of our study certainly brought new knowledge to this important topic. Indeed, this study represents the first, evaluating both iron absorption and serum hepcidin levels in IBD pediatric patients. Our data showed that IBD children with active disease tend to have impaired iron absorption, driven through hepcidin pathway. Indeed, serum hepcidin levels were significantly higher in IBD patients with moderate to severe activity as compared to all other groups, including patients with mild activity or in remission, celiac patients, and healthy controls. In addition, a significant inverse correlation was found between hepcidin levels and iron absorption. These findings are in agreement with the paper from Semrin and colleagues, the only paediatric study investigating the relationship between iron absorption and hepcidin, while the authors used urinary hepcidin as a proxy of serum hepcidin [179].

The prevalence of anaemia among IBD patients was 34% and, not surprisingly, the majority of the anaemic patients of our study population showed a combined IDA and ACD (70.5%). Therefore, in most of our patients we could have been doubtful, whether or not to advise an oral iron cycle. The treatment with oral iron has significant limitations in IBD, being certainly less efficacious than intravenous route [174]. In addition, absorption of iron from the GI tract is limited, and unabsorbed iron is exposed to the intestinal surface. Studies in animal models of colitis indicate that luminal iron may exacerbate disease activity [184, 185]. In a more recent study, iron supplementation affected microbiota and

increased faecal calprotectin [186]. Taking into account this possible warning, the recently published ECCO guidelines clearly state that intravenous route should be preferred in those patients with a suspicion of ACD [173]. On the other side, giving intravenous iron is not always feasible, both for high costs and patients' compliance. It is therefore important to screen the patients needing intravenous iron. On the basis of our study, serum hepcidin may represent a useful surrogate marker to distinguish patients with impaired iron absorption. With regards to the traditional markers, not surprisingly serum ferritin resulted to be the most strictly correlated to hepcidin [187]. However, 58.3% of patients, with a diagnosis of combined IDA+ACD and normal ferritin values, showed an increased hepcidin, demonstrating that ferritin is not sufficiently adequate in the differential diagnosis of IBD related anaemia.

In conclusion, our data clearly demonstrate that serum hepcidin is increased in IBD children with active disease and plays an important role in the process of iron malabsorption. ACD is significantly prevalent in paediatric IBD and it should be taken in consideration before starting an oral iron therapy. If confirmed by further studies, serum hepcidin may serve as a useful, sensitive, surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as first-line option, avoiding wasting time with a possibly harmful cycle of oral iron.

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# Serum Hepcidin and Iron Absorption in Paediatric Inflammatory Bowel Disease

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## Abstract

**Background and Aims**: We sought to correlate hepcidin levels in inflammatory bowel disease [IBD] children with disease activity, inflammatory markers, and iron load test [ILT] and to compare IBD patients with coeliac and healthy patients.

**Methods**: Between December 2012 and June 2013, 145 subjects [50 IBD patients, 45 coeliac patients and 50 healthy controls] were included in the study. All patients underwent the following examinations: blood count, iron status, erythropoiesis parameters, serum hepcidin, C-reactive protein [CRP], and erythrocyte sedimentation rate [ESR]. In order to evaluate the efficacy of iron absorption, ILT was performed in IBD patients. Disease activity indexes and IBD duration, localisation, and therapy were also evaluated, and a faecal sample for calprotectin collected.

**Results**: Serum hepcidin was significantly higher in IBD patients with active disease compared with both coeliac and healthy patients [$p = 0.005$, $p = 0.003$ respectively]. In a multivariate logistic regression model, having a Paediatric Crohn's Disease Activity Index [PCDAI] / Paediatric Ulcerative Colitis Activity Index [PUCAI] ≥ 30 resulted in the only variable independently associated with a positive serum hepcidin (odds ratio [OR] = 6.87; 95% confidence interval [CI] 1.4–33, $p = 0.01$]]. Patients with iron malabsorption [IM] showed higher values of ESR, CRP, and hepcidin [$p = 0.02$, $p = 0.001$, and $p = 0.06$, respectively]. Eight out of 12 [66.7%] children with IM showed an active disease compared with 6/31 [19.3%] children with normal ILT [$p = 0.01$]. Hepcidin levels correlated negatively with ILT [r = -0.451, $p = 0.002$], and positively with ferritin and CRP [r = 0.442, $p = 0.0001$; r = 0.243, $p = 0.009$, respectively]

**Conclusions**: Our study demonstrates that serum hepcidin is increased in IBD children with active disease and it is responsible for IM.

**Keywords**: Hepcidin; IBD; iron absorption

## 1. Introduction

Anaemia is the most frequent extra-intestinal manifestation of inflammatory bowel disease [IBD], with a great impact on the patient's quality of life.[1,2] The prevalence of anaemia in IBD varies between 15% and 75%, depending on the definition and the subgroup of examined patients.[3,4] The main types of anaemia in IBD are iron deficiency anaemia [IDA], anaemia of inflammatory aetiology, anaemia of chronic disease [ACD], and combined IDA + ACD.[4,5] IDA is mainly the result of the chronic blood loss from the gastrointestinal [GI] tract, due to prolonged inflammation of the small and large intestine epithelium. On the other hand inflammation, through an inflammatory cytokines-mediated mechanism, leads to a decreased iron level in the circulation and thus to a limited availability of iron for erythroid cells.[6] Hepcidin, a 25 amino-acid peptide mainly secreted by hepatocytes, controls the amount of iron entering the blood circulation by binding and downregulating ferroportin, a plasma membrane transporter that pumps iron out of phagocytes and duodenal enterocytes. The hepcidin expression is regulated transcriptionally in response to changing serum iron levels. Elevated serum iron promotes hepcidin expression, leading to downregulation of ferroportin and decreased entry of iron into the circulation. Conversely, low serum iron leads to reduced hepcidin expression, elevated ferroportin, and increased movement of iron into the circulation.[7,8] In addition to iron status, inflammatory cytokines can also influence transcription of the hepcidin gene. Interleukin-6 [IL-6] has been shown to increase hepcidin expression in vitro and in vivo, and IL-6 induced hepcidin upregulation has been proposed to play an important role in the pathogenesis of ACD.[9,10] The role of hepcidin in the mechanisms of anaemia in paediatric IBD is limited and shows conflicting results. Increased urine[11] or serum[12] hepcidin has been reported in two studies, correlating with the rise of IL-6 levels, ferritin, and disease activity. Conversely, Arnold *et al.* found significantly decreased hepcidin levels in IBD patients compared with healthy controls.[13] Hepcidin precursor, pro-hepcidin, has also been evaluated in three different studies in IBD patients, with variable results.[12,14,13,15] Conflicting results may suggest that hepcidin in human IBD is likely to be influenced by various factors such as age, type of disease, and disease activity.

The primary aim of this study was to correlate hepcidin serum levels in patients affected by paediatric IBD with disease activity, inflammatory markers, and iron absorption. The secondary aims were to compare serum hepcidin levels of IBD patients with a group of coeliac and healthy patients, and to establish which iron parameter better correlates with hepcidin.

## 2. Materials and Methods

### 2.1. Study population

We conducted a comparative, cross-sectional, single-centre study in paediatric patients with a diagnosis of IBD. Children and adolescents aged from 2 to 18 years with a diagnosis of IBD were prospectively enrolled between December 2012 and June 2013 at the Department of Translational Medical Science, Section of Paediatrics, University of Naples 'Federico II', Italy. The diagnosis of IBD was established on the basis of clinical, endoscopic, radiological, and histological criteria according to the Porto criteria.[16] During the same study period, we also recruited a group of children who were referred to our centre with suspected coeliac disease due to pathological serum levels of anti-tissue transglutaminase [tTG] [> 7 U/ml] and/or positive anti-endomysium [EMA] antibodies. Only patients with a confirmed diagnosis of coeliac disease or potential coeliac disease [positive serology and normal duodenal architecture] were finally included in the study. In addition, we enrolled a group of healthy children referred to our primary care centre for routine well-child visits. Exclusion criteria from the study were: age ≤ 2 years or > 18 years; patients with suspected coeliac disease without confirmation of diagnosis; the presence of other comorbidities; patients having iron supplementation during the month preceding the enrolment; and inability or unwillingness to give informed consent. At the time of the enrolment, all patients underwent the following examinations: full blood count, reticulocytes, serum iron, ferritin, transferrin, soluble transferrin receptor [STfR], total iron-binding capacity [TIBC], transferrin saturation [Tsat], and inflammatory indexes (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR]). In addition, blood samples from all enrolled patients were also obtained for hepcidin 25 isoform analysis. After being centrifuged, the serum of all patients was stored at -80°C in aliquots in order to avoid multiple-frozen thaw. Once collected, all samples were sent to the Section of Internal Medicine, Department of Medicine, University of Verona for the analysis. Hepcidin 25 isoform was measured through a validated mass spectrometry-based assay, as previously described.[17] Synthetic hepcidin 25 [Peptides International, Louisville, KY] was used for external calibration and a synthetic hepcidin analogue [Hepcidin 24, Peptides International] as an internal standard. The lower sensitivity limit of the assay was 0.55 nM. All samples were measured in duplicate.[18] For the IBD group, type of IBD, anatomical distribution of disease, symptoms, disease activity, and treatments, including surgery, were recorded. For the purpose of this manuscript, disease location was described according to the Paris classification.[19] Disease activity was scored by the Paediatric Crohn's Disease Activity Index [PCDAI] or the Paediatric Ulcerative Colitis Activity Index [PUCAI] for CD and UC, respectively.[20, 21] In addition, a stool sample for faecal calprotectin determination was also obtained. For the coeliac group, tTG, EMA, and duodenal histology [according to Marsh grading] were also evaluated.

### 2.2. Differential diagnosis of anaemia

Anaemia was defined on the basis of World Health Organization Criteria [22]: in boys aged > 15 years, as Hb < 13 g/dl; in non-pregnant girls aged > 15 years, as Hb < 12 g/dl; in children aged 5–11 years, as Hb < 11.5 g/dl; and in children aged < 5 years, as Hb < 11 g/dl. Iron deficiency was defined as a ferritin < 12 ng/ml in children aged < 5 years, or ferritin < 15 ng/ml in children aged > 5 years, when the corresponding CRP was < 1 mg/dl and Tsat < 20%.[22] In the presence of biochemical evidence of inflammation, the diagnostic criteria for ACD were a serum ferritin > 100 ng/ml and TfS < 20%, whereas if the serum ferritin level was ≤ 100 ng /ml, a combination of true iron deficiency and ACD was diagnosed.[23]

### 2.3. Iron absorption in IBD patients

In order to evaluate the efficacy of iron absorption, an iron load test [ILT] was performed in children affected by IBD. We used a previously described protocol.[24] After an overnight fast and baseline serum iron determination, ferrous sulphate [dosed as 1 mg/kg elemental iron with a 60-mg maximum] was administered orally as a liquid preparation, followed by determination of serum iron after 2 h. The change in iron levels between the baseline and the 2-h period (Δ[Fe]2hr) was calculated. Iron malabsorption [IM] was defined using the normative data, when the increase of serum iron after 2 h from the ILT was lower than the fifth percentile.[24]

### 2.4. Ethical approval

All parents or guardians signed a consent form indicating their awareness of the investigative nature of the study and possible risks. When appropriate, we also obtained children's assent. The study was approved by the Institutional Review Board of University of Naples Federico II.

### 2.5. Statistical analysis

Variables were screened for their distribution, and appropriate parametric or non-parametric tests were adopted as necessary. Student's t-test, the ANOVA test, and the Mann-Whitney test for continuous variables, and the chi-square and Fisher's exact tests for categorical variables, were used where appropriate. Multivariate conditional logistic regression analysis was used to explore the odds associated with a positive serum hepcidin and a pathological ILT. Serum hepcidin and ILT were used as dependent variables, and the effect of all the parameters was analysed by a stepwise procedure. Serum hepcidin was considered positive for values higher than the measurable cut-off [0.55 nM]. Correlations between serum hepcidin and Δ[Fe]2hr with continuous variables were evaluated through linear regression and expressed by Pearson's correlation coefficient. Variables not normally distributed [serum hepcidin and ferritin] were log-transformed before performing the correlations. Statistical significance was predetermined as $p < 0.05$. Percentages were rounded to the nearest whole numbers. SPSS version 15 was used for all statistical analyses. The sample size of 50 children in each group was estimated with a 90% power to detect a difference of at least 20% between the three groups, with an alpha of 0.05.

## 3. Results

Initially, 150 subjects were enrolled between December 2012 and June 2013, comprising 50 IBD patients (UC: 28; CD: 20; IBD-unclassified

**Table 1.** Demographic and clinical characteristics of enrolled patients.

| Characteristics [n, %] | IBD patients | Coeliac patients | Healthy controls | *p* |
|---|---|---|---|---|
| | *N* = 50 | *N* = 45 | *N* = 50 | |
| Age, years [range] | 12.6 ± 3.5 [4–18] | 8 ± 3.2 [2–14] | 11.1 ± 4.2[3.2–18] | 0.01 |
| Gender [*n*, %] | | | | |
| Male | 27 [54] | 20 [44.4] | 25 [50] | 0.6 |
| Coeliac disease diagnosis [*n*, %] | | | | NA |
| Coeliac disease | - | 38 [84.4] | - | |
| Potential coeliac disease | - | 7 [15.6] | - | |
| tTG | - | 110.8 ± 87.6 [3.4–200] | - | |
| EMA positive | - | 41 [91.1] | - | |
| Marsh grading [*n*, %] | - | | - | |
| T1 | - | 3 [6.6] | - | |
| T2 | - | 0 | | |
| T3a | - | 0 | | |
| T3b | - | 12 [26.6] | - | |
| T3c | - | 21 [46.6] | - | |
| IBD type [*n*, %] | | | | NA |
| UC | 28 [56] | - | - | |
| CD | 20 [40] | - | - | |
| IBD-U | 2 [4] | - | - | |
| IBD disease location [*n*, %] | | - | - | NA |
| UC | | | | |
| Proctosigmoiditis [E1] | 9 [32] | - | - | |
| Left-sided colitis [E2] | 2 [8] | - | - | |
| Extensive colitis [E3] | 6 [21] | - | - | |
| Pancolitis [E4] | 11 [39] | - | - | |
| CD | | - | - | |
| Ileum only [L1] | 1 [5] | - | - | |
| Colon only [L2] | 5 [25] | - | - | |
| Ileum and colon [L3] | 14 [70] | - | - | |
| Upper gastrointestinal tract [L4a] | 2 [10] | - | - | |
| - | - | | | |
| IBD disease activity indexes | | | | NA |
| PUCAI | 13.8 ± 16.4 [0–47.5] | - | - | |
| PCDAI | 11.8 ± 14.4 [0–60] | - | - | |
| IBD therapy [*n*, %] | | | | NA |
| Steroids | 10 [20] | - | - | |
| Enteral nutrition | 3 [6] | - | - | |
| Azathioprine | 11 [22] | - | - | |
| Methotrexate | 1 [2] | - | - | |
| Biologicals | 2 [4] | - | - | |
| Mesalazine | 31 [62] | - | - | |

All continuous variables values are expressed as means ± standard deviation [range].

CD, Crohn's disease; EMA, anti-endomysium; tTg, anti-tissue transglutaminase; IBD, inflammatory bowel disease; UC, ulcerative colitis; IBD-U, inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; NA, not applicable.

**Table 2.** Laboratory parameters of enrolled patients.

| Parameters | IBD patients [$n$ = 50] | Coeliac patients [$n$ = 45] | Healthy controls [$n$ = 50] | $p$* |
|---|---|---|---|---|
| Hb, g/dl | 12.2 ± 1.7 [8.3–14.9] | 12.1 ± 1 [9.1–14.6] | 12.8 ± 1 [11–16] | 0.04 |
| MCV, fl | 78.6 ± 8.1 [60–94] | 78.5 ± 5.3 [60.3–91] | 82.5 ± 4.1 [74–92] | 0.002 |
| Serum iron, µg/dl | 56.6 ± 32.5 [13–148] | 60.4 ± 33.4 [19–176] | 80.7 ± 26.9 [28–138] | 0.001 |
| Ferritin, ng/ml | 45.8 ± 36.8 [6–217] | 33.8 ± 30.5 [6–157] | 56.8 ± 31 [10–179] | 0.0 001 |
| Transferrin, g/l | 2.5 ± 0.6 [1.3–4.3] | 2.7 ± 0.3 [2.1–3.4] | 1.4 ± 0.3 8 [0.7–2.6] | 0.02 |
| STfR, mg/dl | 1.9 ± 1.2 [0.8–8.5] | 1.7 ± 0.4 [1.1–2.8] | 1.4 ± 0.3 [0.4–2.6] | 0.02 |
| Tsat, % | 16.1 ± 9.8 [3–41] | 15 ± 8.6 [5–42] | 21.4 ± 8.3 [6–44] | 0.01 |
| TIBC, µg/dl | 315.5 ± 77.7 [139–460] | 365.1 ± 72.8 [236–576] | 350.8 ± 43.8 [268–450] | 0.3 |
| Reticulocytes, % | 3.8 ± 3.6 [0.6–9] | 4.0 ± 3.3 [1–4] | 3.2 ± 3.4 [0.6–9] | 0.01 |
| Serum hepcidin, nM | 4.3 ± 8.3 [0.55–49.2] | 2.1 ± 3.1 [0.55–15.5] | 2 ± 2.6 [0.55–11.3] | 0.06 |
| ESR, mm | 12.2 ± 11.8 [1–46] | 9.5 ± 6.1 [2–23] | 6.2 ± 3.9 [1–17] | 0.03 |
| CRP, mg/dl | 1.4 ± 3.1 [0.33–18.6] | 0.35 ± 0 .04 [0.33–0.4] | 0.36 ± 0.2 [0.33–2] | 0.0 001 |

All values are expressed as means ± standard deviation [range].

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; MCV. mean corpuscolate volume; STfR, soluble transferrin receptor; TIBC, total iron binding capacity; TSat, transferrin saturation.

* ANOVA test.

**Table 3.** Serum hepcidin levels in different groups of patients.

| Groups [$n$] | Serum hepcidin* | $p$ |
|---|---|---|
| IBD with PCDAI/PUCAI ≥ 30 [11] | 9.4 ± 15.8 [0.55–49.2] | 0.02 |
| IBD with PCDAI/PUCAI < 30 [39] | 2.8 ± 3.7 [0.55–19.2] | |
| IBD type | | 0.1 |
| UC [28] | 3.3 ± 6.2 [0.55–28.5] | |
| CD [20] | 5.7 ± 10.9 [0.55–49.2] | |
| IBD-U [2] | 1.8 ± 1.7 [0.55–3] | |
| IBD with PCDAI/PUCAI ≥ 30 [11] | 9.4 ± 15.8 [0.55–49.2] | 0.005 |
| Coeliac patients [45] | 2.1 ± 3.1 [0.55–15.5] | |
| IBD with PCDAI/PUCAI ≥ 30 [11] | 9.4 ± 15.8 [0.55–49.2] | 0.003 |
| Healthy controls [50] | 2.1 ± 2.6 [0.55–11.3] | |
| Coeliac patients [45] | 2.1 ± 3.1 [0.55–15.5] | 0.9 |
| Healthy controls [50] | 2.1 ± 2.6 [0.55–11.3] | |
| Coeliac disease group | | 0.3 |
| Coeliac disease [38] | 1.9 ± 3.0 [0.55–15.5] | |
| Potential coeliac disease [7] | 3.1 ± 3.3 [0.55–8.3] | |
| Patients with anaemia | | |
| IDA [8] | 0.55 [0.55] | 0.001 |
| ACD [2] | 25.1 ± 34 [1–49.2] | |
| ACD [2] | 25.1 ± 34 [1–49.2] | 0.001 |
| IDA + ACD [12] | 1.9 ± 4.3 [0.55–15] | |
| IDA [8] | 0.55 [0.55] | 0.06 |
| IDA + ACD [12] | 1.9 ± 4.3 [0.55–15] | |

*Values are expressed as means ± standard deviation [range].

