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DIAGNOSTIC APPROACH TO MONOGENIC INFLAMMATORY BOWEL DISEASE WITH NEXT-GENERATION SEQUENCING TECHNOLOGIES.

Settore scientifico-disciplinare: Scienze mediche

DOTTORANDA
Sara Lega

COORDINATORE
Prof. Paolo Gasparini

SUPERVISORE
Prof. Egidio Barbi

TUTOR
Dott. Alberto Tommasini

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1 INTRODUCTION

1.1 Monogenic inflammatory bowel diseases

1.1.1   From polygenic disorders to single gene defects

Inflammatory bowel diseases (IBD) are chronic, disabling inflammatory disorders of the gastrointestinal tract that include three main disease entities: Crohn's Disease (CD), Ulcerative Colitis (UC) and IBD-Unclassified (IBD-U). CD can affect any part of the gastrointestinal tract and involve the full thickness of the bowel wall leading to complications such as strictures, abscesses, or fistulas, while UC is typically limited to the colon and restricted to the mucosal layer. The term IBD-U identifies those patients with colonic disease who have clinical or endoscopic findings that are atypical for UC(1). Most IBD, also called idiopathic or polygenic IBD, are complex disorders, resulting from the interaction between genetic predisposition and environmental factors (2). Meta-analysis of genome-wide association studies (GWAS) identified over 230 disease loci linked to polygenic IBD, highlight the importance of host–microbe interactions, autophagy and specific inflammatory signaling pathways in IBD pathogenesis. However, the contribution of common variants identified with GWAS can explain only a fraction of the expected heritability in IBD (3,4).

In recent years a diverse spectrum of rare monogenic disorders caused by genetic defects in high-impact genes have been recognized that can present primarily or solely with chronic gastrointestinal inflammation and are referred to as "monogenic IBD ". Also, the terms "IBD-like" or "CD/UC-like" have spread in the literature to define those patients presenting with IBD in the setting of a known or suspect monogenic disorder (5). Monogenic IBD usually manifest in infancy or early

childhood and are associated with high morbidity and mortality (6). The starting point for the identification of monogenic disorders presenting with intestinal inflammation was the identification of genetic defects in the IL10 and IL10 receptor coding genes as the cause of severe infantile IBD by family association studies and candidate sequencing (7). Since the identification of IL-10 and IL10R more than 50 mendelian disorders have been identified, largely by genomic technologies, and the list is constantly expanding. The field of monogenic IBD is an active are of research that offers the possibility to improve our understanding of the mechanisms involved in intestinal homeostasis and inflammation, and to develop pathway-specific therapies.

1.1.2   Epidemiology

The overall frequency of monogenic IBD in the general population is exceedingly low compared to polygenic forms and is estimated to be of 1 in 100.000 to 1 in 10.000.000 (8). Recent data based on small cohort studies suggests that the proportion of monogenic IBD among all IBD patients correlates inversely with the age at disease onset, even though monogenic diseases such as XIAP deficiency or neutrophil defects have been observed in later onset IBD (9).

Amongst 25 children diagnosed with IBD before the age of 6 years that were prospectively recruited over 1 year, Kammermeier et al. found a monogenic IBD in 4 patients (16%)(10). More recently the same authors described a cohort of 62 children with IBD onset before the age of 2 years who underwent genetic screening and reported 19 monogenic diagnoses (31%)(11). Fang et al. reported similar proportions in children with IBD younger than 6 years: in their single center

retrospective study including 54 patients, a monogenic diagnosis could be established in 9 patients (16%), of whom 8 were found in the subgroup of 34 patients (24%) diagnoses with IBD before 2 years of age (12). Very recently, Charbit-Henrion F. et al. described the largest cohort of patients with suspected monogenic IBD who underwent a genetic work-up. The study included 207 patients with chronic diarrhea requiring immunosuppressive therapy or surgical treatment that were diagnosed before the age of 6 years or showed clinical findings considered suggestive of a monogenic disorder. Patients were recruited over 45 pediatric centers across Europe between 2009 and 2015. A molecular diagnosis was achieved in 66 of 207 patients (32%)(13). Altogether these data suggest that approximately 15% of patients with IBD rising before the age of 6 years and defined as Very-Early-Onset IBD (VEO-IBD) may have a rare monogenic disorder and that the proportion raise up to approximately 25 to 30% in patients with IBD onset before 2 years, identified as Infantile-Onset IBD (IO-IBD). Even though no exact estimates are available, these frequencies might be even higher in the subgroup of patients with Neonatal-onset disease (Neo-IBD).

1.1.3   Genetic defects

To date more than 50 genetic variants have been associated with monogenic IBD. Single gene defects have been observed in genes regulating the innate and adaptive immune functions, inflammatory homeostasis and intestinal epithelial barrier functions (5,14). Very recently novel defects have been found to affect pathways implicated in previously identified mendelian disorders (15).

Known genetic defects can be grouped according to their key functional role as proposed by Uhlig et al. as follows:

1.   Epithelial barrier and epithelial response defects: genetic defects affecting the epithelial barrier function lead to increased translocation of commensal bacteria in the lamina propria, causing a physiological inflammatory immune response. This is the case of IBD presenting in patients with Kindler syndrome (KS), an epithelial barrier disorder due to homozygous defects in *FERMT1* gene. Patients with KS manifest with skin blistering, poikiloderma, photosensitivity and carcinogenesis. Some patients also exhibit severe colitis with focal detachment of the epithelium, chronic inflammation and mucosal atrophy (16).

2.   Phagocyte defects: include genetic defects affecting intracellular bacterial killing and transendothelial migration of neutrophil granulocytes and other phagocytes; bacterial clearance impairment results in increased bacterial translocation in the lamina propria and secondary hyperinflammation. The most common cause of IBD due to neutrophil dysregulation is chronic granulomatous disease (CGD). In CGD defects in genes encoding the 5 components of NADPH complex (*CYBB*, *CYBA*, *NCF1*, *NCF2*, and *NCF4*) make the neutrophils unable to produce reactive oxygen species (ROS) and thus to kill phagocytized bacteria or fungi. Mutations in the X-linked *CYBB* gene are the most common cause of CGD, as the other subunits are inherited in an autosomal recessive fashion (17). About 40% of patients with CGD develop CD-like intestinal inflammation at a median age of 5 years (18). Even though the inflammatory process can be found in any part of the gastrointestinal tract, the colon is the most affected part. CGD is diagnosed by the dihydrorhodamine (DHR) test that is a sensitive, specific, and low-priced screening

method. Therapeutically, prophylactic treatment with antibiotics and antifungals is indicated. Due to the life-long risk of life threatening infectious complications, hematopoietic stem cells transplantation (HSTC) is recommended in most cases and leads to a complete reconstitution of neutrophil function and to the resolution of IBD (19). In addition to CGD, other neutrophil defects listed in Table 1, are associated with intestinal inflammation.

3. <u>Hyper- or auto-inflammatory disorders:</u> include genetic defects that impair the down-regulation of the inflammatory cascade such as XLP-2 and mevalonate kinase deficiency (MKD). X-linked lymphoproliferative disease type 2 (XLP-2) is a primary immunodeficiency caused by mutations in the X-linked inhibitor of apoptosis gene (*XIAP*). XIAP protein has numerous functions. Among these, it enhances the NF-κB-dependent immune responses triggered by pathogen binding to NOD2, and promotes the expansion of cytotoxic T cells that inhibits the inflammatory cascade. XLP-2 individuals are susceptible to several specific and potentially fatal infections, such as Epstein-Barr virus (EBV) and may present during childhood with either EBV- or CMV-induced hemophagocytic lymphohistiocytosis (HLH) or with IBD. IBD develops in at least 30% of XIAP-deficient patients and can be the first as the sole clinical manifestation of the defect; it can manifest in infancy as well as in adulthood and tend to have a severe clinical course despite conventional immunosuppressive treatments contributing to the overall mortality in these patients (9,20). Extraintestinal manifestations associated with conventional IBD such as arthritis, uveitis, and erythema nodosum are rather common as well (21). Allogenic HSCT is recommended in most patients with XLP-2 as it prevents the risk of fatal HLH and in patients with IBD it resolves the intestinal inflammation (21).

