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Inflammatory Bowel Diseases: genetic and functional approaches in the NGS era

Settore Scientifico-Disciplinare: Biologia Molecolare

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1. Introduction

Inflammatory Bowel Diseases (IBDs) are a group of pathologies characterized principally by chronic inflammation of the gastrointestinal (GI) tract. The two principal IBDs are Crohn's Disease (CD) and Ulcerative Colitis (UC); there is also a third group of IBDs that represents the unclassified IBDs (IBDUs).

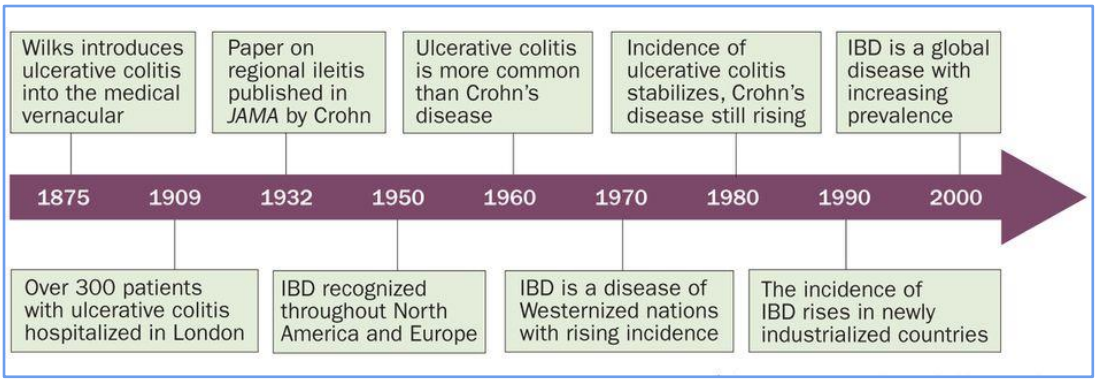


Figure 1.1: Historical timeline of Crohn's disease and ulcerative colitis from first reports to early 00's. Image adapted from Kaplan, G. G. (2015). The global burden of IBD: from 2015 to 2025. *Nature Reviews Gastroenterology & Hepatology*, *12*(12), 720–727. https://doi.org/10.1038/nrgastro.2015.150

Since the fifties of the last century, the incidence of ulcerative colitis and Crohn's disease has increased in the Western world, which includes North America, Europe, Australia and New Zealand[1]. For newly industrialized countries, these observations suggest that as developing countries become westernized, rates of IBDs prevalence and incidence should thrive in parallel to those in the Western world (Figure 1.2).

Epidemiological studies have demonstrated that IBDs affects individuals of all ethnic groups (for example, Hispanic and African American) whose families have lived in the Western world for many generations[2].

Industrialization, and consequent changes in lifestyle, had undoubtedly influenced this trend; in fact, it is well known that, despite genetic risk factors have been recognized, IBDs are not driven by ancestry or ethnicity, but rather by the environment that fosters us.

For instance, it has been reported an association between smoking and Crohn's disease whereas smoking cessation, but not current smoking, is associated with an increased risk of ulcerative colitis. Moreover, dietary fiber (particularly fruits and vegetables), saturated fats, depression and impaired sleep, and low vitamin D levels have all been associated with incident IBDs[3].

In the US estimates of Crohn's disease, incidence varies between 6 and 8 per 100,000, with a prevalence of 100[4] to 200[5] per 100,000. For ulcerative colitis, the incidence in the United States ranges between 9 and 12, with a prevalence of 205 to 240 per 100,000[4–6].

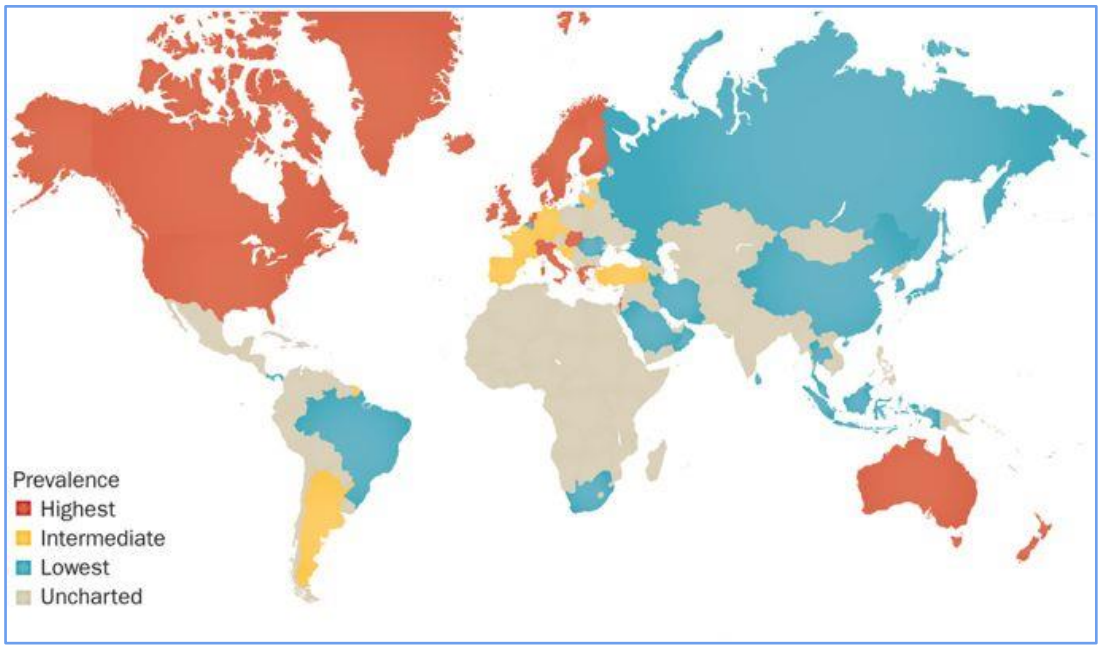


Figure 1.2: Global prevalence of inflammatory bowel diseases in 2015. Data from Kaplan, G. G. (2015). The global burden of IBD: from 2015 to 2025. *Nature Reviews Gastroenterology & Hepatology*, *12*(12), 720–727. https://doi.org/10.1038/nrgastro.2015.150

1.1 Ulcerative Colitis

UC was the first subtype of inflammatory bowel disease to be characterized as a distinct entity. The first one to recognize this novel condition was Sir Samuel Wilks, that in a letter published in 1859 on *The Medical times and gazette* reported the case of a woman with an acute inflammation circumscribed to the terminal part of the intestine, but with peculiar characteristics that differentiated it from bacterial dysentery. In 1875 the term 'ulcerative colitis' was first used (Figure 1.1).

Ulcerative colitis is an idiopathic, chronic inflammatory disease of the colonic mucosa. The main pathogenic mechanism of UC is thought to be an aberrant activation of the immune system in response to a change in the gut environment. However, the cause of this pathological immune system activation is not fully understood. In recent years, it has been

suggested that intestinal microorganisms may play an important role in UC pathogenesis, inasmuch microbiota species in patients with UC differs from that of healthy subjects[7–9].

UC has a bimodal pattern of incidence, with the main onset peak between ages 15 and 30 years, and a second smaller peak between ages 50 and 70 years. Studies have noted either no preference regarding sex, or a slight predilection for men[10].

This disease typically presents with bloody diarrhea, tenesmus, abdominal pain and fatigue. The onset of symptoms can be precipitous or gradual.

The presence of anemia, thrombocytosis, or hypoalbuminemia may suggest inflammatory bowel disease, but most patients with ulcerative colitis do not have these abnormalities[11].

C-reactive protein level and erythrocyte sedimentation rate are relatively insensitive for detecting ulcerative colitis and should not be relied on to exclude inflammatory bowel disease. At the time of diagnosis, less than one-half of patients with ulcerative colitis have abnormal findings on these tests[12]. Endoscopic biopsy with histological confirmation is fundamental to substantiate the diagnosis[13].

Elevated fecal calprotectin and lactoferrin levels have been proven sensitive for the detection of inflammatory bowel disease, but using these tests to exclude patients from endoscopic examination lead to delayed diagnosis in about 6-8% of patients. Tests for perinuclear anti-neutrophil cytoplasmic antibodies (ANCAs) and anti– *Saccharomyces cerevisiae* antibodies are often positive in patients with ulcerative colitis, and can help distinguish the condition from Crohn's disease in those with indeterminate histology[14].

Approximately one-third of patients with ulcerative colitis have extraintestinal manifestations, which may be present even when the disease is inactive.

The differential diagnosis for ulcerative colitis includes Crohn's disease and infectious colitis caused by bacterial, viral, or parasitic pathogens (Figure 1.3).

Table 2. Differential Diagnosis of Ulcerative Colitis and Recommended Testing

Disease	*Findings that suggest diagnosis*	*Evaluation*
Amebic dysentery	Travel to endemic areas or exposure to illness	Anti-amebiasis antibodies, microscopy (ova and parasites)
Bacterial colitis	Should be routinely considered; exposure history may increase suspicion	Stool culture, including testing for *Escherichia coli* O157:H7
Clostridium difficile infection	Recent antibiotic use	Stool studies for *C. difficile* toxin
Crohn's disease	Should be routinely considered; increased suspicion with disease not limited to colon	Endoscopic biopsy
Ischemic colitis	Risk factors for vascular disease	Endoscopic biopsy
Microscopic colitis	Should be routinely considered; nonbloody stools	Endoscopic biopsy
Viral or parasite-induced colitis	Immunocompromised	Endoscopic biopsy

Figure 1.3: Differential diagnosis of ulcerative colitis. UC must be correctly identified and distinguished from other types of colitis induced by pathogens. Faecal culture and endoscopic biopsies are indispensable to reach a diagnosis. Table from Adams, S. M., & Bornemann, P. H. (2013). Ulcerative colitis. *American Family Physician*, 87(10), 699–705.

In contrast with that of Crohn's disease, the inflammation of ulcerative colitis is limited to the mucosa surface, and the specific portion of the colon affected can vary. Some patients have inflammation that is limited to the rectum (ulcerative proctitis), whereas others have more proximal disease; there are also cases of pancolitis, namely, ulcerative colitis that affects the entire colon. (Figure 1.4)

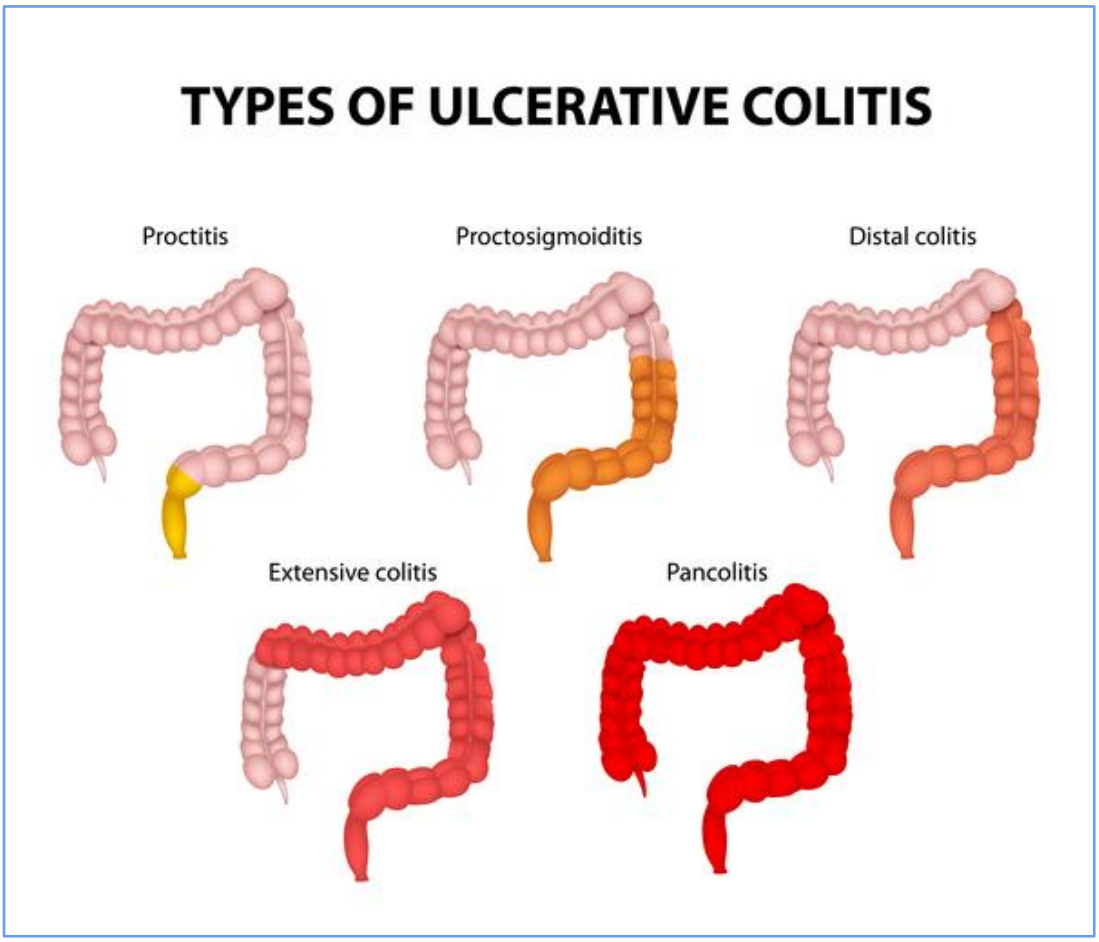


Figure 1.4: Categorization of ulcerative colitis based on inflammation extension in various colonic tracts.

Some patients with UC have persistent disease activity despite diagnosis and medical therapy, and a small number of patients present with the rapid-onset progressive type of colitis known as fulminant disease.

The clinical course is unpredictable, marked by alternating periods of exacerbation and remission. In this perspective, the goals of treatment in ulcerative colitis are to induce remission of active disease and prevent relapse.

The preferred method of treatment for active disease is determined by the endoscopic extent and clinical severity of disease. The same agent used for remission is generally used for maintenance, but at a lower dosage.

For active disease distal to the descending colon, topical 5-aminosalicylic acid (5-ASA), including suppository and enema formulations, is the preferred treatment[15].

Corticosteroid foams are also effective, although less so than 5-ASA. Infliximab, an intravenously administered monoclonal antibody against tumor necrosis factor-α (TNF-α), is effective for those patients who do not respond to corticosteroids.

Also Azathioprine (AZA) is an established treatment for ulcerative colitis, especially for patients who fail 5-aminosalicylates in both the short and long term.
For patients with severe acute or chronic colitis who do not improve with medical therapy, surgical proctocolectomy is recommended.
Proctocolectomy with ileal pouch-anal anastomosis is most common, but also permanent ileostomy is an option. Other indications for surgery are exsanguinating hemorrhage, perforation, or documented or strongly suspected carcinoma[16].

Furthermore, faecal microbiota transplantation (FMT) may be of therapeutic value in patients with UC by contributing to the repopulation of healthy intestinal flora [17–19]. However, conflicting results have been reported regarding the efficacy of this treatment.
In a preliminary study by Costello S. et al., a group of adults with mild to moderate UC received a 1-week treatment with anaerobically prepared donor FMT. Comparison with autologous FMT resulted in a higher likelihood of remission at 8 weeks[20].
Also the group of Tian Y. et al. reported abdominal pain score, diarrhea score, bloody stool score, intestinal mucosal lesion, and Mayo score significantly decreased after treatment with FMT[21].
However, even if FMT studies in ulcerative colitis have shown improved remission rates compared to placebo, relatively small study sample sizes and varied treatment methods limit definitive conclusions. With clear evidence of a dysbiosis in IBD gut, novel therapies to treat and prevent disease relapse will surely require a microbiome-modulating approach.

1.2 Crohn's Disease

CD was first described by Burril Bernard Crohn, an American gastroenterologist that in 1932 published a paper on *Journal of the American Medical Association* (*JAMA*), in which he reported this novel condition[22].
He described a disease affecting mainly young adults, with subacute or chronic necrotizing and cicatrizing inflammation. In the analyzed intestines, it was observed an abnormal growth of connective tissue, with consequent stenosis of intestine lumen and formation of fistulas. He noticed common signs with ulcerative colitis, but the localization was not the same, since UC location is limited to the colon.

In contrast, CD can affect any part of the gastrointestinal tract (most commonly, the terminal ileum or the perianal region) in a non-continuous type. In terms of distribution of the disease, 25% of the patients have colitis only, 25% is ileitis only and 50% have ileocolitis[23].
CD is also commonly associated with different complications, such as abscesses, fistulas and strictures[24].

In terms of clinical presentation, CD patients often show a UC-like clinical phenotype. Blood or muco-purulent exudates within the stool are present in up to 40% to 50% of patients with Crohn's colitis, but less frequently than in UC subjects[25]. By contrast, chronic diarrhea is the most common presenting symptom in classical ileo-colonic CD, followed by abdominal pain and weight loss[26,27]. Systemic symptoms such as fever, tachycardia and weight loss are usually restricted to severe and extensive disease in UC, but occur more frequently in CD, particularly in the case of severe relapse or transmural complications.
The most common extraintestinal manifestation is represented by enteropathic arthritis, with a prevalence of 11% in UC and 20% in CD. Conversely, primary sclerosing cholangitis occurs more frequently in UC (2%-4%) than in CD (1%-2%)[28].

Table 3. Differential Diagnosis of Crohn's Disease

Celiac disease	Irritable bowel syndrome
Chronic pancreatitis	Ischemic colitis
Colorectal cancer	Lymphoma of small bowel
Diverticulitis	Sarcoidosis
Infection (e.g., *Yersinia, Mycobacterium*)	Ulcerative colitis

Figure 1.7: Differential diagnosis of CD. Image from Wilkins, T., & Jarvis, K. (2011). *Diagnosis and Management of Crohn's Disease* (Vol. 84).

Crohn's disease is classified based on disease location, behavior and patient's age at onset, as reported in figure 1.5. The Vienna classification was proposed in 1998, in occasion of the Working Party for the World Congresses of Gastroenterology[29]; this criteria were then slightly updated in 2005 in the Montreal World Congress of Gastroenterology.

Clinical factors	Vienna	Montreal
Age at onset	A1: <40 years A2: ≥40 years	A1: below 16 years A2: between 17 and 40 years A3: above 40 years
Disease location	L1: terminal ileum L2: colon L3: ileocolon	L1: ileal L2: colonic L3: ileocolonic L4: isolated upper disease†
Disease behavior	B1: inflammatory B2: stricturing B3: penetrating	B1: nonstricturing, nonpenetrating B2: stricturing B3: penetrating 'p': perianal disease modifier

†L4 is a modifier that can be added to L1–3 when concomitant upper GI disease is present.
'p' is added to B1–3 when concomitant perianal disease is present.
Adapted with permission from [12].

Figure 1.5: Vienna and Montréal classification of Crohn's disease. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut. 2006;55(6):749–753. doi:10.1136/gut.2005.082909

In 2011, a group of IBD experts proposed a paediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification[30].

This reclassification was based on several findings indicating that children diagnosed with IBD before 10 years of age develop a somewhat different disease phenotype compared to adolescents or adults[31]. Paris classification is reported in figure 1.6:

Characteristics	Montreal	Paris
Age at diagnosis	A1: <17 yr	A1a: 0 to <10 yr
	A2: 17–40 yr	A1b: 10 to <17 yr
	A3: >40 yr	A2: 17–40 yr
		A3: >40 yr
Location	L1: Terminal ileal±limited cecal disease	L1: Distal 1/3 ileal±limited cecal disease
	L2: Colonic	L2: Colonic
	L3: Ileocolonic	L3: Ileocolonic
	L4: Isolated upper disease	L4a: Upper disease proximal to ligament of Treitz
		L4b: Upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum
Behavior	B1: Non-stricturing non-penetrating	B1: Non-stricturing non-penetrating
	B2: Stricturing	B2: Stricturing
	B3: Penetrating	B3: Penetrating
	p: Perianal disease modifier	B2B3: Both penetrating and stricturing disease either at the same or different times
		p: Perianal disease modifier
Growth	NA	G0: No evidence of growth delay
		G1: Growth delay

Figure 1.6: Paediatric modification of Montréal IBDs classification, named Paris classification. Moon J. Clinical Aspects and Treatments for Pediatric Inflammatory Bowel Diseases. *PGHN* 2019 Jan; 22(1): 50–56. Doi: 10.5223/pghn.2019.22.1.50

1.2.1 CD etiology: environmental factors

Based on the epidemiological, genetic and immunological data, Crohn's disease must be considered a heterogeneous disorder with multifactorial etiology in which genetics and environment interact to manifest the disease.

The most important environmental factor is probably smoking: it is associated with a two fold increase in the risk for CD and this included early life exposure and passive smoking[32].

Diet is another relevant factor: there has been reported a higher incidence of IBD in those who consumed larger amounts of refined carbohydrates. [Martini] It has also been observed that newly diagnosed IBD patients consumed less dietary fiber, less raw fruits and vegetables when compared with healthy individuals[33].

Moreover, the three most important lifestyle activities that has an effect on CD include sleep, stress and exercise. Disturbed sleep in more common in CD and has been associated with active disease[34].

It has also been shown that IBD patients have reduced diversity in gut microbiota when compared with the healthy individuals and this change is more pronounced in CD than UC[7,35].

Some medications such as antibiotics, aspirin, nonsteroidal anti-inflammatory drugs (NSAID) and oral contraceptives have been implicated as potential risk factors for CD[36].

One important factor that can influence CD susceptibility is the intestinal barrier integrity; the first line of defense of the mucosal immune system is a polarized single layer of epithelial cells covered by mucus biofilm secreted from goblet cells with interspersed bacteria[37].

Numerous components are fundamental to maintain the structural and functional integrity of this barrier, and modification in some of these components were found to be associated to CD development: for instance, mucin gene MUC1 was noticed to have a decreased expression in the inflamed terminal ileum of CD patients in a paper by Buisine MP et al.[38]; variation in the tight junction of epithelial intestinal cells were reported too[39,40]. Also abnormalities in Paneth cells, which produce large amounts of α-defensins and other antimicrobial peptides, have been reported in CD patients[41].

It has been hypothesized that particular viral or bacterial infections could be causative of CD; in effect Crohn's disease frequently occurs after acute infectious gastroenteritis[42], and patients shows increased numbers of intramucosal bacteria[37]. Furthermore, adherent-invasive *Escherichia coli* strains were found to be associated specifically with ileal mucosa in CD[43].

Also mycobacteria have been identified in tissues and blood of adult and paediatric patients with Crohn's disease and they remain an important differential diagnosis in endemic areas[44,45].