ACD, anaemia of chronic disease; CD, Crohn's disease; IDA, iron deficiency anaemia; IBD, inflammatory bowel disease; IBD-U: inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; UC, ulcerative colitis.

[IBD-U]: 2; mean age ± standard deviation [SD:] 12.6 ± 3.5; range 4 to 18 years; M/F: 27/23), 50 children with a suspicion of coeliac disease, and 50 healthy controls [mean age ± SD: 11.1 ± 4.2 years; range 3.2 to 18 years; M/F: 25/25]. Among the coeliac disease group, 5 patients [10%] were subsequently excluded from the study for non-confirmed positive coeliac serology and therefore only 45 coeliac patients were included in the study [mean age ± SD: 8 ± 3.2 years; range 2 to 14 years; M/F: 20/25]. Of these 45 children, 38 [84.4%] were affected by coeliac disease and 7 [15.6%] by potential coeliac disease. The baseline and laboratory characteristics of all subjects included in the study are described in Table 1 and Table 2.

### 3.1. Prevalence of anaemia among different study groups

The prevalence of anaemia in IBD patients was significantly higher compared with both the coeliac group and the healthy controls (17/50 [34%] versus 5/45 [11.1%]; OR = 4.1; 95% CI 1.3 to 12.3, $p$ = 0.01; 17/50 [34%] versus 0/50 [0%]; $p$ = 0.001; OR 2.5; 95% CI 1.9 to 2.3, respectively]. In detail, 3 out of 17 IBD patients [17.6%] were affected by IDA, 2/17 [11.7%] by ACD, and in 12 out of 17 [70.5%] IBD children a combination of IDA and ACD was identified. All five anaemic coeliac patients were affected by IDA. In addition, an iron deficiency status without anaemia was found in 1/50 [2%] IBD patients, 7/45 [14%] coeliac patients, and 2/50 [4%] healthy controls [$p$ = 0.03].

### 3.2. Serum hepcidin among different groups

Serum hepcidin was significantly higher in IBD patients with a PCDAI/PUCAI ≥ 30 compared with patients with PCDAI/PUCAI < 30, coeliac patients, and healthy controls [$p$ = 0.02, $p$ = 0.005, $p$ = 0.003, respectively] [Table 3]. In IBD patients with PCDAI/PUCAI < 30, serum hepcidin values were higher than in coeliac or in healthy children, but statistical significance was not reached [$p$ = 0.3, $p$ = 0.2, respectively]. No difference was observed when comparing coeliac patients with healthy controls [$p$ = 0.9] [Table 3]. In addition, serum hepcidin was significantly higher in patients with ACD compared with children with IDA and ACD + IDA [$p$ = 0.001, $p$ = 0.001, respectively]. Patients with ACD + IDA showed higher values of serum hepcidin compared with patients with IDA, with a trend toward statistical significance [$p$ = 0.06] [Table 3]. In detail, none of the patients with pure IDA showed hepcidin values higher than the lower limit of the assay [0.55 nM], whereas 7 out of 12 [58.3%] patients with combined IDA and ACD showed values > 0.55 nM. Both of the two patients [100%] with pure ACD showed values higher than the cut-off.

#### 3.2.1. Multivariate analysis

In a multivariate logistic regression model, serum hepcidin was considered positive for values > 0.55 nM. Having a PCDAI/PUCAI ≥ 30 resulted in the only variable independently associated with a positive serum hepcidin (9/11 [81.8%] patients with a PCDAI/PUCAI ≥ 30 versus 53/134 [39.5%] of the remaining patients; OR = 6.87; 95% CI 1.4–33, $p$ = 0.01). None of the other variables was associated with positive serum hepcidin.

### 3.3. Iron absorption in IBD patients

Off 50 IBD patients, 43 [86%] performed the ILT. Twelve of the 43 [27.9%] patients showed a pathological ILT. Iron absorption was not associated with patient age or gender [$p$ = 0.6 and $p$ = 0.3, respectively] [Table 4]. Specific type of IBD and duration and extension of disease did not associate with the IM [$p$ = 0.1, $p$ = 0.1, and $p$ = 0.7, respectively]. Patients with IM showed significant higher values of ESR and CRP compared with patients with normal iron absorption [$p$ = 0.02 and $p$ = 0.001, respectively]. Active disease was more frequent in children with a pathological ILT when compared with children with normal iron absorption (8/12 [66.7%] versus 6/31 [19.3%] [$p$ = 0.01]. In particular, a PUCAI/PCDAI ≥ 30 was more common in children with pathological ILT when compared with children with normal ILT (5/12 [41.6%] versus 1/31 [1.3%]; $p$ = 0.004). Mean PUCAI and PCDAI are shown in Table 4. In addition, IBD children with abnormal ILT were significantly more often taking immunosuppressive therapy (9/12 [75%] versus 11/31 [35.5%]; $p$ = 0.03). Baseline serum iron, haemoglobin, transferrin, and Tsat values were significantly lower in patients with IM [$p$ = 0.001, $p$ = 0.005, $p$ = 0.04, and $p$ = 0.003, respectively] [Table 4]. Although baseline ferritin and faecal calprotectin were found to be higher in patients with IM, these differences were not statistically significant [$p$ = 0.2 and $p$ = 0.6, respectively] [Table 4]. Serum hepcidin was higher in patients with pathological ILT, with a trend toward statistical significance [$p$ = 0.06] [Table 4]. In a multivariate logistic regression model, being affected by active IBD resulted in the only variable independently associated with a pathological ILT [OR = 15.4; 95% CI 1.4–160.2, $p$ = 0.007].

### 3.4. Correlations of serum hepcidin and Δ[Fe]2hr

In order to determine correlations, we included all the patients [$n$ = 145] in the analysis, except for Δ[Fe]2hr, PUCAI, PCDAI, calprotectin, and tTg, which were determined only in IBD patients or in coeliac patients. Log-tranformed hepcidin levels correlated negatively with Δ[Fe]2hr [r = -0.451, $p$ = 0.002] [Figure 1A], and positively with log-transformed ferritin and CRP [r = 0.442, $p$ = 0.0 001; r = 0.243, $p$ = 0.009, respectively] [Figure 1B and C]. An inverse relationship was found with serum transferrin [r = -0.249, $p$ = 0.005, respectively] [Figure 1D]. A direct correlation with a trend toward statistical significance was found between log-transformed hepcidin and ESR [r = 0.158, $p$ = 0.09]. No specific correlation was found comparing log-transformed hepcidin with age, duration of IBD, serum iron, STfR, TIBC, or reticulocytes. Among coeliac patients, no significant correlation was identified between log-transformed hepcidin levels and tTG titres [r = -0.201, $p$ = 0.2]. Δ[Fe]2hr was found to be inversely correlated with log-transformed ferritin and CRP [r = -0.588, $p$ = 0.0001; r = -0.585, $p$ = 0.0001, respectively] [Figure 2A and B]. A direct correlation was found between Δ[Fe]2hr and transferrin [r = 0.613, $p$ = 0.0001] [Figure 2C]. An inverse correlation with a trend toward statistical significance was found with ESR, PUCAI, and faecal calprotectin [r = -0.296, $p$ = 0.07; r = -0.292, $p$ = 0.06; r = -0.325, $p$ = 0.08, respectively]. None of the other variables correlated with Δ[Fe]2hr.

**Table 4.** Characteristics associated with iron malabsorption in IBD children.

| Characteristics | Pathological ILT [$n$ = 12] | Normal ILT [$n$ = 31] | $p$ |
|---|---|---|---|
| Mean age [years, range] | 12.9 ± 3.6 [2–18] | 12.3 ± 3.5 [2–18] | 0.6 |
| Gender [$n$, %] | | | 0.3 |
| Male | 4 [33.3] | 17 [54.8] | |
| Duration of disease [months] | 25.5 ± 29.6 [0–82] | 43.8 ± 36.3 [1–113] | 0.1 |
| IBD type [$n$, %] | | | 0.1 |
| CD | 6 [50] | 10 [32.3] | |
| UC | 5 [41.7] | 20 [64.5] | |
| IBD-U | 1 [8.3] | 1 [3.2] | |
| Active disease [$n$,%] | 8 [66.7] | 6 [19.4] | 0.01 |
| PCDAI/PUCAI ≥ 30 [$n$, %] | 5 [41.7] | 1 [3.2] | 0.004 |
| Disease activity indexes | | | |
| PCDAI | 17.1 ± 17.4 [0–47.5] | 3.7 ± 4.6 [0–10] | 0.03 |
| PUCAI | 25.8 ± 15.3 [0–40] | 7.5 ± 5.1 [0–35] | 0.002 |
| Immunosuppressants [$n$,%] | 9 [75] | 11 [35.5] | 0.03 |
| Laboratory parameters | | | |
| Haemoglobin, g/dl | 11.3 ± 1.5 [9.1–14.5] | 12.8 ± 1.5 [8.3–14.9] | 0.005 |
| Pre-load sideraemia, µg/dl | 29.7 ± 14.4 [13–52] | 71.6 ± 31 [18–148] | 0.001 |
| Ferritin, ng/ml | 46.8 ± 28 [6–99] | 36.7 ± 23.9 [10–115] | 0.2 |
| Serum hepcidin, nM | 4.8 ± 7.8 [0.55–28.5] | 2.7 ± 3.9 [0.55–19.2] | 0.06 |
| Transferrin, g/l | 2.3 ± 0.8 [1.3–4.3] | 2.7 ± 0.4 [1.6–3.6] | 0.04 |
| Tsat, % | 9.9 ± 5.7 [4–21] | 19.8 ± 9.5 [3–41] | 0.003 |
| STfR, mg/dl | 1.8 ± 0.5 [0.8–2.4] | 1.9 ± 1.5 [0.9–8.5] | 0.8 |
| TIBC, µg/dl | 321 ± 51 [220–370] | 327.6 ± 78.2 [139–460] | 0.8 |
| Reticulocytes,% | 2.6 ± 3 [0.8–9] | 4.6 ± 3.9 [0.6–9] | 0.1 |
| CRP, mg/dl | 1.7 [0.2–5.1] | 0.4 [0–2.6] | 0.001 |
| ESR, mm | 16.7 ± 11.4 [5–35] | 8.4 ± 9.6 [1–46] | 0.02 |
| Calprotectin, µg/g | 316 [25–485] | 234.1 [30–493] | 0.6 |

All continuous variables values are expressed as means ± standard deviation [range].

CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; IBD-U, inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; STfR, soluble transferrin receptor; TIBC, total iron binding capacity; Tsat, transferrin saturation; UC, ulcerative colitis.

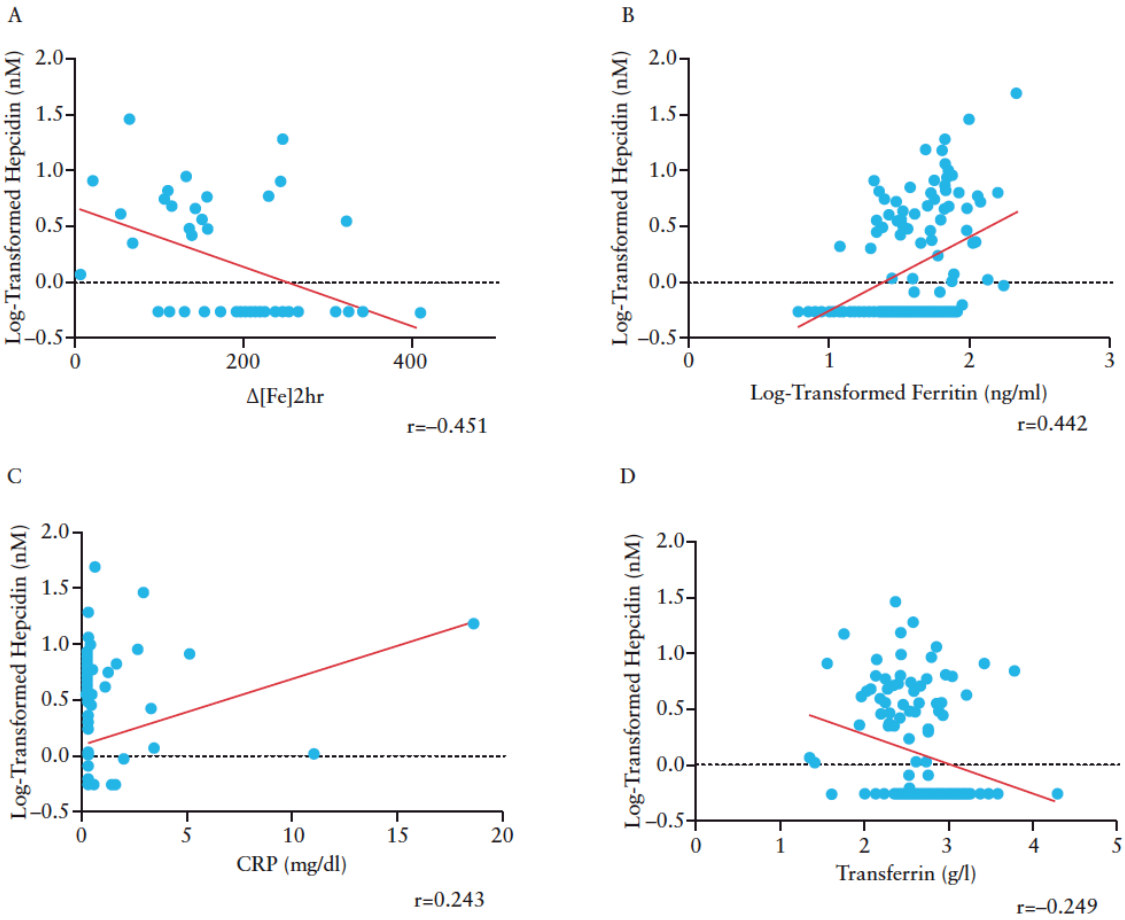


**Figure 1.** Correlations between log-transformed serum hepcidin and Δ[Fe]2hr [r = -0.451, $p = 0.002$] in inflammatory bowel disease [IBD patients]. [A] Correlations between log-transformed serum hepcidin and log-transformed ferritin [r = 0.442, $p = 0.0$ 001]. [B] C-reactive protein [CRP] [r = 0.243, $p = 0.009$] and [C] transferrin [r = -0.249, $p = 0.005$], [D] in all the enrolled patients.

## 4. Discussion

To the best of our knowledge, this is the first paediatric study evaluating iron absorption and serum hepcidin levels in IBD paediatric patients. Our data show that IBD children with active disease tend to have impaired iron absorption, driven through the hepcidin pathway. Indeed, serum hepcidin levels were significantly higher in IBD patients with moderate to severe activity as compared with all other groups, including patients with mild activity or in remission, coeliac patients, and healthy controls. In addition, a significant inverse correlation was found between hepcidin levels and iron absorption. Our results are in agreement with the paper from Semrin and colleagues, the only paediatric study investigating the relationship between iron absorption and hepcidin.[11] The authors, using urinary hepcidin as a proxy for serum hepcidin, enrolled 19 paediatric patients with CD and found that hepcidin was increased in those with active disease and inversely correlated with iron absorption.[11]

Anaemia is a relevant problem frequently occurring during IBD management. Despite its relative high prevalence, costs, and impact on patient quality of life, it is rarely considered and adequately treated.[2,25,26] In our study population, the prevalence of anaemia among IBD patients was 34%. It is well known that the origin of IBD-related anaemia is usually multifactorial.[23] Not surprisingly, the majority of the anaemic patients in our study population showed a combined IDA and ACD [70.5%]. This finding once more highlights the difficulties in the therapeutic management of IBD-related anaemia. Indeed, in those cases with a mixed pathogenesis, the clinician usually faces the dilemma whether or not to use oral iron. As previously reported, the treatment with oral iron has significant limitations in IBD, being less efficacious than the intravenous route.[27] In addition, absorption of iron from the GI tract is limited, and unabsorbed iron is exposed to the intestinal surface. Iron mucosal harm has been described in IBD.[28] Studies in animal models of colitis indicate that luminal iron may exacerbate disease activity.[29,30] In a more recent study, iron supplementation affected microbiota and increased faecal calprotectin.[31] Taking into account this possible warning, the recently published ECCO guidelines state that the intravenous route should be preferred in those patients with suspected ACD.[23] It is therefore important to detect ACD in order to avoid an unnecessary and possibly harmful oral iron therapy.

The development of ACD is influenced by numerous factors, among which hepcidin is now considered the leading actor. Indeed, the decrease in ferroportin expression, which results from elevated hepcidin levels, would block entry of iron into the circulation, with consequent erythropoiesis impairment. Furthermore, since intestinal absorption of iron is inhibited by downregulation of ferroportin on enterocytes, the anaemia would be resistant to oral iron supplementation.[7,8] Therefore, serum hepcidin may represent an useful

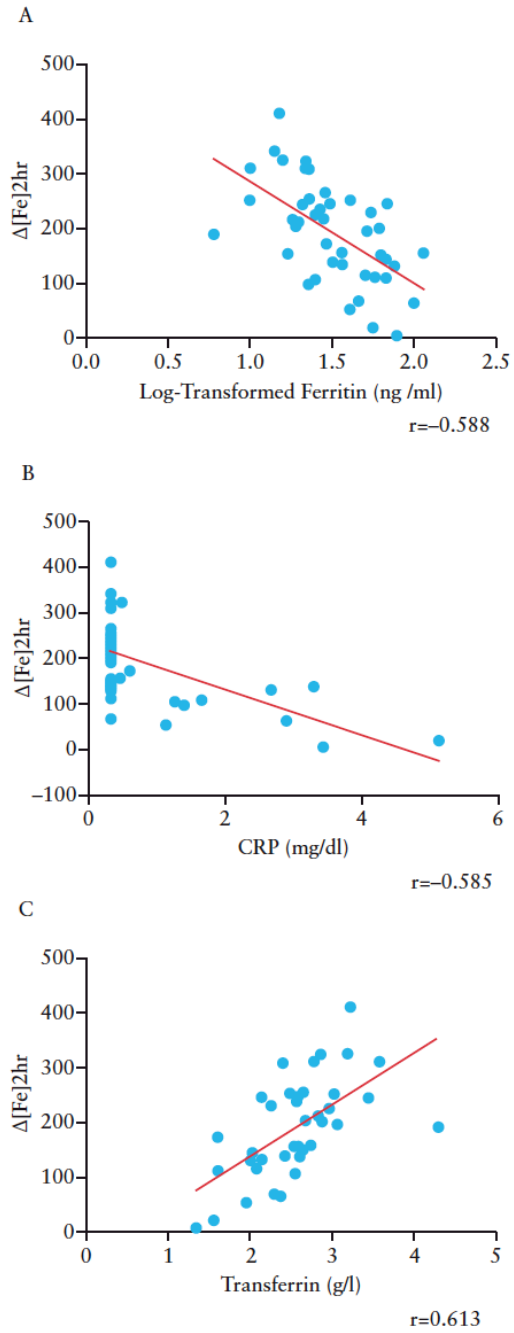


**Figure 2.** Correlations between Δ[Fe]2hr and log-transformed ferritin [r = -0.588, $p$ = 0.0 001]. [A] C-reactive protein [CRP] [r = -0.585, $p$ = 0.0 001] and [B] transferrin [r = 0.613, $p$ = 0.0 001], [C] in inflammatory bowel disease [IBD] patients.

surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as first-line option. To date, no paediatric study has evaluated serum hepcidin sensitivity and specificity in IBD-related anaemia. As previously reported in adult populations, the low specificity together with the lack of standardisation should be considered the main limitations of this marker.[17,32] Although strongly limited by the small sample of anaemic patients, our data suggest that patients with pure IDA have undetectable hepcidin levels. This finding, if confirmed by larger series, may at least allow hepcidin use in the differential diagnosis of pure IDA from ACD. On the other hand, hepcidin's role in the diagnosis of combined IDA and ACD is still questionable and needs to be addressed by further studies.

