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

Background and aims

Crohn's disease is an incurable chronic disease, with very high costs both for the individual and the society, both in terms of quality of life and economic, in particular, for pediatric patients. The project aimed to identify a new strategy for long-term treatment, in order to modify the natural history of the disease thus reducing complications, hospitalization, the use of surgery and the need for other invasive treatments. Recent top-down approaches using immunosuppressive therapies as first line therapy have shown promising results. It has been hypothesized that thalidomide may have the ability to slow disease progression when used as an early therapy. In order to verify this hypothesis, a randomized controlled study was planned to compare thalidomide with the “standard” treatment.
The project had set three objectives, the achievement of which was entrusted to the 3 Participating Operational Units: the Institute for Maternal and Child Health IRCCS Burlo Garofolo, the Civil Hospitals of Brescia and the Oncology Reference Center of Aviano.
The first objective was to evaluate the efficacy and safety of thalidomide against "standard" therapies in bringing about a change in the natural history of Crohn's disease. The second objective concerned the evaluation of the immunological mechanisms of Crohn's disease and of therapy with thalidomide. Finally, the third objective concerned the evaluation of the pharmacokinetics, metabolomics and pharmacogenomics of thalidomide and their impact on the efficacy and safety of the drug.
The study was conceived as a unitary project at the top of which was the Maternal and Child Health IRCCS Burlo Garofolo as coordinator of the clinical study.
The assessments necessary to achieve objectives 2 and 3 were thought of as dependent on the enrollment of patients in the clinical study.
Following the presentation of the protocol of the clinical study to the Italian Medicines Agency (AIFA), some changes to the original project were requested. Those changes have limited:

- The possibility of comparing the efficacy and safety of thalidomide against "standard" therapies defined as all drugs available in the therapeutic armamentarium of Crohn's disease. Comparison was limited to thalidomide versus infliximab in patients with disease onset and the presence of negative prognostic factors.
- The request to limit enrollment to patients with negative prognostic factors (identified in accordance with the international ESPGHAN / ECCO guidelines of 2014) at the onset of the disease, resulted in a significant restriction in the population potentially eligible for the study. Patients with negative prognostic factors at the onset of the disease who in clinical practice are deserving of aggressive therapy (top down) from the beginning, represent only about 20-30% of the total. It follows that this limitation led to the

exclusion of about 70-80% of the patients who had been foreseen in the study design and in the calculation of the sample size.

- The age of enrollment for patients over 6 years of age to allow the comparison between the off-label investigational drug and an in-label comparator drug, infliximab (off-label under 6 years of age) thus excluding patients with onset at the age of less than 6 years representing 10% of cases.
- In order to evaluate the immunological mechanisms of Crohn's disease, the possibility of collecting biological samples from patients with onset at the age of less than 6 years has been maintained regardless of the therapy. In fact, it is thought that immunological disorders with a defined monogenic basis can globally justify 10% of cases of CD, and up to 21% of cases occurring under the age of 6 years.

Applied methodology

1. Work package 1

Randomisation lists were produced for each participating center in collaboration with the Complex Research Structure of Epidemiology and Statistics of the IRCCS Burlo Garofolo.
Epidata databases have been prepared for the collection of clinical, laboratoristic and pharmacoeconomic data.
The experimental drug was distributed and kits containing the material for the collection of biological samples and the relative instructions were prepared and sent.
Patients eligible for the study, after obtaining informed parental consent, were enrolled in the study and randomized to therapy with thalidomide or the standard reference drug.
Clinical and laboratoristic data of the enrolled patients were collected as required by the protocol.

2. Work package 2

DNA, RNA and mononuclear cells were isolated from the collected biological samples to perform immunological, genetic and histological studies as indicated in the protocol.
All enrolled subjects were tested with DHR123. This test is used to identify defects in the oxidative burst of neutrophils, such as in chronic granulomatous disease (CGD). Up to 50% of CGD patients present with diarrhea, abdominal pain, and failure to thrive, usually before age 5; Since the differential diagnosis of CGD includes Crohn's disease, it is imperative to correctly identify these patients in order to provide them with adequate 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, the granulocytes were controlled by frontal and lateral dispersion parameters (physical gate). Only CD16 + cells were selected (immunological gate) and analyzed for their fluorescence in the green channel. The DHR123 test was applied to all patients enrolled in this study and all but one patient identified in Genoa tested negative.