To date, there is no specific pathogen that has been addressed as a cause of CD itself. However, animal research suggests that viral infections, as an environmental factor, might convert genetic susceptibility to disease outbreak.

1.2.2 CD etiology: genetic factors

A number of studies in twins in northern Europe indicated the existence of a genetic component in Crohn's disease, as the concordance rate in monozygotic twins was estimated at between 20% and 50%, whereas the concordance rate in dizygotic twins brought up in the same environment is less than 10%[46–48].

These data have been confirmed in other studies: a German nationwide study[46] showed that 35% of monozygotic pairs, but only 3% of dizygotic pairs were concordant for the disorder.

Moreover, prevalence in Ashkenazi Jews is higher than in any other ethnic group and Jewish descent is an independent risk factor for the disorder[49].

In contrast, literature reports also a case of monozygotic twins that have developed IBD discordant in overall type, disease distribution, clinical course, response to therapy, and extraintestinal manifestations[50]. Additionally, a paper by Halfvarson J.[51] argues that genetic component in CD has been overestimated in twins studies, due to methodological limitations. In effect, this component still explains only a little more than 20% of the heritability of Crohn's disease, which together with the relatively low concordance rates in monozygotic twins, emphasizes the importance of other factors, such as the environment.

Large genome-wide association studies (GWAS) of IBD have found more than 200 common loci associated with disease[52,53]. The most common variants found in CD patients interested NOD2, IL23R and ATG16L1 genes.

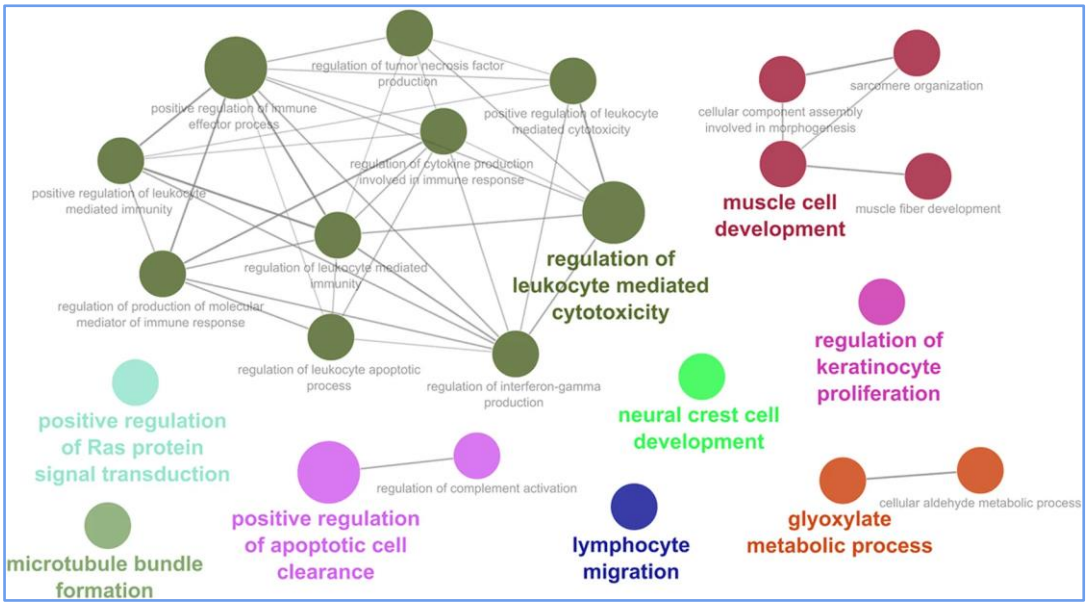


Figure 1.7: Pathway enrichment of the top 200 most significant common mutated genes in pediatric IBD patients. Shaw, K.A., Cutler, D.J., Okou, D. et al. Genetic variants and pathways implicated in a paediatric inflammatory bowel disease cohort. *Genes Immun* 20, 131–142 (2019).

1.3 Very Early Onset IBDs

Patients diagnosed with IBD occurring before the age of 6 are a unique population, known as Very Early Onset (VEO)-IBD and can be phenotypically and genetically distinct from older-onset IBD. In fact pediatric inflammatory bowel disease has higher variability of clinical presentation, resistance to conventional immunosuppressive therapy, and unique complications; often patients present with failure to thrive and delayed puberty in addition to classic IBD symptoms such as abdominal pain and diarrhea, whereas in adult IBD, the main clinical presentation is diarrhea.

Approximately 6–15% of the pediatric IBD population is less than 6 years old and disease in the first year of life is rare[31,54].

This population of paediatric IBDs has increased in incidence and prevalence over the last few years, as reported in different epidemiological studies[55–57]. This increase accounts for the presence of environmental risk factors; for VEO-IBD in particular, incidence has gone from 1.3 to 2.1 per 100,000 children from 1994 to 2009, with a mean annual augment of 7.4%[57].

Evidence reported in epidemiological studies in Canada also suggests that although immigrants to the western world have a lower incidence of IBD than that of nonimmigrants,

children of individuals immigrating from some low-prevalence regions in Asia have a similarly high incidence of IBD compared with the children of nonimmigrants[54,57].
In addition, a role for the gut microbiota has been suggested by its development between birth and 3 years of life, coincident with the age of onset of many VEO-IBD cases.

Nonetheless, due to the usually aggressive phenotype, the early age of onset, and the strong familiar history, the subset of VEO-IBD is thought to be a monogenic disease, often involving genes associated with primary immunodeficiencies[58,59].
The phenotypic presentation of VEO-IBD patients can be heterogeneous, ranging from children with mild disease to others with extensive colonic involvement and elevated severity[60,61].

Due to lack of specific guidelines, usually the diagnostic approach to VEO-IBD is the same as in older children: assessment of intestinal and extraintestinal disease phenotypes by clinical, laboratory, radiologic, and endoscopic evaluation.
Yet, there are important features to note in the patient's clinical history that can direct further investigative tests, as age of onset, history of consanguinity, family history of inflammatory or autoimmune conditions, history of disease in male family members (X-linked disease), or a history of severe or recurrent infections, skin disease, or autoimmunity. These features may suggest a monogenic defect.
For those with a high suspicion for monogenic disease, immunology support should be sought to allow for vaccine titers, immunoglobulin profiles, analyses of B-cell and T-cell function, analysis of oxidative burst by neutrophils (using the nitro blue tetrazolium test or a dihydrorhodamine-123 flow cytometry assay), and if necessary, more targeted profiling of the systemic and mucosal immune system.
In case an IL-10 deficiency is suspected, IL-10 functional assay is recommended. (Figure 1.8)

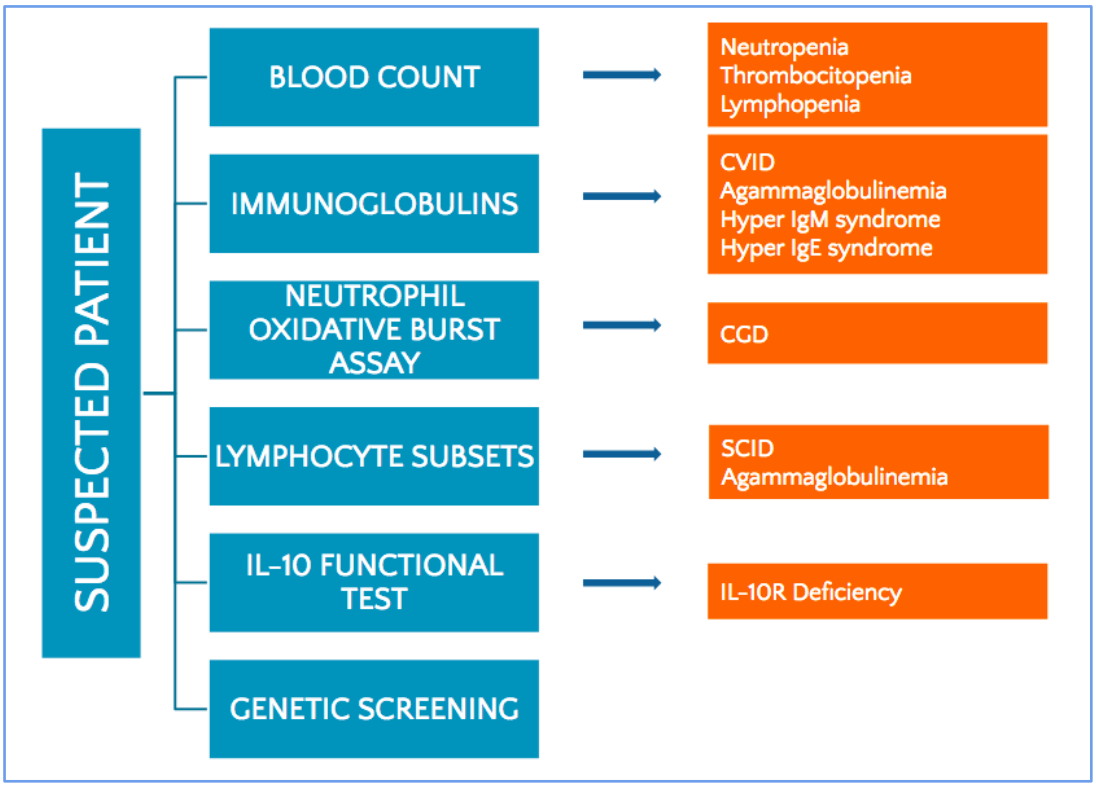


Figure 1.8: Diagnostic tests for VEO-IBD. In case of ambiguous diagnosis from endoscopic and histological examinations, these tests can be useful to correctly identify pathologies underlying the bowel inflammation. Adapted from Uhlig, H. H. et al. The Diagnostic Approach to Monogenic Very Early Onset Inflammatory Bowel Disease. *Gastroenterology* 147, 990–1007.e3 (2014).

Next to all these assays, genetic analysis could be useful to identify the alleged monogenic defect; depending on the specific situation, the approach can vary from candidate gene sequencing to panel sequencing, to whole exome sequencing (WES), up to whole genome sequencing (WGS). If a genetic variant is identified, functional validation is important to confirm the variant before taking clinical action.

It is clear that dealing with such a complex pathology in children requires an interdisciplinary support including pediatric gastroenterologists, immunologists, geneticists and infectious disease specialists.

Primary immunodeficiencies, also known as Inborn Errors of Immunity (IEIs) are a heterogeneous group of more than 330 different disorders; to date, around 300 genes have been identified to be associated with monogenic, Mendelian forms[62]. Moreover, current consensus estimates that about 20% of genetic defects underlying IEIs can develop bowel inflammation[63].

Patients with these disorders often develop symptoms during infancy or early childhood, along with endoscopic or histologic features of Crohn's disease, ulcerative colitis or IBD unclassified.

Monogenic defects have been found to alter intestinal immune homeostasis via several mechanisms (figure 1.9). These include disruption of the epithelial barrier and epithelial response, and reduced clearance of bacteria by neutrophil granulocytes, and other phagocytes. Other single-gene defects induce hyper- or auto-inflammation, or disrupt T- and B-cell selection and activation. Hyper-activation of the immune response can result from defects in immune inhibitory mechanisms, such as defects in IL-10 signaling or dysfunctional T regulatory cell activity.

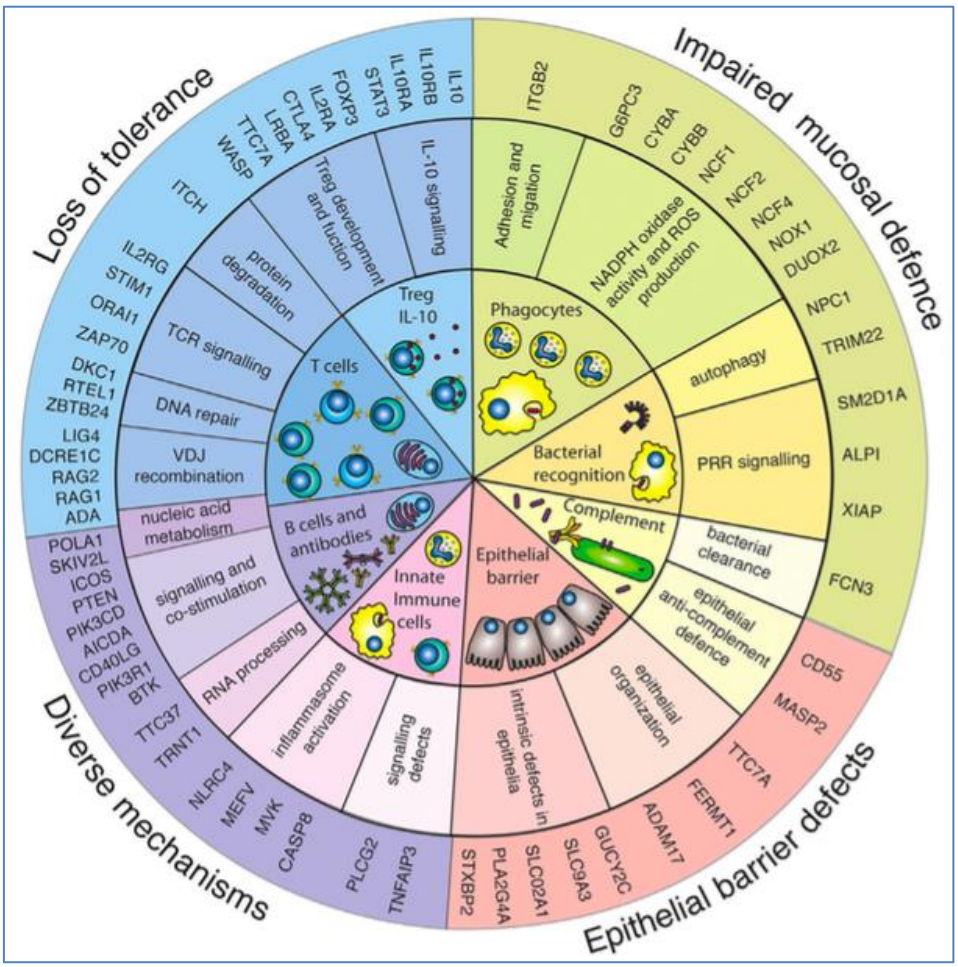


Figure 1.9: Representation of monogenic diseases that provokes IBD. The genes are grouped based on the function impaired in the specific pathology and the cell types involved. image from Pazmandi J, Kalinichenko A, Ardy RC, Boztug K. Early-onset inflammatory bowel disease as a model disease to identify key regulators of immune homeostasis mechanisms. *Immunol Rev*. 2019;287(1):162-185. doi:10.1111/imr.12726

1.3.1 VEO-IBD genetic defects: epithelium defects

In the intestine wall, epithelium layer constitute a physical and biochemical barrier between gut lumen microbiota and immune cells present in the mucosa. Consequently, dysregulation of this epithelium can lead to immune overactivation that culminates in bowel inflammation. Here are reported a few examples of genetic defects related to IBDs.

One of the genetic defects causing VEO-IBD that provokes epithelial abnormalities is mutation in TTC7A gene. It encodes a protein with diverse functions in cell cycle control, protein transport, phosphate turnover, and protein trafficking and secretion. Patients with TTC7A deficiency typically present with features of severe combined immunodeficiency (SCID), along with severe exfoliative apoptotic enterocolitis[63].
The creation of TTC7A-deficient patient-derived organoids showed defective apicobasal polarity and increased apoptosis, that may cause a physical breach of the epithelium therefore aggravating the bowel inflammation[64].

Another mutation that causes IBD is that of FERMT1 gene, that in Kindler syndrome lead to lack of Kindlin 1 protein and induction of inflammatory response in keratinocytes.
Kindlin 1 is involved in integrin signaling and the linkage of the actin cytoskeleton to the extracellular matrix. Patients with Kindler syndrome have been reported to have ulcerative colitis,[65,66] and Fermt1$^{-/-}$ mouse model shows gut epithelial detachment due to a lack of epithelial integrin activation[67]. This was hypothesized to cause epithelial barrier breach, which culminated in bowel inflammation in this model.

Mutations in gene GUCY2C (Guanylate Cyclase 2C), an intestinal receptor for bacterial heat-stable enterotoxins, cause relatively mild early-onset chronic diarrhea and is associated with increased susceptibility to IBD, small-bowel obstruction, and esophagitis[68]. The exact molecular mechanism behind the familial diarrhea is still unknown; however, it has been shown that the expression of mutant GUCY2C results in increased production of cGMP, possibly underlying the hyperactivation of the chloride channel protein CFTR (Cystic Fibrosis Transmembrane conductance Regulator), leading to increased chloride and water secretion from enterocytes.

1.3.2 VEO-IBD genetic defects: neutrophil defects

Emerging evidence suggests that an important role in intestinal integrity is played by neutrophils, as highlighted by IBD in patients with either quantitative or qualitative neutrophil deficiencies. Neutrophil function in the gut is not restricted to the killing of bacteria that have translocated across mucosal epithelium. During the inflammatory response, neutrophils also contribute to the recruitment of other immune cells and facilitate mucosal healing by releasing mediators necessary for the resolution of inflammation[69].

A protein complex which function is fundamental to neutrophils is the nicotinamide adenine dinucleotide phosphate oxidase (NOX), which allows these cells to produce reactive oxygen species (ROS) during respiratory burst.
Mutations in genes encoding the cytosolic subunits of NOX (CYBB, CYBA, NCF1, NFC2, and NCF4) abrogate its activity and compromise host immunity against certain bacteria and fungi. These defects cause chronic granulomatous disease (CGD), which is characterized by immunodeficiency and can cause IBD-like intestinal inflammation[70].

Mutations in G6PC3, encoding the catalytic subunit of glucose-6-phosphatase (G6Pase) cause severe congenital neutropenia type IV (SCN IV) and predispose patients to IBD[71,72].
SCN IV has been linked to glycogen storage disease type 1b as both disorders involve disruption of the glucose-6- phosphatase/glucose-6-phosphate transporter complex, leading to developmental or functional defects in neutrophils. In effect, the function of NOX in phagocytes from patients with G6PC3 is diminished, abrogating normal ROS production[73].

Mutations in the integrin beta chain 2 (ITGB2) gene cause leukocyte adhesion deficiency type 1 (LAD1). This gene encodes an integrin (also known as CD18) participating in cell adhesion and cell surface-mediated signaling.
Clinically the disease is characterized by delayed umbilical cord separation, recurrent severe infections, impaired pus formation, poor wound healing, and persistent leukocytosis. These clinical features are consequences of defective leukocyte adhesion to endothelial cells, the absence of transmigration into inflamed tissues as well as deficient phagocytosis and chemotaxis of granulocytes, monocytes, and lymphoid cells[74].
Some patients develop an IBD-like phenotype, most likely due to the complex pathology caused by dysregulation in the recruitment of leukocytes into the intestine, and abrogation of mucosal defense[75].

1.3.3 VEO-IBD genetic defects: bacterial sensing defects

A correct interaction between gut and commensal microbiota is fundamental to maintain homeostasis at intestinal level; defects in genes involved in bacterial sensing or clearance are known to interrupt this imbalance, possibly leading to pathological inflammation.

X-linked lymphoproliferative (XLP) disease is a rare immunodeficiency caused by mutations in the SH2D1A (SH2 Domain Containing Protein 1A, or SAP) or XIAP (X-Linked Inhibitor Of Apoptosis) genes. Clinical manifestations are varied and include hemophagocytic lymphohistiocytosis (HLH), lymphoma and dysgammaglobulinemia, often triggered by Epstein-Barr virus infection[76].

Mutations that disrupt SAP protein impair proper signaling to induce immune response toward viral infection and led to the development of lymphomas due to defective lymphocytes apoptosis.

Large deletions of XIAP have been associated with GI symptoms of colitis and gastritis[Booth].XIAP is involved in the function of cytotoxic lymphocytes and is an important regulator of T and NK cells function, as well as NKT cell ontogeny[77,78].

Recently, biallelic inherited loss of function mutations in ALPI gene have been reported to cause IBD. ALPI is an intestinal alkaline phosphatase that is thought to function in the detoxification of lipopolysaccharide (LPS) and prevention of bacterial translocation in the gut. Mutations in ALPI abrogate the regulation of host-microbiota interactions and restrain host inflammatory responses causing early-onset severe diarrhea, weight loss, and severe ulcerations from transverse colon to the rectum[79].

1.3.4 VEO-IBD genetic defects: T cell defects

Gene defects that disturb adaptive immune cell selection, activation, and differentiation can all manifest in immunodeficiency, autoimmunity, and intestinal inflammation. Mutations in any of the genes that underlie SCID can cause an IBD-like pathology. In particular, hypomorphic mutations where the proteins and/or molecular functions are impaired but residual activity can be observed often lead to IBD.

Omenn syndrome, an autosomal recessive form of SCID, is caused by impaired V(D)J recombination due to mutations in RAG1 and RAG2 genes, and defective DNA repair after

V(D)J recombination by mutations in DCLRE1C/ARTEMIS. Patients present erythroderma, desquamation, alopecia, eosinophilia, hepatosplenomegaly, elevated serum IgE levels, and often, colitis[80].

Moreover, defects in DNA ligase 4 (LIG4) encoding an ATP-dependent DNA ligase that joins double stranded breaks during non-homologous end joining pathway, and is essential for V(D)J recombination, can cause SCID and IBD[81].

Interleukin receptor common gamma chain (IL2RG), is a cytokine receptor subunit that is common to the receptor complexes of at least six different interleukin receptors: IL-2, IL-7, IL-9, IL-15, and IL- 21. Lack of IL2RG activity due to genetic defects results in the near-complete absence of T and NK lymphocytes and nonfunctional B lymphocytes. The phenotype presents as SCID with often chronic diarrhea, a phenotype very similar to Omenn syndrome[82,83].

DOCK2 (Dedicator Of Cytokinesis 2), an activator of Rho GTPases such as RAC1 (Ras-related C3 botulinum toxin substrate 1), when mutated can lead to develop combined immunodeficiency, with consequent early-onset invasive bacterial and viral infections, lymphopenia, and various defective T cell, B cell, and NK cell responses. DOCK2 mutations impair RAC1 activation in T cells and chemokine-induced migration and actin polymerization in lymphocytes. This impaired T-cell activation may account for the immune dysregulation in DOCK2 deficiency, leading to bowel inflammation[84].

ZAP70 (Zeta-Chain-Associated Protein kinase 70) is a protein normally expressed on the surface membrane of T cells and NK cells. It is part of the T-cell receptor signaling cascade, crucial in the context of TCR signaling. ZAP70 deficiency leads to CD4 and CD8 T cell deficiency due to defective T-cell receptor signaling; it can present with IBD as well, potentially due to the dysregulation of T cell-mediated immune processes[85].