Based on a recent paper published by Wang and colleagues, hepcidin may also act beyond the simple role of iron regulator.[33] The authors, inhibiting hepcidin expression in a mouse model of colitis, not only corrected IBD-related anaemia but also reduced colonic inflammatory cytokine expression.[33] This finding suggests that hepcidin may be some way involved in perpetuating inflammation, representing a new potential IBD therapeutic target. Our study does not provide information about hepcidin's role in the inflammatory process. Nevertheless, IBD children with pathological ILT showed higher values of disease activity indexes and acute phase reactants and were more often under immunosuppressive therapy, indicating the more severe phenotype of disease. In addition, the positive correlations of hepcidin with ESR and CRP, confirming that inflammation plays a major role in hepcidin induction, may also indicate that hepcidin could be directly implicated in the IBD inflammatory cascade. Anyhow, targeted studies are still necessary to confirm hepcidin's role in the pathogenesis of IBD's inflammatory course.

Our study was also meant to find out which iron parameters better correlate with hepcidin levels, and serum ferritin was found to be the most strictly correlated. This finding is in agreement with previous literature,[17,34] and once more demonstrates that ferritin is the primary biochemical marker correlated to hepcidin concentration. However, 58.3% of patients, with a diagnosis of combined IDA + ACD and normal ferritin values, showed an increased hepcidin, demonstrating that the ferritin value is not sufficient in the differential diagnosis of IBD-related anaemia.

This study has some limitations. First of all, intestinal iron absorption was simply determined using the ILT based on the increment of iron level 2 h after administrating an iron load, and this constitutes a methodological limitation. However, studies using dual stable iron isotope techniques, which are considered the gold standard for iron absorption determination, have shown a similar inverse correlation between serum hepcidin and iron absorption in healthy controls.[35,36] In addition, ILT has already been correlated with urinary hepcidin in IBD.[11] Finally, due to the earlier diagnosis, coeliac children were not age-matched with IBD and healthy controls and this may have influenced differences in hepcidin levels.

### 4.1. Conclusion

In conclusion, this comparative, cross-sectional study demonstrates that serum hepcidin is increased in IBD children with active disease and plays an important role in the process of iron malabsorption. ACD is significantly prevalent in paediatric IBD and should be taken in consideration before starting oral iron therapy. If confirmed by further studies, the negative correlation between hepcidin levels and iron malabsorption during the ILT may have important practical implications for a tailored management of anaemia in children with IBD-associated IDA. Indeed, serum hepcidin may serve as a useful, sensitive, surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as first-line option, avoiding the waste of time with a futile and possibly harmful cycle of oral iron. Further studies are needed to better elucidate the role of hepcidin in both iron metabolism and inflammation in IBD, in order to design new therapeutic strategies for ACD in paediatric IBD.

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## Conflict of Interest

The authors declare no conflict of interest to disclose regarding this paper; AS is a speaker for Valeas Angelini, Milte Italia, and Danone, and consultant of D.M.G. Italy and Sucampo.

## Author Contributions

MM: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. CS: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. AA: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. FR: substantial contributions to conception and design, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published. RA: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. NC:substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. DG: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. BN: revising the article critically for important intellectual content and final approval of the version to be published. AS: revising the article critically for important intellectual content and final approval of the version to be published. SP: revising the article critically for important intellectual content and final approval of the version to be published. EM: substantial contributions to conception and design, interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

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### 5.2 Pancreatic involvement in paediatric IBD

Pancreatic involvement (PI) in patients with IBD includes all the spectrum of clinical presentations from asymptomatic hyper-enzinemia to chronic pancreatitis (CP) with development of pancreatic insufficiency [188]. Although this EIM seems to be much more frequently observed than reported, data about the prevalence and the incidence of PI in IBD are not well defined both in adults and children. The incidence of PI varies widely in IBD adult patients, ranging from 5% to 21% [189, 190]. According to several studies, hyperamylasemia (HA) and exocrine pancreatic insufficiency are found in 6–16 and 21–80% of adult patients, respectively, whereas histological changes are observed in 38–53% of post-mortem pathological examinations [189-192]. With regards to IBD paediatric patients there are only limited published data on the incidence of acute pancreatitis (AP) [193, 194]. There are many possible etiological factors, which link IBD and PI [195]:

- Drugs assumption;
- Biliary lithiasis, which is known to be more frequent in IBD;
- CD duodenal involvement;
- Sclerosing cholangitis.

Furthermore, it is worth citing, the rare variant of autoimmune pancreatitis, which has been described to occur more frequently among IBD patients [196]. With regards to drugs, several case reports about drug-induced pancreatitis have been published [197]. Although it is always difficult to establish a causal role for medications, few drugs are clearly associated with a high risk for drug-induced pancreatitis. The medications used for the IBD management, associated with the PI development, are 5-ASA compounds, AZA, metronidazole and CCS [195, 198]. Nevertheless, besides these well-known etiological factors, it appears that IBD itself may contribute to the pathogenesis of PI [188].

As above-mentioned, in addition to the more classical clinical manifestations of PI, children with IBD may often present an asymptomatic hyper-amylasemia (HA) and hyper-lipasemia

(HL), which may be completely misdiagnosed. Various possible explanations for asymptomatic HA and HL in IBD patients have been proposed. The pancreatic enzyme elevation observed in more extensive or active disease can represent the abnormal passage of the enzyme from the gut lumen to the blood, due to the increased permeability of the inflamed mucosa [199]. In addition, there are several potential mechanisms for the suggested enzyme leakage from the pancreas. First, the pancreas might be affected in some way directly by the extent of IBD. Another explanation could be an enzyme increase related to the pancreatic effects of inflammatory mediators and cytokines released from the inflamed gut. A third mechanism might be associated with inflammation of pancreatic ducts [189]. With regards to outcomes of PI and treatment, few limited data are available. As well known, there is no specific therapy for PI. Therefore, the supportive treatment, including fasting, rehydration, proton pump inhibitor and antibiotic therapies, is also used in PI associated to IBD [195].

Despite limited reports, the relationship between PI and IBD has not been further investigated, particularly in paediatrics. For these reason, we conducted a retrospective study, using the IBD web-registry of the Italian Society for Paediatric Gastroenterology and Nutrition (SIGENP) to address the following objectives:

1. To investigate prevalence and disease course of IBD paediatric patients presenting with pancreatitis;
2. To evaluate the clinical significance of exclusive HA and HL in IBD children.

From 2009 to 2012 649 patients with a diagnosis of IBD were identified, of whom 27 (4.1%) resulted to be affected by PI. Among patients diagnosed with PI, 11 (1.6%) fulfilled diagnostic criteria for AP, while the remaining 16 showed only an asymptomatic HA and HL. One interesting finding is that up to 18% of AP cases was already present at IBD onset. Broide and colleagues reported that AP may even precede the diagnosis of IBD

[193]. These data may suggest that at least in a subset of patients the intestinal and the pancreatic inflammation may be part of the same autoimmune disorder. As a matter of fact, 81.2% of patients with hyperenzinemia and 90.8% of children with AP showed an active intestinal disease. One of the hypotheses is that the epithelial cells of the GI tract and the pancreatic tissue may share similar target molecular or cellular structures vulnerable to injury. This thesis is also supported by a mouse model [200]. In 2005, Barthet and colleagues reported that mice affected by trinitrobenzene sulfonic acid-induced colitis, showed concurrently to intestinal lesions, specific pancreatic lesions. The authors concluded that in some cases PI and IBD may be different manifestations of the same disease [200]. We tried also to find out if there was any risk factor, allowing discriminating children with simple asymptomatic hyperenzinemia from patients developing an actual AP. The only significant factor resulted female gender. Bermejo et al. have already reported this finding in an adult population looking at the risk of AP in patients undergoing AZA treatment [201]. In addition, it looked like that patients with PI tend to have a major colonic involvement. The association between colonic disease and PI remains obscure. One possible explanation may be that the colon is considered a major source of the bacteria causing pancreatic necrosis in AP. Supporting this hypothesis subtotal colectomy before AP in rats was found to reduce mortality [202].

The results of our study give also important information regarding the clinical management of PI in IBD children. As well known, nausea and vomiting with abdominal pain are the most usual features associated with a diagnosis of acute or chronic pancreatitis. In IBD children, these symptoms need to be considered non-specific, since they may easily overlap with IBD flares. Indeed, abdominal pain during an episode of pancreatitis may be falsely attributed to active IBD and serum levels of amylase or lipase never tested resulting in an underestimation of AP incidence [195]. Our data confirmed that symptoms were not able to discriminate patients with exclusive increase of pancreatic

enzymes from patients with AP, suggesting that pancreatic imaging should be routinely evaluated in all IBD patients with a PI suspicion.

Another important issue is those related to the medications. As already mentioned, AZA e 5-ASA may be responsible of PI in children with IBD, although it is always difficult to establish a clean causal relationship [203-205]. Our study did not provide insights regarding this topic, but it gives an idea of the real practice in Italian centres. Indeed, we demonstrated that among children needing therapeutic measures, 85% of subjects withdrew AZA and/or suspended 5-ASA. This confirms that although the supportive literature is poor, clinicians tend to be very prudent in the management of these medications in the presence of PI.

Finally, our study is the first reporting data regarding the natural history of disease in IBD children with PI. Although in all our cases the severity of AP episodes was mild and self-limiting, some of them tend to recur during the follow up. Furthermore, at one-year follow-up, 1 patient showed a rapid evolution toward CP and consequent pancreatic insufficiency, suggesting that, in a subgroup of IBD patients, pancreatic dysfunction may not have a favourable course. Although the clinical significance of exclusive HA/HL has been questioned, the disease course of our patients seems not to be always benign. In our cohort, at 6 months follow-up, 25% of children with exclusive increase of pancreatic enzymes at the enrolment developed AP and at 12 months follow-up, 32.3% had persistent HA/HL.

In conclusion, the prevalence of AP in children is similar to that reported in adults. Our data support the thesis that in at least a subset of patients, IBD and PI may be part of the same systemic disorder. PI is more common in colonic disease and female gender seems to be significantly associated with the development of AP in IBD children. This study underlines that specific attention has to be paid to the monitoring of pancreatic

function in IBD children, considering that in a proportion of patients, PI tends to persist and in some cases the pancreatic damage may evolve.

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Alimentary Tract

# Natural history of pancreatic involvement in paediatric inflammatory bowel disease

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ABSTRACT

*Background:* Few case reports describe the clinical features of pancreatic involvement in inflammatory bowel disease.
*Aim:* To investigate prevalence and disease course of inflammatory bowel disease children with pancreatitis and with exclusive hyperamylasemia and hyperlipasemia.
*Methods:* We used a web-registry to retrospectively identify paediatric inflammatory bowel disease patients with hyperamylasemia and hyperlipasemia. Participants were re-evaluated at 6 months and 1 year.
*Results:* From a total of 649 paediatric patients, we found 27 with hyperamylasemia and hyperlipasemia (4.1%). Eleven patients (1.6%) fulfilled diagnostic criteria for acute pancreatitis. Female gender was significantly associated with acute pancreatitis ($p = 0.04$). Twenty-five children (92.5%) had colonic disease. At 6 months 1/11 children with acute pancreatitis (9%) showed acute recurrent pancreatitis, while 1 patient (9%) had persistent hyperamylasemia and hyperlipasemia. At 12 months, 1 patient showed chronic pancreatitis (9.1%). Of the 16 children with exclusive hyperamylasemia and hyperlipasemia, 4 developed acute pancreatitis (25%), while 1 patient (6.2%) still presented exclusive hyperamylasemia and hyperlipasemia at 6 months. At 12 months, 11/16 patients (68.7%) reached a remission of pancreatic involvement, whereas 5 remaining patients (32.3%) had persistent hyperamylasemia and hyperlipasemia.
*Conclusions:* In inflammatory bowel disease children, acute pancreatitis is more common in colonic disease and in female gender. Pancreatic function should be monitored, considering that pancreatic damage may evolve.


## 1. Introduction

Inflammatory bowel disease (IBD), characterized by chronic, relapsing immune-mediated inflammation of the gastrointestinal tract is often associated with extra-intestinal manifestations (EIMs) affecting multiple organs. EIM are reported to occur in 18–47% of paediatric and adult patients with IBD [1–5]. Acute and chronic

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pancreatitis as well as pancreatic insufficiency have been reported as one of EIMs in IBD [6].

Acute pancreatitis (AP) in children is a costly and increasingly recognized disease. Several studies have documented an increase during the past 10–15 years [7]. Estimated incidences range from 3.6 to 13.2 cases per 100,000 children per year [8,9]. The reasons for the increase are not entirely clear and may be multifactorial. An Australian study suggests that the increasing number is mainly due to the complications of systemic illness [9]. Patients with IBD are at increased risk of developing both acute and chronic pancreatitis. Clinical symptoms of IBD-associated pancreatitis are found in about 2% of patients but the actual frequency of the disease could be much higher. According to several studies, hyperamylasemia and exocrine pancreatic insufficiency are found in 6–16 and 21–80% of adult patients, respectively, whereas histological changes are observed in 38–53% of postmortem pathological examinations [6,10–12]. There are only limited published data on the incidence of acute pancreatitis in paediatric patients with IBD [13,14]. Although pancreatitis can be seen in association to drugs assumption, biliary lithiasis, Crohn's disease (CD) duodenal involvement or sclerosing cholangitis, the contribution of these etiological factors to histopathology-proved pancreatitis appears to be low and IBD itself seems to contribute to the pathogenesis [5]. In addition, a previous study also indicates that the rarer variant, autoimmune pancreatitis, occurs more often among IBD patients [15]. Regards to drugs, several case reports about drug-induced pancreatitis have been published [16]. Nevertheless, it is always difficult to establish a causal role for medications in the pathogenesis of pancreatitis, but a few medications are clearly associated with a high risk for drug-induced pancreatitis. This is true with regard to some medications used in IBD management. Of the medications, the possible agents inducing pancreatitis include sulfasalazine, 5-aminosalicylic acid (ASA) compounds, azathioprine (AZA), metronidazole and steroids [17,18]. Complicating the scenario it appears that IBD-associated pancreatic involvement may be often a silent disease in children. Various possible explanations for asymptomatic hyperamylasemia and hyperlipasemia in IBD patients have been proposed. The pancreatic enzyme elevation observed in more extensive or active disease can represent the abnormal passage of pancreatic amylase from the gut lumen to the blood due to increased permeability of the inflamed mucosa [19]. In addition, there are several potential mechanisms for the suggested enzyme leakage from the pancreas. First, the pancreas might be affected in some way directly by the extent of IBD. Another explanation could be an enzyme increase related to the pancreatic effects of inflammatory mediators and cytokines released from the inflamed gut. A third mechanism might be associated with inflammation of pancreatic ducts [6].

Despite scattered case reports, the relationship between pancreatic involvement and IBD has not been further investigated. The primary aim of the present study was to investigate prevalence and disease course of paediatric IBD patients presenting with pancreatitis; secondary aim was to evaluate the clinical significance of exclusive hyperamylasemia and hyperlipasemia in children with IBD.

## 2. Subjects and methods

We retrospectively reviewed data collected in the IBD web-registry of the Italian Society for Paediatric Gastroenterology, Hepatology and Nutrition (SIGENP). Paediatric gastroenterologists from all the Italian paediatric IBD centers belonging to the SIGENP, established in 2008 a prospective registry to collect demographic, clinical, and epidemiologic data from paediatric patients with IBD. The registry started the 1st January 2009 and included patients less than 18 years with a new diagnosis of IBD. Data of all paediatric patients enrolled and stored in the registry from January 1, 2009 to November 30, 2012 (data retrieval date) were used for this study. Nine sites participated to this study; trained investigators at each centre obtained information from the medical records (electronic and paper charts) and standardized information was entered into the registry. Eligible subjects included all patients with any form of IBD [ulcerative colitis (UC), CD and inflammatory bowel disease unclassified (IBD-U)]. Diagnosis of IBD was based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies, according to Porto criteria [20]. All patients presenting with serum amylase ≥100 IU/L (normal range: 28–100 IU/L) and serum lipase ≥60 IU/L (normal range: 13–60 IU/L) were included in the study. Participants were additionally evaluated within 6 months, and 1 year from enrolment. AP was defined as the presence of 2 of the following criteria: (a) abdominal pain compatible with AP, (b) serum amylase and/or lipase values ≥3 times upper limits of normal, (c) imaging findings of AP [21]. Acute recurrent pancreatitis (ARP) was defined as: ≥2 distinct episodes of AP with intervening return to baseline. The severity of AP episodes was assessed with the Paediatric Acute Pancreatitis Score (PAPS), developed by DeBanto and colleagues [22]. The system has eight parameters, scored at admission and at 48 h. The admission criteria include: age <7 years, weight <23 kg, white blood cell count >18,500/mm$^3$, and LDH > 2000 U/L. The 48-h criteria are trough calcium < 8.3 mg/dl, trough albumin < 2.6 mg/dl, fluid sequestration > 75 ml/kg/48 h, and a rise in BUN > 5 mg/dl. One point is assigned for each criterion met; a score of ≥3 is predictable of a severe course of disease [22]. Chronic pancreatitis (CP) was diagnosed if one of the following criteria was present: (a) typical abdominal pain plus characteristic imaging findings; (b) exocrine insufficiency plus imaging findings; (c) endocrine insufficiency plus imaging findings. Exclusive hyperamylasemia and hyperlipasemia was used to describe those patients who did not meet diagnostic criteria for pancreatitis [20].

The information retrieved for the purpose of this study included demographic features (age, gender), IBD type (CD, UC, IBD-U), median lag time period between the diagnosis of IBD and pancreatic involvement episodes, and disease location. The disease location at the diagnosis and at follow-up was established by endoscopic and imaging evaluations in all patients according to the availability of individual methods for each centre and reported in the registry. For the purpose of this manuscript, disease location was described according to Paris classification [23]. Disease activity at the diagnosis was scored by the Paediatric Crohn's Disease Activity Index (PCDAI) [24] or the Paediatric Ulcerative Colitis Activity Index (PUCAI) [25] for CD and UC, respectively. Laboratory tests included full blood count, C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR), nutritional, renal, and liver function parameters. In addition, pancreatic laboratory studies including serum amylase and lipase, were collected. Data on imaging methods used for the diagnosis of pancreatic involvement including transabdominal ultrasound (US), magnetic resonance cholangiopancreatography (MRCP), abdominal computed tomography scan (CT) or endoscopic retrograde cholangiopancreatography (ERCP), were evaluated. In patients with CP if available, details on genetic testing (CFTR, SPINK1, PRSS1) or on exocrine pancreatic function assessed with the faecal elastase, were recorded. In addition, pancreatic involvement episode characteristics, including drug exposure, severity, complications, in-hospital stay, actions taken post-pancreatic involvement were reported.

Institutional review board approval for the registry protocol and the informed consent and assent forms were obtained at each site before subject enrolment and data collection. Signed parental and patient informed consent and signed youth assent when appropriate were required from all patients enrolled.

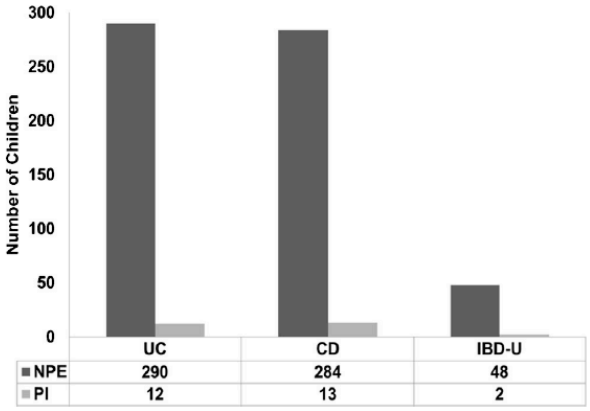


**Fig. 1.** Prevalence of pancreatic involvement in different inflammatory bowel disease type. CD: Crohn's disease; UC: ulcerative colitis; IBD-U: unclassified-IBD; NPE: normal pancreatic enzymes; PI: pancreatic involvement.