4.   <u>Defects of T and B cell function:</u> include multiple genetic defects that disturb T and/or B cell activation and selection. Disorders associated with IBD-like phenotypes include B cell defects such as Hyper IgM syndrome (HIGM), Common Variable Immunodeficiency (CVID) and Wiskott-Aldrich syndrome (WAS). WAS is caused by the absence of the cytoskeletal regulator WASP, which is associated with defects in hematopoietic cells and typically presents in infant males with microthrombocytopenia, eczema, recurrent infections, and an increased incidence of autoimmunity and malignancies (22,23). Patients with WAS typically develop UC-like intestinal inflammation due to the loss of magrophage-mediated mucosal immune tolerance mechanisms (24). IBD-like manifestations rising in early childhood are commonly observed also in atypical severe combined immunodeficiencies (SCID) and in telomeropathies. Telomeropathies, including X-linked dyskeratosis congenita and the more severe hoyeraal-hreidarsson syndrome, are caused by defective variants of the gene DKC1. As telomeropathies preferably affect tissues with high cell turnover, the gastrointestinal manifestations are common including inflammatory enteropathy or enterocolitis with villous atrophy and marked apoptosis (25).

5.   <u>Defect of down-regulation of the inflammatory process</u>: include loss of function defects of the anti-inflammatory cytokine IL-10 and its receptor (encoded by IL10RA and IL10RB) and defects in regulatory T cells responsible for the X-linked immune dysregulation, polyendocrinopathy, enteropathy syndrome (IPEX syndrome and IPEX-like defects). Defects in IL-10 signaling present with a colitis dominant phenotype, severe perianal disease, folliculitis and predisposition to B-cell lymphoma (26). IPEX is caused by mutation in *FOXP3* gene that affect natural and induced regulatory T cells causing autoimmunity and immunodeficiency. Patients with IPEX

develop a form of inflammatory enterocolitis resembling graft versus host disease (GVHD)(27). Defects in IL2 signaling (due to IL2 receptor defects or defects in STAT1) associated with an IPEX-like syndrome also manifest with intestinal involvement (28).

6.	Other defects without clear pattern: this is the case of Tricohepatoenteric syndrome (THES) due to *TTC37* or *SKIV2L* defects. Patients with THES show facial dysmorphism, hair abnormalities, intractable diarrhea, and immunodeficiency with antibodies deficiency. The occurrence of IBD has been hypothesized to be a consequence of a presumed defect in epithelial cells as well as of the adaptive immune defect (29,30).

The list of genes associated with monogenic IBD, ordered by functional group and related clinical manifestations are summarized in Table 1.

Table 1. Genetic defects and phenotypes in monogenic IBD

Group	Gene	Inheritance	Syndrome/disorder	Gastrointestinal manifestations	Extraintestinal manifestations
Epithelial barrier defects	COL7A1	AR	Dystrophic epidermolisis bullosa	Ileo-colic, strictures, apoptosis on histology	Epidermolysis bullosa
	FERMT1	AR	Kindler Syndrome	UC-like, strictures, apoptosis on histology	Epidermolysis bullosa
	IKBKG	X	X-linked ectodermal immunodeficiency	CD-like, ileo-colic, apoptosis on histology	Arthritis , vasculitis
	TTC7A	AR	TTC7A deficiency	Ileo-colic, apoptosis on histology	
	ADAM17	AR	ADAM17 deficiency	Ileo-colic, apoptosis on histology	Nail & air abnormalities, eczema, pustular rash
	GUCY2C	AD	Familial diarrhea	CD-like, ileo-colic	
Phagocyte defects	CYBB	X	CGD		
	CYBA	AR	CGD	CD-like, ileo-colic, oral, perianal, granulomas on histology	Infections, deep abscesses, granulomas, skin eczema
	NCF1	AR	CGD		
	NCF2	AR	CGD		
	NCF4	AR	CGD		
	SLC37A4	AR	Glycogen storage disease type Ib (GSD1)	CD-like, ileo-colic, oral, perianal, structuring	Hepatomegaly, huperuricemia
	G6PC3	AR	Congenital neutropenia	CD-like, ileo-colic, perianal	Visible superficial veins, renal agenesis, hepatomegaly
	ITGB2	AR	Leukocyte adhesion deficiency 1	CD-like, ileo-colic, oral, perianal, structuring	Staphylococcal and gram - infections, periodontitis
Hyperinflammatory and autoinflammatory disorders	MVK	AR	Mevalonate kinase deficiency	Ileo-colic , stricturing	Arthritis, fever, rash , HLH
	PLCG2	AD	Phospholipase C-γ2 defects	Ileo-colic	Arthritis, interstitial pneumonitis
	MEFV	AR	Familial Mediterranean Fever	UC-like	Serositis
	STXBP2	AR	Familial HLH type 5	Ileo-colic	
	XIAP	X	X-linked lymphoproliferative syndrome 2 (XLP2)	CD-like, ileo-colic, perianal, fistulizing	HLH, lymphoma, complicated EBV infection
	SH2D1A	X	X-linked lymphoproliferative syndrome 1 (XLP1)	Ileo-colic	
	HPS1	AR	Hermansky-Pudlak 1	CD-like, ileo-colic, perianal, granulomas on histology	Oculo-cutaneous albinism, bleeding diathesis
	HPS4	AR	Hermansky-Pudlak 4		
	HPS6	AR	Hermansky-Pudlak 6	Ileo-colic	
T and B cells defects	ICOS	AR	CVID 1	Colonic	Arthritis
	LRBA	AR	CVID 8	Ileo-colic	Autoimmune hemolytic anemia, erythema nodosum
	IL21	AR	CVID-like	CD-like, granulomas on histology	-
	BTK	X	Agammaglobulinemia	CD-like, colic	-
	PIK3R1	AR	Agammaglobulinemia	Colic	-

Table 1. Continued

Group	Gene	Inheritance	Syndrome/disorder	Gastrointestinal manifestations	Extraintestinal manifestations
T and B cells defects	CD40L	X	Hyper IgM syndrome (HIGM)	Colic, perianal	Opportunistic infections, hemolytic anemia
	AICDA	AR	Hyper IgM syndrome (HIGM)	Ileo-colic	Bacterial infections
	WAS	X	WAS	UC-like	Arthritis, eczema, thrombocytopenia with small platelets, autoimmune hemolytic anemia
	DCLRE1C	AR	Omenn syndrome	CD-like, ileocolic	Eczema
	CTLA4	AR	CTLA4 deficiency	Ileocolic , lymphocytic infiltrate	Autoimmune cytopenias, splenomegaly
	ZAP70	AR	SCID	UC-like	-
	RAG2	AR	SCID/ Hyper IgM syndrome	UC-like	Arthritis
	IL2RG	X	SCID	Ileo-colic	-
	LIG4	AR	SCID	-	Autoimmune neutropenia
	ADA	AR	SCID	-	Autoimmune hemolysis
	CD3γ	AR	SCID	CD-like, colic	-
	DKC1	X	Dyskeratosis congenita	Ileo-colic, strictures, apoptosis on histology	Nail & air abnormalities
	RTEL1	AR	Dyskeratosis congenita	Colic, strictures, apoptosis on histology	Nail & air abnormalities
	DOCK8	AR	Hyper IgE syndrome	Colic, CD-like, granulomas	Eczema, primary sclerosing cholangitis
Immune regulatory functions	FOXP3	X	IPEX	Ileo-colic	Thyroiditis, diabetes mellitus type 1, hemolytic anemia, muco-cutaneous candidiasis
	IL2RA	AR	IPEX-like	Ileal	
	STAT1	AD	IPEX-like	Ilael	
	IL10	AR	IL-10 signalling defect	CD-like, ileo-colic, severe perianal, fistulizing	Arthritis, folliculitis/pyoderma, lymphoma
	IL10RA	AR	IL-10 signalling defect		
	IL10RB	AR	IL-10 signalling defect		
Others	MASP2	AR	MASP deficiency	UC-like	Arthritis
	SKIV2L	AR	Trichohepatoenteric syndrome	Ileocolic	Air abnormalities
	TTC37	AR	Trichohepatoenteric syndrome		