ORAI1 (Calcium release-activated calcium channel protein 1) is a calcium selective ion channel encoded by the ORAI1 gene. Together with STIM1 (Stromal Interaction Molecule 1) protein, it forms a complex vital to maintain cytoplasmic-endoplasmic reticulum calcium homeostasis of cells, and is particularly important in the context of $Ca2^{+}$-dependent T cell activation[86].

Patients with deficiency in ORAI1 or STIM1 present with variable expression of CID that is characterized by severe T cell activation defects, with GI manifestations previously reported in ORAI1 deficiency. These findings illustrate that impaired calcium signaling can result in gut inflammation through reduced number of Treg cells and/or aberrant T-cell thymic selection[87].

In addition, T regulatory cell function impairment can lead to the development of IBDs.
IPEX (Immune dysregulation, Polyendocrinopathy, and Enteropathy X-linked) syndrome is caused by mutations in the FOXP3 (Forkhead box P3) gene, a master regulator of the development and function of Tregs. In IPEX, the lack of or mutant FOXP3 protein causes abnormal Treg function, with consequent systemic autoimmunity and severe enteropathy associated with eosinophilic inflammation[88].
Similarly, mutations in CD25 gene encoding IL-2RA, results in an IPEX-like syndrome. The protein constitute the high affinity IL-2 receptor, expressed on the surface of activated T and B cells. The patients exhibit defective IL-10 expression from CD4 lymphocytes, highlighting the importance of IL-2 in IL-10 production, and the priming of Treg for immunosuppressive functions[89].

CTLA-4 (Cytotoxic T-Lymphocyte Associated Protein 4) is a critical inhibitory checkpoint of immune responses. The crucial role of the negative regulation by CTLA-4 is illustrated by the lethal autoimmunity developed by CTLA-4-deficient mice[90].
CTLA-4 resides in intracellular vesicles in Treg cells and is released and mobilized to the cell surface after TCR stimulation; it can bind to either CD80 or CD86 on the surface of APCs and downregulates activating signals. CTLA-4 haploinsufficiency or impaired ligand binding results in a complex syndrome presenting with features of both autoimmunity and immunodeficiency[91], as autoimmune thrombocytopenia and abnormal lymphocytic infiltration of non-lymphoid organs, including the lungs, brain, and gastrointestinal tract, resulting in enteropathy.

An important molecule involved in the expression, function, and trafficking of CTLA-4 from intracellular vesicles to the cell surface is LRBA (LPS Responsive Beige-Like Anchor Protein). In fact, patients with LRBA mutations show CTLA-4 loss and immune dysregulation and can present with VEO-IBD[92,93].
Mutations in the IL-21 gene leads to defective phosphorylation of STAT1, STAT3, and STAT5, and cause early-onset IBD and common variable immunodeficiency-like disease[94,95].
In the context of IL-21 deficiency, the IBD phenotype could be explained by the lack of anti-inflammatory action of IL-21 in inducing IL-10 production through a STAT3-mediated signaling axis. However, this might not be the only mechanism as IL-21R deficient patients have not been reported to develop IBD.

1.3.5 VEO-IBD genetic defects: complement defects

The complement system consists of a high number of plasma proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses in order to oppose infections. A lot of complement proteins are proteases, activated themselves by proteolytic cleavage. Such enzymes are called zymogens and were first found in the gut. For example, the digestive enzyme pepsin is stored inside cells and secreted as pepsinogen, an inactive precursor enzyme, which is only cleaved to pepsin in the acid environment of the stomach.

Deficiencies in complement proteins mostly manifest as recurrent bacterial infections, due to defective bacterial clearance, and autoimmunity such as systemic lupus erythematosus. However, multiple cases of complement deficiency presenting with IBD or IBD-like symptoms have been sporadically reported, pointing to a possible role of complement pathway in IBD pathogenesis[96].

The mechanism of IBD pathogenesis in complement deficiencies has been hypothetically directed toward defective bacterial clearance and potential defective epithelial defense against complement attack.

MASP2 (Mannan-binding Lectin-associated Serine Protease 2) can bind to carbohydrate structures on microorganisms, and autoactivate, leading to cleavage of the complement factors C4 and C2, generating the C3 convertase C4bC2b.

The identification of MASP2 deficiency highlighted the potentially vital role of proper activation of the complement system in colitis. In one patient a homozygous mutation in the MASP2 gene caused defective activation of the complement system through the mannan-binding lectin (MBL) pathway, and resulted in a presentation of ulcerative colitis and later on erythema multiforme bullosum[97].

Other variants in MASP2 have been reported Therefore, MASP2 might be a modulator of IBD pathogenesis and possibly requires further triggers to result in an IBD presentation.

Ficolin-3 is a protein encoded by the FCN3 gene; it is structurally closely related to MBL and can activate the lectin pathway of complement independently of MBL. While low levels of MBL occur in 10% of Caucasians, ficolin-3 deficiency is extremely rare, suggesting it exerts crucial functions for the human immune system. Ficolin-3 deficiency was first reported in a patient with immunodeficiency and recurrent infections, clinical manifestations that are in line with complementopathies. In was reported in 2011 of 2 patients with congenital FCN3 deficiency suffered from severe, potentially fatal necrotizing enterocolitis; it has been

suggested that this was due to defective control of intestinal microbiota leading to local inflammation[98].

1.3.6 VEO-IBD genetic defects: IL-10 and IL-10R deficiency

Interleukin 10 (IL-10) was first discovered in 1989 upon the observation that a factor produced by mouse Th2 clones inhibited the synthesis of several cytokines by Th1 clones[99]. This newly discovered cytokine was first named cytokine synthesis inhibitory factor (CSIF) but the name IL-10 was already applied in the follow-up publication from the same group where they described that the Epstein-Barr virus (EBV) gene BCRFI showed extensive homology with IL-10[100].

IL-10 is a pleiotropic regulatory cytokine produced by all leukocytes, with $CD4^+$ T cells and monocytes/macrophages being the most important sources. Some non-immune cells such as keratinocytes or epithelial cells can also produce IL-10.
The overall activity of this cytokine is to limit and terminate the immune response in order to prevent damage caused by the host's inflammatory response.

The main biological function of IL-10 is exerted on dendritic cells, macrophages and neutrophils, inhibiting MHCII expression, differentiation of monocytes, expression of proinflammatory cytokines, phagocytosis and reactive radical species production[101].
IL-10 anti-inflammatory activities are not only limited to the innate branch of the immune system. It also directly inhibits proliferation of $CD4^+$ T cells[102], IL-2 and IFNγ synthesis by Th1 cells and IL-4 and IL-5 synthesis by Th2 cells[103]. Again, it limits the secretion of other proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and IL-12[104].
IL-10 has also stimulatory properties on specific cell types: it activates B cells, promotes their survival and proliferation, and contributes to class switching and antibody secretion; IL-10 can also stimulate natural killer (NK) cell proliferation and cytotoxic activity as well as proliferation of specific subsets of $CD8^+$ T cells. Summarizing, IL-10 has an important role in the termination of inflammation and restoration of homeostasis helping the development of long-lived memory cells to face future infections.

IL-10 is a homodimer that binds to its heterotetrameric receptor: the receptor complex is made of two subunits of IL-10RA that bind the cytokine and two subunits of IL-10RB that initiate signal transduction. (Figure 1.10)

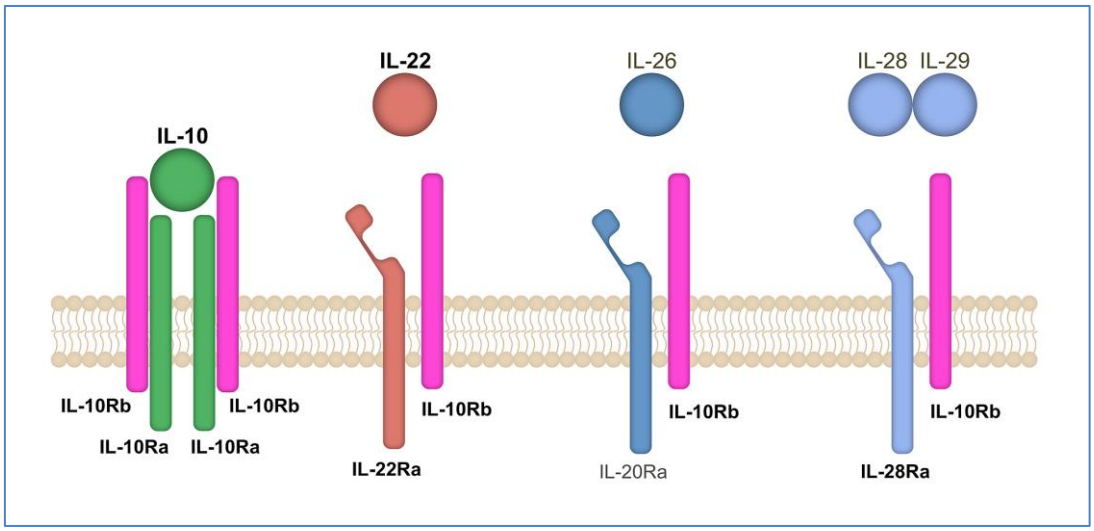


Figure 1.10: Schematic representation of IL-10 receptor complex and other cytokine receptors using subunit IL-10RB. Image from Duque-Correa MA, Karp NA, McCarthy C, Forman S, Goulding D, et al. (2019) Exclusive dependence of IL-10Rα signaling on intestinal microbiota homeostasis and control of whipworm infection. PLOS Pathogens 15(1): e1007265.

IL-10RA is specific and has high affinity for IL-10 while IL-10RB can also act as co-receptor for other cytokines. Both receptors belong to the class II cytokine receptor family (CRFB), characterized by the presence of two particular disulfide bridges and the absence of the so-called "WSXWS" motif in the C-terminal part of the extracellular domain.

Upon activation of the IL-10R complex, a JAK/STAT signaling pathway is initiated, generally triggered by the activation of JAK1 and TYK2 followed by the subsequent phosphorylation of the transcription factor STAT3.
The homodimer formed by two phosphorylated STAT3 molecules then translocates into the nucleus to mediate the transcription of target genes; among other two important target genes are SOCS3 and IL-1RN.

SOCS3 (Suppressor Of Cytokine Signal 3) is a member of SOCS family protein, which modulate signaling include inactivation of Jaks, blocking the access of STAT proteins to receptor binding sites, and enabling ubiquitination of signaling proteins and their subsequent targeting to the proteasome[105].

Induction of SOCS3 by STAT3 results in a negative feedback loop, *via* binding to the gp130 subunit of the IL-6 receptor, which results in transient STAT3 activation with a rapid decline in phosphorylation and nuclear localization[106].

IL-1RN gene encodes a protein known as IL-1RA (Interleukin-1 Receptor Antagonist), that binds non-productively to the cell surface IL-1R, preventing IL-1 from sending a signal to that cell. IL-10 activation pathway is reported to strongly up-regulate IL-1RA production in neutrophils activated with both IL-4[107] and LPS[108].

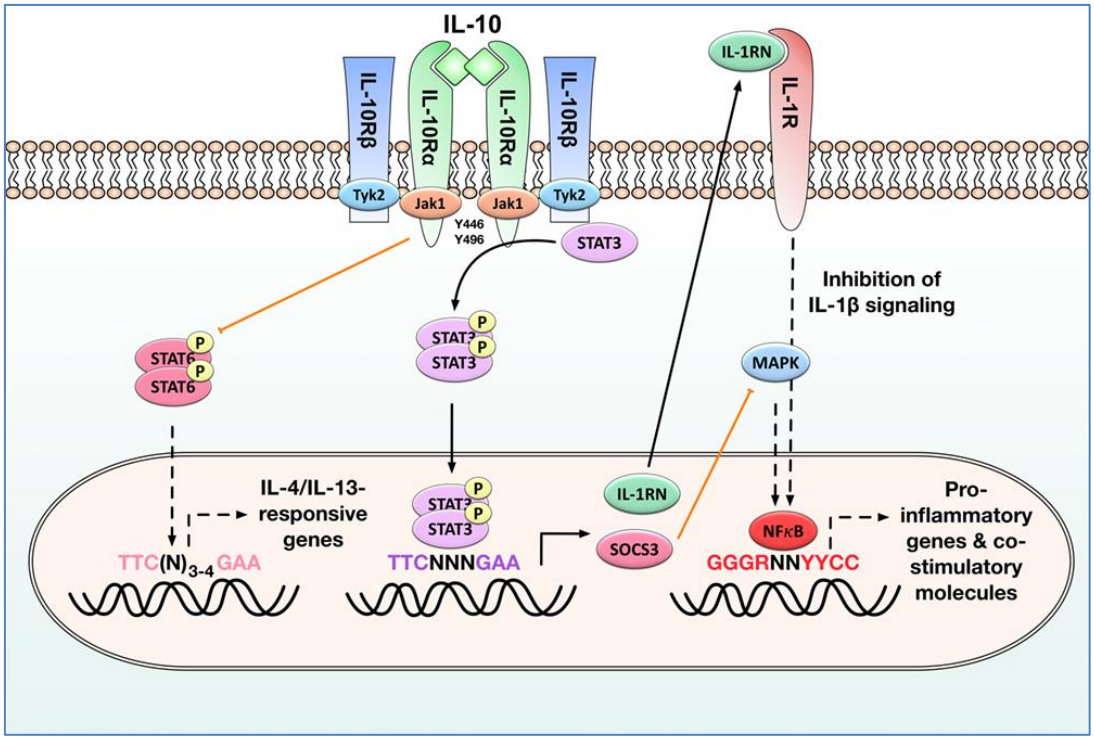


Figure 1.11: IL-10R signaling pathway. Image from1. Schülke S. Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. *Front Immunol*. 2018;9:455. doi:10.3389/fimmu.2018.00455.

In 1993 Kühn et al. were the first to generate, by gene targeting, a IL-10 knockout mouse; they observed that lymphocyte development and antibody responses were normal, but most animals displayed growth retardation, anemia and suffered from chronic enterocolitis[109].

Studies on the role of IL-10 and IL-10R in intestinal immunity went on, and in 2009 Glocker et al. reported a patient which presented in her first year of life with severe enterocolitis, associated with enteric fistulas, perianal abscesses, and chronic folliculitis, consistent with a

diagnosis of Crohn's disease[61]. The girl underwent multiple surgical interventions, including colectomy and ileostomy.

By genetic analysis, researchers found a mutation in IL-10RA gene; the loss of functionality in the mutated allele was confirmed with western blot assays, displaying no STAT3 phosphorylation in patient's PBMC stimulated with increasing doses of IL-10. From this first publication, there has been reported nearly 80 cases of VEO-IBD patients with mutation in IL-10 or IL-10R genes. To date, the real frequency of these mutations in VEO-IBD patients in still unknown, due to lack of multicentric studies; however it seems not so low as previously believed[110].

While VEO-IBD patients present a more complicated clinical course than adults patients, children with autosomal recessive IL-10 signaling defects appear to be even more problematic to deal with.

In fact, they present a more severe and intractable disease, with repeated bouts of bloody diarrhea, marked weight loss, growth retardation, and recurrent perianal inflammation with abscesses, fistulas, and significant fissures [111].

Furthermore, folliculitis and refractory pneumonia are frequent complications in patients with IL-10RB mutations which involves also the response to IL-22, IL-26, IL-28 and IL-29, leading to problems in skin and lung epithelial immunity due to abnormal IL-22 signaling pathway [112,113].

Patients with IL-10R mutation were also reported to develop B-cell lymphoma[114].

In addition to the severe and intractable nature of VEO-IBD with IL-10 or IL-10R mutations, this disease is also usually resistant to a variety of immunosuppressive therapies, including azathioprine, methotrexate, corticosteroids, thalidomide and infliximab, either as single agent therapies or in combination. Treatment with the above agents usually results in no or only mild improvement of clinical manifestations.

A few patients have had to undergo bowel resection and ileostomy or colostomy due to poor treatment efficacy and resistance to therapy[115].

IL-10 predominant function occurs on hematopoietic and immune cells, allogeneic hematopoietic stem cell transplantation (HSCT) has been attempted as a curative therapy for VEO-IBD patients with IL-10 or IL-10R mutations[112,113,116–119].

The reported results appear to support this therapeutic approach, which has been adopted as current curative therapy.

1.3.7 IL-10 biological functions

Our immune system is organized with different cell lineages to mediate response against different pathogen classes; $CD4^+$ T cells differentiate into subsets such as Th1, Th2, Th17, Treg, Th9, Th22, and T follicular helper cells2. Th1 cells rely on the expression of T-bet and eliminate intracellular pathogens through IFN-γ production, which activates macrophages.

Th2 cells play a role in the presentation of allergens, promote immunity against parasites through production of IL-4, IL-5, and IL-13, and are regulated by transcription factor GATA35. Th2 differentiation is also mediated by IL-4, creating a positive feedback loop to reinforce proliferation[120,121].

Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22, and CCL20, express master transcription factor RORγt, encoded by RORC gene, and promote inflammation in response to infections[122].

Regulatory T cells (Tregs) suppress effector function through secretion of inhibitory cytokines such as IL-10 and TGF-β or through cell-mediated engagement of inhibitory checkpoint molecules such as TIGIT and CTLA-4[123]. The relevance of Th17 cells has been documented in promoting autoimmunity, carcinogenesis, and antitumor immunity, whereas Treg cells are essential for immune tolerance and have been shown to dampen autoimmunity and antitumor immunity[124,125].

Most recently, Th9, Th22, and T follicular helper cells (Tfh) have been described as distinct helper populations. As such, knowledge of the programming cytokines and master transcription factors for these subsets is still somewhat under debate.

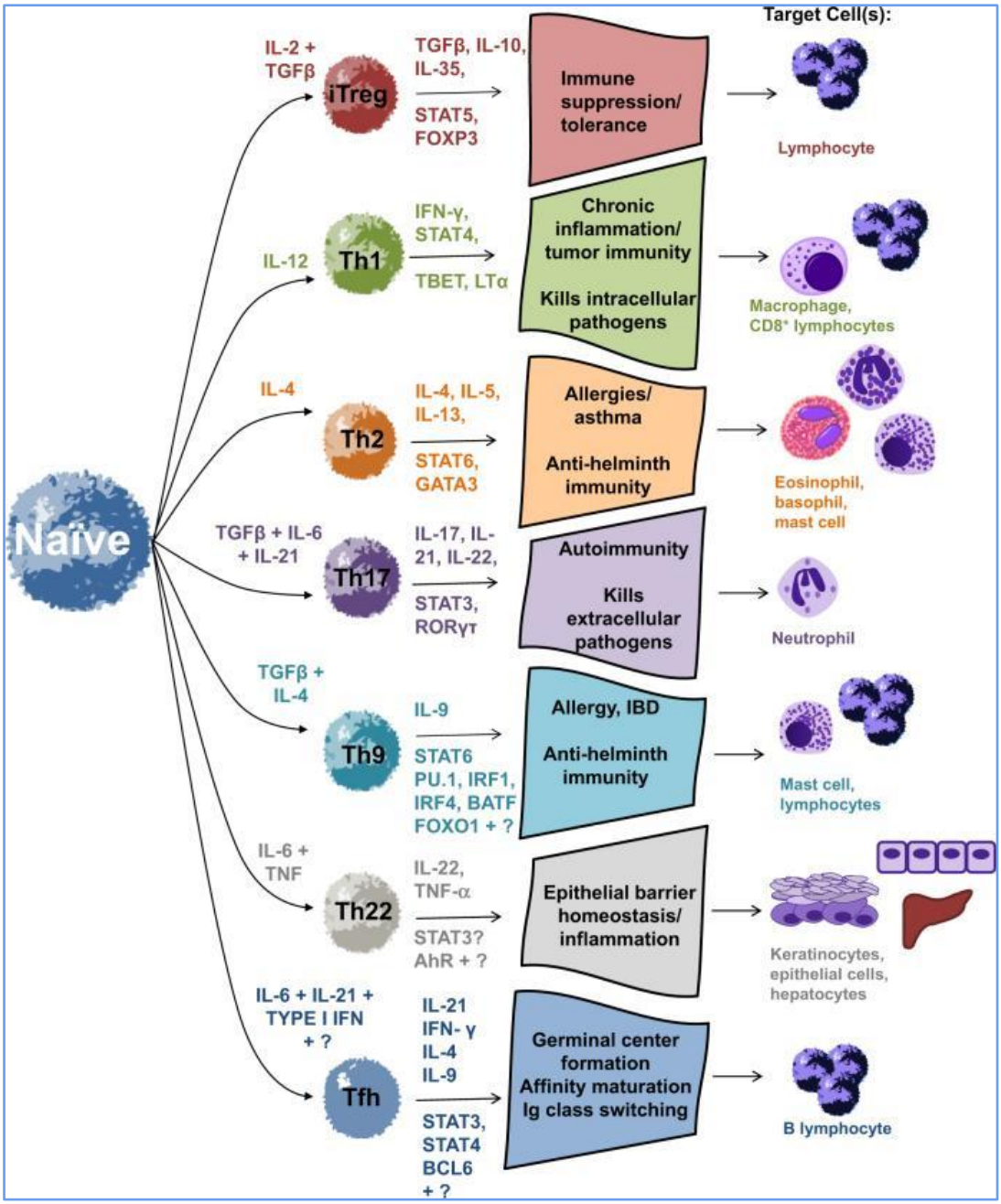


Figure 1.11: Development and functions of T cell subtypes. Due to different cytokine signals received by activated T cells, different pathways and transcription factors will lead to the development of Th1, Th2, Th9, Th17, Th22, Treg or Tfh lineages. Image from Knochelmann HM, Dwyer CJ, Bailey SR, et al. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cell Mol Immunol.* 2018;15(5):458–469.

STAT transcription factor family members activation downstream of corresponding cytokine receptors is critical for the generation of effector CD4+ T cell subsets.

For example, activation of STAT3 downstream of IL-6, IL-23, and IL-21 receptors is required for efficient generation of highly inflammatory Th17 cells essential for protective immunity against yeast, fungi, and extracellular bacteria.

Nevertheless, next to inflammatory cell types intricate negative regulation mechanisms are required to restrict host tissue damage caused by inflammation. These regulatory mechanisms consist of both secreted molecules with autocrine and paracrine activity and specialized cell types.

1.3.8 IL-10 and Tr1 cells

IL-10 in particular has been addressed to have an important role in limiting inflammation at environmental interfaces, especially in the gut and lung mucosa[126,127]. A particular $CD4^+$ T regulatory cell subtype, named Tr1, is well known to secrete high levels of IL-10 and play a major role in maintaining immune tolerance through its strong immune regulatory activity. Tr1 cells are characterized by coexpression of CD49b and LAG-3, high secretion of IL-10, and the lack of FOXP3 expression[128–130]. These population, together with $FOXP3^+$ Treg is reported to be able to inhibit mature a Th17 cells via IL-10, suggesting that the protective function of $FOXP3^+$ Treg and Tr1 cells is likely to be of selective benefit to the host when confronted with an inflammatory insult at its mucosa[131].