### 2.1. Statistical analysis

Statistical analysis was performed using SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). Means and medians were calculated for dimensional variables after controlling for normality of distribution. Categorical data were expressed as frequencies and percentages. The Student's *t*-test and the Mann–Whitney test for continuous variables and the $\chi^2$ and Fisher's exact tests for categorical variables were used where appropriate. A *p* value of 0.05 or less was considered significant.

## 3. Results

### 3.1. Patients characteristics

From 2009 to 2012 we identified 649 paediatric patients with a diagnosis of IBD, of whom 27 met the inclusion criteria and were enrolled in the study (4.1%, Fig. 1). Demographic and clinical characteristics for the study group are reported in Table 1. Median lag time between the diagnosis of IBD and pancreatic involvement was 7 months (range 0–65 months). In 5/27 children (18.5%), pancreatic involvement was present at time of IBD diagnosis (Table 2). Median serum amylase level was 196 (range 61–413 IU/L), and median serum lipase level was 299 (range 37–2565 IU/L). Eleven patients (40.7%) fulfilled diagnostic criteria for AP (6 CD, 4 UC, 1 IBD-U). On the basis of PAPS all 11 patients presented with an episode of mild AP (Table 3). The remaining 16 patients (60.3%) presented with exclusive hyperamylasemia and hyperlipasemia. Median serum amylase level and median serum lipase level were significantly higher in AP patients compared with patients with exclusive hyperamylasemia and/or hyperlipasemia ($p = 0.009$ and $p = 0.001$ respectively; Table 2). Comparing the total IBD registry population with patients with pancreatic involvement, female gender resulted to be significantly associated with AP ($p = 0.04$; OR: 4.8; 95% confidence interval 1–22), whereas no significant difference in gender was observed in patients with exclusive hyperamylasemia and/or hyperlipasemia ($p = 0.7$). IBD type, ongoing treatments, and extension of disease were not significant risk factors when comparing patients with hyperamylasemia/hyperlipasemia and children with AP (Table 2). Twenty-three patients (85.1%) with pancreatic involvement presented with active disease, but no significant difference was found among children with exclusive hyperamylasemia and/or hyperlipasemia and subjects with AP ($p = 0.6$; Table 2). Twenty-five patients (92.5%) with pancreatic involvement had colonic disease [11 CD, 12 UC and 2 IBDU]. Eight of the 12 children with UC and pancreatic involvement (66.6%) were affected by pancolitis (Table 2). Regarding symptoms associated with pancreatic involvement, 21 patients (77.8%) reported epigastric pain as the dominant clinical characteristic, followed by nausea and/or vomiting (22.2%), and fever (22.2%). No patient experienced jaundice. No statistical significant difference was observed when comparing symptoms in patients with exclusive increase of pancreatic enzymes and AP (Table 2).

**Table 1**
Baseline characteristics of 27 inflammatory bowel disease children with pancreatic involvement.

| | |
|---|---|
| Characteristics | |
| Patients (*N*) | 27 |
| Median age (years; range) | 12.3 (5.4–15.9) |
| PI onset (years; range) | 12.2 (0–65) |
| Gender (*N*, %) | |
| Males | 13/27 (48.1) |
| IBD type (*N*, %) | |
| CD | 13/27 (48.2) |
| UC | 12/27 (44.4) |
| IBD-U | 2/27 (7.4) |
| Paris classification | |
| CD (*N*, %) | |
| Ileum only (L1) | 2/13 (15.3) |
| Colon only (L2) | 0/13 (0) |
| Ileum and colon (L3) | 11/13 (84.6) |
| Upper gastrointestinal tract (L4a) | 5/13 (38.4) |
| UC (*N*, %) | |
| Proctosigmoiditis (E1) | 2/12 (16.6) |
| Left-sided colitis (E2) | 2/12 (16.6) |
| Extensive colitis (E3) | 2/12 (16.6) |
| Pancolitis (E4) | 6/12 (50) |
| IBD therapy (*N*, %) | |
| 5-ASA | 16/27 (59.3) |
| CCS | 3/27 (11.1) |
| AZT | 12/27 (44.4) |
| MTX | 1/27 (3.7) |
| Biologic therapy | 2/27 (7.4) |

PI: pancreatic involvement; CD: Crohn's disease; UC: ulcerative colitis; IBD-U: unclassified-IBD; 5-ASA: 5-aminosalicylic acid; CCS: corticosteroids; AZT: azathioprine; MTX: methotrexate.

### 3.2. Imaging

All 11 patients with AP underwent abdominal US imaging, 6 also underwent MRCP (54.5%) and 2 a CT scan (18.1%). Pancreatic pathological findings were found in all subjects with AP. Main pancreatic findings were: enlargement of the head and the body ($n = 2$); tail and head enlargement ($n = 2$); tail enlargement ($n = 3$); diffuse oedema ($n = 3$) and peripancreatic/pancreatic fluid collections ($n = 1$). Primary sclerosing cholangitis (PSC) was diagnosed in 1 (9%) out of 11 patients. None of the patients with exclusive hyperamylasemia and/or hyperlipasemia showed pathological findings at imaging studies.

### 3.3. Treatment

Nine patients (33.3%) were receiving mesalamine (5-ASA), 14 (51.8%) were receiving immunomodulatory therapy [1 steroids, 6 AZA, 5 AZA + 5-ASA, 1 methotrexate, 1 infliximab (IFX), and 1 IFX + ASA + AZA], and 4 patients (14.8%) were not treated. Nine of 11 (81.8%) patients with AP needed therapeutic measures compared to only 5/16 patients with serum hyperamylasemia and/or hyperlipasemia (31.2%; $p = 0.04$). Therapeutic measures in children with AP and hyperamylasemia/hyperlipasemia are reported in Table 4. None of the patients with AP developed early complications.

**Table 2**
Clinical differences between patients with exclusive hyperamylasemia/hyperlipasemia and acute pancreatitis.

| Characteristics | Exclusive HA/HL $n=16$ | Pancreatitis $n=11$ | $p$ |
|---|---|---|---|
| Median age (years; range) | 14 (6–17) | 14.4 (10–16.7) | 0.07 |
| Gender | | | 0.01 |
| Males | 11 (68.8) | 2 (18.2) | |
| Females | 5 (31.2) | 9 (81.8) | |
| PI at IBD disease onset | 2 (18.2) | 3 (18.8) | 1 |
| Symptoms | | | |
| Epigastric pain | 11 (68.8) | 10 (90.9) | 0.3 |
| Fever | 2 (12.5) | 4 (36.4) | 0.2 |
| Nausea/vomiting | 3 (18.3) | 3 (27.3) | 0.6 |
| Amylase (median, range) | 160 (61–320) | 248 (150–413) | 0.009 |
| Lipase (median, range) | 140.5 (37–508) | 817 (512–2565) | 0.001 |
| IBD type | | | 0.7 |
| CD | 7 (43.7) | 6 (54.5) | |
| UC | 8 (50) | 4 (36.3) | |
| IBD-U | 1 (6.2) | 1 (9) | |
| Median disease duration (months, range) | 9 (0–33) | 7 (0–65) | 0.4 |
| Active disease | 13 (81.2) | 10 (90.9) | 0.6 |
| PUCAI (median, range) | 20 (0–45) | 22.5 (15–35) | 1 |
| PCDAI (median, range) | 30 (18–54) | 28.7 (10–70) | 1 |
| Colonic involvement | 16 (100%) | 9 (81.8) | 0.1 |
| Disease location | | | |
| Ileum only (L1) | 0 (0) | 1 (9) | 0.4 |
| Colon only (L2) | 0 (0) | 0 (0) | 1 |
| Ileum and colon (L3) | 7 (43.7) | 4 (36.3) | 1 |
| Upper GI tract (L4a) | 1 (6.2) | 3 (27.3) | 0.1 |
| Upper GI tract (L4b) | 0 (0) | 1 (9) | 0.4 |
| Proctosigmoiditis (E1) | 1 (6.2) | 1 (9) | 1 |
| Left-sided colitis (E2) | 1 (6.2) | 1 (9) | 1 |
| Extensive colitis (E3) | (6.2) | 1 (9) | 1 |
| Pancolitis (E4) | 5 (31.2) | 1 (9) | 0.3 |
| IBD therapy | | | |
| 5-ASA | 10 (62.5) | 6 (54.5) | 0.7 |
| CCS | 3 (18.3) | 1 (9) | 0.6 |
| AZT | 5 (31.2) | 6 (54.5) | 0.3 |
| MTX | 0 (0) | 1 (9) | 0.4 |
| BIO | 2 (12.5) | 0 (0) | 0.5 |
| Imaging findings | | | NA |
| Enlargement of the head and the body | – | 2 (18.1) | |
| Tail and head enlargement | – | 2 (18.1) | |
| Diffuse oedema | – | 3 (25) | |
| Peripancreatic/pancreatic fluid collections | – | 1 (9) | |

5-ASA: 5-aminosalicylic acid; AP: acute pancreatitis; AZT: azathioprine; BIO: biologic therapy; CCS: corticosteroids; CD: Crohn's disease; HA/HL: hyperamylasemia/hyperlipasemia; IBD: inflammatory bowel disease; IBD-U: unclassified-IBD; MTX: methotrexate; PI: pancreatic involvement; PUCAI: Paediatric Ulcerative Colitis Activity Index; PCDAI: Paediatric Crohn's Disease Activity Index; UC: ulcerative colitis.

**Table 3**
Paediatric acute pancreatitis score criteria.

| | Patients with AP |
|---|---|
| Admission criteria | |
| Median age (years; range) | 14.5 (10–17) |
| Median weight (kg, range) | 43 (26–63) |
| White blood cell count, $\times 10^3$/mm (median, range) | 6.9 (4.1–14.8) |
| LDH, U/L (median, range) | 377.5 (206–639) |
| 48-h criteria | |
| Calcium, mg/dl (median, range) | 9.4 (8.5–10) |
| Albumin, mg/dl (median, range) | 4.4 (2.5–4.8) |
| Fluid sequestration, ml/kg/48 h (median, range) | 18.4 (6–54) |
| Rise in BUN, mg/dl (median, range) | 3.2 (1.2–6) |
| Score ($N$, %) | |
| 0–2 points (mild) | 11 (100%) |
| 3–8 points (severe) | 0 |

BUN: blood urea nitrogen; LDH: lactic dehydrogenase.

### 3.4. Natural history

At 6 and 12 months follow-up evaluations, median serum amylase level was 56 (range 8–240 IU/L) and 66.5 (range, 23–480 IU/L), respectively; median serum lipase level was 28 (range 8–633 IU/L) and 51.1 (range, 12–206 IU/L), respectively. Natural history of pancreatic involvement is reported in Fig. 2.

**Table 4**
Therapeutic measures in patients with acute pancreatitis and hyperamylasemia/hyperlipasemia.

| Therapeutic measures | AP (11) | HA/HL (16) |
|---|---|---|
| Fasting with rehydration ($n$, %) | 6 (54.5) | 0 (0) |
| Antibiotic therapy ($n$, %) | 3 (27.2) | 0 (0) |
| PPIs ($n$, %) | 1 (9) | 0 (0) |
| Octreotide ($n$, %) | 1 (9) | 0 (0) |
| AZT suspension | 5 (45.5) | 4 (25) |
| 5-ASA suspension | 2 (18.1) | 1 (6.3) |
| None | 2 (18.1) | 11 (68.7) |

AP: acute pancreatitis; HA/HL: exclusive hyperamylasemia/hyperlipasemia; TPN: total parenteral nutrition; PPIs: proton pump inhibitors; AZT: azathioprine; 5-ASA: 5-aminosalicylic acid.

#### 3.4.1. Acute pancreatitis

At 6 months of follow-up 1/11 children with AP (9%) was diagnosed with ARP, while 1 patient presented with hyperamylasemia/hyperlipasemia. At 6 months, patients presenting with recurrent pancreatic involvement showed higher values of PUCAI/PCDAI scores compared with the remaining patients with a trend towards statistical significance (median: 17.5 vs. 5; range: 0–47.5; $p=0.07$). At 12 months follow-up 10 patients (90.9%) had a remission from AP, while 1 patient (9.1%) showed laboratory

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| Gender | | | 0.01 |
| Males | 11 (68.8) | 2 (18.2) | |
| Females | 5 (31.2) | 9 (81.8) | |
| PI at IBD disease onset | 2 (18.2) | 3 (18.8) | 1 |
| Symptoms | | | |
| Epigastric pain | 11 (68.8) | 10 (90.9) | 0.3 |
| Fever | 2 (12.5) | 4 (36.4) | 0.2 |
| Nausea/vomiting | 3 (18.3) | 3 (27.3) | 0.6 |
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| Colonic involvement | 16 (100%) | 9 (81.8) | 0.1 |
| Disease location | | | |
| Ileum only (L1) | 0 (0) | 1 (9) | 0.4 |
| Colon only (L2) | 0 (0) | 0 (0) | 1 |
| Ileum and colon (L3) | 7 (43.7) | 4 (36.3) | 1 |
| Upper GI tract (L4a) | 1 (6.2) | 3 (27.3) | 0.1 |
| Upper GI tract (L4b) | 0 (0) | 1 (9) | 0.4 |
| Proctosigmoiditis (E1) | 1 (6.2) | 1 (9) | 1 |
| Left-sided colitis (E2) | 1 (6.2) | 1 (9) | 1 |
| Extensive colitis (E3) | (6.2) | 1 (9) | 1 |
| Pancolitis (E4) | 5 (31.2) | 1 (9) | 0.3 |
| IBD therapy | | | |
| 5-ASA | 10 (62.5) | 6 (54.5) | 0.7 |
| CCS | 3 (18.3) | 1 (9) | 0.6 |
| AZT | 5 (31.2) | 6 (54.5) | 0.3 |
| MTX | 0 (0) | 1 (9) | 0.4 |
| BIO | 2 (12.5) | 0 (0) | 0.5 |
| Imaging findings | | | NA |
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| Octreotide ($n$, %) | 1 (9) | 0 (0) |
| AZT suspension | 5 (45.5) | 4 (25) |
| 5-ASA suspension | 2 (18.1) | 1 (6.3) |
| None | 2 (18.1) | 11 (68.7) |

AP: acute pancreatitis; HA/HL: exclusive hyperamylasemia/hyperlipasemia; TPN: total parenteral nutrition; PPIs: proton pump inhibitors; AZT: azathioprine; 5-ASA: 5-aminosalicylic acid.

#### 3.4.1. Acute pancreatitis

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and radiological signs of CP with reduced faecal elastase. The same patient resulted to carry a F1052V CFTR mutation in heterozygosis.

*3.4.2. Hyperamylasemia/hyperlipasemia*

At 6 months follow-up 4/16 children with exclusive increase of pancreatic enzymes developed AP (25%), while 1 (6.2%) still presented hyperamylasemia/hyperlipasemia. At 12 months follow-up, 11/16 patients (68.7%) reached a complete remission of pancreatic involvement, whereas in the 5 remaining patients (32.3%) exclusive hyperamylasemia/hyperlipasemia persisted. At 12 months, patients with recurrent pancreatic involvement had higher PUCAI/PCDAI scores compared to children who did not, without reaching statistical significance (median: 13 vs. 5; range: 0–30; $p = 0.08$).

## 4. Discussion

IBD patients are affected by an increased incidence of pancreatic involvement, including AP and exclusive hyperamylasemia/hyperlipasemia, when compared with the general population [26]. The incidence of pancreatic involvement varies widely in IBD adult patients, ranging from 5% to 21% [6,11]. A possible explanation for this wide range is that diagnosis of mild disease may be easily missed. There are only limited and not recent published data on the incidence of pancreatic involvement in paediatric patients with IBD. To the best of our knowledge our paper represents the first paediatric multicentre IBD registry-based study, characterizing the natural history of pancreatic involvement. Consistent with the published literature in the present cohort, the prevalence of pancreatic involvement was 4.1% and AP was diagnosed in 1.6% of cases.

The question of whether pancreatitis is an EIM of IBD remains unclear [26]. The aetiology and pathogenesis of AP are elusive and seem to be multifactorial in the majority of IBD patients. It is possible that epithelial cells of the gastrointestinal tract and pancreatic tissue may share similar target molecular or cellular structures vulnerable to injury. To support this hypothesis in our cohort of patients AP was present at IBD onset in 18%, suggesting that in some cases pancreatic dysfunction is part of a common immune disorder. The mouse model of trinitrobenzene sulfonic acid-induced colitis was shown to have concurrent pancreatic lesions [27]. Nausea and vomiting as associated features often suggest a diagnosis of acute or chronic pancreatitis. More often in CD than in UC, recurrent abdominal pain is the presenting feature [18]. Unfortunately, an elevation of serum amylase and lipase without symptoms or signs of pancreatitis is more frequent in IBD patients than in controls [6,11]. Various hypotheses for exclusive hyperamylasemia and hyperlipasemia in IBD patients have been proposed and are still matter of discussion [19]. Complicating the picture, symptoms of pancreatitis often overlap with IBD flares. Indeed abdominal pain in an episode of pancreatitis may be falsely attributed to active IBD and serum levels of amylase or lipase never tested resulting in an underestimation of AP incidence [18]. According to this finding, in our cohort of patients symptoms were not able to discriminate patients with exclusive increase of pancreatic enzymes from patients with AP, suggesting that pancreatic imaging should be routinely evaluated in IBD patients.

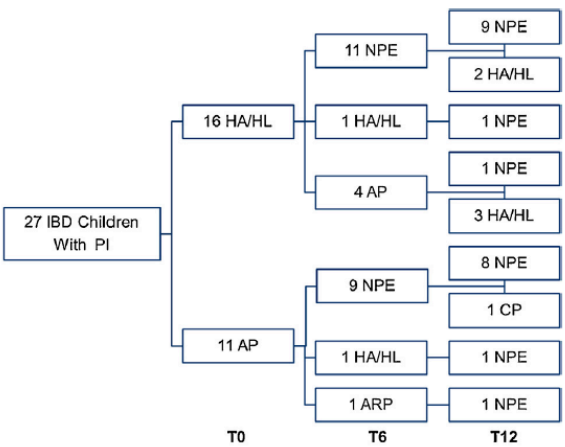


**Fig. 2.** Natural history of 27 children with inflammatory bowel disease and pancreatic involvement at one-year follow-up. AP: acute pancreatitis; ARP: acute recurrent pancreatitis; CP: chronic pancreatitis; HA/HL: hyperamylasemia/hyperlipasemia; IBD: inflammatory bowel disease; NPE: normal pancreatic enzymes; PI: pancreatic involvement; T0: baseline; T6: 6 months; T12: 12 months.

AP in adults has been reported much more commonly in CD than in UC [18]. As previously described in paediatric literature, in our study AP incidence was not different according to the IBD type, either CD or UC [13]. The relationships between pancreatitis and the extent and severity of the disease led to controversial conclusions in the recent literature [10]. According to Heikius et al. who demonstrated a correlation between the lipase increase and the histological activity of the disease, 91% of our patients with pancreatic involvement presented active disease [6]. Furthermore, patients with recurrent episodes of AP and hyperamylasemia/hyperlipasemia showed higher PCDAI/PUCAI scores at 6 and 12 months. These data may suggest that pancreatic involvement could be strictly related to the activity of disease at least in a subset of patients.