Exome sequencing: Patients with very early onset inflammatory bowel disease were analyzed by whole exome sequencing, patients with onset after 6 years were sequenced by clinical exome (SOPHiA GENETICS). This kit is designed to provide complete coverage of all types of genomic variants in almost 4500 disease-related genes. In addition, the SOPHiA DDM software is a useful tool to prioritize variants due to their probable pathogenicity.

3. Work package 3
Preliminary pharmacokinetic, pharmacodynamic and drug genomic analyzes were performed.

Overall results obtained and of the individual operating units:
D.1. Overall Results

At March 31 2019, 7 patients had been enrolled in the randomized parallel-arm clinical trial (3 in the thalidomide arm, 4 in the infliximab arm) compared to the 87 patients expected within 18 months of starting enrollment.
As noted in the Interim Report, the delay in enrollment was due to several factors:

- the long approval times by the ethics committees of the participating Centers and the signing of contracts between the various centers.
- the restriction of the inclusion criteria in the clinical trial with respect to what was initially foreseen. This restriction, as required by AIFA, concerned both the age of the patients and the characteristics of the disease: the clinical study was in fact limited to patients over 6 years of age and to patients with disease onset with negative prognostic factors.
- the possible reduction in the incidence of Crohn's disease as also reported in the international literature. In fact, a recent systematic review showed that in 16 (72.7%) of 22 epidemiological studies concerning Crohn's disease, a decrease in the incidence of the disease was observed in the period between 1990 and 2016 in the European and North American regions (Lancet 2018; 390: 2769-2778). Referring to the Italian situation, an evaluation of the national register of pediatric inflammatory bowel diseases of the Italian Society of Gastroenterology, Hepatology and Pediatric Nutrition (SIGENP), which collects data from the pediatric gastroenterology centers of the national territory, highlighted the inclusion of 63 new patients with Crohn's disease in the 2018. Taking into account that only about 20% of these diagnoses may have had a severe disease phenotype with a negative prognosis, the patients meeting the study inclusion criteria could have been 12. Estimating that the participating centers in the study covered 50% of the new diagnosis in the Italian territory, the number of cases that could be enrolled in the study could have corresponded to 6. During the drafting phase of the project (September 2014) it was instead planned to be able to enroll, on the basis of previous experience and the number of new diagnoses indicated different centers involved, on average about 8/10 cases per center per year.

- from the start of European studies to which some of the participating centers had adhered and which had an eligible population overlapping with the present study (ie: REDUCE-RISKinCD-PIBD-TRIAL; coordinator: F. Ruemmele, Paris).

In order to increase the sample size and allow the completion of the clinical trial and also of the Work Packages related to immunological, genetic and pharmacological investigations, it was proposed to modify the inclusion criteria in the clinical trial not limiting the inclusion to naive patients but expanding the possibility of enrollment also to patients refractory to conventional first-line therapies. In particular, to pediatric patients with Crohn's disease with inflammatory phenotype and without stenotic or fistulising complications (therefore definable in the "early disease" phase), already treated with induction therapy with exclusive enteral nutrition or steroids and immunosuppressive maintenance therapy with azathioprine or methotrexate. These are the patients to whom, based on clinical practice in accordance with international guidelines, an anti-TNFalpha biologic would currently be proposed. In other words, these patients are offered randomization to infliximab or thalidomide.
The following recruitment criteria were therefore proposed:

- Age at diagnosis between 6 and 18 years;
- New diagnosis of Crohn’s disease, defined on the basis of Porto's criteria, therefore after exclusion of infectious causes of enteritis.
- Uncomplicated disease (non-stenosing, non-fistulizing) or that does not require surgery except for the treatment of perianal fistulas;
- Presence of at least one risk factor for negative prognosis as indicated by the international guidelines and other international studies (REDUCE-RISKinCD-PIBD-TRIAL; coordinator: F. Ruemmele, Paris): perianal fistulising disease; panenteric disease (defined as L3 with L4b or L3 with deep ulcers in the esophagus, stomach or duodenum, according to the Paris classification); disease extended for a length> 60 cm; severe growth impairment (height z-score <-2 SD) due to Crohn's disease; severe osteoporosis (z score <-2 SD); hypoalbuminemia (<3 g / dL) or increase in C reactive protein (2 times higher than the normal range); acceptance by reproductive age patients of the contraceptive measures envisaged for the reduction of teratogenic risk;
- Uncomplicated Crohn's disease (non-stenosing non-fistulising) in clinical or laboratory activity not responsive to induction therapy with exclusive enteral or steroid nutrition and maintenance therapy with azathioprine or methotrexate.

By changing the inclusion criteria, it was expected to be able to enroll about 5-10 patients per center per year.
This modification did not involve a change in the purpose and nature of the study as the patients thus included present an inflammatory state not different from the patients at onset and represent a population in which the natural history of the disease is still modifiable through the use of thalidomide.
On 11 June 2019 the request to change the enrollment criteria was approved.

Since then, despite the proposed new inclusion criteria, enrollment has not progressed significantly. The reasons for the non-progression of enrollment were:
- the persistence of restrictive recruitment criteria in particular when compared with the initial project.
- the occurrence of the pandemic linked to the COVID 19 infection which led to the concentration of human resources in care activities and to changes in the pharmacological management of patients.
At 30 July 2020 9 patients were enrolled in the clinical trial compared to 124 patients foreseen in the original project.
The enrollment of patients for WP2 has instead reached the number of 40 patients.

D.2 Single UO Results:

1 - Institute fo Maternal and Child Health IRCCS Burlo Garofolo.
From the start date of the study, eligible patients were enrolled, and clinical data and biological samples were collected according to the protocol.
The coordinating center and participating centers enrolled 9 patients.
Five patients were enrolled in the thalidomide arm. Four patients were enrolled in the infliximab arm, identified as standard treatment by AIFA.
None of the patients enrolled in the thalidomide arm achieved complete mucosal remission after 52 weeks of therapy against a patient enrolled in the thalidomide arm. The reduction in SES at week 52 from diagnosis was greater in the thalidomide group than in the infliximab group (-8.3 versus -4.7 points).
Two of 5 patients enrolled in the thalidomide arm versus 4 of 4 patients enrolled in the infliximab arm achieved clinical remission after 12 and 52 weeks of therapy. They all had a clinical response.
Both groups showed an overlapping reduction in inflammation indices (ESR and CRP) and nutritional parameters (albumin).
Two patients enrolled in the infliximab arm required dose escalation against no patient enrolled in the thalidomide arm.
Three out of 5 patients enrolled in the thalidomide arm had to discontinue therapy due to adverse effects (2 cases of peripheral neuropathy, 1 case of pseudotumor cerebri) against no patient enrolled in the infliximab arm. No patient required hospitalization or surgery during the follow-up. Pharmacoeconomics comparisons were not conducted.

2 - A.O. Civilian Hospital of Brescia
1.1 Patients with inflammatory bowel disease
Forty patients were enrolled, coming from different centers in Italy. All had signs and symptoms of chronic gastrointestinal inflammation and did not have a clear diagnosis.
Thirty of these patients had onset before age six, so they were enrolled for the project. For patients with adult or adolescent onset, the clinical exome sequencing was performed, in order to identify risk factors related to inflammatory bowel disease, while for VEO-IBD patients we performed whole exome sequencing (WES). The clinical exome sequencing