Moreover, IL-10 together with prolonged TCR triggering is known to have an important role driving Tr1 differentiation[128,132]. This crucial role of IL-10 for Tr1 cells function strongly supports the hypothesis that patients with IL-10/IL-10R deficiency may have a reduction or reduced functionality, and that these abnormalities can contribute to the disease phenotype[133].

This hypothesis would be in accordance with findings in animal models: it has been reported that an adoptive transfer in SCID mice of *in vitro*-generated antigen-specific Tr1 cells can prevent colitis induced by pathogenic T cells[126], whereas co-administration of wild-type Tr1 cells could prevent colitis in another murine IBD model induced by transferred $CD45RB^{high}$ or Th17 cells[134].

These data from animal models are also supported by a study in which human intestinal Tr1 cells, identified as the IFN-γ^+IL-$10^+$$CCR5^+$PD-$1^+$ subset, displayed reduced IL-10 production in patients with inflammatory bowel disease (IBD), thereby associating an impairment of Tr1 function with IBD in human subjects[135].

1.3.9 IL-10 and Th17 cells

Under physiologic conditions, Th17 cells are present predominantly in the intestine and associated lymphoid tissues, where they facilitate production of antimicrobial peptides, enforce integrity of the epithelial barrier, and recruit and activate granulocytes and macrophages to restrain pathogenic bacteria[136].

The mechanism used by IL-10 to downregulate immune responses are not fully understood; in the case of intestinal inflammation driven by Th17 it is reported that IL-10 can act via STAT3 signaling to limit Th17 responses[137–139]. Moreover, a paper by Huber S. et al., (2011) showed how Th17 cells express IL-10RA, thus can be directly inhibited by IL-10[131].

This essential function of Treg lineages in maintaining the control of Th17-mediated immune response via IL-10 is confirmed by other findings, in fact a specific deletion of STAT3 results in a fatal Th17 cell-driven colitis: in Treg cells, activated STAT3 and FOXP3 cooperatively regulate a subset of genes, which likely endows Treg cells with the ability to suppress Th17 cell-mediated inflammation[140].

Furthermore, in mice, intact IL-10R signaling is reported to be important in Treg cells for their suppressive function including prevention of colitis, and in T effector cells for preventing exaggerated Th17 cell responses in mucosal compartments[131,141–143].

Given the fundamental role of IL-10 in regulating the imbalance between pro-inflammatory Th17 cells and T regulatory cells, it is interesting to verify how this system can be impaired in patients with a deficiency in IL-10 signaling pathway.

Shouval DS et al in a paper published in 2017[144] studied the role of IL-10 signaling in IBD patients mutated in IL-10 or IL-10R genes. They showed that IL-10 suppresses $CD4^+$ T cell proliferation and limits generation of Th17 cells: T cells obtained from IL10R-deficient patients exhibited enhanced proliferative capacity and Th17 cell generation. This hyperproliferation of Th17 in the intestine may contribute to the pathogenesis of the disease. Contrarily, $FOXP3^+CD25^+$ Treg demonstrated frequencies similar to controls, both in peripheral blood and intestine[144].

Similar exaggerated Th17 cells response was reported in mice with a $IL10RA^{-/-}$ Treg-specific mutation[145] and in IL-10 knockout mice, where lack of IL-10 signaling lead to enhanced Th17 response[146].

Targeting key cytokines required for the generation of Th17 cells may have a role in suppressing intestinal inflammation in patients with IL-10 and IL-10R deficiency.

2. Aims of the study

Inflammatory Bowel Diseases (IBDs) are a group of pathologies characterized by chronic inflammation of the gut, that have a growing incidence in western countries in the last decades. They are principally divided into two subtypes: ulcerative colitis (UC) and Crohn's disease (CD). UC interests the colonic tract and only the mucosal layer, while CD can affect every tract from the mouth to the anus, and can lead to transmural inflammation with complication as fistulae.

Etiology of IBDs is still unclear, even if it is known that an important role is played by microbiota modification, environmental factors such as lifestyle and diet, and genetic predisposition.

A defined group of patients with peculiar genetic and phenotypic characteristics is that of very early onset IBDs (VEO-IBDs): these patients usually experience a more severe pathology and often multi-resistance to therapeutic treatments.

A conspicuous amount of VEO-IBD patients was found to have a monogenic disease, mainly an inborn defect of the immune system, that has provoked the gastrointestinal inflammation.

One of this immunodeficiency causing IBD is IL-10 signaling deficiency, generated by loss of function mutation of IL-10 or IL-10R genes. Patients with such a mutation generally present with a severe inflammation and perianal disease in the first weeks of life. The mechanisms beneath gut inflammation development are still unclear, even if dysregulation of immune Th17 cells is thought to be involved.

We have selected patients both with VEO-IBDs and adult onset IBDs with an unclear diagnosis, and investigated the genetic basis of their disease with NGS techniques. On a specific patient bearing a novel IL-10RA homozygous mutation we performed FACS and RealTime PCR to better understand the immunological basis of his pathology.

3. Materials and methods

3.1 Genetic Analysis

3.1.1 DNA extraction from whole blood

DNA was extracted from patients' whole blood using Qiagen QIAmp DNA Blood Mini Kit (50)® as described by the protocol:

- Incubate 200 µl of whole blood with 20 µl od Proteinase K and add 200 µl of AL buffer
- Vortex for 15 seconds
- Incubate at 56°C for 10 minutes
- Add 200 µl of Ethanol 96-100% and vortex for 15 seconds
- Apply the mixture to the QIAmp Mini Spin Column without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 minute. Place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate
- Add 500 µl of AW1 Buffer to the column and centrifuge at 8000 rpm for 1 minute. Place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate
- Add 500 µl of AW2 Buffer to the column and centrifuge at 14000 rpm for 3 minutes. Place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate
- Place the column in a clean 2 ml collection tube and centrifuge at 14000 rpm for 1 minute, discard the tube containing the residues of filtrate
- Place the column in a clean 1.5 ml microcentrifuge tube, add 200 µl of Buffer AE or distilled water and then centrifuge at 8000 rpm for 1 minute

The DNA obtained is quantified by the spectrophotometer UV/Vis Infinite M200 TECAN at the wavelength of 260nm. The purity is estimated reading the OD at 280nm and making the ratio between the two wavelengths. DNA is stored at 4°C.

3.1.2 Library Preparation for Clinical Exome Sequencing

Library preparation, capture and library preparation for sequencing were performed using the Clinical Exome Solution™ by SOPHiA GENETICS.

A fixed amount of genomic DNA (gDNA), 200ng, is diluted in IDTE buffer to reach the volume of 30 µl; after that, gDNA is prepared for the fragmentation reaction by adding 5 µl of FX Enhancer and kept on ice.

FX reaction pre-mixes are then prepared as follows:

FX REACTION PRE-MIX		
Number of reactions	4	16
FX Buffer 10x	23.6 µl	95 µl
FX Enzyme Mix	47.1 µl	190 µl

LIGATION PRE-MIX		
Number of reactions	4	16
DNA Ligation Buffer 5X	95 µl	380 µl
DNA Ligase	47.5 µl	190 µl
Nuclease-free water	71.3 µl	285 µl

PCR PRE-MIX		
Number of reactions	4	16
HiFi PCR Master Mix 2X	115 µl	460 µl
Primer Mix Illumina Library Amp	6.9 µl	27.6 µl
Nuclease-free water	16.1 µl	64.4 µl

All the pre-mixes and samples must be kept on ice before and after the incubation to block the enzymatic reaction; gDNA is added to 15 µl of FX Reaction pre-mix and then placed in a thermal cycler pre-cooled at 4°C. The program has the following settings:

	TEMPERATURE	TIME
Lid	70°C	
Step 1	4°C	1 min
Step 2	32°C	5 min
Step 3	65°C	30 min
Step 4	4°C	Hold

After the fragmentation reaction, the Dual Index Adapters plate was thawed on ice; the whole volume of FX fragmentation reaction product was mixed with 5 µl of Dual Index Adapters stock and 45 µl of Ligation pre-mix. The reactions are then incubated in the thermal cycler at 20°C for 15 minutes to activate the ligase and connect the indexes with the fragments.
The reaction product is then cleaned up with the AMPure XP magnetic beads following this protocol:

- add 80 µl of AMPure XP beads to the 100 µl ligation reaction product. Mix thoroughly by pipetting up and down 10 times
- incubate at room temperature for 5 minutes and spin briefly if required
- place the 4-tube strip(s) on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully discard supernatant, keeping tubes on the magnetic rack for the following steps
- add 200 µl of 80% ethanol to the beads, let them stand for 30 seconds to 1 minute
- carefully discard the ethanol. Repeat once
- remove the residual ethanol using a P10 or P20 multichannel pipette
- air-dry the beads at room temperature for 5 minutes
- remove tubes from the magnetic rack
- add 105 µl of nuclease-free water to the beads. Mix thoroughly by pipetting up and down 10 times. Incubate at room temperature for 5 minutes and spin briefly if required
- place the tubes on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully transfer 100 µl of the supernatant to new labeled tubes
- proceed to Dual Size Selection

Dual Size Selection allow us to eliminate fragments that are too long or too short, by modifying the beads concentration. The procedure was:

- add 60 µl of AMPure XP beads to the 100 µl ligated reaction product. Mix thoroughly by pipetting up and down 10 times
- incubate at room temperature for 5 minutes and spin briefly if required

- place the tubes on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully transfer 140 µl of the supernatant to new labeled tubes containing 20 µl of AMPure XP beads. Mix thoroughly by pipetting up and down 10 times
- incubate at room temperature for 5 minutes and spin briefly if required
- place the tubes on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully discard 150 µl of the supernatant. Keep tubes on the magnetic rack for the following steps
- add 200 µl of 80% ethanol to the beads. Let them stand for 30 seconds to 1 minute
- carefully discard ethanol using a multichannel pipette. Repeat once.
- remove the residual ethanol using a P10 or P20 multichannel pipette
- air-dry the beads at room temperature for 5 minutes
- remove tubes from the magnetic rack
- add 20 µl of IDTE to the beads. Mix thoroughly by pipetting up and down 10 times and spin briefly
- proceed to Library Amplification.

The Dual Size selected ligation products is PCR amplified by adding 30 µL of PCR pre-mix and placing them in the thermal cycler with the following settings (12 cycles):

	TEMPERATURE	TIME (s)
Lid	99°C	
Initial Step	98°C	120
1st Step	98°C	20
2nd Step	60°C	30
3rd Step	72°C	30
Final Step	72°C	60
Hold	10°C	∞

The PCR reaction product is then purified following this protocol:

- add 50 µl of AMPure XP beads to your 50 µl PCR product. Mix thoroughly by pipetting up and down 10 times.
- incubate at room temperature for 5 minutes and spin briefly if required
- place the tubes on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully discard supernatant. Keep tubes on the magnetic rack for the following steps.
- add 200 µl of 80% ethanol to the beads. Let them stand for 30 seconds to 1 minute.
- carefully discard ethanol using a multichannel pipette. Repeat once.
- remove the residual ethanol using a P10 or P20 pipette. Air-dry the beads at room temperature for 5 minutes. Remove tubes from the magnetic rack.
- using a multichannel pipette add 20 µl of nuclease-free water to the beads. Mix thoroughly by pipetting up and down 10 times.
- incubate at room temperature for 5 minutes and spin briefly if required
- place the tubes on a 96-well plate format magnetic rack for 3 minutes or until liquid becomes clear
- carefully transfer 18 µl of the supernatant (transferring two times 9 µl is recommended at this step) to a new labeled library storage tube. It can be stored at 4°C overnight or at -20°C for longer.

Before going on with the protocol, libraries are quantified using PicoGreen kit. A calibration curve is made quantifying serial dilution of standard DNA, using the microplate reader Infinite® 200 PRO (Tecan).
1 µl of library sample is mixed with 99 µl of TE Buffer and 100 µl of PicoGreen working solution (1:200 in TE Buffer) and quantified.

3.1.3 Capture

As each individual library is ligated with a unique index, they can now be pooled in one reaction: 300 ng of every library are added in a DNA low-binding tube, together with 2 µl of Blocking oligos xGen Universal Blockers – Ts Mix and 5 µl of Human Cot DNA. The mix is then dried using a vacuum DNA concentrator until mix is completely lyophilized.
Hybridization is performed as follows:

- pre-warm the thermal cycler to 95°C (set lid to 99°C)
- after the 10-minutes denaturation switch directly to 65°C (set lid to 75°C)
- resuspend the lyophilized pellet in 13 µl of the hybridization mix (8.5 µl of 2x Hybridization Buffer; 3.4 µl of Hybridization Buffer Enhancer; 1.1 µl of nuclease-free water). Prepare a pre-mix in advance according to the amount of capture reaction(s).
- transfer the resuspended pellet to a PCR tube (one tube per capture reaction)
- incubate in the thermal cycler at 95°C for 10 minutes
- directly add 4 µl of probes to the mix. Mix thoroughly by pipetting up and down 5 times.
- incubate in the thermal cycler at 65°C for 4 hours
- prepare the different wash buffers 1x working solutions as follows in advance to allow them to reach equilibrium during the hybridization reaction:

BUFFER	STOCK BUFFER	WATER	FINAL VOLUME 1X
10X Wash Buffer I	33 µl	297 µl	330 µl
10X Wash Buffer II	22 µl	198 µl	220 µl
10X Wash Buffer III	22 µl	198 µl	220 µl
10X Stringent Wash Buffer	44 µl	396 µl	440 µl
2X Bead Wash Buffer	275 µl	275 µl	550 µl

- pre-warm 1x Stringent Buffer and an aliquot of 110 µl of 1x Wash Buffer I at 65°C in a heat block or water bath for at least 2 hours. Keep the remaining Wash Buffer I at room temperature.

Capture is performed using streptavidin-coated beads, that have to be prepared as follows:

- mix the beads by vortexing them for 15 seconds
- transfer 100 µl of beads per capture (4 reactions 400 µl) to a single 1.5 ml tube
- place the tube on a magnetic rack and let it stand until the solution becomes clear
- carefully remove and discard the supernatant
- add 200 µl of 1x Bead Wash Buffer per capture (4 reactions 800 µl) to the tube. Vortex for 10 seconds

- place the tube on a magnetic rack and let it stand until the solution becomes clear
- carefully remove and discard the supernatant. Repeat once.
- add 100 µl of 1x Bead Wash Buffer per capture (4 reactions 400 µl) to the tube. Vortex for 10 seconds
- transfer 100 µl of cleaned beads to a new PCR tube
- place tube on a 96-well plate format magnetic rack and let it stand until the solution becomes clear
- carefully remove and discard the supernatant
- do not allow the beads to dry and proceed immediately to binding the hybridized targets to the beads
- working quickly to ensure that the temperature remains close to 65°C, remove the hybridization reaction from the thermal cycler and briefly spin down the tube
- for each reaction, transfer 17 µl of the hybridization reaction solution to one PCR tube containing cleaned beads. Resuspend the beads by pipetting up and down until the solution is homogeneous
- bind the DNA to the beads by placing the tube into a thermal cycler set at 65°C (lid at 75°C).
- incubate for 45 minutes. During the incubation, gently pipette up and down the tube every 15 minutes to ensure that the beads remain in suspension.

At this point a wash procedure is performed in order to remove unbound DNA:

- add 100 µl of 1x Wash Buffer I (at 65°C) to each of your hybridized target/streptavidin beads tube. Transfer the mix to new DNA low-binding 1.5 ml tube
- place tube on a magnetic rack and let it/them stand until the solution becomes clear. Carefully remove and discard the supernatant
- add 200 µl of 1x Stringent Wash Buffer (at 65°C) to each of your tube(s). Gently resuspend the beads by pipetting up and down (strong mixing of beads with the stringent wash buffer could decrease the quality of the capture)
- incubate at 65°C for 5 minutes
- place tube on a magnetic rack and let it/them stand until solution becomes clear. Carefully remove and discard the supernatant. Repeat once.

- work at room temperature: add 200 µl of 1x Wash Buffer I (at room temperature) to each tube. Vortex for 2 minutes.
- place tube on a magnetic rack and let it stand until the solution becomes clear. Carefully remove and discard the supernatant
- add 200 µl of 1x Wash Buffer II to each tube. Vortex for 1 minute.
- place tube on a magnetic rack and let it stand until the solution becomes clear. Carefully remove and discard the supernatant
- add 200 µl of 1x Wash Buffer III to each tube. Vortex for 30 seconds. Spin briefly to collect all the liquid
- place tube on a magnetic rack and let it stand until the solution becomes clear. Carefully remove and discard the supernatant
- add 200 µl of 1x IDTE and resuspend the beads. Spin briefly to collect all the liquid
- place tube on a magnetic rack and let it stand until the solution becomes clear. Carefully remove and discard the supernatant.
- remove the remaining liquid by using a P10 or P20 pipette. It is crucial at this step to remove all the remaining IDTE.
- add 20 µl of nuclease-free water, resuspend and transfer the beads/water mix to a new PCR tube.

The library is now amplified using a high fidelity polymerase, in order to minimize errors during the PCR reaction. The protocol follows:

- prepare the PCR pre-mix mixing 25 µl of KAPA HiFi HotStart ReadyMix with 2,5 µl of 10X Library Amplification Primer Mix and 2,5 µl of nuclease-free water
- add 30 µl of PCR pre-mix to the bead suspension. Mix thoroughly by pipetting up and won 10 times and spin briefly.
- place the tube in the thermal cycler and run this program (12 cycles):

	TEMPERATURE	TIME (s)
Lid	99°C	
Initial Step	98°C	45
1st Step	98°C	15
2nd Step	60°C	30
3rd Step	72°C	30

Final Step	72°C	60
Hold	10°C	∞

The PCR product is then processed to perform the AMPure beads clean-up procedure, as described before; at this step the sample can be stored at 4°C overnight or at -20°C for longer storage.

Before preparing the sample for sequencing, it has to be quantified with a fluorometric method, for example with the Qubit 4 Fluorometer.

The pool molarity is determined as follows:

$$\text{Library molarity (nM)} = \frac{\text{Library concentration (ng/µl)}}{\text{Average size in base pairs} \times 649.5} \times 10^6$$

The pool is now diluted to 4nM, and a 10pM dilution is loaded to MiSeq (Illumina) sequencer.

3.1.4 Clinical Exome Sequencing

The sequencer used was a MiSeq machine that employs the Illumina technology; the steps are showed in figure 1. After library preparation, single DNA molecules are bound to the surface of a flow cell and then bridge-amplified to form clusters; sequencing is made by synthesis, using fluorescently labeled reversible terminators to detect bases as they are incorporated.

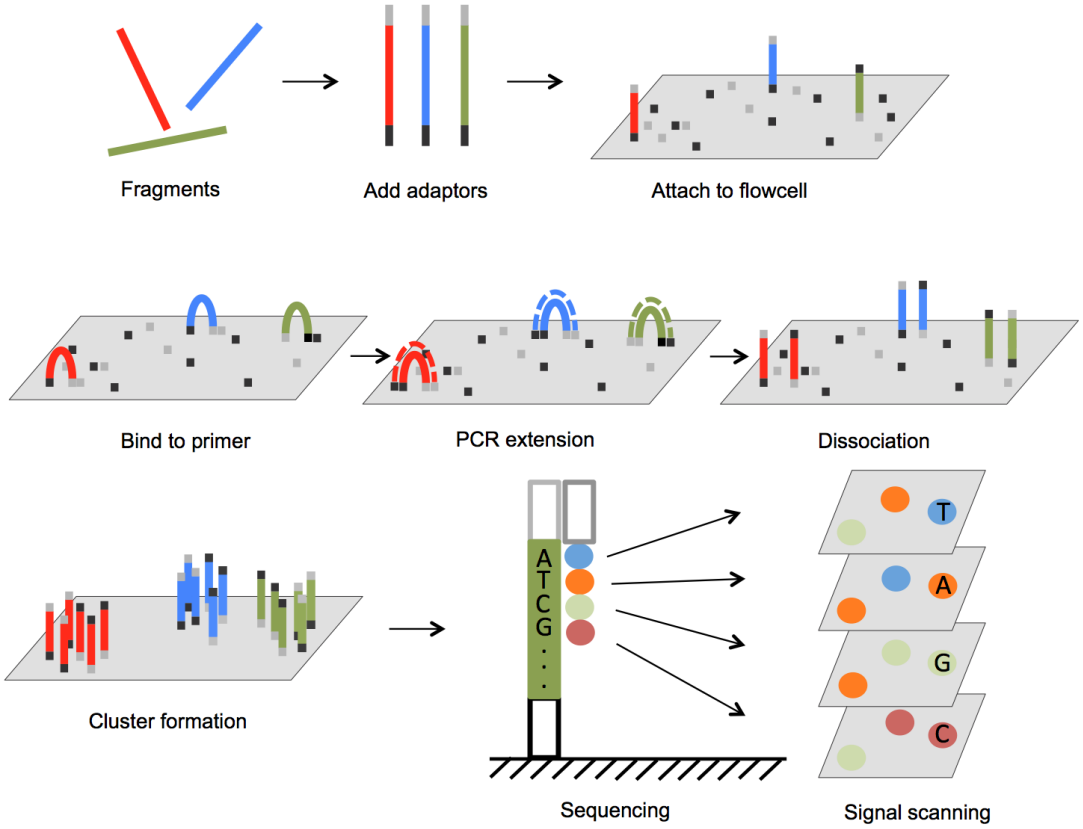


Fig.1: Illumina NGS technology: linkers bound to DNA fragments pairs with oligos on the flow cell and act as primers for the polymerase. Fragments are PCR amplified to form clusters and then sequenced by synthesis.

Libraries (600 µl) are loaded in a reagent cartridge, previously thawed in a room temperature water bath. Flow cell has to be cleaned first in distilled water and then with an alcohol wipe. The MiSeq machine can now be loaded with flow cell, waste bottle and PR2 bottle, and at the end with reagent cartridge. Start the run choosing the appropriate protocol.

The row data generated by the MiSeq sequencer are VCF files, and they have been analyzed with R software.

For Whole Exome Sequencing, 1µg of genomic DNA was sent to Personal Genomics Srl, which provided us the row VCF file to be analyzed.

3.2 Cellular Biology

3.2.1 PBMC Isolation

Peripheral Blood Mononuclear Cells are isolated in order to obtain T and B lymphocytes. The separation is made on a gradient of Lympholyte H, according to the brand protocol:

- blood is diluted with an equal volume of PBS
- blood is stratified on an equal volume of Lympholyte, avoiding the mixture of the volumes
- centrifuge at 2000 rpm for 20 minutes at RT
- the PBMC ring is withdrawn and washed with PBS at 1200 rpm for 10'
- cells obtained are counted with a Burker chamber and diluted in RPMI 10%FBS.