Interestingly, the majority of our patients with CD and AP showed a colonic involvement, as previously described [13]. Nevertheless, It is well known that paediatric patients with CD present with more colonic involvement [28]. The association between colonic disease and AP remains obscure. One possible explanation may be that the colon is considered a major source of the bacteria causing pancreatic necrosis in AP. Supporting this hypothesis subtotal colectomy before AP in rats was found to reduce mortality [29].

Female gender resulted to be significantly associated with the onset of pancreatitis in our patients, also when comparing with the total IBD registry population. Bermejo et al. reported that female gender was a risk factor for AZA/Mercaptopurine-associated acute pancreatitis [30]. Female predominance in the majority of autoimmune disorders may be one of the possible explanations.

Among IBD children with pancreatic involvement needing therapeutic measures, 85% of subjects withdrew AZA and/or suspended ASA. Indeed, AP is a well-recognized adverse effect occurring in 2–4% of IBD patients receiving thiopurines and it is usually considered as an absolute contraindication to reintroduction of a thiopurine [30–32]. However, the actual impact of thiopurines in AP episodes of IBD children is still questioned. In fact, pancreatic involvement is much rarer or not associated with AZA therapy in many other conditions, including rheumatoid arthritis, systemic lupus erythematosus, or in post-transplant patients [33]. This finding may suggest that AP could be more likely IBD-related rather than drug induced. Concerning mesalazine, its association with AP remains controversial. Munk et al. found no increased risk for mesalazine using data from a Danish hospital discharge registry [34]. In contrast, the UK study on AP mentioned above showed a nine-fold increased risk in patients receiving mesalazine up to 3 months before the onset of the disease [35].

No paediatric data are reported about the clinical course of IBD patients with AP. Although in all our cases the severity of AP episodes was mild and self-limiting, some of them tend to recur during the follow up. Furthermore, in our cohort, at one-year follow-up, one showed a rapid evolution towards CP and

consequent pancreatic insufficiency, suggesting that, in a subgroup of IBD patients, pancreatic dysfunction has an unfavourable course. However, the severe evolution of the patient developing CP may be partially explained by the concomitant CFTR mutation in heterozygosis, not causing cystic fibrosis, but associated with pancreatic disorders in a subgroup of patients [36]. This finding highlights the need for checking other causes of pancreatic involvement in IBD children. Although the clinical significance of exclusive hyperamylasemia/hyperlipasemia has been questioned, the natural history of our patients seems not to be always benign. In our cohort, at 6 months follow-up, 25% of children with exclusive increase of pancreatic enzymes at the enrolment developed AP and at 12 months follow-up, 32.3% had persistent hyperamylasemia/hyperlipasemia.

Our study has some limitations besides the retrospective nature. Firstly, we did not have a control group of patients with AP without IBD; second, we did not evaluate serological markers of autoimmune pancreatitis. One could argue that the number of IBD patients with pancreatic involvement is too small to draw definitive conclusions; furthermore, in this subset of patients it is difficult to clearly define the aetiology of AP, as IBD itself is a predisposing factor, and development of pancreatitis is certainly multifactorial. Nevertheless our data, based on a large paediatric IBD population, highlight once again that pancreatic involvement has a relatively low prevalence.

In conclusion, this multicentre, retrospective registry-based study suggests that prevalence of AP in children is similar to that reported in adults. AP is more common in colonic disease and female gender seems to be significantly associated with the development of AP in IBD children. This study underlines that specific attention has to be paid to the monitoring of pancreatic function in IBD children, considering that in a proportion of patients the pancreatic involvement tends to persist and in some cases pancreatic damage may evolve. Future studies on the pathogenesis of pancreatitis and its relationship to the long-term outcome in IBD are required.

## Conflict of interest

None declared.

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# CHAPTER 6

# IMPROVING THERAPEUTIC STRATEGIES

As widely discussed within Chapter 1, the therapeutic approach has profoundly evolving in the last decades with a growing trend to use a more aggressive "top-down" approach in order to reach and maintain a stable remission, and above all to modify the natural history of disease and reduce complications. In line with this topic within this chapter we will revise the SIGENP multicentre paper ***"Effect of Early Versus Late Azathioprine Therapy in Pediatric Ulcerative Colitis"*** published in Inflammatory Bowel Disease *(Inflamm Bowel Dis. 2016; 22:1647-54).* This paper was an attempt to verify if the early introduction of AZA in paediatric UC may positively change the course of disease. Finally, we will also discuss the results of the paper ***"Pediatric ulcerative colitis surgery: Italian survey"*** published in Journal of Crohn's and colitis *(J Crohns Colitis. 2015; 9:558-64).* This paper describes the role and the outcomes of surgery in UC. Indeed, despite the great medical armamentarium, surgery keeps still its own role in the therapeutic management of UC, as possible curative means after the drugs' failure. Understanding the exact timing to send our children to the surgeon is often crucial for their prognosis.

## 6.1 Role of early immunosuppression in UC

As well underlined in chapter 1 and 3, paediatric UC is characterized by a more severe disease course when compared to adults [8, 115, 131]. Nevertheless, current guidelines still do not recommend any kind of top-down approach. At the same time, an accelerated step-up approach using AZA from the diagnosis is only suggested in ASC [50]. Although, it is now well known that the conventional "step-up" approach does not impact on the risk of surgery overtime, there is a lack of evidences and RCTs on the efficacy of a more aggressive approach starting from the diagnosis. In addition, risk stratification of UC children has been poorly defined. To date only early onset disease, female gender,

extensive colitis at diagnosis, and a systemic CCS therapy at disease presentation have been identified as possible predictors of worst disease outcomes [206-208]. As a matter of fact, the role of early immunosuppression in paediatric UC has not been deeply investigated. The idea that starting immunosuppressive drugs, and in particularly thiopurines, early from the diagnosis, with the so-called accelerated "step-up" approach, may ameliorate the long-term outcomes of the disease, comes from studies on paediatric CD. The first data were published by Markowitz et al. early in 2000 [209]. The authors conducted a RCT, enrolling 55 children with newly diagnosed moderate-to-severe CD who were randomized to receive an initial course of prednisone and either 6-mercaptopurine (6-MP) or placebo and were followed-up till 18 months [209]. Patients taking 6-MP had a reduced total duration of corticosteroid usage, and their cumulative steroid dose received was also less when compared to the placebo group. Astonishingly only 9% of the 6-MP group relapsed during the study period compared with 47% of the controls [209]. However, this study had several limitations, including the fact that it was not sufficiently powered. Following studies, although not replicating the same efficacy, were anyway able to demonstrate the efficacy of early AZA introduction at least as a steroid-spare agent [210, 211]. Therefore, an "accelerated" step-up approach, based on the initiation of thiopurines, became commonly used in clinical practice for the treatment of CD. Translating data from CD, many centres started to do the same in children affected by severe UC. Nevertheless, data on the short- and long-term efficacy of an accelerated step-up strategy in UC is lacking and studies are strongly needed in order to define new efficacious therapeutic strategies in paediatric UC.

Therefore, we conducted a retrospective analysis of the IBD web-registry of the Italian Society for Paediatric Gastroenterology and Nutrition (SIGENP) and collected the data of all children with UC treated with AZA within 24 months of diagnosis, from January 1, 2009, to January 5, 2015. The primary aim of this analysis was to describe the efficacy

of AZA in newly diagnosed paediatric UC, comparing the outcomes of early (0-6 months of diagnosis) versus late (6-24 months) initiation of therapy.

One-hundred-twenty-one children were included in the analysis. Seventy-six patients (63%) were treated with AZA within 6 months of diagnosis ("early" group), 45 (37%) between 6–24 months after diagnosis ("late" group).

The results of this study evaluating the impact of the timing of initiation of AZA on sustainable clinical remission and mucosal healing in paediatric UC, failed to demonstrate the superiority of early treatment compared to classical step-up approach. Moreover, our data suggest that an accelerated use of thiopurines has no steroid-sparing effect compared to conventional treatment, since no difference in CCS use was found between groups at any time point of follow-up. No significant differences were also noticed with regards to colectomy. Indeed, at 2 years of follow-up the rate of surgery was comparable between early and late group.

To our knowledge, no other reports on the effect of early immunomodulatory therapy in paediatric UC have been published so far. Only recently two population-based studies on the impact of thiopurines questioned their early use, reporting no efficacy in reducing the risk of colectomy in adults and adolescents with UC [212, 213].

Regarding MH, we found an overall rate of 37% and 40% at 1- and 2-year follow-up, respectively. These results are similar to those reported in literature [214, 215]. When comparing MH in early and late patients we didn't find any difference for MH rates both at 12 and 24 months, suggesting that the timing of AZA introduction has no impact on the ability of AZA to heal the intestinal mucosa in paediatric UC. Also, when evaluating other secondary outcomes, including the number of hospitalizations, need of treatment escalation and episodes of ASC, no difference was found based on the timing of AZA initiation.

As said, thiopurines have their own established role as maintenance therapy in both

adult and paediatric UC. However, taken together, our results strongly discourage their use as an early-step-up accelerated treatment in UC. Nevertheless, it has to be underlined that our study had several limitations, including the retrospective nature and the enrolment within the early treatment group of many patients starting AZA 6 months after the diagnosis. Indeed, starting AZA at the onset of disease, contemporarily with the initiation of CCS, may have different outcomes. Therefore, well-designed randomized controlled trials are needed to confirm or not our findings and to select those patients who may benefit from an early immunosuppressive therapy.

# Effect of Early Versus Late Azathioprine Therapy in Pediatric Ulcerative Colitis

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**Background:** We aimed at describing the efficacy of azathioprine (AZA) in pediatric ulcerative colitis, comparing the outcomes of early (0–6 months) versus late (6–24 months) initiation of therapy.

**Methods:** Children with ulcerative colitis treated with AZA within 24 months of diagnosis were included. Corticosteroid (CS)-free remission and mucosal healing (MH), assessed by endoscopy or fecal calprotectin, at 12 months were the primary outcomes. Patients were also compared for CS-free remission and MH, need for treatment escalation or surgery, number of hospitalizations, and adverse events during a 24-month follow-up.

**Results:** A total of 121 children entered the study (median age 10.5 ± 4.0 years, 59% girls). Seventy-six (63%) started AZA between 0 and 6 months (early group) and 45 (37%) started between 6 and 24 months (late group). Seventy-five percent and 53% of patients in the early and late group, respectively, received CS at the diagnosis ($P = 0.01$). CS-free remission at 1 year was achieved by 30 (50%) of the early and 23 (57%) of the late patients ($P = 0.54$). MH occurred in 37 (37%) patients at 1 year, with no difference between the 2 groups (33% early, 42% late; $P = 0.56$). No difference was found for the other outcomes.

**Conclusions:** Introduction of AZA within 6 months of diagnosis seems not more effective than later treatment to achieve CS-free remission in pediatric ulcerative colitis. MH does not depend on the timing of AZA initiation; however, because of the incomplete comparability of the 2 groups at the diagnosis and the use of fecal calprotectin as a surrogate marker of MH, our results should be further confirmed by prospective studies.

*(Inflamm Bowel Dis 2016;22:1647–1654)*

**Key Words:** ulcerative colitis, azathioprine, children, mucosal healing

Ulcerative colitis (UC), one of the inflammatory bowel diseases (IBD) with Crohn's disease (CD) and unclassified IBD, is a chronic relapsing disorder of the large bowel, characterized by an uncontrolled inflammatory process of the intestinal mucosa.[1] Pediatric UC is known to be more extensive and severe than adult-onset disease and to require more aggressive treatment, as suggested by the higher rates of acute severe colitis and colectomy (28% and 30%, respectively), compared with adult UC (15% and 10%–17%, respectively).[2–4]

The conventional "step-up" approach, using corticosteroids (CSs), immunomodulators, and biologics sequentially in patients not responding to the "mildest" drug, has been demonstrated not to affect the risk of surgery overtime.[5] For this reason, an "accelerated" step-up strategy, based on the initiation of immunomodulators—mainly thiopurines (azathioprine [AZA] and 6-mercaptopurine)—already at the diagnosis, is commonly used in clinical practice, although data on its short- and long-term efficacy is lacking and this treatment option is mainly supported by reports in pediatric CD.[6,7] Additionally, 2 very recent population-based studies on the impact of thiopurines on the risk of colectomy in adults and adolescents with UC reported no superior efficacy of early therapy.[8,9] These data, along with increasing concerns about the toxicity of thiopurines[10] and the need of prolonged immunomodulatory treatments in children with a lifelong disease, emphasize the importance

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of assessing the effectiveness and safety of this therapeutic approach in pediatric UC.

The primary aim of our study was to describe the efficacy of AZA in children newly diagnosed with UC, comparing the outcomes of early (0–6 months of diagnosis) versus late (6–24 months) initiation of therapy.

## PATIENTS AND METHODS

In 2008, the Italian Society for Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP) established a prospective registry of all pediatric patients diagnosed with IBD from January 1, 2009. The methodology of the Italian Society for Pediatric Gastroenterology, Hepatology and Nutrition IBD registry has been previously described in detail.[11] For this study, data for all children with UC treated with AZA within 24 months of diagnosis, and enrolled and recorded in the registry from January 1, 2009, to January 5, 2015, (data retrieval date) were used. The study end date was defined as the date of the most recent clinic visit before January 5, 2015. Appendix 1 lists the 10 centers involved in data collection. Eligible subjects included all patients younger than 18 years diagnosed with UC, treated with a thiopurine within 2 years of diagnosis, and a minimum follow-up of 6 months. A diagnosis of UC was made according to the Porto criteria and was based on the clinical history, physical examination, and endoscopic, histological, and radiological findings.[12] Patients with any other cause of colitis (infection, eosinophilic colitis, immunodeficiency) were excluded. Extrapolated data for this study included demographic features (age, sex), a family history of IBD (defined as a diagnosis of CD or UC in first-grade relatives only), disease distribution, therapy at diagnosis and concomitant treatment at follow-up, body mass index z-score, and height velocity. An endoscopic assessment was available for all patients at diagnosis for the definition of disease location, which was reported according to the Paris classification[13]: proctitis (E1, disease limited to the rectum), left-sided colitis (E2, inflammation of the portion of the colorectum distal to the splenic flexure), extensive colitis (E3, involvement distal to the hepatic flexure), and pancolitis (E4). Disease activity at diagnosis and during follow-up was defined by the Pediatric Ulcerative Colitis Activity Index (PUCAI).[14] Clinical remission was defined as a PUCAI below 10. Laboratory tests included full blood count, C-reactive protein, erythrocyte sedimentation rate, perinuclear antineutrophil cytoplasmic antibodies, albumin (nutritional status), pancreatic and liver parameters, and fecal calprotectin (FC), which were recorded at diagnosis and every 6 months during follow-up. Mucosal healing was assessed at 12 and at 24 months by endoscopy performed at the center where the patients were followed or by FC for patients with no endoscopy data available. The degree of endoscopic activity was determined using the Mayo score[15] as follows: 0, normal or inactive; 1, mild (erythema, absent vascular pattern, mild friability); 2, moderate (marked erythema, absent vascular pattern, friability, erosions); and 3, severe (spontaneous bleeding, ulceration). Patients were classified on the basis of the maximum Mayo score recorded in any area of the colon. MH was defined as a value of 0 in Mayo score. For all Mayo scores above 0 (1, 2, and 3), MH was deemed not to have been achieved. A cutoff level of FC of 250 μg/g was used as a surrogate marker of MH in patients with no endoscopic reassessment available at 12 months and 24 months. This cutoff has already been demonstrated to have a sensitivity of 71% and a specificity of 100% (positive predictive value 100.0%, negative predictive value 47.1%) for active mucosal disease activity (Mayo >0).[16] Patients who received AZA within 6 or between 6 and 24 months of diagnosis were classified as early and late, respectively. Patients who started AZA after 2 years of diagnosis were excluded, as well as patients receiving other immunomodulators or biologics already at diagnosis. The clinical, demographic characteristics and laboratory data at diagnosis were analyzed in the 2 groups. Measurement of thiopurine metabolites was not a mandatory item in the registry and was not used for this study. CS-free remission, defined as clinical remission (PUCAI <10) free from any intravenous or oral CS treatment at the time of the evaluation, and mucosal healing at 12 months in early and late groups were the primary outcomes assessed. As secondary outcomes CS-free remission at 6, 18, and 24 months and mucosal healing at 24 months, as well as episodes of acute severe colitis, the need for surgery and treatment escalation, rates of hospitalizations, and therapy-related adverse events at a 24-month follow-up were compared between patients.

### Statistical Methods

All data were summarized and displayed as mean ± SD for the continuous variables. Categorical data were expressed as frequencies and percentages. Comparison of groups was performed using Student's *t* test for unpaired data in a 2-group comparison and 1-way analysis of variance with Bonferroni's test for multiple group comparisons. Chi-square tests with Fisher's correction was used to address any differences for categorical variables, as needed. A *P* value of 0.05 or less was considered as significant. Linear regression analysis was used to identify significant predictors of early AZA treatment (dependent variable) and to determine the correlation coefficient (r) and 95% confidence intervals. Independent variables in the regression model were age, sex (female), pancolitis (E4), PUCAI, growth failure, albumin level, C-reactive protein level, and the need of steroid therapy at disease onset. *P* values of 0.05 or less were considered as statistically significant. The Kaplan–Meier survival method was used to estimate the interval free from colectomy and the cumulative probability of remaining under AZA during follow-up. Differences between curves were tested using the log-rank test. GraphPad statistical package (GraphPad Software, Inc., La Jolla, CA) was used to perform all statistical analyses.

## RESULTS

### Demographics

A total of 447 patients with UC were identified: 250 (56%) were excluded because they were never treated with AZA, 16 (4%) because of AZA initiation after 2 years of diagnosis, 55 (12%), owing to follow-up duration less than 6 months, and

5 (1%) because of start of biologic therapy already at the diagnosis. Of the 250 patients excluded for no AZA treatment, 211 (84%) received 5-aminosalicylic acid, 20 (8%) biological therapy, 8 (4%) sulfasalazine, 3 (1%) methotrexate, and 8 (3%) cyclosporine. Therefore, 121 children were included for efficacy analyses (median age 10.54 ± 4.05 years, 58% females) (Fig. 1). Of these 76 patients (63%) were treated with AZA within 6 months of diagnosis (early group) and 45 (37%) between 6 and 24 months after diagnosis (late group). Among the latter, 20 (44%) started AZA 6 to 12 months from diagnosis and 25 (56%) after 12 to 24 months. The demographic, clinical, and laboratory data at the diagnosis are listed in Table 1. No difference was found for median age, sex, disease location, disease activity, clinical symptoms, growth parameters, and laboratory data between the 2 groups. No significant difference was reported for the mean AZA dose in early and late patients during the first 6 months of the study (1.82 ± 0.46 and 1.82 ± 0.55, respectively; $P = 0.96$).

## Outcomes

Seventeen patients (14%) discontinued AZA at 6 months of treatment, 4 (3%) owing to lack of efficacy, and 13 (11%) for adverse events. Four patients were lost to follow-up after 6 months; thus efficacy analyses at 12 months included 100 patients (60 early, 40 late).

Rates of CS-free remission were similar between both groups at all 4 time points (6 months: 51 [67%] versus 32 [71%], $P = 0.68$; 12 months: 30 [50%] versus 23 [57%], $P = 0.54$; 18 months: 28 [65%] versus 19 [65%], $P = 1.0$; 24 months: 30 [73%] versus 21 [72%], $P = 1.0$) after diagnosis (Fig. 2). When evaluating CS use, a significantly higher percentage of early patients received CS at the diagnosis, compared with the late ones (75% versus 53%; $P = 0.01$). CS use decreased during follow-up with no difference between the 2 groups (6 months: 18 [24%] and 9 [20%], $P = 0.82$; 12 months: 13 [21%] and 8 [20%], $P = 1$; 18 months: 12 [28%] and 6 [21%] $P = 0.58$; 24 months: 10 [24%] and 7 [24%], $P = 1.0$).