Modified from Uhlig et al , Gastroenterology 2014 (5)

1.1.4   Clinical presentation

Clinical presentation of monogenic IBD is widely heterogeneous, reflecting the
diversity of the genetic background. In most patients the intestinal and extraintestinal
manifestations present in infancy or early childhood. Intestinal involvement may be
severe and refractory to conventional treatment or extraintestinal manifestations
that are a consequence of a defect of the immune system, may prevail (5,11).
Endoscopic and histologic findings more often do not allow differentiating patients
with monogenic IBD from patients with polygenic forms. However, some histological
findings may be found in certain monogenic conditions. Approximately 78% of
patients with CGD have at least 2 characteristic hystopathological features that
include epithelioid granulomas, pigmented macrophages, and increased eosinophils
(31,32). When apoptotic cells are abundant, the pathology may resemble graft versus
host disease and can be associated with dyskeratosis congenita or epithelial barrier
defects (25).   Among known genetic defects, some can present with classical
syndromic or immunological phenotypes such as WAS (male-predominance,
thrombocytopenia with small platelets and extraintestinal autoimmunity) or IL10
defects (infantile enterocolitis with severe perianal fistulizing disease and folliculitis).
However, the full phenotype might not be present at diagnosis and often develops
over time.   A list of clinical and laboratory findings that should prompt the diagnosis
of monogenic IBD have been proposed by Uhlig et al. The acronym "YOUNG AGE
MATTERS MOST" has been proposed by the same authors to summarize the key
findings while emphasizing the increase likelihood of a monogenic IBD within the
younger age groups. The key findings are: 1) **YOUNG AGE** onset, the likelihood
increase below 2 years of age at disease onset, 2) **M**ultiple family members affected,

3) **A**utoimmunity associated, 4) **T**hriving failure, 5) **T**reatment failure, 6)**E**ndocrine concerns, 7) **R**ecurrent infections or unexplained fever, 8) **S**evere perianal disease, 9) **M**acrophage activation, 10) **O**bstruction and atresia of intestine, 11) **S**kin lesions or dental/hair anomalies, 12) **T**umors (5).

The main clinical characteristics of known monogenic defects with IBD are reported in Table 1.

1.1.5 <u>Diagnosis</u>

Early genetic diagnosis is particularly important in patients with monogenic IBD as they may benefit from specific treatment strategies that are not part of the standard therapeutic repertoire for patients with conventional IBD, including HSCT. However, due to the wide phenotypic and genetic heterogeneity of these conditions, it is often difficult to reach a genetic diagnosis. The classical approach to suspect monogenic IBD has been relying on laboratory tests followed by single candidate gene analysis oriented by the clinical and immunological phenotype. A limited set of laboratory test that include 1) differential blood count, 2) immunoglobulin levels, 3) lymphocyte subsets and 4) neutrophil function test, can orientate toward some of the most common genetic defects for subsequent candidate gene sequencing analysis as illustrated in Table 2.

Additional functional tests are useful to characterize particular subgroups of defects such as XIAP, FOXP3 and IL10 defects, but these are usually available only at specialized laboratories.

In recent years, next generation sequencing technologies (NGS), have been increasingly used as the first line molecular diagnostic tool for identifying patients

with monogenic IBD as discussed further. NGS offers the advantage to look simultaneously at multiple genes and might allow for an earlier diagnosis compared to sequential candidate gene sequencing, particularly in patients presenting non-specific clinical phenotype.

Table 2. **Differential diagnoses following basic immunological work-up**

Test	Finding	Differential diagnosis
Complete blood cell count	Thrombocytopenia	WAS
	Neutropenia	GSD1, congenital neutropenia
	Hemolytic anemia	IPEX, IPEX-like, CVID, WAS
IgG, IgA, IgM, IgE	Low	Atypical-SCID
	High-IgM	HIGM (CD40L, AICDA, RAG2)
	High-IgE	Omenn, WAS, Hyper-IgE syndrome, IPEX
Lymphocyte subsets	Defective	Atypical-SCID, HIGM
DHR test	Defective	CGD

1.1.6 <u>Therapies</u>

Therapeutic options for patients with monogenic IBD include mainly HSCT and anti-infective prophylaxis in patients with defective immune functions. Moreover, non-conventional biologic therapies that interfere with the pathogenic molecular pathway have been identified. HSTC is a potentially curative treatment option for intestinal and extraintestinal manifestations in patients with XIAP deficiency, IL10 defects, CGD, HIGM syndrome, among others. HSCT also prevents the occurrence of hematologic complications in patients with increased susceptibility (such as IL10 defects, XIAP, WAS) (23,33,34). HSTC is a less suitable option in patients with epithelial barrier defects such as NEMO deficiency since it does not resolve the associated epithelial dysfunction and, as a consequence, the intestinal disease may worsen (35).

Biologic therapies that have been found to be effective in controlling IBD include IL-1β blockers in patients with CGD, MVK deficiency and WAS (36,37), and Abatacept in CTLA4 and LRBA deficiency (38).

1.2 <u>Next generation sequencing technologies</u>

Next-generation sequencing (NGS) include a number of different modern sequencing technologies that allow for the analysis of multiple regions of the genome in one single reaction. NGS has been shown to be a cost-effective and an efficient tool in investigating patients with mendelian diseases.

NGS can be used to sequence a defined number of genes that are preselected and included in gene panels (targeted gene panels sequencing, TGPS), the fraction of the genome that is transcribed into proteins (whole exome sequencing, WES) or the entire genome (whole genome sequencing, WGS). Gene panels contain a select set of genes or gene regions that have known or suspected associations with the disease under study. They are designed to include genomic regions of interest and offer the advantage of a high diagnostic accuracy at moderate costs. WES and WGS offer the possibility to reveal causative genetic variants in novel genes but are usually more expensive and less accurate compared to TGPS. Moreover, they produce extensive amounts of data requiring a substantial bioinformatics expertise and numerous variants of unknown significance whose relevance need to be established with functional studies. WES and WGS might therefore be less likely to have an immediate impact on patient management compared to TGPS. A possible, more practical, application of WES and WGS is performing "targeted" exome/genome analysis, meaning that the analysis is initially focused on the set of known genes associated

with the phenotype of interest and if this proves negative, further genes or the entire exome/genome are analyzed to search for novel candidate genes. This approach minimizes the risk of incidental findings. NGS can be deployed either after single candidate gene testing has returned negative, or as first line, if the condition under study is genetically heterogeneous and multi-gene Sanger sequencing would thus be costly and time consuming. Clinical use of NGS in patients with suspected mendelian disorder who remained undiagnosed after previous genetic studies, has been shown to reveal a molecular diagnosis in 25 to 52% of cases (39).

Depending on the tested population and the availability of additional family members for concurrent sequencing (Trio testing), diagnostic rates of up to 60% have been reported in selected cohorts with WES or WGS (39).