3.2.2 EBV culture

The infection with Epstein-Barr virus lead us to immortalize B lymphocytes in vitro:

- $5x10^6$ PBMCs are incubated with 1ml of cell culture supernatant of Marmoset Clone 95.8 infected with Epstein-Barr virus
- cells are left at 37°C with 5% CO2 for 18h
- cells are washed with PBS and pellet is resuspended in 1ml of RPMI 1640 (Euroclone®), 1% L-Glutamine (Euroclone®) and 1% Pennicillin-Streptomicin (Euroclone®)
- in order to activate lymphocytes 15µg/ml of phytohemagglutinin (PHA) are used. Cell are cultured in 24 wells plates and split until they fill 8 wells; after that, cells are transferred into a 75 cm^2 flask.

3.2.3 Th17 cell culture

In order to obtain a TH17 cell culture CD4 cells need to be isolated from PBMC.

- PBMC are counted and washed with MACS Buffer (PBS, 0,5% FCS, 2mM EDTA) at 1200rpm for 10'

- supernatant is discarded and pellet is incubated with beads anti-CD4 (20µl for 107 cells), mixed well and incubated at 4°C for 15 minutes
- wash cells with 2ml of MACS Buffer and centrifuge at 1200 rpm for 10 minutes
- resuspend the pellet in 500µl of MACS Buffer and proceed with magnetic separation by MS Columns (Miltenyi Biotec)
- the column is moisturized with 500µl of MACS Buffer and fixed on the magnetic support, a 15 ml falcon is used to receive the negative selection
- the cellular suspension is uploaded on MS column and then washed 3 times with 500µl of MACS Buffer
- the column is removed from the magnetic support and put on a new tube to collect the positive selection
- add 1 ml of MACS Buffer to the column and push with the piston: $CD4^+$ cells are collected

In order to expand Th17 cells from $CD4^+$ T lymphocytes we coated a 24-well plate with anti-CD3 antibody (5µg/ml in PBS) at 37°C for 3h or at 4°C overnight. After that, PBS containing the unbound antibody is removed and the plate washed with PBS. $3x10^5$ cells/ml are seeded together with 750ng/ml antibody anti-CD28 and 20U/ml IL-2.
Cells are incubated for 5 days at 37°C, 5% CO^2. Cells in one of the wells are stimulated the day before fluorescent staining with Phorbol Myristate Acetate (PMA) 100ng/ml, Ionomycin 750ng/ml and Brefeldin A 10µg/ml.

3.2.3.1 Intracellular staining of TH17 cells

Intracellular staining for cytokines is performed using the Fix & Perm kit by Caltag Medsystem:

- cells are collected and washed with PBS
- pellet is resuspended with 100 µl of Buffer A, in order to fix cells, and incubated for 15' at RT in the dark
- cells are washed with PBS and pellet is resuspended with 100 µl of Buffer B plus 5µl of anti IFN-ɣ/PE (eBiosciences ®) and 5µl of anti-IL17A/FITC (eBiosciences ®)
- incubation for 20 minutes at RT in the dark
- cells are washed with PBS and resuspended in 300 µl of PBS

- acquisition by flow cytometry

3.2.4 Staining of phosphorylated intracellular proteins

This analysis requires an intracellular staining because the phosphorylated STAT1 protein has a nuclear localization. The protocol follows:

- 100 µl of whole blood is incubated with 5 µl of anti CD3-FITC antibody
- for the stimulation IFN-α (40000U/ml) is added
- incubation for 15 minutes at 37°C
- wash with 2 ml of PBS 1%FBS and centrifuge at 1600 rpm for 4 minutes
- cells are incubated with 2 ml of Lyse/Fix Buffer 1X (Becton Dickinson)® at 37°C for 12 minutes and then washed with PBS-1%FBS 2 times
- resuspend the pellet with 1ml of Perm Buffer III (BD)® and leave the samples at -20°C for 30 minutes
- wash cells with PBS 1%FBS and stain with anti p-STAT1 PE antibody for 20 minutes at RT
- after the incubation with antibodies wash with PBS and acquire by flow cytometry

3.2.5 Treg cells intracellular and extracellular staining

Usually T regulatory cells are identified by staining with antibodies recognizing CD25 and CD127. In order to evaluate a more specific population, we used the True-Nuclear™ Human Treg Flow™ Kit (Biolegend). This kit contains antibodies for intracellular staining (FOXP3/Alexa Fluor®488) and for surface staining (CD4/PE-Cy5, CD25/PE. The protocol follows:

- isolate PBMC with Lympholyte H gradient separation
- distribute cells into FACS tubes (100 µl volume)
- add 20 µl of CD4 PE-Cy5/CD25 PE cocktail to each tube, vortex and incubate at RT in the dark for 20 minutes
- wash once with 1 ml of PBS 5% FBS, centrifuge at 100 rpm for 5 minutes and discard supernatant

- add 1 ml of 1X FOXP3 Fix/Per solution to each tube, vortex and incubate at RT in the dark for 20 minutes
- spin down and remove supernatant
- wash once with PBS 5% FBS, centrifuge at 1400 rpm for 5 minutes and discard supernatant
- wash once with 1X FOXP3 Perm buffer
- resuspend cells in 1 ml of FOXP3 Perm buffer, incubate at RT in the dark for 15 minutes
- spin down, discard supernatant and resuspend pellet in 100 µl of FOXP3 Perm buffer
- add 5 µl of Alexa Fluor®488 anti-human FOXP3 antibody and 5 µl of APC anti-human ROR gamma (t) monoclonal antibody (eBioscience™)
- incubate for 30 minutes at RT in the dark
- wash twice with PBS 5% FBS
- resuspend in PBS and acquire at BD FACScalibur™.

3.2.6 DHR123 Assay

DHR123 (dihydrorhodamine 123) is a nonfluorescent molecule that can diffuse across cell membranes thanks to its lipophilicity. The molecule is oxidized to rhodamine123, that emits green fluorescence, and is trapped within cells in this form.
Neutrophil granulocytes do not show intracellular oxidation of DHR123 in the absence of stimulation, whereas the addition of PMA (phorbol 12-myristate 13-acetate) induces a more than 100-fold increase of rhodamine 123 fluorescence.
The protocol follows:

- three FACS tubes for the healthy donor and three for patients are prepared and marked as W (white), US (unstimulated), PMA
- 100 µl of whole blood are aliquoted in each tube
- add 3 µl of antibody anti-CD16/PerCP-Cy5.5 (BD Biosciences) in tubes marked as US and PMA
- add 25 µl of PBS in tubes W and US
- add 25 µl of PMA (final concentration 500 µg/ml) in tube PMA
- incubate at 37°C for 15 minutes

- add 4,3 µl of DHR123 (final concentration 100 µM) in US and PMA tubes
- incubate at 37°C for 5 minutes
- add 2 ml of 1X Lyse/Fix Buffer (BD Biosciences) pre-warmed at 37°C, agitate well and incubate at 37°C for 10 minutes
- centrifuge at 1600 rpm for 4 minutes
- discard supernatant
- wash twice with PBS
- acquire at cytometer

3.3 Molecular Biology

3.3.1 RNA extraction

PBMC were seeded with the appropriate stimuli or used as such for RNA extraction. We used the RNeasy kit by Qiagen®, according to the manufacturer's instruction:

- cells are pelleted and the supernatant is discarded. The pellet is lysed with 350 µl of RLT Buffer supplemented with 10% β-Mercaptoethanol
- add 350 µl of Ethanol 70% and mix to homogenize the suspension
- carefully apply the solution to the RNeasy Column and spin for 30'' at 14000rpm
- place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate
- add 350 µl of Buffer RW1 and spin for 30'' at 14000rpm
- place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Repeat this step twice.
- add 500µl of Buffer RPE and spin for 30'' at 12000rpm. Place the column in a clean 2 ml collection tube, and discard the tube containing the filtrate
- add 500µl of Buffer RPE and spin for 2' at 12000rpm.
- place the RNeasy column into a new 1,5 ml collection tube, add 50µl of RNase-Free water and centrifuge at 14000rpm for 1'
- RNA concentration was assessed using a spectrophotometer (Infinite M200, Tecan). The extracted RNA was stored at -80°C until use.

3.3.2 Retrotranscription

Retrotranscription was performed using the IMPROM-II Reverse Transcriptase Kit (Promega ®). The RT mix was prepared as follows:

ImProm-II 5X Reaction Buffer	4 µl
MgCl2	2,4 µl
dNTPs 10mM	1 µl
Recombinant RNasin Ribonuclease Inhibitor	0,5 µl
ImProm II Reverse Transcriptase	1 µl
Nuclease Free Water	X
FINAL VOLUME	15 µl

The mix was kept on ice prior to dispensing into the reaction tubes. The reaction tubes were prepared by mixing 1 µl of Random Primers with the appropriate volume of RNA (at least 100 ng of RNA); tubes are placed into a preheated 70°C heat block for 5' and then immediately chilled in ice. 15µl of RT mix are added to each reaction tube to reach the final volume of 20 µl. The PCR protocol follows:

	TEMPERATURE	TIME (m)
1st Step	25°C	5
2nd Step	42°C	60
3rd Step	70°C	15
Hold	10°C	∞

The cDNA obtained was stored at -20 °C.

3.3.3 RealTime PCR

Quantitative reverse transcription polymerase chain reaction (qPCR) was performed using the TaqMan Gene Expression Master Mix by Applied Biosystems and probes by IDT.

We prepared a reaction for each probe by mixing 10 µl of Master Mix with 1 µl of the probe and 4 µl of nuclease-free H_2O. In case of multiplex, the volume of the housekeeping probe was subtracted from water volume. Every sample was repeated twice at least. Plates were designed with Bio-Rad CFX Manager Software and PCR was performed on CFX96 Touch System (Bio-Rad) platform.

Data are presented as relative normalized expression, using one or two housekeeping genes. The used probes follows, all of them were from IDT:

Hs.PT.58.3568944	ICOS	6-FAM/ZEN/IBFQ
Hs.PT.58.19821900	CTLA4	6-FAM/ZEN/IBFQ
Hs.PT.58.849873	HELIOS	6-FAM/ZEN/IBFQ
Hs.PT.58.40542617	GITR	6-FAM/ZEN/IBFQ
Hs.PT.58.1099131	ISG54	6-FAM/ZEN/IBFQ
Hs.PT.58.19416416	TRAIL	6-FAM/ZEN/IBFQ
Hs.PT.58.15498666.g	IKBA	6-FAM/ZEN/IBFQ
Hs.PT.58.4381999	IL1RA	6-FAM/ZEN/IBFQ
Hs.PT.39a.22214847	ACTB	HEX/ZEN/IBFQ
Hs.PT.39a.22214836	GAPDH	Cy5/IBRQ

4. Results

4.1 IBD Patients

In the context of the project "Thalidomide, a novel immunological treatment to modify the natural history of paediatric Crohn's disease: a new proposal from a well-established paediatric research network" there have been enrolled 40 patients in two years, from different centers in Italy.

All of them presented signs and symptoms of chronic gastrointestinal inflammation, and did not have a clear diagnosis.

Thirty of these patients have had an onset before six years of age, thus they have been enrolled in the WP-2 project. The aim of WP-2 is to investigate the genetic and immunological basis of very early-onset IBDs, with molecular biology and next generation sequencing (NGS) techniques.

For patients with adult or adolescent onset, we have chosen to perform clinical exome sequencing, in order to identify risk factors related to IBDs, while for VEO-IBD patients we have preferred whole exome sequencing.

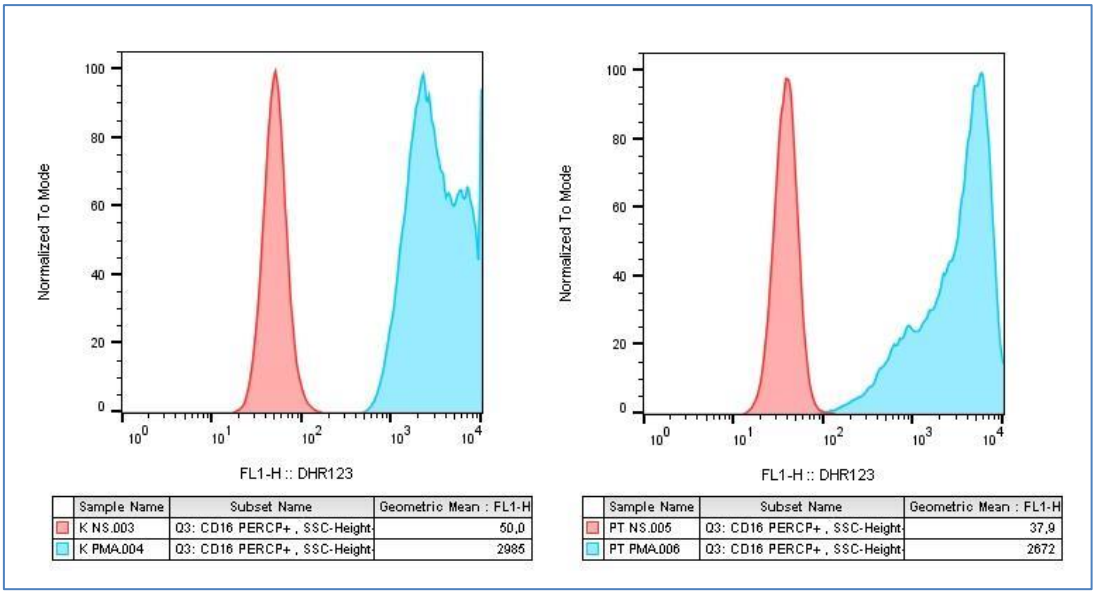
4.1.1 DHR123 Assay

This test can be used to identify defects in the oxidative burst of neutrophils, as in Chronic Granulomatous Disease (CGD). Up to 50% of CGD patients present with diarrhea, abdominal pain, and failure to thrive, usually before the age of 5; since differential diagnosis of CGD includes Crohn's disease, it is fundamental to correctly identify these patients, in order to give them the appropriate treatment.

Fresh blood samples were collected from all patients, then stimulated with PMA (phorbol 12-myristate 13-acetate) for 15 minutes and stained with the addition of DHR123.

During the analysis with FlowJo software, granulocytes were gated by forward and side scatter parameters (physical gate), than only $CD16^{+}$ cells were selected (immunological gate) and analyzed for their fluorescence in the green channel.

DHR123 test was applied to all the patients enrolled in this study, and all of them resulted to be negative for it, as illustrated in exemplificative figure 4.1:



	Sample Name	Subset Name	Geometric Mean : FL1-H
	K NS.003	Q3: CD16 PERCP+ , SSC-Height-	50,0
	K PMA.004	Q3: CD16 PERCP+ , SSC-Height-	2985

	Sample Name	Subset Name	Geometric Mean : FL1-H
	PT NS.005	Q3: CD16 PERCP+ , SSC-Height-	37,9
	PT PMA.006	Q3: CD16 PERCP+ , SSC-Height-	2672

Figure 4.1: Representation of DHR123 test results. In the histograms, red lines shows fluorescent intensity in unstimulated patients, while light blue lines stand for PMA-stimulated samples. The activation of granulocytes following the addition of PMA lead to release of reactive oxygen species (ROS) and the consequent oxidation of DHR123 in rhodamine-123 that emits fluorescence in the green channel. The clear increase of fluorescence indicates that patient (left graph) does not have a deficiency in neutrophil oxidative burst.

4.1.2 Clinical Exome Sequencing

While very early onset IBD patients were analyzed through whole exome sequencing, patients with an onset after 6 years of age were sequenced by the use of Clinical Exome Solution (SOPHiA GENETICS). This CES kit is designed to provide a comprehensive coverage of all types of genomic variants in nearly 4500 disease-related genes. Furthermore, SOPHiA DDM software is a useful tool to prioritize the variants due to their probable pathogenicity.

We have already performed clinical exome sequencing on 7 out of 9 non-VEOIBD recruited patients; the output was a large table that reported every variant with a series of related information, as the population frequency, the inheritance of the eventual OMIM inheritance, the prediction scores of Polyphen2 and HumDiv. For every single patient, we had a mean of 130 variants as row data.

In order to filter and prioritize this amount of data, we have chosen to set some cutoffs:

- population frequency <0.05
- variants with homozygous inheritance that were heterozygous in our patients were not considered

In addition, variants found to be on UTR regions were considered, but only if reported to be potentially pathogenic. The filtered variants were then ordered according to their predictive software scores and their predicted clinical consequences.

In the following image there are represented the amounts of variants in common between two or more patients analyzed. All these variants have already been reported in literature as risk factors; in particular, the circled numbers refer to the underlined variants reported on the right side of the picture.

These are the most interesting risk factors, in common between at least 3 patients: the involved genes are all important for various immune system functions.

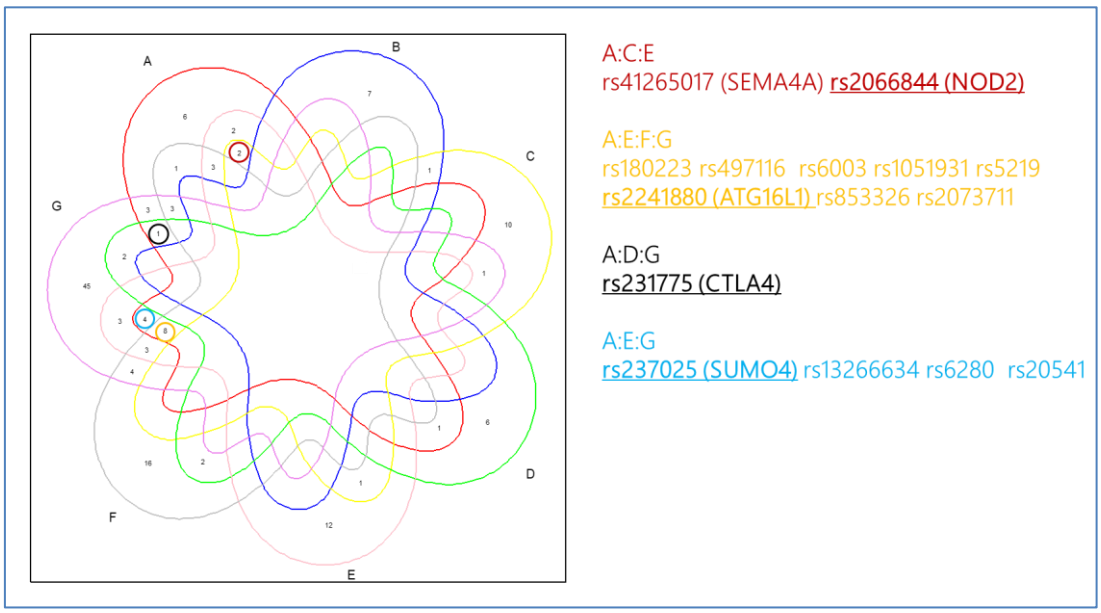


Figure 4.2: Venn diagram of variants found with CES analysis. Data from clinical exome sequencing were analyzed through SOPHiA DDM software and R software. Numbers in the diagram stand for the amount of variant in common between patients. Patients are identified with capital letters from A to G.

The first one is a variant in NOD2 gene (Nucleotide-binding oligomerization domain-containing protein 2), which encodes a protein member of the Nod1/Apaf-1 family; it is characterized by two caspase recruitment (CARD) domains and six leucine-rich repeats (LRRs). NOD2 protein is primarily expressed in the peripheral blood leukocytes, and plays a role in the immune response to intracellular bacterial lipopolysaccharides (LPS) by recognizing the muramyl dipeptide (MDP) derived from them and activating the NF-κB protein.

The variants reported in figure 4.3 are the most common NOD2 mutations found in CD patients; this gene has been extensively associated with an increased risk to develop Crohn's disease[147–149], in particular individuals carrying two of these mutated NOD2 alleles have a 20–40–fold increased risk[149,150].

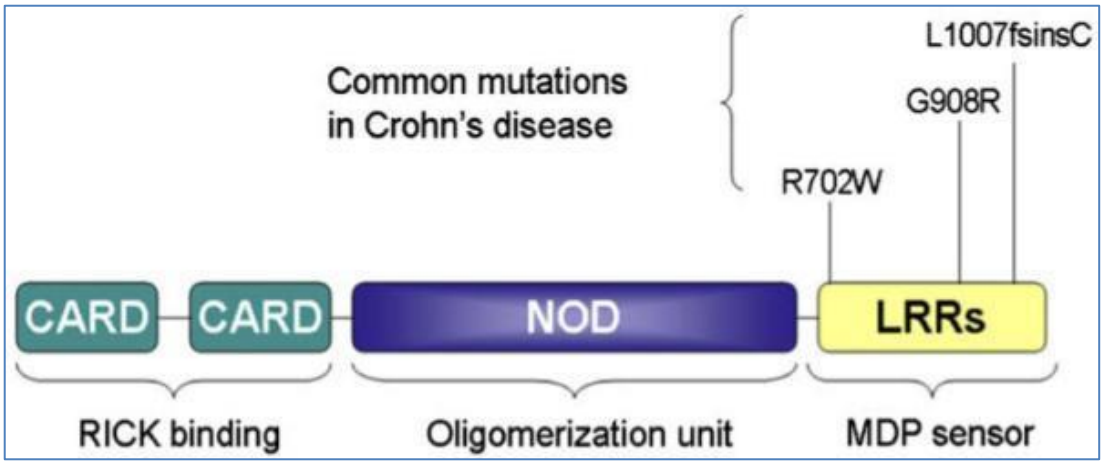


Figure 4.3: NOD2 protein domains and positions of the most common variants found in CD patients. (Yamamoto S, Ma X. Role of Nod2 in the development of Crohn's disease. *Microbes Infect*. 2009;11(12):912-918.)