was has been performed on 7 patients. In order to filter and prioritize this amount of data, some limits have been set:
- population frequency <0.05
- variants with homozygous inheritance that were heterozygous were not considered. Furthermore, variants found in the UTR regions were considered, but only if reported as potentially pathogenic.
The filtered variants were then sorted based on predictive software scores and predicted clinical consequences. There were multiple variants in common between two or more patients analyzed. All these variants have already been reported in the literature as risk factors.
These are the most interesting risk factors, shared by at least 3 patients: the genes involved are all important for various functions of the immune system. The first is a variant of the NOD2 gene (protein 2 containing nucleotide-binding oligomerization domain), which encodes a protein member of the Nod1 / Apaf-1 family; it is characterized by two caspase recruitment domains (CARD) and six leucine-rich repeats (LRR). The NOD2 protein is mainly expressed in 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 are the most common NOD2 mutations found in patients with CD; this gene has been widely associated with an increased risk of developing Crohn's disease, particularly people carrying two of these mutated NOD2 alleles have a 20–40-fold increased risk. Another gene that has already been associated with CD is ATG16L1 (Autophagy 16 as 1): it is a homolog of ATG16 and together with AT5 and ATG12 it is necessary to form autophagosomes. Furthermore, it is known to interact with NOD2 in an autophagy-dependent antibacterial pathway. Specifically, we found the rs2241880 variant in four out of seven patients; these variants lead to the replacement of a threonine with an alanine in position 300. This specific variant is reported in various articles and is a recognized risk factor for CD, as it causes abnormalities in Paneth cells, decreased selective autophagy, increased release of cytokines and reduction of intracellular bacterial clearance. The molecular mechanism appears to be an increased sensitivity of the mutated ATG16L1 protein to caspase 3 cleavage. We also found a recurrent variant (three out of seven patients) in the SUMO4 gene (Small Ubiquitin Like Modifier 4); this gene is part of the SUMO family, which codes for small ubiquitin-linked modifiers that are attached to proteins, controlling the localization, stability or activity of target proteins. SUMO4 is found in the cytoplasm and specifically modifies IκBα, leading to negative regulation of the NF-κB-dependent transcription of the IL12B gene. The specific variant found in our patients (rs237025 or M55V) has been reported to be associated with type 2 diabetes but there appears to be no correlation between the variant and IBDs158. Therefore, patients with CD have been reported to have a significantly higher risk of diabetes than non-IBD controls, although this relationship is not yet clear. Another recurrent variant we reported was found in the CTLA4 gene (cytotoxic T-Lymphocyte Associated Protein 4), in three patients. CTLA4 is an immune checkpoint and its function is critical for the inhibition of immune responses.

An association between the rs231775 variant and variants in the IBD5 locus has been reported in a Hungarian population of Crohn's disease patients.

Sequencing of the entire exome.
Patients with onset before age 6 were studied using whole exome sequencing (WES) technology. In our patient cohort, a total of 30 out of 40 were VEO-IBDs. Of these patients, pathogenetic variants have been identified in the STAT3 and IL10 receptor genes. For a further 24 exomes, bioinformatics analyzes have been completed and variant screening is ongoing.
Among patients with pathogenetic variants, we report a 7-year-old boy who presented with severe early-onset enteropathy and diffuse eczematous dermatitis from birth. Gastrointestinal and skin manifestations were initially attributed to food allergy with a fairly good response to the amino acid formula. Due to the association of severe early onset enteropathy and autoimmune manifestations, X-linked polyendocrinopathy syndrome (IPEX) or other inherited disorders similar to IPEX was suspected. But the search for mutations in FOXP3, CD25 and STAT5b turned out to be negative Subsequently, WES was performed, and a de novo gain of function (GOF) mutation in STAT3 was identified in heterozygosity that localized to the DNA-binding domain and caused substitution of methionine-arginine (c.1986T>G) in position 329 (p.M329R).
In another 1 year old child of Moroccan descent born to consanguineous parents, a causative variant was identified in the IL10RA gene. At two weeks of age, the baby presented a series of signs and symptoms indicative of an inflammatory gastrointestinal disease. In particular, gingivostomatitis, enteritis and neonatal pustular dermatitis with feeding difficulties. During first-level laboratory tests, he showed a slight increase in the population of eosinophils and IgE, while other values were normal. In the following period she also developed recurrent diarrhea, vomiting, and subsequent failure.
At subsequent follow-up with endoscopic examination, it was found that he had perianal disease with anal fistula and ulcerative proctosigmoiditis. He also had an 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 consanguinity of the patients' parents, NGS analysis was performed which revealed a homozygous mutation in IL-10RA, never reported in the literature: NM_001558: cT569C: p.F190S. The mutation is a single nucleotide variant located on exon 5 and causes a change of amino acids: the wild form at position 190 has a phenylalanine, while the mutated form has a serine. The mutation was confirmed by Sanger sequencing.
The analysis on the other exomes is in the screening phase of the identified variants.