A few studies evaluated the clinical utility of NGS in terms of implications for medical management. Overall these studies showed that NGS have an impact on clinical management in 25% to 70 % of patients, depending on the clinical setting (40–42).

1.2.1   NGS application in suspect monogenic IBD

NGS use in children with suspect monogenic IBD have been recently evaluated. In a small prospective study that included 25 patients with VEO-IBD, Kammermeier et al. assessed the clinical usefulness of TGPS as a first-line molecular diagnostic tool and compared the sequencing accuracy of TGPS with respect to WES. In 4 patients (16%) TGPS revealed a pathogenic defect that could not be anticipated by the phenotype, and directly influenced clinical decision making, by supporting or avoiding HSCT, in 2 patients. In the same study TGPS had a significantly deeper coverage and improved variant detection across established VEO-IBD genes compared to WES(10). In

another study, Petersen et al. evaluated the applicability of TGPS as routine screening for genetic causes of IBD or chronic diarrhea in children younger that 10 years, and compared the quality of variants detection between TGPS and WES. Using a custom-made TGPS that included 28 genes, disease-causing variants were identified in 5 out of 71 patients enrolled (7%). TGPS had a significantly deeper coverage and lower costs compared with WES. WES was performed in approximately 1/3 of the cohort population and was not useful in identifying additional causative mutations other than those revealed by TGPS (43).

Very recently Charbit-Henrion et al. reported a comparable diagnostic yield of candidate gene sequencing orientated by abnormal functional tests and NGS (TGPS or WES of parents-child trios). By combining the two diagnostic strategies 66 out of the 207 children (32%) with suspected monogenic IBD obtained a genetic diagnosis. TGPS was particularly efficient when used as the first line molecular diagnostic tool (showing a diagnostic rate of 26.5%) in patients presenting with isolated colitis, without specific clinical findings (68% of monogenic defects were identified by TNGS in this group)(13).

2 RESEARCH PROJECT

2.1 <u>Objectives</u>

In the present study we aimed to describe the diagnostic approach to suspected monogenic IBD in a real clinical setting during a ten-year period, discuss the genetic findings and therapeutic implications and suggest a practical diagnostic approach.

2.2 <u>Materials and Methods</u>

2.2.1   <u>Patients Population and Study Design</u>

This is a multicenter observational cohort study. Patients diagnosed with Very Early onset IBD (VEO-IBD) and patients with Early-onset IBD with severe/atypical phenotypes (EO-IBD s/a) managed at two main tertiary Gastroenterology Centers (Burlo Garofolo Pediatric Institute in Trieste, Bambino Gesù Hospital in Rome in the last ten years (2008-2017) were included. Patients referred to Burlo Garofolo Pediatric Institute for a genetic workup from 8 external Gastroenterology facilities, namely Genoa, Messina, Torino, Brescia, Bologna, Milano, Lubiana, Roma La Sapienza, were also included.

The definition of severe/atypical phenotype was applied when the following clinical findings were present: severe perianal disease, recurrent/ atypical infections, skin/annexes abnormalities, abnormal immune status, associated multiple/severe autoimmunity, history of macrophage activation syndrome or hemophagocitic lymphohystiocytosis, intestinal atresia, early developement of tumors. Demographic information and information on gastrointestinal disease, extraintestinal manifestations and treatments were retrieved from medical records. Written

informed parental consent was obtained for genetic analysis. In the first part of the study information of interest were retrospectively collected and included in a dedicated database. Starting from 2015, newly diagnosed patients with VEO-IBD, EO-IBD s/a and patients without a previous definite genetic diagnosis were prospectively recruited for genetic work-up (Figure 1).
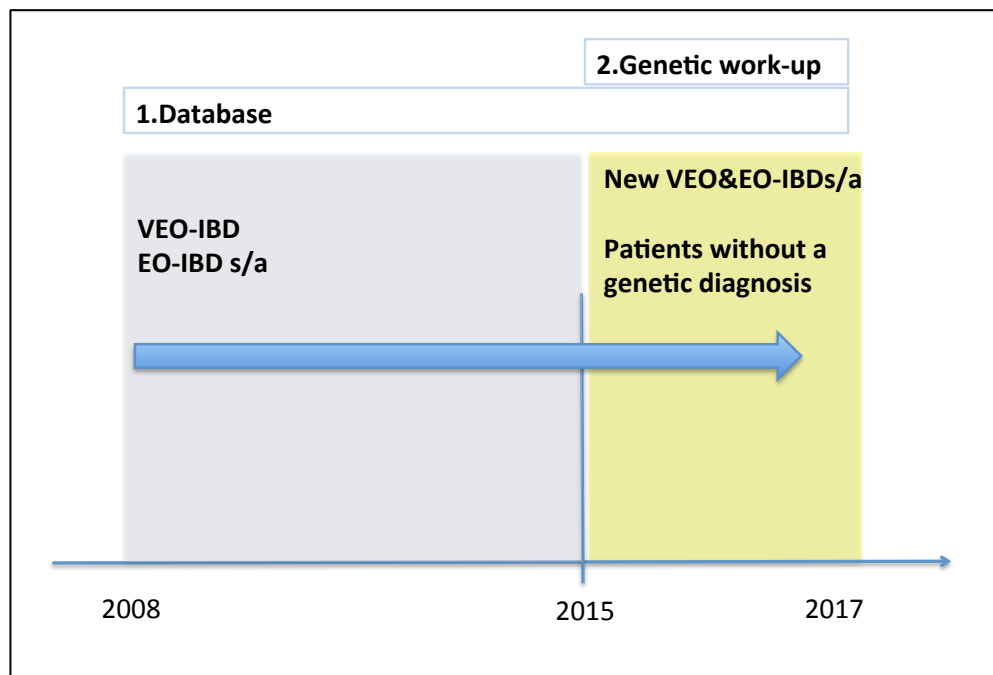

Figure 1: Project workflow. The research project was started in 2015. Data of patients managed between 2008-2015 (gray area) were retrospectively collected into a dedicated database. From 2015 new patients and patients who had remained undiagnosed were recruited fro genetic work-up (yellow area). VEO-IBD: very early onset IBD; EO-IND s/a: Early onset IBD with severe/atypical phenotypes.

2.2.2 Diagnostic work-up

In the prospective phase of the study, patients enrolled for genetic work-up were screened using NGS technologies with the exception of patients with well-defined phenotypes suggestive of a specific monogenic disorder and for whom single gene sequencing was chosen (Figure 2). A targeted gene panel sequencing (TGPS) analysis

was performed in the majority of patients as the first line diagnostic tool. Due to a significant decrease in WES costs, from 2017 WES replaced TGPS. Trio-WES was used in selected cases of patients with IO-IBD and severe disease, when parental DNA was available.