Another gene that has already been associated with CD is ATG16L1 (Autophagy related 16 like 1): is a homolog of ATG16, and together with AT5 and ATG12 is required to form autophagosomes. Moreover, it is known to interact with NOD2 in an autophagy-dependent antibacterial pathway[151]. In particular, we found the variant rs2241880 in four out of seven patients; this variants lead to the substitution of a threonine with an alanine in position 300. This specific variant is reported in various papers, and is a recognized risk factor for CD, since causes abnormalities in Paneth cells, decreased selective autophagy, increased cytokine release, and decreased intracellular bacterial clearance[151–155]. The molecular mechanism seems to be an augmented sensitivity of the mutated ATG16L1 protein to caspase3 cleavage, as reported by Murthy A. et al[156] (figure 4.4):

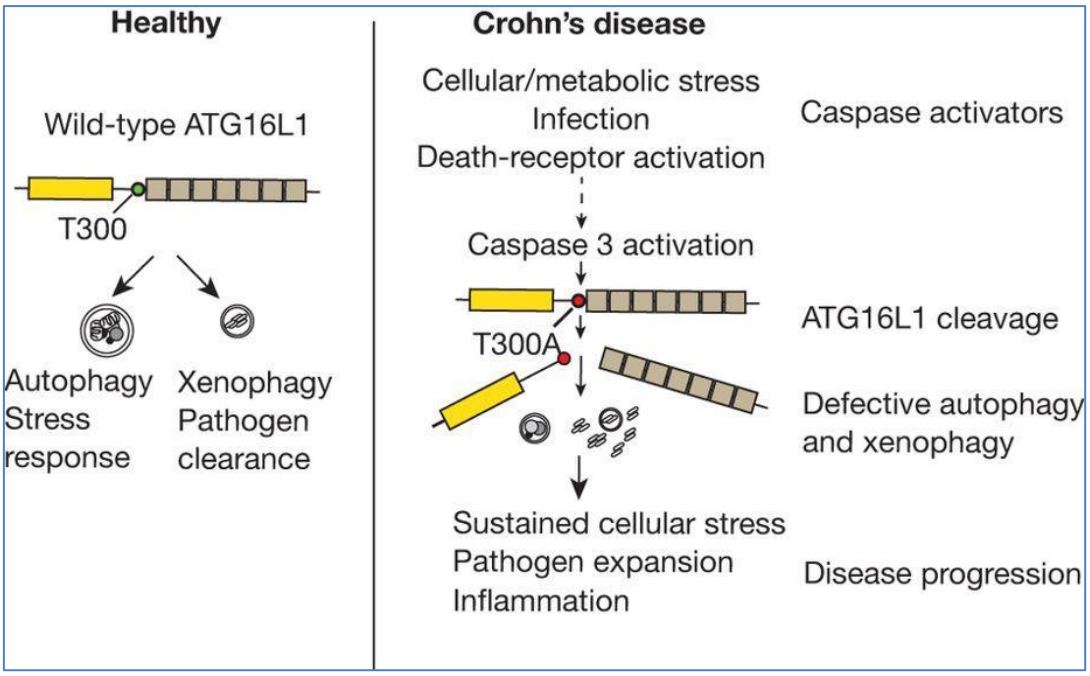


Figure 4.4: An inflamed intestinal environment induces cellular stress and caspase activation, thereby enhancing caspase-3-mediated cleavage of the T300A variant of ATG16L1. This results in defective stress-induced autophagy and bacterial clearance by xenophagy, establishing a chronic inflammatory state. Image adapted from Murthy A, Li Y, Peng I, et al. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature*. 2014;506(7489):456-462.

We also found a recurrent variant (three out of seven patients) in SUMO4 (Small Ubiquitin Like Modifier 4) gene; this gene is a member of SUMO family, that encodes small ubiquitin-related modifiers that are attached to proteins, controlling the target proteins[147] subcellular localization, stability, or activity. SUMO4 is located in the cytoplasm and specifically modifies IκBα, leading to negative regulation of NF-κB-dependent transcription of the IL12B gene.
The specific variant found in our patients (rs237025, or M55V) was reported to be associated with type 2 diabetes[156,157], but it seems there is no correlation between the variant and IBDs[158]. Thus, it has been reported that patients with CD have a significantly increased risk of diabetes compared with non-IBD controls[159], albeit this relation is still unclear.

One more recurrent variant we reported was found in CTLA4 (Cytotoxic T-Lymphocyte Associated Protein 4) gene, in three patients. CTLA4 is an immune checkpoint, and its function is fundamental to downregulate immune responses. An association between the variant rs231775 and variants in IBD5 locus was reported in a Hungarian population of patients affected by Crohn's disease[160].
A summary of the variants found in our patients follows in table 4.1.

Patient_ID	Gene_ID	dbSNP	Position	Protein
Patient A	LRRK2	rs33995883	40740686	p.(Asn2081Asp)
Patient A	SEMA4A	rs41265017	156146640	p.(Arg713Gln)
Patient A	GBA	rs76763715	155205634	p.(Asn409Ser)
Patient A	MYO1A	rs33962952	57431402	p.(Gly662Glu)
Patient A	SDHB	rs33927012	17027802	p.(Ser163Pro)
Patient A	NOD2	rs2066844	50745926	p.(Arg675Trp)
Patient A	ATG16L1	rs2241880	234183368	p.(Thr216Ala)
Patient A	TERT	rs35719940	1254594	p.(Ala999Thr)/c.3184G>A (p.Ala1062Thr)
Patient A	CTLA4	rs231775	204732714	p.(Thr17Ala)
Patient A	SUMO4	rs237025	149721690	p.(Val55Met)
Patient A	FFAR4 (GPR120)	rs116454156	95347041	p.(Arg254His)
Patient A	KCNJ11	rs5219	17409572	p.(Lys23Glu)
Patient A	CHRNA5	rs16969968	78882925	p.(Asp398Asn)
Patient A	AURKA	rs2273535	54961541	p.(Phe31Ile)
Patient A	ENPP1	rs1044498	132172368	p.(Lys173Gln)
Patient A	NOS3	rs1799983	150696111	p.(Asp298Glu)
Patient A	TG	rs180223	133900252	p.(Ser734Ala)
Patient A	TG	rs853326	133900252	p.(Ser734Ala)
Patient A	IL13	rs20541	131995964	p.(Gln144Arg)
Patient A	XRCC3	rs861539	104165753	p.(Thr241Met)
Patient A	SLC30A8	rs13266634	118184783	p.(Arg276Trp)
Patient A	GHRL	rs696217	10331457	p.(Leu71Met)
Patient A	CASP12	rs497116	104763117	Non Coding Transcript Variant
Patient A	DRD3	rs6280	113890815	p.(Gly9Ser)
Patient A	F13B	rs6003	197031021	p.(Arg115His)
Patient A	NPSR1	rs324981	34818113	p.(Asn96Ile)
Patient A	HFE	rs1799945	26091179	p.(His63Asp)
Patient A	TGFB1	rs1800470	41858921	p.(Pro10Leu)
Patient B	TGFBI	rs121909217	136062674	p.(Arg666Ser)
Patient B	ND1	rs41460449	3394	p.(Tyr30His)
Patient B	KLKB1	rs3733402	186236880	p.(Ser143Asn)
Patient B	FGFR4	rs351855	177093242	p.(Gly338Arg)
Patient B	GATA4	rs3735819	11748803	Intronic variant
Patient B	GATA4	rs10503425	11748855	Intronic variant
Patient B	GATA4	rs745379	11758186	Intronic variant
Patient B	STOX1	rs10509305	68885620	p.(Glu608Asp)
Patient B	HOGA1	rs185803104	97600168	Intronic variant
Patient B	CST3	rs1064039	23637790	p.(Ala25Thr)
Patient B	C1GALT1C1	rs17261572	120626774	p.(Asp131Glu)
Patient B	CCDC170	rs6929137	151615542	p.(Val604Ile)
Patient B	PAH	rs1522296	102917009	Intronic variant
Patient C	SLC4A1	rs45562031	42338993	p.(Glu40Lys)

Patient C	STK11	rs59912467	1223125	p.(Phe354Leu)
Patient C	OPTN	rs11258194	13152400	p.(Met98Lys)
Patient C	TF	rs1799899	133475812	p.(Gly277Ser)
Patient C	NEBL	rs147622517	21177128	p.(Tyr89*)
Patient C	SEMA4A	rs41265017	156146640	p.(Arg713Gln)
Patient C	SERPINA1	rs17580	94847262	p.(Glu288Val)
Patient C	PRODH	rs2904552	18905964	p.(Arg323His)
Patient C	GCH1	rs41298442	55310817	p.(Lys224Arg)
Patient C	PKD1	rs199476099	2168022	p.(Arg324Leu)
Patient C	NOD2	rs2066844	50745926	p.(Arg675Trp)
Patient C	GHRL	rs34911341	10331519	p.(Arg50Gln)
Patient C	TLR5	rs5744168	223111858	p.(Arg392*)
Patient C	HABP2	rs7080536	115348046	p.(Gly508Glu)
Patient C	GABRD	rs41307846	1959699	p.(Arg220His)
Patient D	SEC23B	rs121918221	18496339	p.(Glu109Lys)
Patient D	MBL2	rs5030737	54531242	p.(Arg52Cys)
Patient D	SOD3	rs1799895	24801834	p.(Arg231Gly)/ R213G
Patient D	NLRP12	rs34971363	54313707	p.(Phe402Leu)
Patient D	TGFB1	rs1800470	41858921	p.(Pro10Leu)
Patient D	NLRP1	rs12150220	5485367	p.(Leu155His)
Patient D	CTLA4	p.(Ile544Leu)	204732714	p.(Thr17Ala)
Patient D	CFH	rs800292	196642233	p.(Val62Ile)
Patient D	IL10RB	rs2834167	34640788	p.(Lys47Glu)
Patient D	PPARA	rs1800206	46614274	p.(Leu162Val)
Patient D	PCSK1	rs6232	95751785	p.(Asn221Asp)
Patient D	FRZB	rs288326	183703336	p.(Arg200Trp)
Patient E	SDHB	rs33927012	17354297	p.(Ser163Pro)
Patient E	SEMA4A	rs41265017	156146640	p.(Arg713Gln)
Patient E	KCNJ1	rs59172778	128709126	p.(Met357Thr)
Patient E	LRP5	rs4988321	68174189	p.(Val86Met)
Patient E	NOD2	rs2066844	50745926	p.(Arg675Trp)
Patient E	TRAF3IP2	rs33980500	111913262	p.(Asp10Asn)
Patient E	IRF5	rs2070197	128589000	3' UTR
Patient E	TG	rs853326	133909974	p.(Met1028Val)
Patient E	TG	rs180223	133900252	p.(Ser734Ala)
Patient E	TG	rs2076740	133984058	p.(Arg1999Trp)
Patient E	CASP12	rs497116	104763117	stop_lost
Patient E	HNF1A	rs1169288	121416650	p.(Ile27Leu)
Patient E	AURKA	rs2273535	54961541	p.(Phe31Ile)
Patient E	LRP8	rs5174	53712727	p.(Arg893Gln)
Patient E	AKAP10	rs203462	19812541	p.(Ile588Val)
Patient E	DRD3	rs6280	113890815	p.(Gly9Ser)
Patient E	PTPN1	rs16989673	49199355	3' UTR
Patient E	A2M	rs669	9232268	p.(Ile1000Val)
Patient E	XRCC3	rs861539	104165753	p.(Thr241Met)
Patient E	TOR1A	rs1801968	132580901	p.(Asp216His)
Patient E	IRS2	rs1805097	110435231	p.(Gly1057Asp)
Patient E	ATG16L1	rs2241880	234183368	p.(Thr216Ala)
Patient E	GABRD	rs41307846	1959699	p.(Arg220His)

Patient E	SUMO4	rs237025	149721690	p.(Val55Met)
Patient E	SLC30A8	rs13266634	118184783	p.(Arg276Trp)
Patient F	TSPAN7	rs104894951	38535032	p.(Pro172His)
Patient F	PRB3	rs71455367	11421038	p.(Arg49Cys)
Patient F	MBL2	rs5030737	54531242	p.(Arg52Cys)
Patient F	WDR36	rs35703638	110441839	p.(Ala449Thr)
Patient F	WNT10A	rs121908120	219755011	p.(Phe228Ile)
Patient F	CLEC7A	rs16910526	10271087	p.(Tyr192*)
Patient F	SLC6A20	rs17279437	45814094	p.(Thr199Met)
Patient F	EPOR	rs62638745	11488727	p.(Asn487Ser)
Patient F	ATRX	rs45439799	76856021	p.(Asn1860Ser)
Patient F	ELAC2	rs5030739	12899902	p.(Ala501Thr)
Patient F	FRZB	rs288326	183703336	p.(Arg200Trp)
Patient F	FRZB	rs7775	183699584	p.(Arg324Gly)
Patient F	CASP12	rs497116	104763117	stop_lost
Patient F	SIAE	rs78778622	124530664	p.(Met54Val)
Patient F	AURKA	rs2273535	54961541	p.(Phe31Ile)
Patient F	PLA2G7	rs1051931	46672943	p.(Val379Ala)
Patient F	COL11A1	rs1676486	103354138	p.(Ser1496Pro)
Patient F	TG	rs853326	133909974	p.(Met1028Val)
Patient F	TG	rs180223	133900252	p.(Ser734Ala)
Patient F	ATG16L1	rs2241880	234183368	p.(Thr216Ala)
Patient F	STOX1	rs1341667	70641860	p.(Tyr153His)
Patient F	PRNP	rs1799990	4680251	p.(Met129Val)
Patient F	F7	rs6046	113773159	p.(Arg413Gln)
Patient F	F13B	rs6003	197031021	p.(Arg115His)
Patient F	VEGFA	rs2010963	43738350	5'UTR
Patient F	IRS2	rs1805097	110435231	p.(Gly1057Asp)
Patient F	CHRNA5	rs16969968	78882925	p.(Asp398Asn)
Patient F	AGT	rs699	230845794	p.(Met268Thr)
Patient F	TLR1	rs4833095	38799710	p.(Asn248Ser)
Patient F	NPSR1	rs324981	34818113	p.(Asn96Ile)
Patient F	NOS3	rs1799983	150696111	p.(Asp298Glu)
Patient F	XRCC3	rs861539	104165753	p.(Thr241Met)
Patient F	HNMT	rs11558538	138759649	p.(Thr105Ile)
Patient F	IRS1	rs1801278	227660544	p.(Gly971Arg)
Patient F	PON1	rs662	94937446	p.(Gln192Arg)
Patient F	OAS1	rs1131454	113348870	p.(Gly162Ser)
Patient F	KCNJ11	rs5219	17409572	p.(Lys23Glu)
Patient F	DNASE1	rs1053874	3707747	p.(Arg244Gln)
Patient F	CCR5	rs333	46414943	p.(Ser185Ilefs*32)
Patient F	CILP	rs2073711	65494212	p.(Ile395Thr)
Patient G	ROR2	rs41277835	91723689	p.(Asp935Glu)
Patient G	G6PC3	rs113416399	44076045	3' UTR
Patient G	DKC1	rs878854453	154776814-154776836	indel
Patient G	ABCA4	rs1801581	94047009	p.(Arg943Leu)
Patient G	MYO1A	rs33962952	57037618	p.(Gly662Glu)
Patient G	AMPD1	rs17602729	114693436	Stop gained

Patient G	SARS2	rs34050897	38930489	p.(Ser83Leu)
Patient G	FANCF	rs61752920	22625438	p.(Asp125Asn)
Patient G	CPN1	rs61751507	100069757	p.(Gly178Asp)
Patient G	SLX4	rs114472821	3590714	p.(Pro975Leu)
Patient G	APOB	rs1801695	21001981	p.(Ala4481Pro)
Patient G	ALG6	rs35383149	63406361	p.(Tyr131His)
Patient G	DUOX2	rs530719719	45101228-45101233	p.(Phe966fs)
Patient G	CYP21A2	rs7755898	32040421	p.(Gln319Ter)
Patient G	IRS2	rs1805097	109782884	p.(Gly1057Ala)
Patient G	F13B	rs6003	197061891	p.(Arg115His)
Patient G	TLR5	rs2072493	223111257	p.(Asn592Ile)
Patient G	PRNP	rs1799990	4699605	p.(Met129Val)
Patient G	VEGFA	rs2010963	43770613	5' UTR
Patient G	APOA5	rs3135506	116791691	p.(Ser19Leu)
Patient G	TYR	rs1126809	89284793	p.(Arg402Gln)
Patient G	IL13	rs20541	132660272	p.(Gln79Arg)
Patient G	NPHS2	rs61747728	179557079	p.(Arg229Gln)
Patient G	CHRNA5	rs16969968	78590583	p.(Asp398Asn)
Patient G	ATG16L1	rs2241880	234183368	p.(Thr216Ala)
Patient G	DRD3	rs6280	113890815	p.(Gly9Ser)
Patient G	GHR	rs6180	42719137	
Patient G	SLC30A8	rs13266634	118184783	p.(Arg276Trp)
Patient G	PLA2G7	rs1051931	46672943	p.(Val379Ala)
Patient G	COL11A1	rs1676486	103354138	p.(Ser1496Pro)
Patient G	SUMO4	rs237025	149721690	p.(Val55Met)
Patient G	CTLA4	rs231775	204732714	p.(Thr17Ala)

Table 4.1: Summary of the rare variants found in our patients with potential clinical significance.

4.1.3 Whole Exome Sequencing

Patients with an onset before 6 years of age have been investigated through whole exome sequencing (WES) technology. In our cohort of patients, a total of 30 on 40 were VEO-IBD. Here we report WES results for the first 3 patients analyzed.

Patient VEO-1 was a 4 years old male with a history of eczema, recurrent vomiting and dyspnea episodes from birth. He was diagnosed with Poland syndrome II (underdeveloped chest muscle and short webbed fingers on one side of the body), and presented frequently respiratory distress, and had food allergies. Then he has developed an eosinophilic gastroenteritis (histological examination), with diarrhea and anemia.

Genetic analysis with WES did not reveal any mutation causative of IBD. Nevertheless, we found a heterozygous variant in gene GLI2 (rs114814747), encoding a transcription factor

with zinc-finger motifs. GLI2 affects ventroposterior mesodermal development, and various mutations have been reported, comprising our patient's one, to cause a broad and variable range of clinical manifestations, including polydactyly, abnormalities involving eye, orbits, nose, first branchial arch, and a variable degree of gyral development[161,162]. This variant could have had a role in the development of Poland syndrome of our patient, since he presented hand abnormalities as GLI2 mutated patients.
Moreover, our patient was found to be carrier of a mutation (rs35152987) on HBD gene, encoding the subunit delta of hemoglobin; homozygous variants in this gene can lead to develop delta thalassemia[163].

Patient VEO-2 was an 8 years old girl, which presented bloody diarrhea, anal-rectal ulceration and aphthae at colonic level; furthermore, she had recurrent infective episodes.
At WES genetic analysis we found a heterozygous mutation in TGFB1 gene (rs121909217), reported in ClinVar as a pathogenic variant causing corneal epithelial dystrophy. TGFB1 has been recently associated with severe early onset IBD and central nervous system disease[164]; though the variant found in our patient is not reported to be IBD causing, we can hypothesize it can be a variant which presence increases the risk for IBD, together with other genetic and environmental triggers.

Patient VEO-3 was a 5 years old female, which at the age of 3 started having daily evacuation of malformed feces mixed with blood. At the endoscopic examination, she showed signs of inflammation in all GI tracts (pancolitis).
Through NGS exome sequencing, we have noticed a heterozygous variant in FCN3 gene (rs532781899). The variants is a single nucleotide deletion that leads to frameshift and consequent premature stop codon; the mutation is located in the C-terminal globular fibrinogen-like domain, which function is important for PAMP recognition on pathogens[165], while N-terminal domain function is responsible for signaling and to induce an immune response. Ficolin3, also known as Ficolin-H, is mainly expressed and secreted by hepatocytes, but is also highly expressed in type II alveolar and bronchial epithelial cells [166].

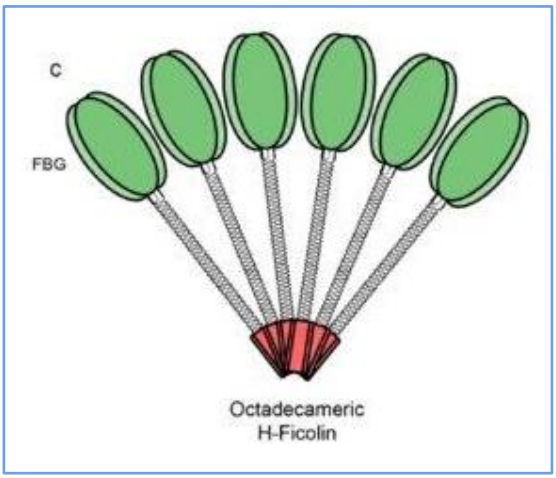


Figure 4.5: Schematic structure of Ficolin3 protein. The monomers associate to form an octadecameric structure. Image adapted from Mason CP, Tarr AW. Human lectins and their roles in viral infections. *Molecules*. 2015;20(2):2229–2271. Published 2015 Jan 29. doi:10.3390/molecules20022229

FCN3 deficiency has been described in one patient as a complement deficiency with recurrent infections[167], but in that case the mutation was homozygous. Nonetheless, in that report the heterozygous parents were found to have a 50% decrease of Ficolin3 serum level; we can speculate that in our patient may be a factor, which increases the risk of developing infections and maybe IBD.

We have found also a variants in genes GJB2 and GRIP1, but they did not have relevance in our patient clinical history: the first variant is reported to be related to deafness[168], and the second is associated with brain morphology abnormalities.

4.2 IL-10R mutated patient

Patient 1 (P1) is a 1-year-old boy of Moroccan origin, born from blood-related parents. At two weeks of age, he presented with a series of signs and symptoms suggestive of an inflammatory disease, concentrated in particular the gastrointestinal tract. In particular, he had gingivostomatitis, Rotavirus enteritis and neonatal pustular dermatitis and feeding difficulties. At the first level laboratory exams, he showed a slight increase in eosinophils population and IgE, while other values were normal. In the following period, he developed also recurrent diarrhea, vomiting and consequent failure to thrive.

At subsequent controls with endoscopic examination, he was found to have perianal disease with anal fistula and ulcerative proctosigmoiditis. He also had one episode of pneumonia and recurrent upper respiratory tract infection.

The clinical response to treatment with antibiotics and a short course of steroids was good.

Due to the clinical presentation and the consanguinity of patient's parents, a panel of genes have been analyzed through NGS technology; the panel included genes related to SCID, CID plus IL-10, IL-10R, XIAP, LAD1. He was found to have a homozygous mutation in IL-10RA, never reported in literature: NM_001558:cT569C:p.F190S. The mutation is a single nucleotide variant located on exon 5 and provokes an amino acid change: the wild-type form in position 190 has a phenylalanine, while the mutated form has a serine.

The mutation was confirmed through Sanger sequencing.

To assess the effects of this novel variant, we first consulted different *in silico* pathogenicity prediction tools, in order to evaluate the effect of amino acid substitutions on the structure or function of the protein before conducting functional studies.

One of the easier approaches to have an estimate of possible consequences deriving from the amino acid substitution is to consult the Grantham matrix[169] (Figure 4.6): in this scheme, a number from 0.0 to 215 is given for every possible combination of substitution. Grantham's distance between two amino acids depends on three properties: composition, polarity and molecular volume. Increasing values express increasing chemical dissimilarity: conservative (0-50), moderately conservative (51-100), moderately radical (101-150), or radical (≥151).