Mean PUCAI dropped from baseline (total mean PUCAI 34.73 ± 18.26) to 6 months of therapy in both groups (17.34 ± 21.45; $P < 0.0001$), with no significant differences between early and late patients at any next time point; this decrease continued through the 24 months of follow-up (total mean PUCAI 9.4 ± 17.85).

Data on MH were available for all patients under AZA at 12 months, as endoscopy findings for 49 patients (49%) and as FC level for 51 patients (51%). MH was reported in 37 patients (37%) of the entire population at 12 months, with no significant differences based on the timing of AZA initiation (20 [33%] early and 17 [42%] late; $P = 0.56$). The rate of MH at 24 months was comparable in both groups as well (14 [34%] and 14 [48%]; $P = 0.12$).

No significant difference was found for the number of hospitalizations, need for treatment escalation, disease extension,

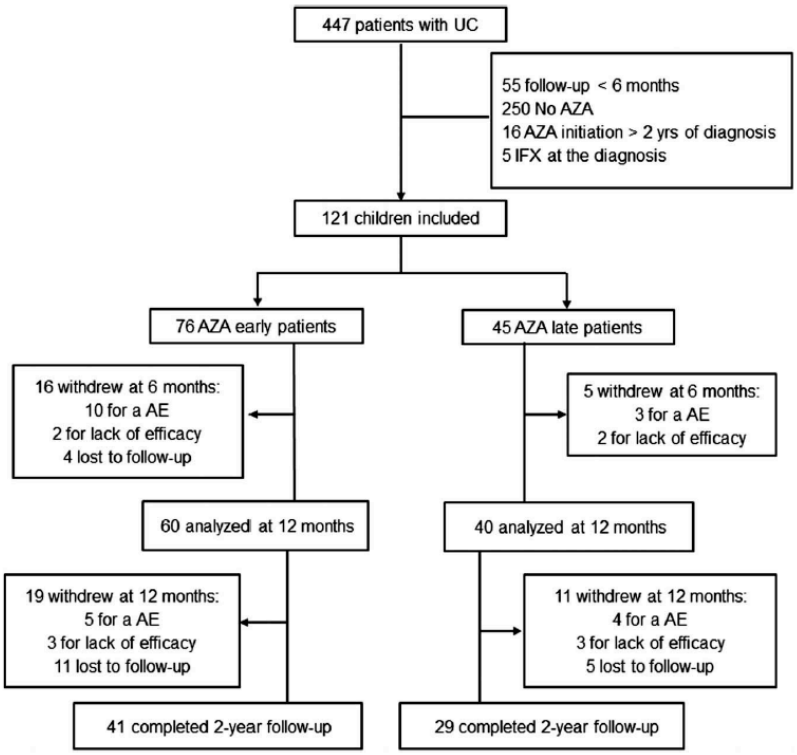


FIGURE 1. Flow chart of the study.

**TABLE 1.** Baseline Characteristics of Early and Late Patients

| | Total | Early | Late | *P* |
|---|---|---|---|---|
| Subjects, n | 121 | 76 | 45 | — |
| Age, median ± SD | 10.54 ± 4.05 | 11.77 ± 4.14 | 10.5 ± 3.83 | 0.25 |
| Female, n (%) | 70 (58) | 44 (58) | 26 (58) | 1 |
| Family history of IBD, n (%) | 16 (13) | 10 (13) | 6 (13) | 1 |
| Disease location, n (%) | | | | |
| E1 | 5 (4) | 5 (7) | 0 (0) | 0.15 |
| E2 | 25 (20) | 16 (21) | 9 (20) | 1 |
| E3 | 14 (11) | 8 (10) | 6 (13) | 0.77 |
| E4 | 77 (64) | 47 (62) | 30 (78) | 0.69 |
| PUCAI, mean ± SD | 34.4 ± 18 | 35.9 ± 19.5 | 32.7 ± 17.6 | 0.88 |
| Growth failure, n (%) | 9 (7) | 4 (5) | 5 (11) | 0.29 |
| BMI, median ± SD | 18.64 ± 3.3 | 18.7 ± 3.3 | 18.6 ± 3.3 | 0.91 |
| Laboratory, mean ± SD | | | | |
| ESR | 33 ± 26.6 | 33.4 ± 26.8 | 32.2 ± 26.8 | 0.83 |
| CRP | 3.4 ± 8.0 | 6.1 ± 13.3 | 2.9 ± 5.4 | 0.22 |
| HB | 11.1 ± 1.9 | 11.0 ± 1.9 | 11.3 ± 1.9 | 0.52 |
| pANCA+, n (%) | 35 (29) | 24 (32) | 11 (24) | 0.53 |
| WBC | 10.8 ± 5.0 | 12.5 ± 13.6 | 10.9 ± 4.5 | 0.54 |
| LYM | 2.9 ± 2.0 | 2.9 ± 2.3 | 2.9 ± 1.2 | 0.98 |
| Albumin | 4.1 ± 0.5 | 4.1 ± 0.6 | 4.2 ± 0.4 | 0.48 |
| Concomitant 5-ASA therapy | 89 (73.5) | 54 (71) | 35 (78) | 0.53 |
| AZA dose, mean ± SD | 1.82 ± 0.49 | 1.82 ± 0.46 | 1.82 ± 0.55 | 0.96 |
| PUCAI, mean ± SD | 34.7 ± 8.2 | 35.9 ± 17.61 | 32.72 ± 19.3 | 0.35 |

5-ASA, 5-aminosalicylic acid; BMI, body mass index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HB, hemoglobin; LYM, lymphocytes; pANCA, perinuclear antineutrophil cytoplasmic antibodies; WBC, white blood cell.

and episodes of acute severe colitis during follow-up (Fig. 3). Fourteen (11.5%) patients stopped AZA at 12 months: 8 (8%) because of adverse events and 6 (6%) because of lack of efficacy. At 24 months of follow-up, 9 (12%) patients discontinued AZA (8 [11%] for lack of efficacy and 1 [1%] for side effect). There were no significant differences between early and late patients in the cumulative probability of remaining under AZA therapy at follow-up (log-rank 1.25, $P = 0.81$) (see Fig. 1, Supplemental Digital Content 1, http://links.lww.com/IBD/B281).

At 2 years' follow-up, 7 patients had undergone colectomy, 4 in the early (5%) and 3 (7%) in the late group, resulting in a colectomy rate of 6% overall. No significant difference in surgical risk was reported between the 2 groups ($P = 0.71$) (Fig. 4). Serious adverse events related to AZA therapy occurred in 8 (6%) patients (2 fungal pneumonia, 2 sepsis, 4 pancreatitis), 6 (8%) in the early and 2 (4%) in the late group ($P = 0.70$). Overall, mild side effects were recorded in 28 patients (23%), with no significant differences between the 2 groups. In all, 22 (18%) patients discontinued AZA because of adverse effects (Table 2).

The univariate analysis of clinical and laboratory predictors of early AZA therapy in the entire population showed CS therapy at diagnosis to be significantly related with early AZA treatment ($r = 0.18$; $P = 0.05$), whereas no correlation was found with disease location, age, PUCAI, growth failure, albumin, and C-reactive protein levels at diagnosis (see Table 1, Supplemental Digital Content 2, http://links.lww.com/IBD/B282).

## DISCUSSION

Data from this large prospective multicenter registry reflects "real-life" treatment practice for pediatric UC. Of the 447 children diagnosed with UC during the period 2009 to 2015, 44% were treated with thiopurines and 2% with other immunomodulators and 49% were administered 5-aminosalicylic acid and 4% biologic therapy. This figure is in keeping with previous studies on the management of pediatric and adult UC and highlights the wide use of 5-aminosalicylic acid at the diagnosis in children affected with UC.[11,17–19]

Although thiopurines have an established role as maintenance therapy in adult and pediatric UC,[20,21] their efficacy in reducing the surgical risk is not clear. Several adult studies suggest that colectomy risk has not been reduced by the increased use of thiopurines,[8,22] as opposed to data reported in CD.[10,23,24] Additionally, a few reports indicate that the early use of AZA is

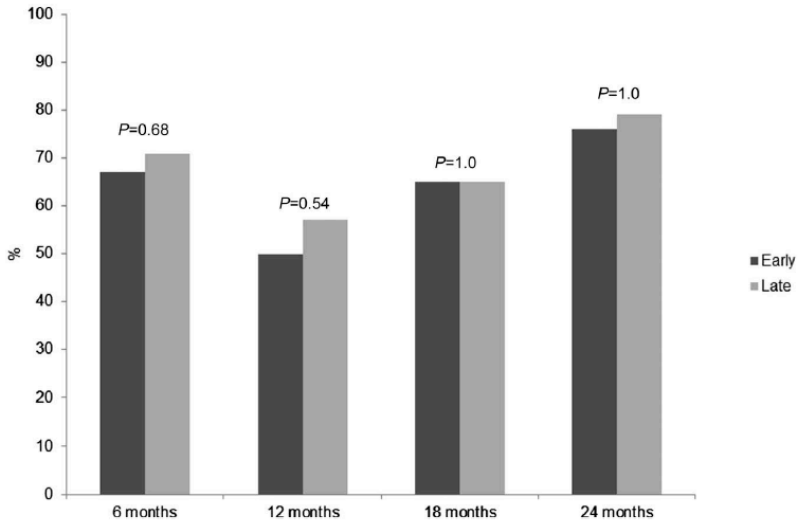


FIGURE 2. CS-free remission in early and late patients during 24 months' follow-up.

associated with a higher surgical risk in adult UC, probably because of a more severe disease phenotype in patients requiring this approach.[25]

Several studies have evaluated the impact of an early immunomodulator treatment in CD, whereas there is very sparse data on this topic in UC. In pediatric CD, the trial by Markowitz and et al,[6] conducted in 52 children newly diagnosed with CD treated with CSs and 6-mercaptopurine or placebo within 8 weeks of diagnosis, showed a significant reduction of disease relapse and need of CS in those treated with 6-mercaptopurine, compared with those on placebo. Since then, the so-called accelerated step-up approach, based on the introduction of thiopurines already at diagnosis, has become very common among pediatric gastroenterologists, both in CD and UC. Nevertheless, 2 recent randomized, double-blind prospective trials conducted in adults with CD showed no added efficacy of early thiopurines compared with placebo in modifying the natural history of the disease.[26,27] As far as UC is concerned, a very recent population-based study aiming at evaluating the impact of the timing and the duration of thiopurine therapy on surgical risk in IBD reported no benefit of early thiopurine (i.e., within the first year of diagnosis) compared with late use in adolescents and young adults with UC.[9] To our knowledge, no other reports on the effect of early immunomodulatory therapy in pediatric UC have been published so far.

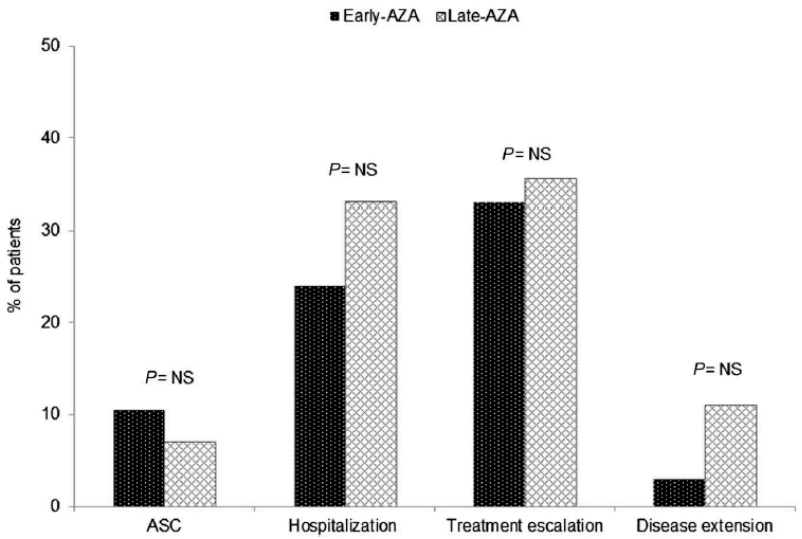


FIGURE 3. Hospitalizations, episodes of acute severe colitis, need of treatment escalation, and disease extension in early and late patients. ASC, acute severe colitis; NS, not significant.

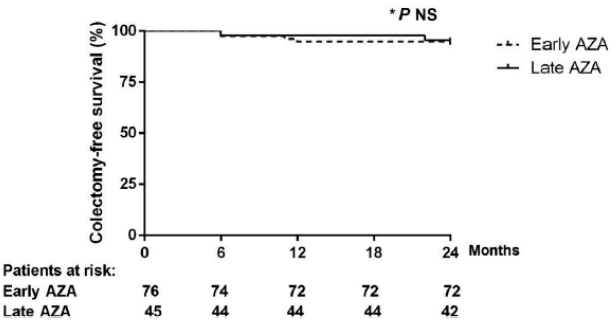


FIGURE 4. Colectomy-free survival curve in early and late patients. NS, not significant.

Our study, evaluating the impact of the timing of initiation of AZA on sustained clinical remission and mucosal healing in pediatric UC failed to demonstrate the superiority of early treatment over the classical step-up approach. Moreover, our data suggest that an accelerated use of thiopurine has no steroid-sparing effect compared with conventional treatment, because no difference in CS use was found between groups at follow-up. Nevertheless, concomitant CS treatment was significantly more common in early than in late patients at the diagnosis, indirectly suggesting a more severe phenotype in the former, and possibly making the comparison between the 2 groups not quite fair.

One strength of our study is to have data on mucosal healing at 1 and 2 years' follow-up in both groups. Mucosal healing has emerged as a specific treatment endpoint in UC, both in clinical trials and in clinical practice, because it is associated with a reduced risk of disease exacerbations in the long term, treatment escalations, and colectomy.[28–31] Although there is no internationally accepted definition of MH,[30] the Mayo endoscopic score is the most commonly used evaluation system,[29,32] with a score of both 0 and 1 used as a definition of MH in different studies and clinical trials.[33] The efficacy of thiopurines in leading to mucosal healing has been described by some clinical trials.[34]

**TABLE 2.** Adverse Events in Early and Late Patients at a 24-Month Follow-up

| | Early | Late | *P* |
|---|---|---|---|
| Subjects, n | 76 | 45 | — |
| Serious adverse events, n (%) | 6 (8) | 2 (4) | 0.70 |
| Fungal pneumonia | 2 | 0 | |
| Sepsis | 2 | 0 | |
| Pancreatitis | 2 | 2 | |
| Mild side effects, n (%) | 19 (12) | 9 (20) | 0.65 |
| Elevated liver enzymes | 3 | 1 | |
| Elevated pancreatic enzymes | 7 | 4 | |
| Leukopenia | 6 | 3 | |
| Vomit | 2 | 1 | |
| Rash | 1 | 0 | |

We found an overall mucosal healing rate of 37% and 40% at 1 and 2 years' follow-up, respectively. These results are similar to those reported in a prospective randomized controlled trial evaluating the efficacy of combination therapy with infliximab and AZA compared with infliximab or AZA monotherapy in UC. Mucosal healing at week 16 occurred in 28 of 76 (36.8%) patients treated with AZA monotherapy.[35] Other small trials, conducted in adults with longer follow-up, reported a rate of 45% to 57% MH in UC treated with thiopurines.[36–38] When comparing MH in early and late patients we did not find any difference for MH rates both at 12 and 24 months, suggesting that the timing of AZA introduction has no impact on the ability of AZA to heal the intestinal mucosa in pediatric UC.

One important limitation of our study is the use of FC as a surrogate marker of MH in about half of the patients. Indeed, FC is the most commonly used fecal biomarker reflecting disease activity in UC, and, because of its noninvasiveness, is better accepted by children with UC and their families, with respect to repeat endoscopy. Moreover, FC has been proved to be reliable in assessing the endoscopic severity,[16,39,40] in predicting disease relapse in patients in clinical remission,[40,41] and in evaluating response to treatments.[42,43] Several studies have reported a significant correlation of FC and MH (Mayo 0). We used a cutoff of 250 μg/g, because it was proved to have a sensitivity of 71% and a specificity of 100.0% and a positive predictive value of 100% for active mucosal disease activity (Mayo >0).[16] Future studies are needed to define the role of routine use of FC instead of endoscopy with biopsies to monitor the deep effect of therapies in UC.

Only a few predictive factors of aggressive UC course have been described so far, including young age, female gender, extensive colitis at diagnosis, and a systemic CS therapy at disease presentation.[18,44,45] In our series, linear regression analysis found CS treatment at diagnosis to be the only predictive factor of early AZA treatment. We cannot exclude that the small size of our study cohort might have limited the identification of further predictive factors.

In line with previous adult and pediatric data,[2,4,46] the crude colectomy rate in this population of thiopurine-treated children was 6% at 2 years' follow-up, with no differences based on timing of immunomodulatory initiation. In contrast to the small adult data suggesting that early AZA would be related to high surgical risk,[25] we did not find any difference between early and late users. As the small number of children needing colectomy in this cohort may have biased this finding, further studies are recommended to clarify this aspect. Furthermore, the outcome figures in the classical step-up group have to be interpreted cautiously, bearing in mind that 44% of these patients were also treated with AZA rapidly (within 1 year of diagnosis).

When evaluating other secondary outcomes, including the number of hospitalizations, need of treatment escalation, and episodes of acute severe colitis, no difference was found based on the timing of AZA initiation.

Adverse events resulting in AZA discontinuation occurred in 18% of children, which is consistent with previous studies,[47] with no differences based on the timing of initiation. Most of the

adverse events occurred in the first 6 months of therapy, suggesting a good drug tolerance beyond this period.

There are several limitations to our study. First, as with any registry-based study, our results could be biased by site-specific dissimilarities in patient definition and approach. In fact, as the registry is conceived to describe the real-life disease history and management, no attempt to standardize the therapeutic strategy was made. Second, we used a surrogate marker of MH for about half of the patients, thus making the results not absolutely homogeneous. However, given previous published data showing significant correlation between FC and endoscopic findings, we consider that our results may well reflect the ability of AZA to lead to mucosal healing. Third, we may have overlooked small differences in efficacy and safety of both approaches, because of the relatively small number of patients in each group and the follow-up duration of 2 years. Moreover, as data on thiopurine drug monitoring were not mandatory in the registry, we could not use this technology to assess AZA efficacy and safety. The need remains for prospective trials to reduce such possible biases. In conclusion, our study demonstrated that an early therapy with AZA has no added benefit, compared with conventional treatment, in pediatric UC. This result is noteworthy, particularly in children with a lifelong disease, because of the increasing concerns on the adverse effects of thiopurines, in particular the risk of lymphomas, skin cancers, and possible other tumors, as well as liver toxicity.[10,19,48]

Further data are warranted to select patients who should receive early therapy and to identify better timing and optimization of thiopurine use. Prospective, multicenter, and controlled trials may provide further evidence, allowing to balance the efficacy of an early immunomodulator therapy with the risks of prolonged treatments, in the perspective of providing patients and their families with an accurate risk-to-benefit analysis.

## ACKNOWLEDGMENTS

The authors thank their colleagues who provided data for the IBD Registry that improved the quality of the results of this study.

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## APPENDIX I: List of Participating Centers

1. Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, Roma, Italy
2. Pediatric Department, Gastroenterology and Nutrition Unit, Institute for Maternal and Child Health IRCCS "Burlo Garofolo," Trieste, Italy
3. Department of Pediatric Gastroenterology, University of Padua, Padua, Italy
4. Pediatric Gastroenterology, Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Naples, Italy
5. Pediatric Department, Maggiore Hospital, Bologna, Italy
6. Pediatric Gastroenterology and Endoscopy Unit, Spirito Santo Hospital, Pescara, Italy
7. Pediatric Gastroenterology and Endoscopy, University of Messina, Messina, Italy
8. Pediatric Gastroenterology, University of Messina, Messina, Italy
9. Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy
10. Pediatric Gastroenterology Unit, Institute "Giannina Gaslini," Genoa, Italy

### 6.3 Role and outcomes of surgery in UC

The progresses of medical therapy and the increase of options for second-line treatment of UC children have significantly reduced the need for colectomy. As reported in chapter 3, this is clearly demonstrated by the decrease of surgery rate from 1990s [216].