Basic immunological work-up included: complete blood count, Ig subclasses, lymphocyte subset, neutrophil function studies.
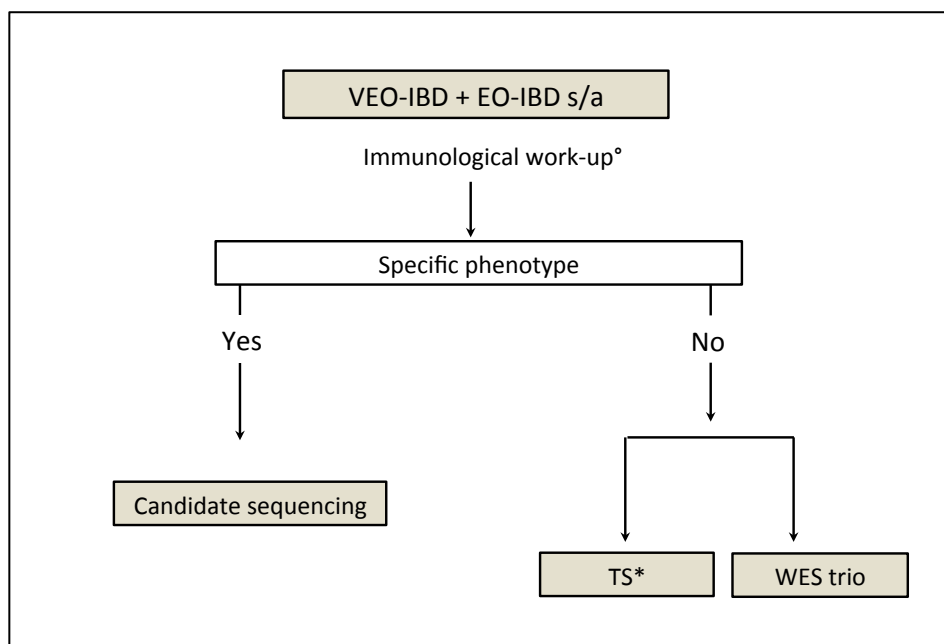
Figure 2: Diagnostic strategy. °differential blood count, Ig, lymphocyte subset, neutrophil function; * TS: TGPS panels *or* WES (400 genes).

2.2.3 DNA preparation

Genomic DNA was extracted from peripheral blood cells. Nanodrop ND 1000 spectrophotometer (Thermo Fisher scientific, DE, USA) was used to measure DNA concentration.

2.2.4 Targeted gene panel design

Two custom-made Target Gene Panel Sequencing (TGPS) were designed. The first panel designed at Burlo Garofolo included 30 genes (Panel A, Table 3), the second panel designed at Bambino Gesù Hospital included 37 genes (Panel B, Table 3). Gene's selection for both panels was based on lists of genes suggested by Uhlig (5), Christodoulou (44) and Kammermeier (10).

Table 3: Selection of genes within the two panels adopted for TGPS and gene coverage

PANEL A			PANEL B		
Gene	Chr	coverage %	Gene	Chr	coverage %
ADAM17	2p25	98,78	ADAM17	2p25	100
GUCY2c	12p13	98,75	GUCY2c	12p13	100
FERMT1	20p12	98,29	CDH1	16q22	100
EGFR	7p11	97,13	EPCAM	2p21	100
ITGB2	21q22	99,71	CYBA	16q24	97,55
G6PC3	17q21	99,17	CYBB	Xp21.1	97,19
PLCG2	16q23	98,25	NCF1	7q11	99,24
STXBP2	19p13	98,02	NCF2	1q25	100
XIAP	Xq25	80,84	NCF4	22q12	94,15
HPS1	10q23	92,71	RAC2	22q31	95,26
HPS4	22q12	95,21	XIAP	Xq25	80,84
HPS6	10q24	85,16	SH2D1A	Xq25	100
NLRC4	2p22	100,00	IL6	7p15	100
MVK	12q24	100,00	IRGM	5q33	100
FOXP3	Xp11	98,93	FOXP3	Xp11	100
IL10	1q32	79,99	IL10	1q32	100
IL10RA	11q23	96,46	IL10RA	11q23	100
IL10RB	21q22	85,85	IL10RB	21q22	100
ITCH	20q11	96,64	TTC37	5q15	97,11
MASP2	1p36	100,00	SKIV2L	6p21	97,5
TTC37	5q15	97,11	TTC7A	2p21	97,5
SKIV2L	6p21	97,50	NOD2	16q12	100
TTC7A	2p21	97,50	NOD1	7p14	100
NOD2	16q12	100,00	IL23R	1p31	100
TRIM22	11p15	99,14	ATG16L	2q37	100
ATG16L1	2q37	100,00	MST1	3p21	95,04

Table 3: continued

IL23R	1p31	90,43	**IL21**	4q27	100
MST1	3p21	93,72	**RIPK2**	8q21	100
PEPD	19q13	89,28	**IL12B**	5q33	100
DKC1	Xq28	96,76	**IL17**	6p12	100
			IFNG	12q15	100
			LRBA	4q31	100
			STAT5B	17q21	100
			ICOS	2q33	100
			RET	10q11	99,95
			SEC61A1	3q21	100
			IL18RAP	2q11	100
			CUL2	10p11	99,09
			PTPN22	1p13	100
			CD39	10q24	100
			LAMB1	7q31	99,88
			CARD9	9q34	92,71
			MEFV	16p13	100

2.2.5   DNA Library preparation

Ion torrent NGS system was used for TGPS. DNA library were generated using Ion Ampliseq Library Kit 2.0 and each sample was labeled with an Ion Xpress Barcode Adapters Kit (Life Technology, CA, USA) according to the manufacturer's protocol. The sequencing step was performed on Ion Torrent PGM system™ platform after libraries amplification on Ion Sphere Particles (ISP) using Ion OneTouch™ 2 system (Life Technology, CA, USA). WES-was carried out in outsource service from Macrogen Inc (Korea). (Exomes were enriched with SureSelect Human All Exon v4 Kits (Agilent Technologies, Santa Clara, CA, USA) and the sequencing of 2 X 150bp were made in Illumina HiSeq 2500 systems.).

2.2.6   Data Analysis

For TGPS the sequencing data were analyzed by Torrent Suite™ v 5.10. This software performs the base calling, the alignment of the trimmed reads to the human genome reference (GRch38/hg38) and the variant calling. The output VCF files were further annotated using wANNOVAR software (http://wannovar.wglab.org/) (45). WES data were analyzed using Genome Analysis Toolkit (GATK), SAMtools and Picard, according to documented best practices (https://software.broadinstitute.org/gatk/best-practices/). The annotation of VCF was performed using Annovar (46). WES analysis was initially restricted to a set of 400 genes that included all the genes selected in Panel A and Panel B plus the list of genes associated with primary immunodeficiency and related pathways, described by Kelsen et al. (14).

2.2.7   Variant selection

Data were filtered selecting non-synonymous, nonsense, frameshift, splicing (about 10 nucleotides from the splice site) and novel variants that were either absent or had a minor allele frequency (MAF) < 0.02 or MAF < 0.001 in case of recessive or dominant inheritance model, respectively. For MAF selection 1000 Genomes Project (http://www.1000genomes.org/) database and ExAC browser were used. Moreover, all variants were interrogated by GERP score as a measure of the conservation of the genomic position (47). Genetic variants were classified according to the American College of Medical Genetics (ACMG) guidelines (48) into "pathogenic", "likely pathogenic" or "variants of uncertain significance" using dedicated tools (49). Non-synonymous variants were further selected according to four different in silico

prediction tools, namely Mutation Taster (50), Polyphen-2 (51) , SIFT (52) and LRT (53). Among the selected variants those with a pathogenic prediction in at least two out of four tools were retained. Human Splicing Finder v3.1 (http://www.umd.be/HSF3/) was used to predict the effect of splicing variants.

The clinical significance of variants already described in public databases and the association with specific phenotypes were investigated using OMIM (https://www.omim.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD (Human Gene Mutation Database) professional. For novel mutations, pathogenicity was established with a functional assay, when available, or inferred from similar mutations with known clinical significance or based on the presence of highly specific clinical features.

2.2.8 Variant validation

Variants considered to be causative according to the clinical phenotype and the mode of inheritance were validated by Sanger Sequencing after visualizing the reads coverage of each mutations using the Integrative Genomics Viewer (IGV) (https://software.broadinstitute.org/software/igv/) (54,55). Primers were designed using Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer- blast/) and synthesized by Eurofins Genomics (www.eurofinsgenomics.eu). DNA regions were amplified by standard PCR protocols and sequenced in both directions. Sequences were evaluated using CodonCode Aligner 6.0.