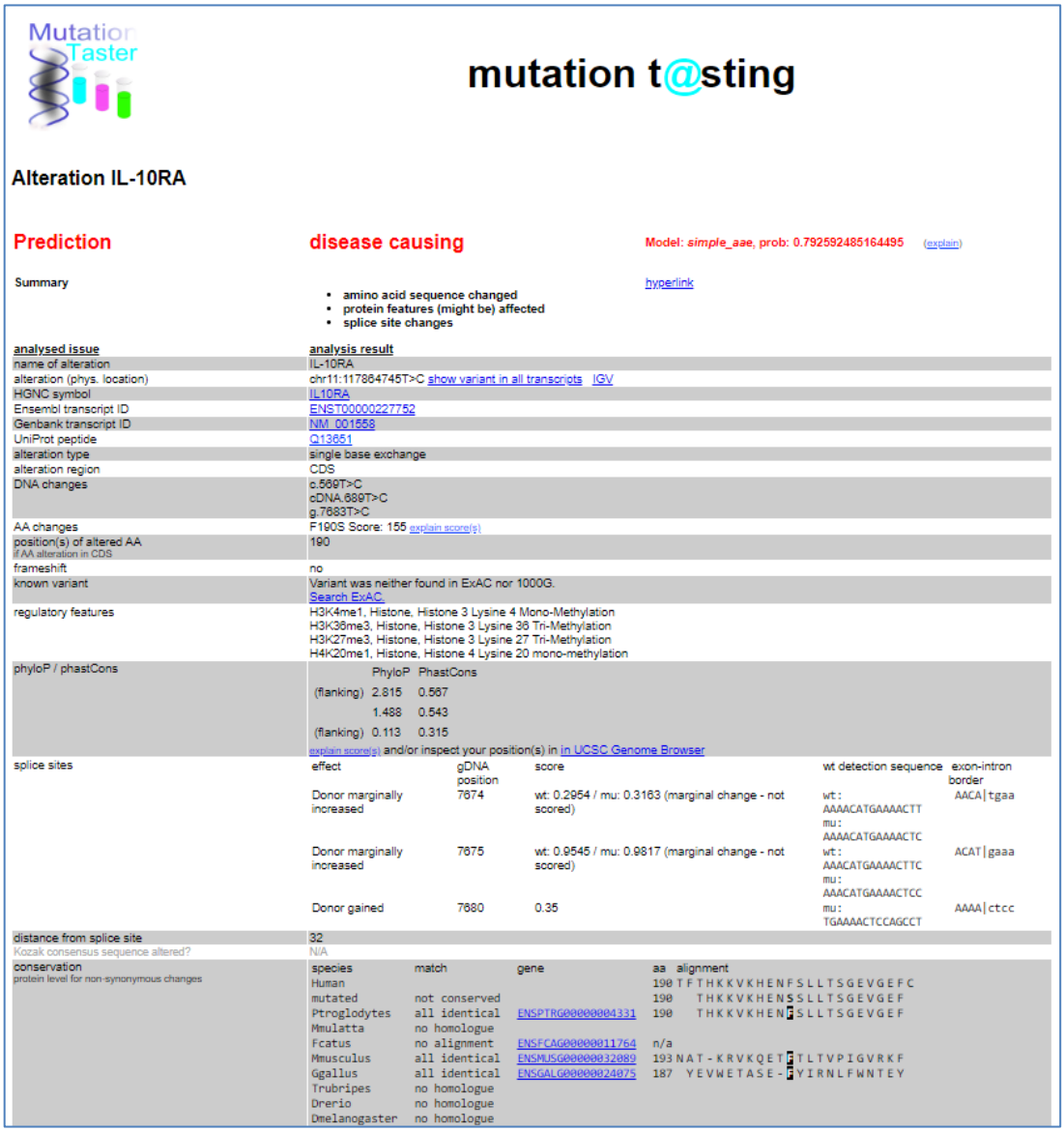
The phenylalanine to serine substitution found in our patient has a score of 155.

Arg	Leu	Pro	Thr	Ala	Val	Gly	Ile	Phe	Tyr	Cys	His	Gln	Asn	Lys	Asp	Glu	Met	Trp	
110	145	74	58	99	124	56	142	155	144	112	89	68	46	121	65	80	135	177	Ser
	102	103	71	112	96	125	97	97	77	180	29	43	86	26	96	54	91	101	Arg
		98	92	96	32	138	5	22	36	198	99	113	153	107	172	138	15	61	Leu
			38	27	68	42	95	114	110	169	77	76	91	103	108	93	87	147	Pro
				58	69	59	89	103	92	149	47	42	65	78	85	65	81	128	Thr
					64	60	94	113	112	195	86	91	111	106	126	107	84	148	Ala
						109	29	50	55	192	84	96	133	97	152	121	21	88	Val
							135	153	147	159	98	87	80	127	94	98	127	184	Gly
								21	33	198	94	109	149	102	168	134	10	61	Ile
									22	205	100	116	158	102	177	140	28	40	Phe
										194	83	99	143	85	160	122	36	37	Tyr
											174	154	139	202	154	170	196	215	Cys
												24	68	32	81	40	87	115	His
													46	53	61	29	101	130	Gln
														94	23	42	142	174	Asn
															101	56	95	110	Lys
																45	160	181	Asp
																	126	152	Glu
																		67	Met

Table 2. Difference *D* for each amino acid pair (*10*). The mean chemical distance from the three-property formula (see text) $\bar{D}_{epp} = 100$ (D_{ij} values have been multiplied by 50.723 to make this mean possible). Linear regression of *RSF* and log *RSF* on these *D* values gives correlation coefficients of −.66 and −.72, respectively. Previous difference indexes give correlation coefficients against *RSF* of −.34 (minimum base changes), −.42 (Sneath difference), and −.49 (Epstein formula). In each case, correlation is between the two sets (difference and *RSF*) of 190 values (*3*, *4*, *7*).

Figure 4.6: Grantham matrix. (Grantham R. Amino Acid Difference Formula to Help Explain Protein Evolution. *Science* (80-). 1974;185(4154):862-864.)

There are many online prediction tools, in particular we focused on MutationTaster, PROVEAN, SIFT and Polyphen2. The first one is an online free tool that gives a prediction of the effects of various mutation, including deletions and insertions. Variants are rated by Bayes classifier to eventually predict the disease potential of an alteration. The output given by this tool is reported in figure below (Figure 4.7).



mutation t@sting

Alteration IL-10RA

Prediction **disease causing** Model: *simple_aae*, prob: 0.792592485164495 (explain)

Summary hyperlink

- **amino acid sequence changed**
- **protein features (might be) affected**
- **splice site changes**

analysed issue	analysis result
name of alteration	IL-10RA
alteration (phys. location)	chr11:117864745T>C show variant in all transcripts IGV
HGNC symbol	IL10RA
Ensembl transcript ID	ENST00000227752
Genbank transcript ID	NM_001558
UniProt peptide	Q13651
alteration type	single base exchange
alteration region	CDS
DNA changes	c.569T>C cDNA.689T>C g.7683T>C
AA changes	F190S Score: 155 explain score(s)
position(s) of altered AA if AA alteration in CDS	190
frameshift	no
known variant	Variant was neither found in ExAC nor 1000G. Search ExAC
regulatory features	H3K4me1, Histone, Histone 3 Lysine 4 Mono-Methylation H3K36me3, Histone, Histone 3 Lysine 36 Tri-Methylation H3K27me3, Histone, Histone 3 Lysine 27 Tri-Methylation H4K20me1, Histone, Histone 4 Lysine 20 mono-methylation
phyloP / phastCons	PhyloP PhastCons (flanking) 2.815 0.567 1.488 0.543 (flanking) 0.113 0.315 explain score(s) and/or inspect your position(s) in in UCSC Genome Browser

splice sites	effect	gDNA position	score	wt detection sequence	exon-intron border
	Donor marginally increased	7674	wt: 0.2954 / mu: 0.3163 (marginal change - not scored)	wt: AAAACATGAAAACTT mu: AAAACATGAAAACTC	AACA|tgaa
	Donor marginally increased	7675	wt: 0.9545 / mu: 0.9817 (marginal change - not scored)	wt: AAACATGAAAACTTC mu: AAACATGAAAACTCC	ACAT|gaaa
	Donor gained	7680	0.35	mu: TGAAAACTCCAGCCT	AAAA|ctcc

distance from splice site	32
Kozak consensus sequence altered?	N/A

conservation (protein level for non-synonymous changes)

species	match	gene	aa	alignment
Human			190	T F T H K K V K H E N F S L L T S G E V G E F C
mutated	not conserved		190	T H K K V K H E N S S L L T S G E V G E F
Ptroglodytes	all identical	ENSPTRG00000004331	190	T H K K V K H E N F S L L T S G E V G E F
Mmulatta	no homologue			
Fcatus	no alignment	ENSFCAG00000011764	n/a	
Mmusculus	all identical	ENSMUSG00000032089	193	N A T - K R V K Q E T F T L T V P I G V R K F
Ggallus	all identical	ENSGALG00000024075	187	Y E V W E T A S E - F Y I R N L F W N T E Y
Trubripes	no homologue			
Drerio	no homologue			
Dmelanogaster	no homologue			

Figure 4.7: Mutation Tester tool output. The page displays information related to the mutation, as position at cDNA and genomic level, eventual information from ExAC and 1000G databases, conservation of the protein across different species.

In figure 4.8 is reported the output from tool PROVEAN by J. Craig Venter Institute. The variant is predicted to be deleterious by this software and damaging by SIFT.

VARIATION		PROTEIN SEQUENCE CHANGE							
ROW_NO.	INPUT	PROTEIN_ID	LENGHT	STRAND	CODON_CHANGE	POS	RESIDUE_REF	RESIDUE_ALT	TYPE
1	11,117864745,T,C	ENSP00000227752	578	1	AAC T[T/C]C AGC	190	F	S	Single AA Change

PROVEAN PREDICTION				SIFT PREDICTION				ANNOTATION	
SCORE	PREDICTION (cutoff=-2.5)	#SEQ	#CLUSTER	SCORE	PREDICTION (cutoff=0.05)	MEDIAN_INFO	#SEQ	dbSNP_ID	GENE_ID
-3.59	Deleterious	102	30	0.000	Damaging	2.92	40		ENSG00000110324

Figure 4.8: PROVEAN tool output. The novel mutation found in our patient is predicted to be damaging even with this prediction tool.

Also the Polyphen2 tool classified the variant as probably damaging, with a score of 1.00, as showed in the following image.

Figure 4.9: PolyPhen2 output.

4.2.1 Functional Assays: STAT3 Phosphorylation

In order to evaluate the molecular consequences of this unreported mutation, we decided to perform a functional phosphorylation test. Whole blood samples from this patient were stained with anti-CD14/FITC antibody and then stimulated with increasing doses of IL-10, in particular 1, 10, 50, 100 ng/ml. Cells were stimulated for 12 minutes at 37°C, then reaction was stopped by adding Lyse/Fix Buffer; cell were permeabilized with PB III before intracellular staining with anti-P-STAT3/PE antibody. In the analysis with FlowJo10, cells were gated on physical parameters forward scatter and side scatter, to eliminate debris and dead cells, than we selected only CD14/FITC-positive cells.

As represented in figure 4.10, the healthy donor shows an augmented rate of phospho-STAT3 in response to IL-10, except for the point with the maximum dose, where saturation point is reached.

In contrast, our patient do not demonstrate any response, either at high dosage. The P-STAT3 mean fluorescence levels is the same in unstimulated and in stimulated samples.

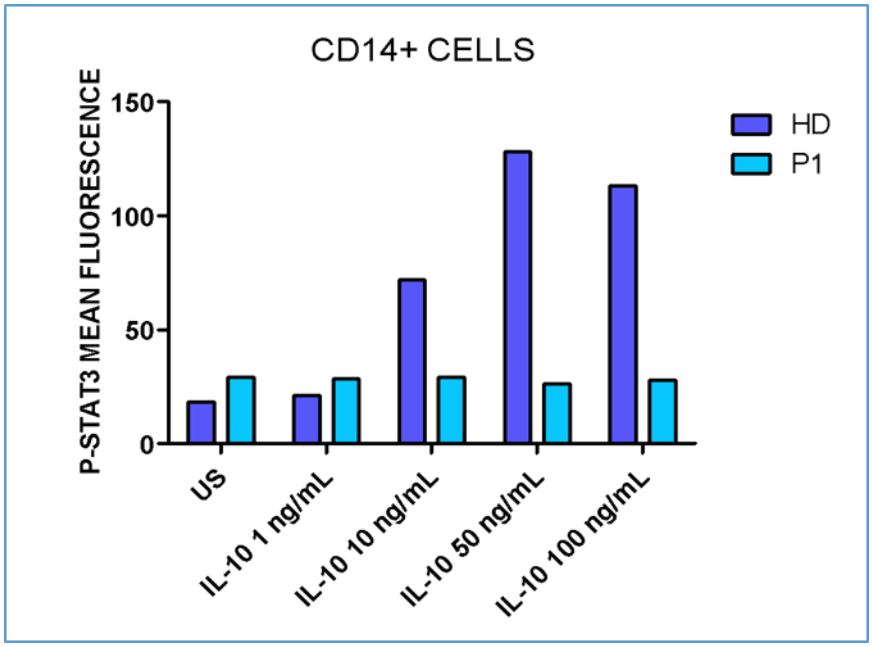


Figure 4.10: IL-10 dose-response curve. The bar diagram represents mean fluorescence values of p-STAT3 in healthy donor and P1, with increasing doses of IL-10. While HD samples shows an augment of STAT3 phosphorylation in response to stimuli, P1 does not have any activation.

4.2.2 Functional Assays: Th17 subpopulation

IL-10 and its signaling pathway is fundamental to maintain the correct imbalance between immune response and its suppression; in particular, IL-10 production and signaling constitute a negative feedback mechanism to damp uncontrolled production of inflammatory cytokines in both innate and adaptive systems, thereby contrasting the development of Th17 cells and excessive inflammation.

Shouval et al. reported an increased rate of Th17 cells in IL-10/IL-10R deficient patients, as a result of the lack of inflammatory response downregulation[144].

Due to the total lack of response to IL-10 as phosphorylation of STAT3 in our patient, we decided to analyze the population of Th17 cells through flow cytometry. $CD4^+$ selected cells

from both patient and healthy donor were seeded in the presence of anti-CD3 and anti-CD28 antibodies; to stimulate cytokine production we added phorbol 12-myristate 13-acetate, together with brefeldin A and ionomycin, the day before the analysis.

Then we proceeded with the intracellular staining to verify the amount of IL-17A produced by the cells.

In the following figure, it is possible to notice the increased percentage of IL-17-producing cells in P1:

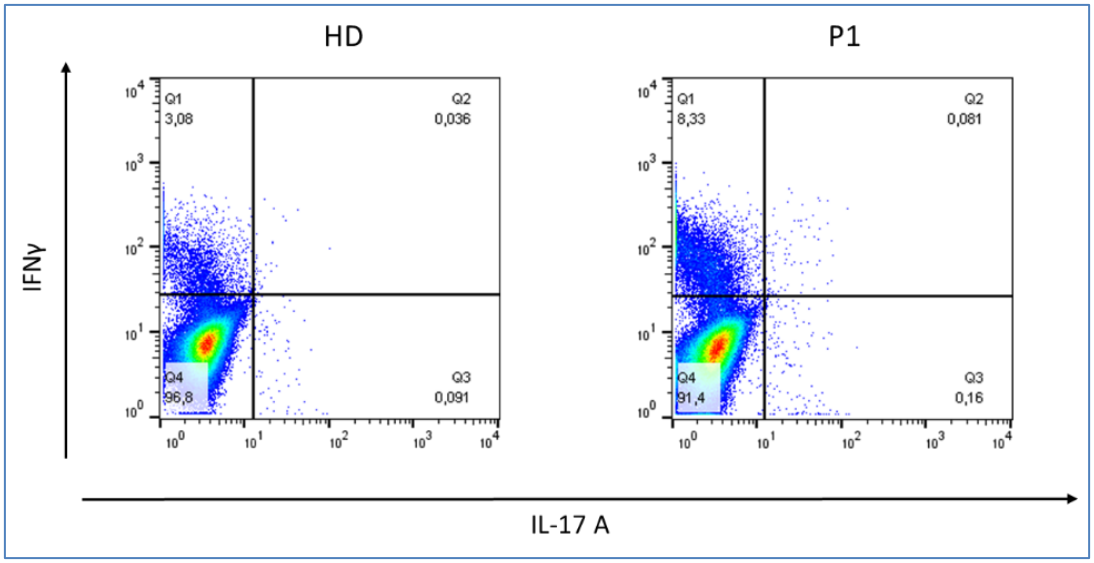


Figure 4.11: IL-17A and IFNγ staining on anti-CD3/anti-CD28 stimulated $CD4^+$ cells. $CD4^+$ cells were selected from PBMC with immunomagnetic beads, seeded anti-CD3 coated plates and added with anti-CD28. The day before the analysis ionomycin, brefeldin A and PMA were added to the culture to induce cytokine production but not exocytosis. IL-17-producing T cells in P1 were slightly increased compared to HD.

4.2.3 Functional Assays: Treg cell subpopulations

IL-10 is reported to be necessary to maintain FOXP3 expression and immune suppressive function in colitis murine models[143]; however, it is still unclear if this non-redundant role is the same in humans. In order to evaluate the presence and the effective amount of T regulatory cells in our patient, we performed a flow cytometry assay using the True-Nuclear™ Human Treg Flow™ Kit (Biolegend). Cells were stained with an anti-CD4/anti-CD25 cocktail for surface markers, and then permeabilized to allow intracellular staining with anti-FOXP3 antibody.

During the analysis with FlowJo 10 software, $CD4^+$ lymphocytes were gated and plotted for their CD25 and FOXP3 expression levels. As shown in the following figure, P1 had a slightly decreased amount of CD25 FOXP3 double positive cells. We can argue that IL-10RA signaling is not fundamental for this subset development.

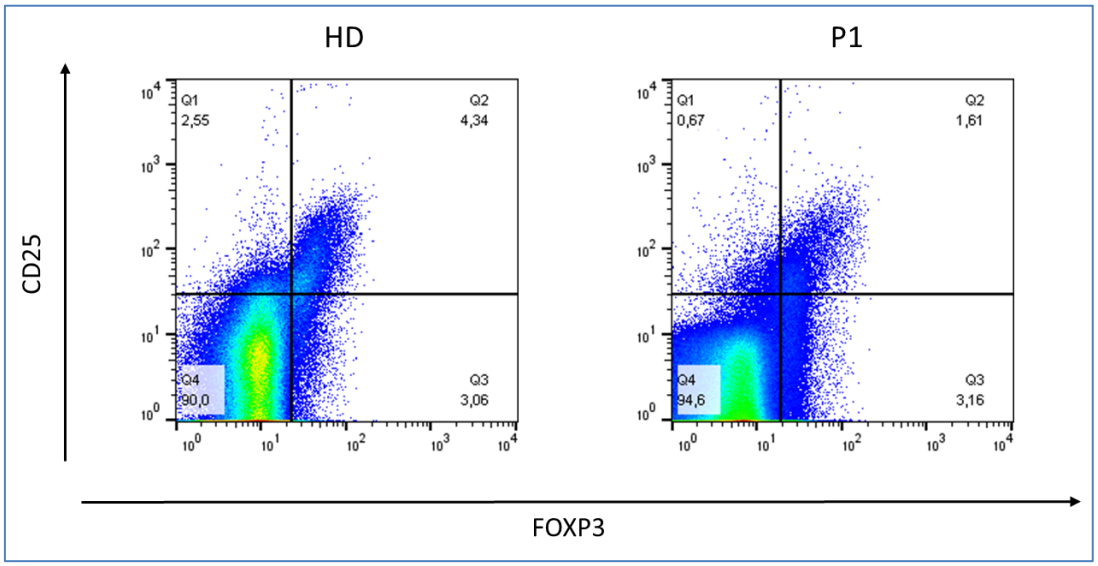
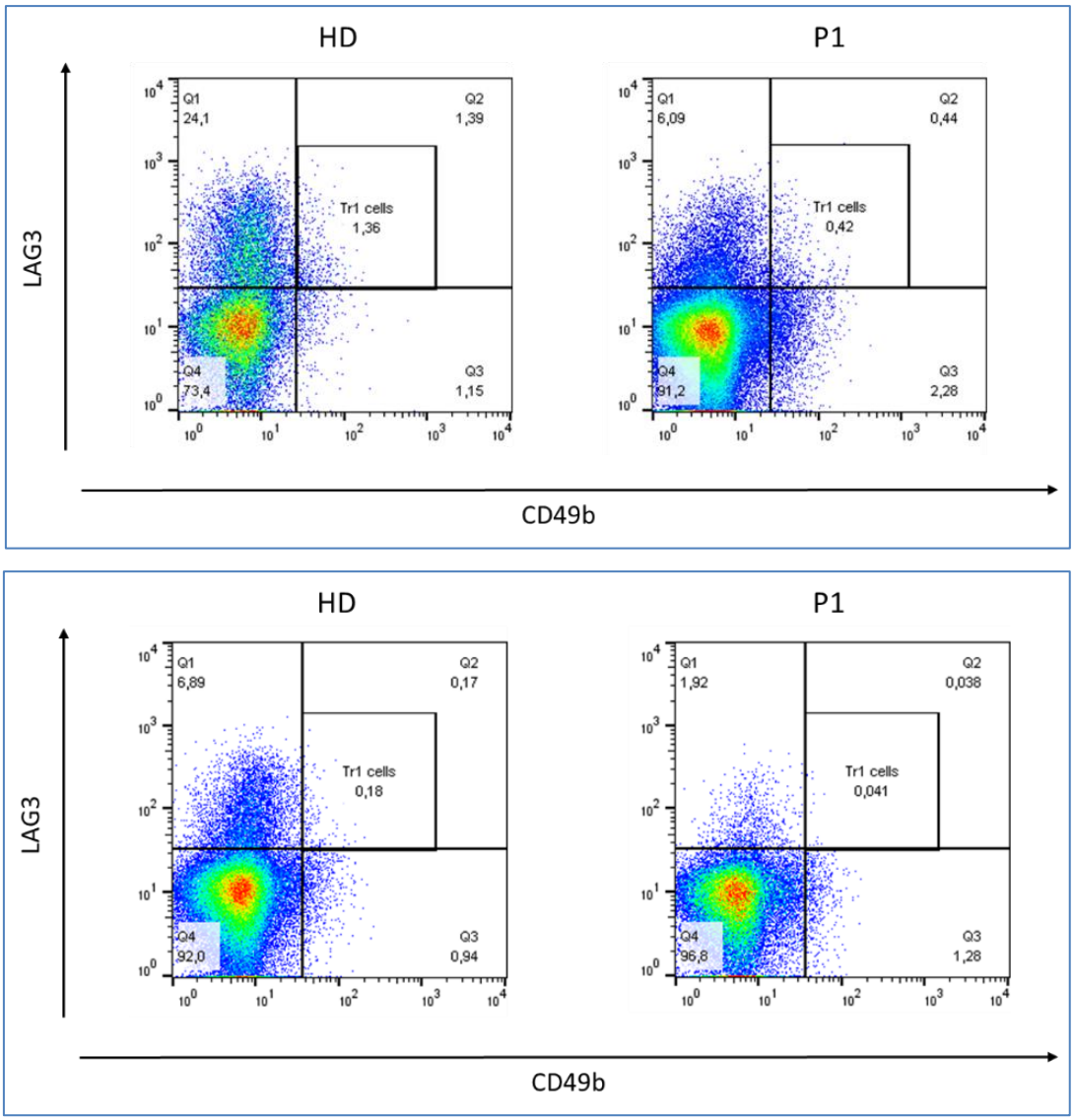


Figure 4.12: $FOXP3^+CD25^+$ T cells. Lymphocytes were selected in the analysis by physical parameters, then $CD4^+$ cells were gated and plotted for their fluorescence on CD25 and FOXP3 channels. Conventional Treg are CD25 FOXP3 double positive cells.