On the basis of the last updated ESPGHAN guidelines on the management of paediatric UC, an elective colectomy may be indicated in children with active or steroid-dependent UC despite maximal treatment with 5-ASA, thiopurines, and anti-TNF therapy, or after the finding of colonic dysplasia [EL5, RG D; adults EL 4, RG C] [50]. Indeed, differently from adults, in whom the main indication to colectomy is represented by dysplasia, children are usually referred to the surgeon for a chronic on-going steroid-dependent disease [50]. In general, most of the paediatric gastroenterologists tries to implement immunomodulatory agents and biologics therapy to their more effective doses before referral to colectomy. However, as suggested by the low level of evidence of the ESPGHAN recommendation, differently from medical therapy much less have been reported on surgical treatment needs of UC children. Several studies tried to identify factors associated with progression to colectomy, but there is still no general agreement on the surgical indication, timing and techniques [217-220]. The timing is a particularly hot topic in paediatrics, considering that children with active UC are at high risk of delayed growth and puberty. An appropriate, prompt surgical intervention may be able to restore growth and improve quality of the life [221].

With regards to the preferred technique, the literature is much more conclusive and there is a quite widespread agreement. Restorative proctocolectomy (ileoanal pouch or ileal pouch-anal anastomosis), especially the J-pouch, should be certainly preferred over straight pull-through (ileoanal) or ileorectal anastomosis for elective surgery of paediatric UC. Most series reported better continence after the pouch procedure [221-223]. In particular, a paediatric meta-analysis consisting of 5 studies and 306 patients suggested

that the straight ileoanal pull-through was associated with a higher failure risk (15% for straight pull through vs 8% for pouch procedure), and perianal sepsis (20% vs 10%), as well as a higher stool frequency [223]. Because the quality of life in children with restorative proctocolectomy is inversely related to stool frequency and continence, this difference is clinically relevant [221]. However, the pouch procedure is associated with risk for pouchitis [223]. Finally, considering the low complications rates and better cosmetics effects, when possible, laparoscopic approach is usually preferred [50].

In order to characterize the practice in terms of surgery of UC children among 7 Italian referral centres for paediatric gastroenterology, we performed a retrospective study, collecting all cases of UC surgery from 2009 to 2013. The aims of the survey were to define the surgical management of paediatric patients affected with UC, focusing on details of surgical technique, postoperative complications and quality of life, and to describe and compare attitudes among paediatric surgeons.

Our study represents the first Italian survey of UC children, focused on the surgical management of this population. After evaluating the attitude of each referring centre, we found a general consensus regarding indications for surgery, in accordance with the guidelines proposed by the ESPGHAN [50]. As previously reported, in our series the main indication for colectomy was failure of medical therapy (56%), followed by ASC (34%) and side effects of medical therapy (9.8%).

With regards to the timing of surgery, as underlined, there is still no consensus among paediatric gastroenterologists and surgeons. Although few paediatric data have been published, some papers suggested that delayed surgery may be associated with an increased risk of complications [224]. In this study colectomy was performed at a median interval of 2.5 years after diagnosis. One of the most intriguing finding is that up to 40% of the cases performed surgery after 1 month from the diagnosis, once more underlining the severity of certain subtypes of paediatric UC. This high percentage of early surgery may

explain also the low rate of complications, in according with the previous literature [221-223]. Concerning the technique, most of the participating centres correctly performed restorative proctocolectomy with ileal J-pouch–anal anastomosis. In addition, centres using a straight anastomosis at the beginning of this study progressively moved towards pouch surgery, demonstrating the adherence to the guidelines [50]. Nevertheless, the study still underlined many differences in terms of laparoscopic approach. As a matter of fact, its use in reconstructive surgery remains limited to a few centres because of the technical skills required at this stage. Centralization of specialized centres should be considered in order to provide these patients with the benefits of a laparoscopic approach.

Finally, we confirmed that proctorestorative colectomy is superior to the straight anastomosis both in terms of continence and stool frequency. Indeed, at 1 year of follow-up, children belonging to the straight anastomosis group showed a higher frequency of daytime and night-time incontinence than the pouch group, and a higher stool frequency with more than 10 daily evacuations. Probably due to the small follow-up (12 months after surgery) data from our series showed a lower rate of pouch inflammation.

In conclusion, this study demonstrated a general agreement to the ESPGHAN guidelines in terms of indications and technique. Restorative proctocolectomy with ileal J-pouch–anal anastomosis resulted the intervention of choice and once more demonstrated its superiority in terms of continence and stool frequency with a low rate of complications. Further follow-up is needed to define the long-term quality of life and pouchitis risk in our population. Larger studies are needed to define the optimum timing for surgery.

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Original Article

Original Article

# Paediatric Ulcerative Colitis Surgery: Italian Survey

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## Abstract

**Background and Aims**: Recent epidemiological studies showed an increase in ulcerative colitis among children, especially in its aggressive form, requiring surgical treatment. Although medical therapeutic strategies are standardized, there is still no consensus regarding indications, timing and kind of surgery. This study aimed to define the surgical management of paediatric ulcerative colitis and describe attitudes to it among paediatric surgeons.

**Methods**: This was a retrospective cohort study. All national gastroenterology units were invited to participate. From January 2009 to December 2013, data on paediatric patients diagnosed with ulcerative colitis that required surgery were collected.

**Results**: Seven units participated in the study. Seventy-one colectomies were performed (77.3% laparoscopically). Main surgical indications were a severe ulcerative colitis attack (33.8%) and no response to medical therapies (56.3%). A three-stage strategy was chosen in 71% of cases. Straight anastomosis was performed in 14% and J-pouch anastomosis in 86% of cases. A reconstructive laparoscopic approach was used in 58% of patients. Ileo-anal anastomosis was performed by the Knight–Griffen technique in 85.4% and by the pull-through technique in 9.1% of patients. Complications after colectomy, after reconstruction and after stoma closure were reported in 12.7, 19.3 and 35% of cases, respectively.

**Conclusions**: This study shows that there is general consensus regarding indications for surgery. The ideal surgical technique remains under debate. Laparoscopy is a procedure widely adopted for colectomy but its use in reconstructive surgery remains limited. Longer follow-up must be planned to define the quality of life of these patients.

**Key Words**: Paediatric ulcerative colitis; IBD; surgery

## 1. Introduction

Inflammatory bowel diseases include idiopathic disorders associated with chronic inflammation of the gastrointestinal tract and include Crohn's disease and ulcerative colitis (UC). The definition of UC is a chronic relapsing inflammatory condition of the colon, extending continuously from the rectum proximally to a varying degree, clinically appearing with bloody diarrhoea, tenesmus, abdominal pain, weight loss, vomiting and fatigue. Acute severe exacerbations (ASCs) of UC are a medical emergency and are defined by a Pediatric Ulcerative Colitis Activity Index (PUCAI) of at least 65 points (maximum PUCAI score 85 points).[1] Recent epidemiological studies suggest that the incidence of UC has increased in the paediatric population, being diagnosed before the age of 20 years in 20% of cases; the incidence of UC is reported to be between 1 and 4/100 000/year in American and European studies.[2–4] Moreover, UC appears to manifest more aggressively in childhood and 60–80% of all cases present with pancolitis, a frequency that is approximately 2-fold higher than that seen in adults. Because of the increased severity of UC in children, even the colectomy rate 10 years after onset is higher when compared with adult data (40 and 20% respectively).[3–6] Additionally, no changes in the colectomy rate have been observed during the last 20 years[7] and, although significant progress has been made in the medical treatment of UC, the colectomy rate in children with steroid-refractory disease is still high (60%).[8] It was also reported that delayed surgical treatment in cases refractory to medical therapy is associated with an increased risk of postoperative complications.[9]

Although the medical management of UC, even in its critical phases, has been standardized[1,3,4] with well-characterized pharmacological treatment steps, surgical treatment needs to be further defined in the absence of uniformity and consensus. Several studies have tried to identify factors associated with progression to colectomy but there is still no general agreement on the surgical indication, timing and techniques.[10–14] Children with active UC are at particular risk of delayed growth and puberty, and correct surgical treatment is necessary to guarantee a better quality of life.

The aims of the study were to define the surgical management of paediatric patients affected with UC in the major national centres for paediatric gastroenterology, focusing on details of surgical technique, postoperative complications and quality of life, and to describe and compare attitudes among paediatric surgeons.

## 2. Methods

We conducted a retrospective cohort observational multicentre national study from January 1, 2009 to December 31, 2013. All SIGENP (Italian Society of Pediatric Gastroenterology Hepatology and Nutrition) centres were invited to participate in the study through the society's mailing list and journal. All patients younger than 18 years who had undergone operation for UC in one of the participating centres were included in the study. The diagnosis of inflammatory bowel disease was established on the basis of clinical, endoscopic, radiological and histological data according to the Porto criteria.[15]

Every responsible referent from each centre collected data on demographics, date of UC onset, indication for surgery, PUCAI before surgery, therapy and age of the patient at the time of the first operation, details about staged surgery, operative technique and other technical aspects, postoperative complications and functional results in terms of number of evacuations, daytime and night-time soiling and incontinence at 3 and 12 months of follow-up. The deadline for data collection was set as January 31, 2014. All data that were collected were added to a database according to the National Data Protection Act and analysed by a team of two physicians.

### 2.1. Statistical analysis

Descriptive statistics were reported as percentages with the 95% confidence interval (CI), when appropriate, for categorical variables. Median and range were used for age, given the wide variability in our series. Differences in the frequencies of each categorical variable were evaluated by the $\chi^2$ test. Comparison of continuous data was performed using the two-tailed unpaired *t*-test. For scant data or non-normal distribution, a non-parametric test (Mann–Whitney) was used. A $p$ value lower than 0.05 was considered statistically significant. Analyses were performed using Stata for Windows (release 9.0, Stata Corporation, College Station, TX).

## 3. Results

Seven gastroenterology units participated in the study. A total of 71 cases were collected (37 males and 34 females; male:female ratio 1.08), all of which progressed at least to colectomy. Median age at diagnosis was 9.41 years (SD 4.27). Sixty-seven patients (94.3%) had available data on preoperative medical therapy (Table 1). In 11 cases (16.4%) the therapies were not specified and 5 patients (7.4%) were out of therapy at the time of surgery. Surgical indications were side effects of steroid therapy in 7 cases (9.8%), ASC in 24 cases (33.8%) and UC not responsive to medical therapy in 40 cases (56.3%). The PUCAI index prior to surgery was evaluated in 70 patients; it was <40 in 12 (17.1%), between 41 and 64 in 34 (48.6%) and ≥65 in 24 (34.3%); the median value was 65 (range 20–80). Surgery was performed by a paediatric surgeon in 52 cases (76.4%) and by a general adult surgeon in 16 cases (23.6%); in 3 cases the surgeon's specialty was unknown.

All patients underwent total colectomy; it was performed by laparoscopy in 51 patients (77.3%) and by laparotomy in 15 cases (22.7%); this information was not reported for 5 patients. Median age at colectomy was 12 years (SD 4.84, range 1.8–17.5) and the operation was performed a median of 2.58 years (SD 2.51, range 0–10.2) after the diagnosis of UC. Twenty-eight patients (39.4%) needed surgery in the first month after diagnosis. Nine patients (12.7%) received only colectomy at the end of the study period without any reconstructive step, 6 laparoscopically and 2 open (data were missing for 1 patient); of these, 4 patients had reconstructive surgery after the study deadline and data were not included. In 1 case reconstructive surgery was delayed beyond the study deadline because of a second operation due to stoma complication. In 4 cases there were insufficient data to determine the cause of the delay in reconstructive surgery.

Sixty-two patients received reconstructive surgery and surgical technical details were reported in 58 cases (93.5%). Only 3 of the participating centres performed mini-invasive surgery at the time of reconstruction and 81.8% of laparoscopic proctectomies and ileoanal anastomoses were performed at a single centre. A single-stage operation was performed in 1 case (1.6%) and a two-stage operation in 15 (24.2%). A three-stage operation was planned in 46 cases (74.2%) and this was completed with stoma reversal in 36 cases (58%). A laparoscopic approach for reconstructive surgery was chosen in 33 patients (57.9%). Nineteen patients (26.8%) still had an ileostomy at the end of the study period; of these patients, 9 did not receive a reconstructive operation, 7 had a reconstructive procedure close to the study deadline and 3 had insufficient data. The stoma was maintained after laparoscopic or open reconstructive surgery in

**Table 1.** Medical therapies used in children with UC at surgery. Total number of patients on 5-aminosalicylic acid (5-ASA) agents was 6 (9%), on steroid therapy 22 (32.8%), on immunomodulators 29 (43.2%) and on biological agents 13 (19.4%).

| Medical therapy | Number of patients | % |
|---|---|---|
| 5-ASA agents | 2 | 2.8 |
| Steroids | 7 | 9.8 |
| Immunomodulators | 11 | 15.5 |
| Biological agents | 11 | 15.5 |
| 5-ASA agents + steroids | 2 | 2.8 |
| Immunomodulators + biological agents | 1 | 1.4 |
| Steroids + immunomodulators | 12 | 16.9 |
| Immunomodulators association | 2 | 2.8 |
| 5-ASA agents + immunomodulators | 2 | 2.8 |
| Steroid + immunomodulators + biological agents | 1 | 1.4 |
| Biological agents + immunomodulators | 1 | 1.4 |
| Steroids + biological agents | 1 | 1.4 |
| 5-ASA agents + steroids + biological agents | 1 | 1.4 |
| 5-ASA agents + biological agents | 2 | 2.8 |

7 and 3 cases, respectively. Ileo-anal anastomosis was performed at a median time of 4.7 months (SD 5.7) from colectomy; an ileal pouch was constructed in 50 cases (86.2%) and a straight anastomosis in 8 (13.8%), while 4 patients had insufficient surgical details. Data on ileo-anal anastomosis were reported for 55 of 62 reconstructions: a double-stapled anastomosis according to Knight–Griffen technique was performed in 47 patients (85.4%) and a pull-through anastomosis in 5 (9.1%). Three patients who had a pouch constructed had mucosectomy of the anal channel (5.4%). The surgical approach was laparoscopic in 33 cases (56.8%), open in 23 (39.6%) and transanal in 2 (3.4%). After three-stage surgery was completed, ileostomy was closed at a median time of 2.85 months (range 0–12) after the reconstructive operation. Medical therapies were required in 31 patients (55.4%) after stoma closure; probiotics were used in 23 (74.2%), faecal thickening agents in 18 (58.1%), steroids or non-steroidal anti-inflammatory agents in 6 (19.3%), immunomodulators in 1 (3.2%), anti-kinetic agents in 4 (12.9%) and other minor therapies in 2 (6.4%). Information on complications after each surgical stage was collected. Postcolectomy complications occurred in 11.2% of cases (8 of 71 procedures), postreconstructive complications in 19.3% (12 of 62 procedures) and postcanalization complications in 26.9% (14 of 52 patients with no stoma); reoperations were required in 2 cases (Table 2). After reconstructive surgery, anastomotic leak was reported in 2 straight procedures (3.2%) and not observed in J-pouch operations ($p = 0.01$); 1 patient receiving a straight procedure had a single-stage and 1 had a three-stage operation. Anastomotic stenosis was observed in 4 cases (6.4%): 3 patients with straight anastomosis and 1 with a J-pouch ($p = 0.006$); the patient with a pouch had a three-stage operation. Among patients from the straight group, the patient who had a single-stage procedure and anastomotic leak also presented a stenosis; the other 2 patients had a two-stage and three-stage operation, respectively. Bowel obstruction after reconstructive surgery occurred in 3 cases (4.8%), 2 after a straight procedure and 1 after pouch anastomosis ($p = 0.04$), all performed by three-stage open surgery. Intestinal occlusion after stoma reversal occurred in 1 patient (1.9%) with a pouch anastomosis. Perianal skin lesions were all described in straight anastomosis procedures (3.8%, 2 versus 0, $p = 0.01$), while 2 patients in the straight group and 1 in the J-pouch group had an anastomotic stenosis after stoma closure (5.7%, $p = 0.04$). Major complication after colectomy refers to patients who present cardiorespiratory failure requiring medical therapy with inotropes, tracheostomy and prolonged total parenteral nutrition. Reoperations were necessary in 2 cases (2.8%). One of these patients presented a bowel obstruction after laparoscopic colectomy because of internal hernia on the stoma site; a second laparoscopic operation was performed, the hernia was reduced and a stoma was recreated. The second patient required the creation of a second stoma because of adhesions between the terminal ileum and uterus after straight anastomosis and stoma closure; anal anastomosis was delayed and a J-pouch anastomosis was performed. Rectal bleeding because of a short tract of affected mucosa was reported in 2 cases (3.8%) but insufficient surgical details were collected.

Clinical follow up-was performed 3 and 12 months after stoma closure, reporting evacuation frequency, daytime and nocturnal soiling and incontinence (Table 3). Among patients with stoma reversal, follow-up data were collected on 47 cases (90.4% of patients with bowel continuity). After 3 months of follow-up, patients in the pouch anastomosis group had a median of 7 daily evacuations (range 3–20, IRQ 5), while patients in the straight anastomosis group had a median of 10 daily evacuations (range 7–30, IRQ 5). Among these 47 patients, 11 (23.4%) had more than 10 daily evacuations (3 patients in the straight group and 8 in the J-pouch; difference not significant). At 12 months of follow-up, patients with a pouch anastomosis had a median of 5 daily evacuations (range 3–10, IRQ 3) while those in the straight anastomosis group had a median of 8.5 daily evacuations (range 5–12, IRQ 6.5). Four patients (8.5%) with more than 10 daily evacuations had follow-up data at 12 months (3 patients with a straight and 1 with a J-pouch anastomosis, $p = 0.006$). Twelve patients (25.5%) reported daytime soiling after 3 months of follow-up (3 in the straight group and 9 in the J-pouch group; difference not significant); the number of cases with daytime soiling decreased to 4 (8.5%) after 12 months of follow-up (3 in the straight group and 1 in the J-pouch group; $p = 0.006$). After 3 months of follow-up, nocturnal soiling was reported by 13 patients (27.6%, 6 with a straight and 7 with a J-pouch anastomosis, $p = 0.0009$); nocturnal soiling decreased to 3 and 1 cases, respectively ($p = 0.006$) after 12 months of follow-up (when these patients constituted 8.5% of the overall follow-up population). Incontinence was observed in 10 cases (21.2%) at 3 months of follow-up (3 in the straight and 7 in the J-pouch group; difference not significant) and in 3 J-pouch cases after 12 months of follow-up (6.3%, not significant). Data on pouchitis were collected for 19 of 40 patients with a J-pouch and stoma closure (47.5%). Among these 19 patients, inflammation of the J-pouch was observed at 6 months of follow-up in 3 cases (15.7%) and at 12 months in 1 case (5.2%).