2.2.9 Statistical analysis

Statistical analysis were made using GraphPad Prism version 8.0.0. Categorical variables were summarized as frequency and percentage and were compared across independent groups by the Fisher's exact test. Numerical variables with asymmetrical distribution were summarized by median and interquartile range (IQR) and were compared by the Kruskal-Wallis test. A p value < 0,05 was considered for significance.

2.3 **Results**

2.3.1 Patients population

A total of 93 patients diagnosed with VEO-IBD and EO-IBD s/a and followed up between 2008 and 2017 were identified; of these, 46 patients (49%) had disease onset before the age of 2 years and 6 patients (6%) above 6 years. Fifty-five patients (59%) were male; 7 patients (8%) had a family history of IBD amongst first-degree relatives, 2 patients (2%) had a sibling who had died in infancy or early childhood.

2.3.2 Genetic work-up and diagnoses

Fourty-seven patients (50%) underwent Sanger sequencing of one or multiple genes over time. In 8 patients single gene sequencing was guided by the presence of specific clinical and immunological feature. NGS was performed in 84 patients (90%): TGPS in 69/84 patients (82%), WES in 16 (19%) and trio-WES in 5 (6%). Of the patients who underwent NGS, 37 (45%) had been studied previously with a single gene approach and had remained without a genetic diagnosis. The proportion of patients who

underwent Sanger sequencing or NGS as the first molecular analysis according to the year of IBD diagnosis is reported in Figure 3.
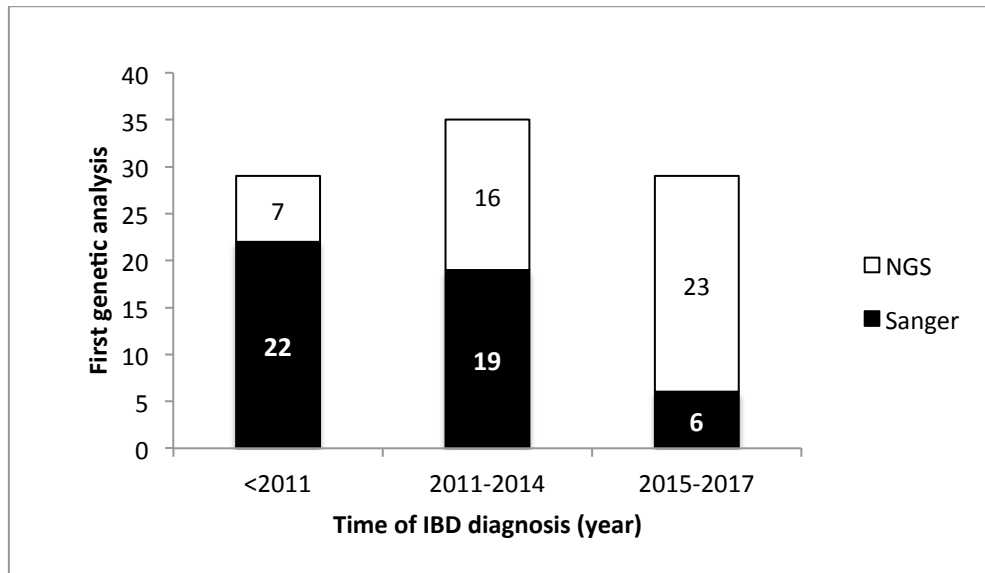
Figure 3 : Proportion of patients who underwent Sanger sequencing or NGS as the first molecular analysis according to the year of IBD diagnosis.

Genetic analysis revealed 14 cases (15%) of monogenic IBD. Likely causative defects were revealed by NGS in 5 patients; namely by TGPS in 3 patients (TTC37, DKC1, XIAP), WES in 1 (NOD2) and Trio-WES in 1 patient (FERMT3). A single gene approach was diagnostic in 8 patients (2WAS, CYBA, CYBB, FOXP3, 2CD40L, XIAP). One patient with WAS, in whom Sanger sequencing did not reveal any mutation, was diagnosed elsewhere-through WAS protein expression analysis followed by WGS that showed a large genomic inversion (36). The clinical and genetic characteristics of patients diagnosed as monogenic IBD are summarized in Table 4. In 7 out of the 8 patients diagnosed with Sanger sequencing, the analysis was guided by the presence of disease specific features as illustrated in Table 4. One patient with XIAP deficiency

who had non-specific presentations underwent sequential sequencing of multiple genes over a period of 15 months, in which failed to respond to several immunosuppressive treatment and underwent colectomy before reaching the diagnosis. Of the 38 patients who underwent NGS as a second step after candidate gene sequencing, one patient with IBD onset at the age of 5 months and associated arthritis was found to carry a rare homozygous variant of NOD2 nucleotide-binding domain. Bioinformatics and functional studies demonstrated that the consequence of the mutation was an auto-activation of NOD2-mediated NF-kB signaling alike that described in patients with Blau Syndrome. Overall, NGS revealed a likely causative mutation in a new gene (FERMT3) in one patient. This was a female infant who presented in the neonatal period with severe jaundice and cholestasis that could be attributed to alfa-1 antitripsyn deficiency (PiZZ) and subsequently developed multiple recurrent ileal perforation, delayed wound healing and recurrent sepsis that could not be explained solely by the same defect. Ileal histopathology showed an eosinophilic infiltrate within the mucosa. Trio-WES analysis revealed 2 rare variants in exons 2 and 3 of FERMT3. The diagnostic flow chart is illustrated in Figure 4. Genetic diagnosis impacted patient management in 11 patients (78%): 7 patients (2XIAP, 2WAS, 2CD40L, FOXP3) underwent HSCT, the patients with WAS gene inversion introduced anti IL-1 antagonist (anakinra) which led to the resolution of a severe pyoderma gangrenosus and arthritis, before undergoing gene therapy (36), two patients introduced anti-infective prophylaxis (2 CGD) and the patient with dyskeratosis congenita (DKC1) introduced danazole as a telomere elongating therapy.
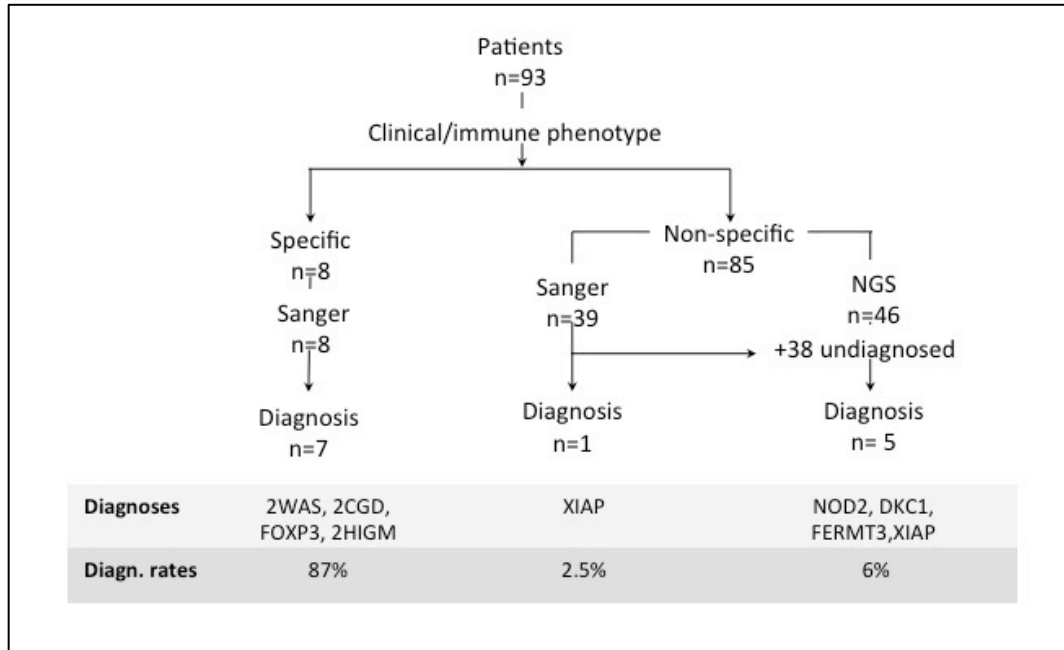
Figure 4: Diagnostic steps and rates of monogenic diagnoses with the different diagnostic approaches.