A well-characterized Treg subpopulation is represented by Tr1 cells, responsible for the largest production of IL-10 within T cells. Brockmann et al. reported that in mice the IL-10 function is not necessary for Tr1 cells development, while it is required for this subset correct functionality, analyzed in terms of IL-10 production[134].

Tr1 cells are currently identified as $CD49b^+LAG3^+$ T cells[128]; to identify this population, we collected whole blood samples from both healthy donor and P1, and incubated them for 24 hours in T cells stimulating condition (addition of anti-CD3, anti-CD28, IL-2). The following day, we performed staining with anti-CD3, anti-CD4, anti-CD49b, anti-LAG3 antibodies.

In the analysis, CD3/CD4 double positive lymphocytes were selected and plotted for their expression of CD49b and LAG3. Here we reported two different experiments on the same patients, at two weeks of distance. In both the experiments P1 showed a mild decrease in Tr1 gated cells, but more interestingly, a clear reduction of LAG3 production itself.



Figures 4.13 and 4.14: Tr1 cells subpopulation and LAG3 expression. Whole blood samples from both HD and P1 stimulated for 24h with anti-CD28, anti-CD3 and IL-2 were stained for CD3, CD4, LAG3 and CD49b. Lymphocytes were selected in the analysis by physical parameters, then $CD3^{+}CD4^{+}$ cells were selected and plotted to verify LAG3 and CD49b expression levels.

A lineage of FOXP3+ Treg lineage can stably express a transcription factor usually considered to be Th17-specific, the retinoic acid-related orphan receptor-γt (RORγt)[170]. B-H Yang et al. demonstrated that this population of regulatory cells have an augmented suppressive capacity compared to $FOXP3^{+}$ $ROR\gamma t^{-}$ lymphocytes in a mouse model of colitis. Due to this peculiar capability and to the intestinal localization of $FOXP3^{+}$ $ROR\gamma t^{+}$ cells, we decided to perform a double intracellular staining to possibly identify and quantitate them in the peripheral blood of our patient.

The kit was the same used to analyze conventional Treg, with the addition of anti-RORγt antibody. PBMC were gradient-isolated, then stained with anti-CD4 and anti-CD25 antibodies for surface staining; after permeabilization, cells were marked with anti-FOXP3 and anti-RORγt antibodies for intracellular staining.

To evaluate FOXP3$^+$ RORγt$^+$ cells, in the analysis we have selected CD4$^+$ lymphocytes then gated cells FOXP3 CD25 double positive; on this population RORγt fluorescence was plotted against forward scatter (FSC). We designed a gate specific for the individual, based on the unstained sample. The result is shown in the following figure:

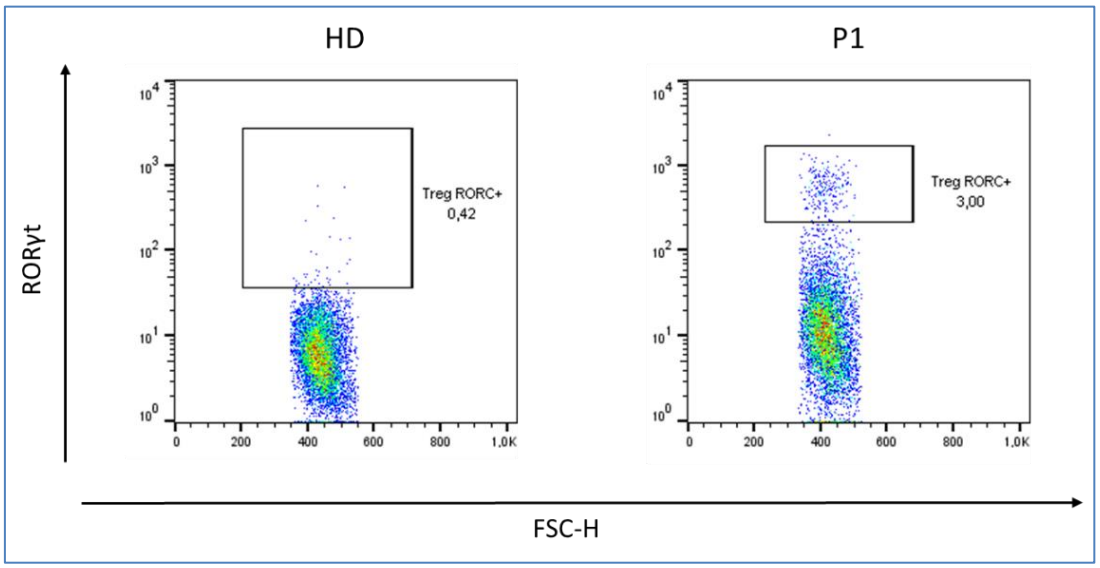


Figure 4.15: FOXP3$^+$RORγt$^+$ cells. Lymphocytes were selected in the analysis by physical parameters, then CD4$^+$ cells were selected and plotted to verify CD25 and FOXP3 expression levels. On this population we evaluated RORγt$^+$ cells.

Despite the difference between basal signals of HD and P1 on RORγt fluorophore channel (represented by the difference in the gate design), there is an evident increase of Treg FOXP3$^+$ RORγt$^+$ population in our patient.

4.2.4 Functional Assays: RealTime PCR

In order to evaluate the expression of different genes related both to Th17 cells and Treg cells, we decided to perform a quantitative RealTime PCR. PBMC from P1 and a healthy donor were treated to isolate CD4$^+$ lymphocytes, and then cells were seeded in presence of anti-CD3, anti-CD28 antibodies plus IL-2. After 5 days of stimulus, cells were collected and RNA

extracted using the RNeasy kit by Qiagen, according to the manufacturer's instructions. At least 100 ng of RNA were retrotranscribed and cDNA used for the Real Time PCR. Probes marked with different fluorophores were used simultaneously in a multiplex experiment; GAPDH and ACTB were used as housekeeping genes, and we made an experimental triplicate of each sample.
Data are presented as relative expression, normalized of housekeeping genes expression and relative to the healthy donor.

We collected patient's blood samples twice:

- at time 1 the patient was in good health conditions, with no signs of perianal disease, regular growth
- at time 2 he presented a recrudescence of his pathology, with fever, diarrhea and perianal disease

The bar graph shows the values for 11 genes: some of them are characteristic, but not exclusive, of T regulatory population (CD25, FOXP3, Helios, GITR, CTLA4, ICOS), and the others are more related to T helper cells (IL1-RA, RORγt, IL-23R, IL-17A).

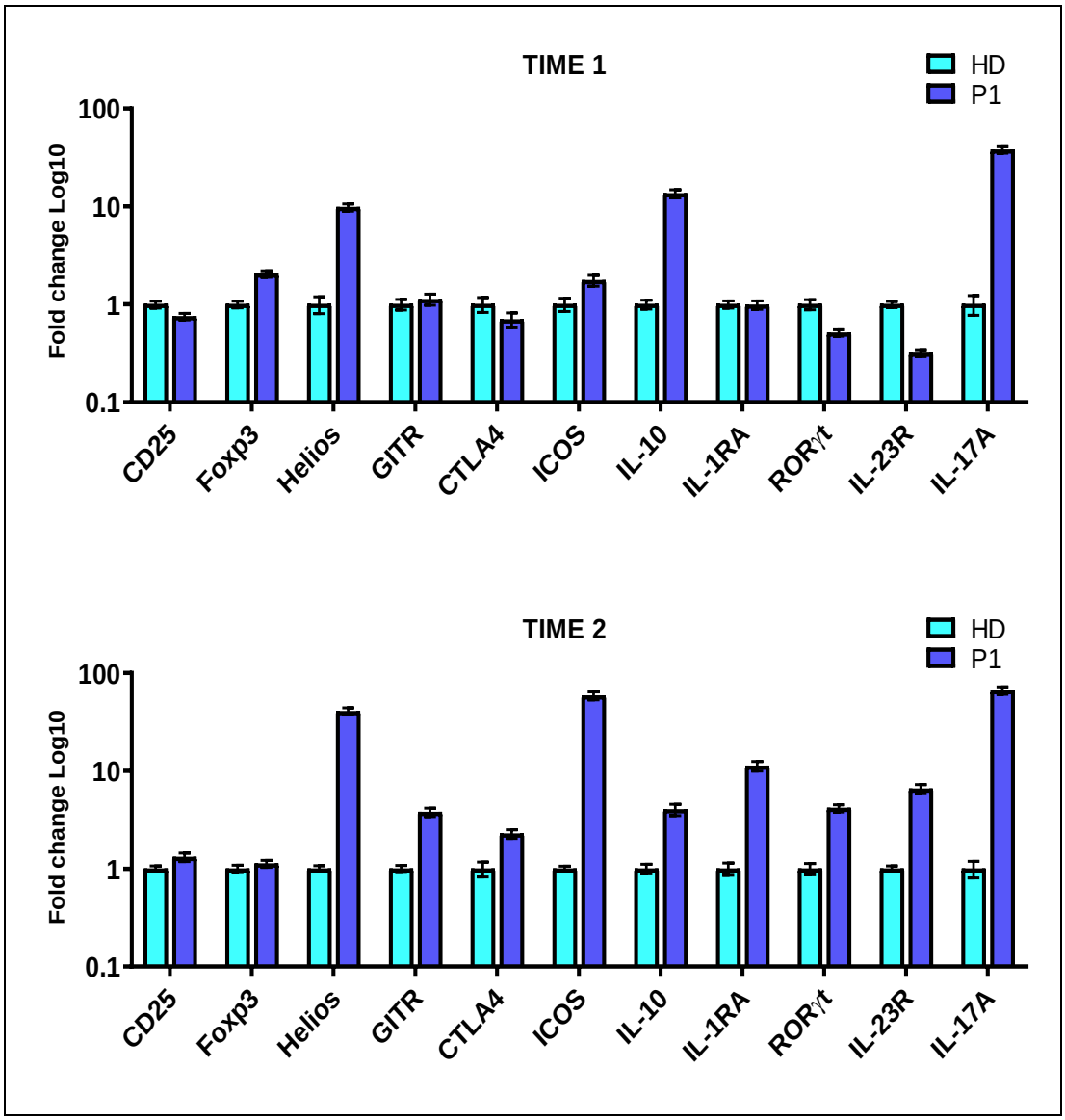


Figure 4.16: RealTime PCR. Eleven genes related to both Th17 and Treg were evaluated for their expression at mRNA level, after 5 days of stimulation in T cell-activating conditions (anti-CD3, anti-CD28, IL-2).

As illustrated in the graph, at time 1 the main differences are in IL-17A, IL-10, and Helios, which expressions are augmented in P1 compared to HD. Moreover, at time 2 P1 presented an evident increase in ICOS, IL-1RA, IL-23R and RORγt.

5. Discussion

Crohn's disease (CD) and ulcerative colitis (UC) are the two major pathologies belonging to the group of inflammatory bowel diseases (IBDs). Typical symptoms of IBDs are abdominal pain and bloody diarrhea.

Understanding the basis of these diseases is of growing importance, due to their augmented incidence in the last decades.

Most factors have been thought to have an important role in IBD development: smoking, infection, drugs, stress, air and water pollution, diet, and food additives have been investigated[1–3].

Next to environmental factors, there have been reported an increasing number of genetic variants which presence predisposes to IBDs.

In our study we have enrolled till now 40 patients: 30 were VEO-IBD patients, thus with onset before six years of age, and 10 were adult or adolescent onset patients. All of them have been tested in order to exclude CGD.

Chronic granulomatous disease (CGD) is an inherited immunodeficiency disorder involving the innate immune system that impairs the neutrophil oxidative burst. The basis of this defect is a loss of nicotinamide adenine dinucleotide phosphate-oxidase (NOX) function in phagocytic cells, which diminishes their ability to produce reactive oxygen species (ROS), with consequent impaired microbial killing[171]. The gastrointestinal tract is the most frequently affected organ in CGD patients, as more than 80% of patients have inflammatory GI disorders[172].

As a first step, we wanted to exclude in our patients chronic granulomatous disease, which clinical presentation can overlap with that of IBDs. With this aim, we performed DHR123 test on all patients to evaluate the oxidative burst activity of neutrophils. Cells were stained with DHR123 and anti-CD16 to identify neutrophils. None of our patients showed a defective response to PMA stimulation, excluding the diagnosis of CGD.

We wanted to evaluate the genetic predisposition weight in our cohort of patients.

It is well known that genetic susceptibility plays an important role in the development of IBDs, and large genome-wide association studies have identified more than 230 risk alleles[173,174]. Many of the genes involved are important in host-microbiome interactions.

Especially with regard to non-VEO patients, the variants found are risk factors that predispose to develop the pathology together with environmental factors, rather than causing variants. For such a group of patients in our cohort, we have chosen to perform clinical exome

sequencing, an NGS technique that allows to enrich specific exome regions in nearly 4500 disease-related genes.

7 out of 10 non-VEO patients have already been analyzed, and our findings highlighted that most patients had shared variants that have been previously reported as risk factors. In particular, the most common variants were found in genes NOD2, ATG16L1, CTLA4 and SUMO4.

All of these genes have been linked to IBD susceptibility.

NOD2 is known to increase risk of CD, and have an important role in the regulation of microbiota in the small intestine through the secretion of antibacterial compounds. In particular, the missense variant rs2066844 found in 3/7 patients have been associated with CD[175,176] and other inflammatory syndromes as Blau syndrome and Yao syndrome [ClinVar accession VCV000004693.2].

ATG16L1 gene encodes a protein involved in autophagy processes, fundamental for bacterial clearance; dysfunctional autophagy is recognized as a contributing factor in many chronic inflammatory diseases, including IBD. The variant rs2241880 is reported to be the most common disease-associate in ATG16L1 gene[177]; we found this variant in 4 out of 7 patients.

CTLA4 is a homologue of the co-stimulatory protein CD28, but with an opposite role: it is an immune checkpoint which function is to downregulate T cell immune responses. Dysregulated expression of CTLA-4 leads to many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and type 1 diabetes. The variant rs231775 have been associated with CD[178] and other autoimmune diseases susceptibility[179]. We have detected this variant in three of our patients.

SUMO4 is an IκBα modifier, thus leads to negative regulation of NF-κB signaling pathway. Increased levels of NF-κB have been reported in IBD patients[180], and its pathway is thought to have an important role in the development of gut inflammation and colorectal cancer[181]. The specific polymorphism found in our patients (3/7) in this SUMO gene leads to the M55V substitution, that has been associated with type I diabetes[182,183]. It is still unclear whether there is a relationship between diabetes and IBDs, but it has been reported that patients with CD have a significantly increased risk of diabetes compared with non-IBD controls, regardless of steroid use[159]. One explanation for the relationship between the two pathologies is that IBD is associated with diabetes in terms of chronic inflammation and dysbiosis.

For VEO-IBDs, that are more likely to have a monogenic disease and it is more feasible to do gene discovery, we have chosen to perform whole exome sequencing (WES). Variants have

been filtered for frequency <0.05 and for reported clinical relevance as first approach. We have reported the results of the analysis of the first 3 patients.

Patient VEO-1 was a 4 years old male a diagnosis of Poland syndrome II that has developed an eosinophilic gastroenteritis with diarrhea and anemia. Our genetic analysis highlighted two variants, even if unrelated to IBD: the first one in GLI2 gene, that has an important function in ventroposterior mesodermal development[161]. Mutation in GLI2 can cause cranio-facial abnormalities and polydactyly with a broad spectrum of phenotypes; since our patient presented hand abnormalities as GLI2 mutated patients, we could argue that his variant have had a role in the development of Poland syndrome.

Patient VEO-2, a 10 year-old girl, carried a heterozygous mutation in TGFB1 gene, reported in ClinVar as a pathogenic variant causing corneal epithelial dystrophy. TGFB1 has been recently associated with severe early onset IBD and central nervous system disease[164]; moreover, mutational inactivation of the TGF-beta pathway can be found in a large proportion of patients affected by colorectal cancer[184]. Even though the variant found in our patient is not reported to be IBD causing, we can hypothesize it can be a variant which presence increases the risk for IBD, together with other genetic and environmental triggers.

Patient VEO-3 was a 5 years old female, which was found to have a heterozygous variant in FCN3 gene. The related protein, Ficolin3, has an important function in PAMP recognition and subsequent complement system activation. FCN3 deficiency has been described as a complement deficiency with recurrent infections in one patient carrying the same mutation, but homozygous[165]. In our patient this variant, even if not causative itself, can be considered a risk factor.

Another IBD patient (P1) was brought to our attention: he was born from consanguineous parents, and since birth had presented gastrointestinal inflammation signs and symptoms. Genetic analysis highlighted a homozygous mutation on IL-10RA gene.
The importance of IL-10 signaling pathway has been extensively demonstrated[101,103], as it is fundamental to restrain immune responses during inflammation processes. This cytokine has a broad spectrum of activity, since its action is directed both to innate immunity cells, as dendritic cells and monocytes, and adaptive immunity. Despite the number of studies in IL-10 signaling field, some aspects of its functions are still unclear: for instance, it is known that IL-10 plays an essential role in Treg cells biology, allowing them to suppress excessive Th1

and Th17 responses[140]. In the gut and lung mucosae a particular subset of regulatory T cells, Tr1, are known to be a source of IL-10[126,127].

The non-redundant role of IL-10 in immune system homeostasis is proved by the phenotype of IL-10 signaling-deficient patients: these children usually develop severe enterocolitis, perianal abscesses, and chronic folliculitis[61,119] within their first year of life. The pathology is often non responsive to common IBD treatment, and bone marrow transplantation is to date the preferred approach[111–113,116,117,185].

P1 mutation (NM_001558:cT569C:p.F190S) was not reported in literature, so we used different *in silico* pathogenicity prediction tools to understand if the mutation can really be causative. We investigated the variant with Polyphen2, MutationTaster, PROVEAN, SIFT software: all the predictions were consistent with a disease-causing mutation.

To assess the *in silico* predictions, we performed a STAT3 phosphorylation assay in flow cytometry on PMBC from patient's whole blood: CD14 stained cells showed a null response even to the highest doses of IL-10, confirming the functional defect.

Another reported defect in patients with IL-10 or IL-10R deficiency is the increased rate of Th17[144];we performed an intracellular staining on $CD4^+$ activated cells, and confirmed the presence of Th17 augment in P1. Also IL-17 transcription was largely augmented in patient's stimulated $CD4^+$ cells, even when his health conditions were good.

Despite Th17 increase in these patients, T regulatory cells are not reported to have a numeric variation[144]; we first analyzed $CD4^+CD25^+FOXP3^+$ cells from patients and noticed that he presented a slight reduction of this population. Moreover, we wanted to identify and quantify specifically IL-10 producing Treg, namely Tr1.

This subset is commonly selected with LAG3 and CD49b surface markers; so we performed a flow cytometry assay on PMBC left for 24 hours under T cells stimulating condition (anti-CD3, anti-CD28).

We gated $CD4^+CD25^+FOXP3^+LAG3^+CD49b^+$ cells and found that the percentage of Tr1 in P1 was reduced of almost two thirds, both when patient was in good health conditions and when he had active inflammation.

IL-10 is known to have an important role driving Tr1 differentiation[128,132]. This crucial role of IL-10 for Tr1 cells function strongly supports the hypothesis that patients with IL-10/IL-10R deficiency may have a reduction or reduced functionality, and that these abnormalities can contribute to the disease phenotype[133].

Moreover, we wanted to evaluate in our patient another Treg population, expressing both FOXP3 and RORγt; this population is reported to be stable and have unique functional properties, that allows them to efficiently suppress gut-specific inflammatory responses[170].
Due to the impossibility to work on an intestinal biopsy, we have selected $CD4^+CD25^+FOXP3^+ROR\gamma t^+$ cells from total PBMC, and surprisingly we found that our patient presented an increased percentage of this population.
These results suggest that IL-10 is not necessary for Treg $FOXP3^+$ and Tr1 cells development, and that the chronic inflammation is not dependent on a lack of these cells, but is more likely due to the inability to sense the produced IL-10. Again, $FOXP3^+ROR\gamma t^+$ subset seems not to be sufficient to control colitis progress in this patient.
Both Treg and Tr1 population have never been investigated in IL-10/IL-10R deficient patients[133]. We can hypothesize that increased Tr1 number is an attempt to recover an imbalance between pro- and anti-inflammatory responses.
These findings suggest that regulatory T cells may have an important role in the onset of intestinal inflammation, but further studies are necessary to better understand the etiopathogenesis of immunodeficiency and IBD caused by IL-10R mutations.

In summary, this study has highlighted the importance of genetic predisposition in inflammatory bowel diseases, even if they are not sufficient to cause the pathology by themselves. Also environmental factors have a fundamental contribution to the etiology of the chronic inflammation.
In some cases, a mutation can be addressed as unique cause of the disease, as in the case of IL-10/IL-10R deficiency. Here we report a novel homozygous variant in IL-10RA gene in a patient with VEO-IBD; through phosphorylation assays we confirmed the functional defect. Furthermore, we investigate Th17, Treg, Tr1 and $FOXP3^+ROR\gamma t^+$ subsets, verifying some differences with healthy controls; these findings can help us to better understand how the chronic intestinal inflammation arises and maintains, albeit further investigations are required.

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