## 4. Discussion

The increased options for second-line therapy due to advances in the medical treatment of UC in children reduced the need for colectomy, despite its curative results. As reported by several studies, the rate of colectomy for UC exacerbations decreased from 40% during the 1990s to 9% during the 2000s, and in the latest published series the figure was 2.7%.[8,10,16] Moreover, the standardization of pharmacological management and the development of an international approved clinical scoring system (PUCAI) and international guidelines have contributed to the success of the medical therapy of paediatric UC.[1,3,4,17] However, surgical treatment is still needed but

**Table 2.** Complications after surgical procedures. The percentage of complications and 95% CI were calculated with respect to the total number of patients and specific procedures (open or laparoscopic surgery, straight or J-pouch ileo-anal anastomosis). The *p* values were calculated comparing open versus laparoscopic procedures and straight versus pouch anastomosis. Percentages were calculated on the total number of patients who underwent each surgical procedure: 71 colectomies, 62 reconstructions and 52 stoma reversals. Among patients with post-stoma closure complications, perianal lesions and recto-anal bleeding were reported after a two-stage procedure; recto-anal stenosis was observed in 2 patients with three-stage surgery and in 1 patient with a two-stage approach. Other complications included persisting nausea and vomiting and stoma wound infection.

| Complications | No. of cases (%) | 95% CI | Open (%) | 95% CI | Laparoscopic (%) | 95% CI | Open versus laparoscopic p | Straight (%) | 95% CI | Pouch (%) | 95% CI | Straight versus pouch p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| After colectomy | | | | | | | | | | | | |
| Bowel obstruction | 2 (2.8%) | 1–13.2 | 0 | – | 2 (3.9%) | 1–13.2 | ns | – | – | – | – | – |
| Leak | 1 (1.4%) | 0.3–10.3 | 0 | – | 1 (2%) | 0.3–10.3 | ns | – | – | – | – | – |
| Stoma complication | 2 (2.8%) | 1–13.2 | 0 | – | 2 (3.9%) | 1–13.2 | ns | – | – | – | – | – |
| Major complication | 1 (1.4%) | 1.1–29.8 | 1 (6.6%) | 1.1–29.8 | 0 | – | ns | – | – | – | – | – |
| After reconstruction | | | | | | | | | | | | |
| Leak | 2 (3.2%) | 0.8–11 | 2 (8.7%) | 2.4–26.7 | 0 | – | ns | 2 (25%) | 7.1–59 | 0 | – | 0.01 |
| Stenosis | 4 (6.4%) | 2.5–15.4 | 3 (13%) | 4.5–32.1 | 1 (3%) | 0.5–15.3 | ns | 3 (37.5%) | 13.6–69.4 | 1 (2%) | 0.3–10.4 | 0.006 |
| Bowel obstruction | 3 (4.8%) | 4.5–32.1 | 3 (13%) | 4.5–32.1 | 0 | – | ns | 2 (25%) | 7.1–59 | 1 (2%) | 0.3–10.4 | 0.04 |
| Stoma complication | 2 (3.2%) | 0.8–11 | 0 | – | 2 (6%) | 1.6–19.6 | ns | – | – | – | – | – |
| After stoma closure | | | | | | | | | | | | |
| Pain/tenesmus | 1 (1.9%) | 0.3–10.1 | – | – | – | – | – | 0 | – | 1 (2.5%) | 0.4–12–8 | ns |
| Recto-anal bleeding | 2 (3.8%) | 1–12.9 | – | – | – | – | – | – | – | – | – | – |
| Perianal lesions | 2 (3.8%) | 1–12.9 | – | – | – | – | – | 2 (25%) | 7.1–59 | 0 | – | 0.01 |
| Recto-anal stenosis | 3 (5.7%) | 1.9–15.6 | – | – | – | – | – | 2 (25%) | 7.1–59 | 1 (2.5%) | 0.4–12–8 | 0.04 |
| Ileal perforation | 1 (1.9%) | 0.3–10.1 | – | – | – | – | – | 1 (12.5%) | 2.2–47 | 0 | – | ns |
| Bowel obstruction | 1 (1.9%) | 0.3–10.1 | – | – | – | – | – | 0 | – | 1 (2.5%) | 0.4–12–8 | ns |
| Other | 2 (3.8%) | 1–12.9 | – | – | – | – | – | 1 (12.5%) | 2.2–47 | 1 (2.5%) | 0.4–12–8 | ns |

ns, not significant.

**Table 3.** Clinical follow-up 3 and 12 months after surgery, comparing patients who received a straight anastomosis with those who received an ileal J-pouch anastomosis ($p < 0.05$ was considered statistically significant). Overall, 52 patients had a stoma reversal or one-stage surgery but 5 were lost to follow-up. Percentages were calculated for the 47 patients with 12 months of follow-up. Pouchitis percentages refer to 19 of 40 patients who received a pouch anastomosis and were checked by endoscopy on follow-up at 6 and 12 months.

| Follow-up | Number of patients (%) | 95% CI | Straight versus pouch | *p* |
|---|---|---|---|---|
| Number of evacuations per day at 3 months | | | | |
| 1–6 | 15 (31.9) | 20.4–46.1 | 0 versus 15 (0 versus 31.9%) | ns |
| 6–10 | 16 (34) | 22.1–48.3 | 4 versus 12 (8.5 versus 25.5%) | ns |
| >10 | 11 (23.4) | 13.6–37.2 | 3 versus 8 (6.3 versus 17%) | ns |
| Number of evacuations per day at 12 months | | | | |
| 1–6 | 32 (68) | 53.8–79.6 | 2 versus 27 (4.2 versus 57.4%) | ns |
| 6–10 | 12 (25.5) | 15.2–39.5 | 3 versus 9 (6.3 versus 19.1%) | ns |
| >10 | 4 (8.5) | 3.3–19.9 | 3 versus 1 (6.3 versus 2.1%) | 0.006 |
| Daytime soiling | | | | |
| 3 months | 12 (25.5) | 15.2–39.5 | 3 versus 9 (6.3 versus 19.1%) | ns |
| 12 months | 4 (8.5) | 3.3–19.9 | 3 versus 1 (6.3 versus 2.1%) | 0.006 |
| Nocturnal soiling | | | | |
| 3 months | 13 (27.6) | 16.9–41.7 | 6 versus 7 (12.7 versus 14.8%) | 0.0009 |
| 12 months | 4 (8.5) | 3.3–19.9 | 3 versus 1 (6.3 versus 2.1%) | 0.006 |
| Incontinence | | | | |
| 3 months | 10 (21.2) | 11.9–34.9 | 3 versus 7 (6.3 versus 14.8%) | ns |
| 12 months | 3 (6.3) | 2.1–17.1 | 0 versus 3 (0 versus 6.3%) | ns |
| Pouchitis | | | | |
| 6 months | 3 (15.7) | 5.5–37.5 | - | - |
| 12 months | 1 (5.2) | 0.9–24.6 | - | - |

ns, not significant.

no standardization of surgical management has been proposed and indications for surgery, timing and surgical technique still depend on the surgeon's professional opinion.

### 4.1. Indications for and timing of surgery

Surgery is indicated in cases of ASC not responding to medical therapy or in cases of toxic megacolon; these could represent indications for urgent colectomy. Indeed, it is well know that severe and extensive colitis is the main presentation of UC in children.[3–6] Other indications for elective colectomy include prolonged steroid therapy complications (growth retardation, osteoporosis, cataract) and poor or absent response to second-line medical therapies.[3,4] Moreover, the increased risk of developing colorectal cancer on long-term follow-up may also be an indication for colectomy. Ekbom et al.[18] demonstrated an incidence of cancer of 5% at 20–years and 40% at 35 years in children with UC diagnosed before 14 years of age; similarly, Griffiths et al.[19] estimated that children affected by UC had an 8% of risk of cancer 10–25 years after UC diagnosis. Two retrospective studies in a large series from McAteer et al.[10] and Kelley-Quon et al.[12] identified factors associated with progression to colectomy in children, stressing the importance of disease severity and associated comorbidities when assessing the need for colectomy (malnutrition, hypoalbuminaemia, total parenteral nutrition, electrolyte imbalance, *Clostridium difficile* colitis, anaemia requiring blood transfusion, sepsis, family history of UC, use of advanced medical therapies). In our series the main indication for colectomy was failure of medical therapy (56%), followed by ASC (34%) and side effects of medical therapy. Nine patients (20%) not responding to medical therapy had colectomy because of the development of ASC. These results are consistent with recent data showing that the main indications for colectomy in children are the failure of second-line medical therapies and ASC.[8] No data on previously reported risk factors were collected in this study, so no negative prognostic factors could be identified. Regarding the timing of surgery, there is still a lack of consensus. A study of adult patients from the UK suggested that delayed surgery is associated with an increased risk of complications[9]; moreover, a large series of paediatric patients from the USA reported an 8% increased risk of colectomy for each successive admission after the primary diagnosis of UC.[10] In this study colectomy was executed at a median interval of 2.5 years after diagnosis, being performed in the first month after diagnosis in almost 40% of cases. This could explain the low rate of complications reported in this series, similar to those reported in the studies mentioned above.

### 4.2. Surgical technique

The benefits of laparoscopic procedures are well known in term of cosmesis, pain control and fast-track principles. Regarding colectomy, almost 80% of patients in this study had the operation performed by mini-invasive surgery. Decisions regarding laparoscopic or open approach to colectomy are completely dependent on the surgical team's preferences and skills and no significant differences were reported in major complications between the two approaches.

Although in the past ileo-anal straight anastomosis has been the procedure of choice, nowadays restorative proctocolectomy and ileal J-pouch–anal anastomosis are the most frequently used operations, although there are few paediatric data to support this. As reported by the ECCO and ESPGHAN guidelines,[3,4] straight anastomosis is associated with a higher failure risk, perianal sepsis and higher stool frequency. It is still debated whether straight anastomosis should be performed in female patients, because of the lower risk of associated reduced fecundity.[20–22] This global trend is also evident in our series, as 86% of patients had restorative proctocolectomy and J-pouch

ileo-anal anastomosis. The cases treated by straight pull-through were mostly reported at the beginning of the study period; this could be explained by a shift in surgeons' preferences towards ileal pouch anastomosis because of the advantages of this procedure.

It was reported that ileo-anal anastomosis could be performed with hand-sewn or stapled sutures.[23,24] Mechanical sutures make it possible to reduce anal manipulation and the need for mucosectomy, which can be difficult because of the inflamed mucosa; moreover stapled suturing is associated with a shorter hospital stay.[25] The intrinsic limitation of this procedure is that it leaves a few centimetres of inflamed mucosa. Otherwise, in hand-sewn anastomosis proctectomy is accomplished by endorectal mucosectomy, permitting a lower anastomosis, just above the columns of Morgagni, avoiding disease recurrence and preserving continence. Some authors have proposed a combined approach, using endorectal mucosectomy prior to mechanical anastomosis.[26] In our opinion, although mucosectomy permits radical treatment of UC, it carries a significant risk of leaving islets of inflamed mucosa *in situ*, which could be difficult to follow up by endoscopic examination. Moreover, it has not been proved that mucosectomy reduces the risk of dysplasia and malignancies.[27] The results of this series highlight the preference for performing a lower anastomosis by a double-stapled technique; although it leaves a few centimetres of affected mucosa with a certain degree of inflammation (cuffitis), it could be easily controlled by medical therapy (systemic or local) and checked periodically by endoscopy. Mucosectomy was performed in only 3 cases from 1 centre, thus reflecting the general opinion that it is not a recommendable procedure because the risk of recurrence that could be difficult to evaluate by endoscopy. Pull-through procedures represent a minority in this series (9%); these data confirm the technical difficulties represented by mucosectomy. Moreover, in pull-through procedures islets of inflamed mucosa could be retained and hidden by the pulled through ileum.

There is still a lack of general consensus among surgeons about the staging of operations. Single-stage procedures have been proposed only for selected cases with no risk factors. Both two- and three-stage surgeries are used more often in emergency surgery than in elective surgery. Data from this series showed that most (70%) surgeons preferred the three-stage approach; this could be explained by the high incidence of colectomy performed for ASC, after prolonged steroid therapies or because of failure of second-line medical therapies. Two-stage surgery was performed mostly in elective surgery; in this study group a single-stage operation was executed only in 1 case, in which the surgical approach was combined with laparoscopic and transanal straight pull-through. This patient reported postoperative complication, again confirming that a single-stage approach must be reserved strictly for selected cases. A two-stage operation could represent the best indication in patients treated other than in an emergency situation and with minimal risk factors.

The feasibility, safety and benefits of the laparoscopic approach are well demonstrated in the literature.[11,14] In this series the laparoscopic approach to reconstructive surgery was used in 58% of cases and more than 80% of these were treated in one centre. These data reflect the need for a learning curve in performing proctectomy and ileo-anal anastomosis by laparoscopy and lead us to speculate about the need to centralize care in specialized centres in order to guarantee the benefits of a full laparoscopic treatment for these patients. Laparoscopic reconstructive surgery was performed mostly after laparoscopic colectomy, but in 6% of cases it was performed after open colectomy and no complications were reported, demonstrating the feasibility and safety of this procedure even after open surgery.

Complications after reconstructive surgery were not significantly different between open and laparoscopic procedures or between pull-through and Knight–Griffen operations. However, there was a significant difference in the complication rate between straight and pouch procedures; anastomotic leak, anal stenosis and bowel occlusion occurred more frequently in patients in the straight anastomosis group compared with those who underwent the pouch procedure. The lack of association with postoperative complications represents another point in favour of pouch procedures. When surgery was performed in three stages, the only significant differences, after ileostomy closure, concerned perianal lesions and anal stenosis, both of which were increased in the straight anastomosis group. These data support the advantages of pouch procedures. Similarly, no differences among surgical techniques were demonstrated in terms of the need for medical therapies after surgery.

### 4.3. Follow-up

When analysing data on continence and frequency of evacuation, no differences were found at 3 months of follow-up between the pouch and straight procedure groups, except for night-time soiling, which was higher in patients in the straight anastomosis group. At 1 year of follow-up there was a general decrease in daily evacuation, daytime and night-time soiling and incontinence. When comparing these data on the basis of surgical treatment received at 1-year of follow-up, the straight anastomosis group showed a higher frequency of daytime and night-time soiling than the pouch group, and had more than 10 daily evacuations. These data support the advantages of the J-pouch reservoir in terms of quality of life, which is inversely related to the number of evacuations and incontinence and is in accordance with data from a meta-analysis on 306 paediatric patients.[28] However, a multicentre study on 112 paediatric patients showed that the difference in stool frequency was less evident with longer follow-up.[29] Pouchitis is the most common complication of pouch anastomosis, occurring with a frequency varying from 30 to 75%.[3] Data from our series showed a lower rate of pouch inflammation, because we focused on immediate postoperative complications and short-term follow-up (12 months). As pouchitis can occur several months after surgery, we speculate that the frequency reported in our series might have been higher with longer follow-up. It appears that these patients must be observed for a longer period in order to better define the real outcome of different surgical options in terms of quality of life.

## 5. Conclusion

This study is the first survey of paediatric patients with UC in Italy and focused on the surgical management of this population. After evaluating the attitude of each referring centre, we found a general consensus regarding indications for surgery, in accordance with the guidelines proposed by ECCO-ESPGHAN. The ideal surgical technique remains debatable, although most of the centres participating in the study performed restorative proctocolectomy with ileal J-pouch–anal anastomosis. Centres using a straight anastomosis at the beginning of this study progressively moved towards pouch surgery. Laparoscopy is a procedure widely adopted for colectomy, but its use in reconstructive surgery remains limited to a few centres because of the technical skills required at this stage. Centralization of specialized centres should be considered in order to provide these patients with the benefits of a laparoscopic approach. Longer follow-up must be planned in order to precisely define the advantages of the ileal J pouch in terms of continence and evacuation frequency.

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## Conflict of interest

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## Author contributions

All authors contributed equally to the realization of the study.

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# CHAPTER 7

# CONCLUSIVE REMARKS

This thesis project clearly outlines the burden, the unique features and the complex management of paediatric IBD. The medical advances made in the last 20 years have certainly optimized the ability to care for these patients, allowing the reach of objectives and goals not imaginable before. The IBD management by paediatric gastroenterologists is progressively moving from symptomatic control to achievement of mucosal healing and deep remission with the use of more targeted agents. This is due to a growing awareness that early use of disease-modifying drugs can alter the natural history and limit disease progression. As a matter of fact, thanks to the technological progresses, we made further steps in the comprehension of genetics and microbiome, allowing a better understanding of IBD pathogenetic mechanisms. This consented the development of more targeted molecules able to counteract specific pathways along the disease inflammatory cascade. These new drugs are slowly coming out and will be soon available also in paediatric age. The increasing therapeutic armamentarium will hopefully lead to more patient-tailored treatment approaches.

Nevertheless, there are still many gaps that research need to address soon. Probably, as previously underlined, the most pressing issue in the management of paediatric IBD is the improvement of patients' risk stratification. Indeed, we are still far from the expected and advocated personalized approach. This implies a further improvement in the understanding and the classification of genetic background, serological markers, endoscopic and imaging features.

On the other hand, the impressive data on the continuous increase of disease incidence, as well as the new reports from less developed countries, highlight the need for developing preventive strategies. To make this objective achievable a further effort is needed to better clarify the influence and the interaction of the exposome with microbiome

and genetics. This last step will allow us to establish preventive strategies and face the current IBD epidemic.

# CHAPTER 8

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# CHAPTER 9

# CURRICULUM VITAE

**Main Research Fields:**

1) Clinical and therapeutic management of children affected by IBD;
2) Characterization of paediatric IBD pathogenesis;
3) Endoscopic assessment of children affected by IBD, intestinal polyposis, gastroesophageal reflux disease and celiac disease;
4) Clinical and therapeutic management of Functional Gastrointestinal Disorders in children;
5) Clinical and therapeutic management of gastrointestinal disorders among children with neurological disabilities.

**List of publications in the years 2014-2017**


1. Urs AN, **Martinelli M**, Rao P, Thomson MA. Diagnostic and therapeutic utility of double-balloon enteroscopy in children. J Pediatr Gastroenterol Nutr. 2014; 58:204-12.
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**Book Chapters**

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**Abstracts and Communications**

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26. F.P. Giugliano, C. Strisciuglio, **M. Martinelli**, M. Andreozzi, S. Cenni, S. Campione, M. D'Armiento, A. Staiano, E. Miele Azathioprine induces endoscopic but not histological healing in children with inflammatory bowel disease: A prospective, observational study. Digestive and Liver Disease 2016, Volume 48, Supplement 4, Page e270. SIGENP Annual Meeting, Milan, September 29-October 1, 2016.
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**Grants and Awards**

- ESPGHAN Young Investigator Award for the communication *"Intestinal Iron Absorption in Pediatric Inflammatory Bowel Disease: a Prospective, Single-Center",* presented at the ESPGHAN 47th Annual Meeting, June 9-12 2014, Jerusalem, Israel.

**Invited as a speaker**

- *Clinical Case Based Discussion. Joint Meeting IG-IBD-SIGENP.* La gestione della terapia con farmaci biologici nel bambino con Malattia Infiammatoria Cronica Intestinale. Palermo, 3 Dicembre, 2015.

- *Problematiche gastroenterologiche tra funzionale ed organico: La Stipsi.* Corso di formazione in Gastroenterologia ed Epatologia Pediatrica. Manfredonia (FG), 12 Dicembre, 2015.
- *Novità in Nutrizione Pediatrica: Malattie Infiammatorie Croniche Intestinali.* Workshop Avanzato di Nutrizione Neonatale e Pediatrica. Cagliari, 30 Giugno, 2016.
- *Le Videocapsula Endoscopica in pazienti pediatrici.* Incontro RAVE: 1° Riunione Annuale Videocapsula Endoscopica. Villa Braida-Migliano Veneto, 16-17 Settembre 2016.
- *La rettorragia: I quesiti del clinico.* Pediatria a Napoli: il quesito del clinic e la soluzione multidisciplinare, Napoli, 26-28 Gennaio 2017.

**Teaching Activities**

- Professor of the Postgraduate Course in Paediatric Gastroenterology, Hepatology and Nutrition held at the Department of Paediatrics of the University of Naples “Federico II”, edition 2014, 2015 and 2016.