2.3.3 Clinical, endoscopic and laboratoristic findings

Sixty-nine of 93 patients (74%) presented with bloody diarrhea; failure to thrive was present in 53 patients (57%). The intestinal disease was isolated to the colon in 48 patients (51%) and involved the colon and the small bowel in 22 patients (21%); perianal disease was present in 20 patients (21%). The initial endoscopic diagnosis was consistent with IBD-U in 26 patients (28%), CD in 21 patients (23%) and UC in 18 patients (19%). Eighteen patients were classified as CD-like phenotypes (19%); 9 patients (10%) were diagnosed as allergic or eosinofilic colitis and 1 patient (1%) received an initial diagnosis of autoimmune enteropathy. Fifty-nine patients (63%) had severe intestinal disease (as defined by specific clinical indexes for CD or UC) and 17/39 patients (44%) with CD/CD-like phenotypes had a complicated disease course (14 structuring disease, 4 internal penetrating disease). Extraintestinal manifestations were reported in 40 patients (43%) and there were severe/atypical/recurrent infections in 20 patients, skin rash or skin/annexes abnormalities in 14 patients, macrophage activation syndrome/HLH in 5 patients, extraintestinal autoimmune manifestations were observed in 9 patients; 12 patients had "classical" IBD-associated extraintestinal manifestations (namely: erithema nodosum, uveitis, arthritis, sclerosing cholangitis, pyoderma gangrenosum), 4 patients had metastatic CD and 4 patients had associated dismorphic features or congenital malformations. The clinical features of patients in whom a monogenic defect was diagnosed versus those in whom no causative genetic defects were observed are summarized in Table 5. Disease onset ≤1month had a PPV of 100% to predict monogenic IBD with a sensitivity of 29% and a specificity of 100%. The

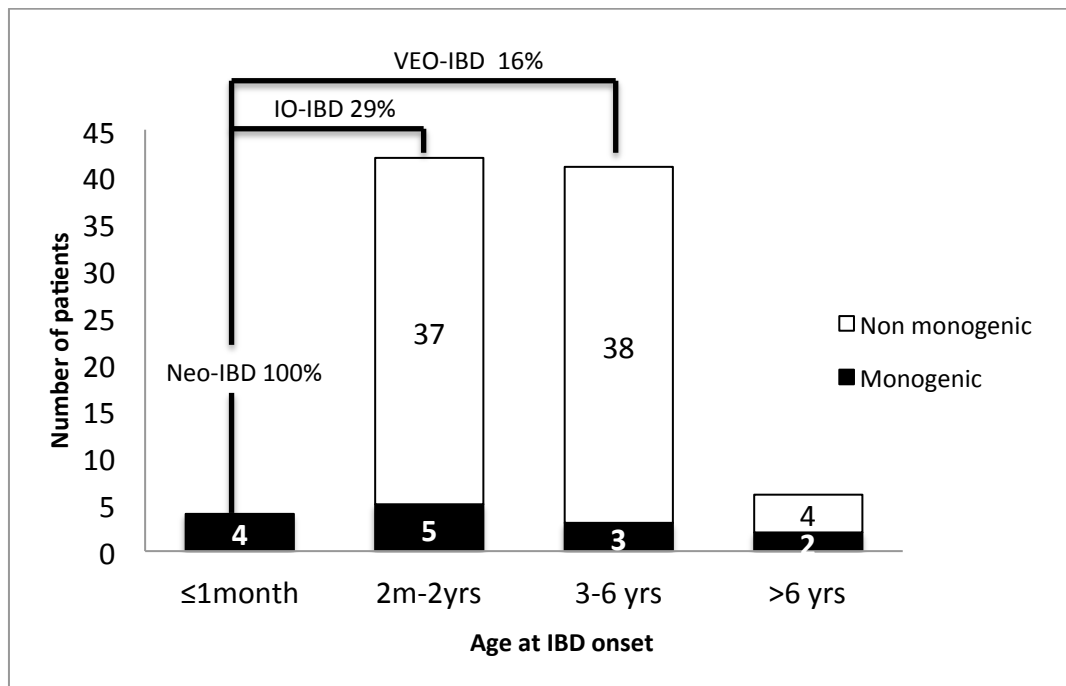distribution of patients with a monogenic diagnosis within different age groups is illustrated in Figure 5.

Table 5: Clinical characteristics of patients with monogenic and non-monogenic IBD

Clinical features	MonoIBD (n=14)	Non-MonoIBD (n=79)	P-value
IBD onset*, median (IQR)	27 (9-48)	24 (8-48)	ns
Age group			
≤ 1 month, n(%)	4 (29)	0	<.001
≤24 months, n(%)	9 (64)	37 (47)	ns
≤6 years , n(%)	12 (86)	75 (95)	ns
Males, n (%)	12 (85)	43 (54)	.004
Family history, n(%)	3 (21)	6 (8)	ns
Endoscopy			
CD/CD-like, n(%)	8 (57)	31 (39)	ns
UC, n(%)	0	18 (23)	.06
IBD-U, n(%)	2 (14)	24 (30)	ns
Other, n(%)	4 (29)	6 (8)	.04
Perianal, n(%)	6 (43)	14 (18)	ns
Severe GI, n(%)	10 (71)	49 (62)	ns
Growth failure, n(%)	12 (86)	42 (53)	.04
Extraintestinal features, n(%)	14 (100)	26 (33)	<.001
Infections, n(%)	10 (71)	10 (13)	<.001
HLH/MAS, n(%)	3 (21)	2 (3)	.02
Skin, n(%)	6 (43)	6 (8)	.002
Autoimmune, n(%)	3 (21)	6 (8)	ns
Low PLT, n (%)	4 (31)	2 (3)	.003
Low Ig, n(%)	4 (31)	0	<.001
Lymph.Subset, n(%)	4/13 (31)	2/50 (3)	.01
TNFfailure, n(%)	2/7 (29)	18/65 (28)	ns
Steroid resistence, n(%)	0/8	13/67 (19)	ns
Surgery, n(%)	4 (28)	20 (25)	ns

*months

Four out of the 88 patients (5%) for whom differential blood count and Ig subclasses were available had low immunoglobulin levels, and all of them were diagnosed with a monogenic IBD (2HIGM, WAS, XIAP). Lymphocytes subsets were available for 64

patients and were altered in 6 (10%), 4 of them received a genetic diagnosis (2HIGM, TTC37, DKC). Neutrophil oxidative burst assay was performed in 59 patients and was diagnostic in each of the 2 patients with CGD.

Figure 5. Distribution of patients with monogenic IBD within different age groups.

2.4 <u>Discussion</u>

The diagnostic approach to suspect monogenic IBD has changed over time. Most of the patients with IBD onset before 2011 underwent a single gene approach. More recently NGS has been used as the first line diagnostic step in most of the patients.

In our cohort the molecular diagnostic sensitivity of NGS was 6%, which is lower than the previous observation in VEO-IBD patients by Kammermeier et al., who reported a diagnostic sensitivity of 16% for a TGPS including 40 genes (10), and Charbit-Henrion et al., who, using a TGPS with 66 genes, reported a variable diagnostic yield of 14% to

up to 26.5% when TGPS was used either as a second line investigation or as a first screening respectively (13). These differences can be explained by a few factors. First, in both cohorts the majority of patients had a disease onset before the age of two years and the study by Charbit-Henrion et al. (13) included only patients with a severe disease course, thus patients in both cohorts might have had a higher pre-test probability for a monogenic disease. Also, it should be noted that the two TGPS used in our study did not include at least part of the genes known to be associated with well recognizable phenotypes or have valid functional tests for which a single gene approach has been used. Including these genes within the target gene panels would probably result in a higher diagnostic yield of NGS. In our study a single gene approach had a good diagnostic performance when oriented by clinical or immunological features that were specific for known monogenic defects such as CGD, WAS or HIGM, but performed poorly in patients with nonspecific phenotypes. In this subgroup only 1 out of 39 patients (2.5%) could reach a molecular diagnosis of XIAP deficiency. The diagnostic process implied multiple single gene sequencing and took 15 months. During this time the patient experienced recurrent bouts of HLH, was dependent on total parenteral nutrition, received several immunosuppressive treatments that were all unable to control the inflammatory process, and ultimately underwent a total colectomy. After the diagnosis of XIAP deficiency, he underwent HSCT, which led to a complete cure. In this patient the use of NGS at the beginning of his symptoms could have helped avoiding treatment failures and surgery.

In our cohort a monogenic diagnosis could be established in 15% of the patients combining different genetic approaches. Monogenic IBD accounted for 16% of patients with VEO-IBD and for almost 30% of patients with IO-IBD. These frequencies

are in agreement with previous reports (10–12). Interestingly we found that all of the patients presenting with an IBD-like disease within the first month of life had a genetic disorder, accounting for half of the cases with a monogenic diagnosis below the 2 years of age. A molecular diagnosis was also identified in 2 out of 6 patients with onset of the disease after 6 years. This age group however included only selected cases and the 2 patients who received a genetic diagnosis, namely one WAS and one TTC37, had developed other signs/symptoms that were specific of their genetic condition earlier that IBD. IBD severity did not seem to differ between patients with monogenic and non-monogenic IBD nor did the frequency of perianal disease. However, growth failure was more frequent in patients with monogenic IBD, probably reflecting a more systemic involvement in this specific group. Extraintestinal features were universally present in patients who received a genetic diagnosis in our cohort. The most represented extraintestinal finding were infections, reported in approximately two thirds of the patients with a genetic diagnosis reflecting the fact that most of monogenic IBD in our cohort are primary immunodeficiencies.

Establishing a genetic diagnosis had an effect on the medical management in the majority of patients. The most frequent consequence was HSCT. HSCT is still the only curative treatment for many primary immunodeficiency disorders and is an option in severe-refractory cases of IBD. Introduction of HSCT as a potentially curative option for intestinal and extraintestinal manifestations of monogenic IBD has changed the clinical practice, thus identifying patients for whom HSCT is indicated as well as excluding those that are unlikely to benefit from such treatment has become crucial. In patients with XIAP deficiency HSCT prevents the development of fatal HLH and

resolves the associated intestinal disease, which is a major cause of morbidity and mortality (21). Also, HSCT has become the standard treatment in patients with IL10 signaling defects, in whom HSCT resolves intestinal inflammation and prevents the development of hematopoietic cancer (34). HSCT is a less amenable option in patients with epithelial barrier dysfunction. Evidence from mouse models and case series have shown that in patients with NEMO deficiency or TTC7A defects, HSCT fail to correct the epithelial defect. In our cohort we identified two patients with genetic defects impacting the epithelial barrier functions, namely one patient with tricohepatoenteric syndrome (TTC37) and one patient with diskeratosis congenita (DKC1). The patient with diskeratosis congenita had severe intestinal disease that was refractory to medical therapy and led to multiple surgeries. HSCT option was considered in this patient but was abandoned based on previous reports describing a poor outcome after HSCT in patients with epithelial barrier dysfunction, including patients with DKC1.

Our study has several limitations: data were collected retrospectively in most of the patients thus the quality of data for such patients might be poor; a selection bias might have been introduced given the fact that multiple Centers participated in the study and probably selected the more severe cases to send for genetic analysis.

However, studies describing the genetic profile of patients with suspect monogenic IBD are still sparse and our cohort represents one of the largest reported so far.

2.5 Conclusions

In conclusion, our data provide evidence that a significant proportion of IBD in infancy are genetic disorders. Early age at disease onset and the coexistence of

extraintestinal manifestation are highly suggestive of a genetic defect. When

monogenic IBD is suspected, Sanger sequencing may be the first choice in patients in

whom clinical and immunological findings orientate toward a specific diagnosis,

however NGS should be preferred in patients with nonspecific phenotypes, especially

in infants in whom the probability of a monogenic condition is higher and timely

diagnosis, before the full phenotype or complication develops, might have an impact

on patient's management.

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Table 4: clinical e genetic characteristics of patients diagnosed as monogenic IBD.

Patient (Sex)	IBDonset (months)	Initial endoscopy	GI disease	Extraintestinal findings	Lab work-up	Treatment	Defective gene	Diagnostic analysis	Impact of genotype
1 (M)	2	AI	Extensive colitis Apoptosis	Persistent fever, CMV infection, HLH	Normal	EN, steroids, AZA, Anti-TNF, tacrolimus, colectomy,	XIAP	Sanger	HSCT
2 (M)	108	CD-like	Colitis, p.	Arthritis, vasculitis, PG, uveitis, nephritis,	↓ PLT, ↑IgA,↓IgM,IgG	Steroids, anti-TNF, MTX cyclosporine, thalidomide, fistulotomy, colectomy	WAS	WGS	Anti IL-1, gene therapy
3 (M)	5	AC	Pancolitis, p	Arthritis	Normal	Steroids, AZA, fistulotomy	NOD2	WES	-
4 (M)	0	EOS	Extensive colitis	CMV infection	↓ PLT	Steroids	WAS	Sanger	HSCT
5 (F)	96	CD-like	Colitis, p	Trichorrhexis nodosa, syndromic facies, epatopathy	↑ Ig A	Anti-TNF	TTC37	TGPS	Genetic counselling
6 (M)	16	CD-like	Enterocolitis,, apoptosis	Leukoplakia, nail dystrophy, skin reticulate	↓ NK, B	Steroids, 5-ASA, anti-TNF, thalidomide, colectomy	DKC1	TGPS	Danazole
7 (F)*	0	EOS	Enterocolitis, small bowel perforations	Severe infections, bleeding diathesis	↑Eos	Ileal resections and diversion, liver transplantation	FERMT3	Trio-WES	Genetic counselling
8 (M)	20	CD-like	Enterocolitis, p, ileal fistulas	Recurrent respiratory infections	↓Treg & B, ↑IgM,↓IgA,IgG	EN, ileostomy	CD40L	Sanger	HSCT
9 (M)	48	IBD-U	Colitis	Sclerosing colangitis, cryptosporidium	↓ B, ↓ Ig, ↑Eos	EN, steroids,	CD40L	Sanger	HSCT, liver transplant
10 (M)	10	IBD-U	Enterocolitis	Liver ascess, eczema	DHR defective	EN, steroids, 5-ASA	CGD	Sanger	Prophylaxis
11 (M)	30	CD-like	Colitis, p	Skin granulomas	DHR defective	EN, steroids, 5-ASA, AZA	CGD	Sanger	Prophylaxis
12 (M)	70	CD-like	Enteropathy	Complicated EBV, HLH	↓ Ig	EN, steroids, AZA, anti-TNF	XIAP	TGPS	HSCT
13 (M)	1	CD-like	Enterocolitis	Candidiasis, psoriasis, opportunistic infections	↓ PLT	EN, steroids	IPEX	Sanger	HSCT
14 (M)	1	CD-like	Enterocolitis	Arthritis, infections	↓ PLT ↓ WBC	EN, steroids, 5-ASA	WAS	Sanger	HSCT