3 - Aviano Oncology Reference Center
Analyzes on the biological materials of the patients in the study have not yet been conducted. However, preliminary studies on cell lines and in vivo have been performed. In particular, the protocols for the quantification of thalidomide and its metabolite 5 '- OH by HPLC analysis with UV detector on control sera have been developed. Stability tests of thalidomide over time (up to 72 hours) under physiological conditions have been

established. Preliminary studies were carried out on two different cell lines: the 3-D (organoid) intestinal cell model, generated from adult intestinal stem cells and on the stabilized monocyte line THP1.
Molecular biology methods have been developed for the analysis of 14 polymorphisms in 6 genes involved in the pharmacokinetics and pharmacodynamics of thalidomide (CYP2C9, CYP2C19, VEGFA, TNFA, IL8 and IFNG). The polymorphisms were then tested on a retrospective series of 77 cases of pediatric patients suffering from inflammatory bowel diseases treated with thalidomide. The molecular results produced were then processed in the last months of activity demonstrating that some variants studied could have a clinical relevance in this group of patients similar for clinical characteristics to that which would have been enrolled in the prospective clinical study. Finally, a panel for NGS analysis of 10 genes was developed for the identification of uncommon genetic variants potentially implicated in the outcome of thalidomide therapy.
The results of the in vitro and molecular studies have not been further tested due to the interruption of the clinical study being researched.

Research products:

1. Todaro F, Tamassia N, Pinelli M, Moratto D, Dotta L, Grassi A, Consonni F, Giacomelli M, Lionetti P, Gardiman E, Cassatella MA, Gambineri E, Canani RB, Badolato R. Multisystem autoimmune disease caused by increased STAT3 phosphorylation and dysregulated gene expression. Haematologica. 2019 Jul;104(7):e322-e325. doi: 10.3324/haematol.2018.202374. Epub 2019 May 9.

2. Tripodi SI, Pinelli M, Dotta L, Cifaldi C, Chiriaco M, Naviglio S, Lega S, Giardino G, Pignata C, Todaro F, Bramuzzo M, Finocchi A, Cancrini C, Fuoti MG, Ravelli A, Tommasini A, Badolato R. Study of IL-10 receptor variants associated with very early onset inflammatory bowel disease: disease pathogenesis and outcome. (submitted).

3. Tripodi SI, Pinelli M, Dotta L, Cifaldi C, Chiriaco M, Naviglio S, Lega S, Giardino G, Pignata C, Todaro F, Bramuzzo M, Finocchi A, Cancrini C, Fuoti MG, Ravelli A, Tommasini A, Badolato R. Aggiornamento dello Studio delle basi genetiche delle malattie infiammatorie croniche dell'intestino (MICI) ad esordio precoce (sotto i 6 anni di vita). Abstract Gruppo di studio AIEOP Ipinet, Bologna Maggio 2019.

4. Lucafò M, Bramuzzo M, Pugnetti L, Gerdol M, Greco S, Paci M, Curci D, Nonnis M, Renzo S, Lionetti P, Tommasini A, Decorti G, Stocco G. New insights into the molecular mechanisms of thalidomide in paediatric inflammatory bowel disease patients. Journal of Crohn s and Colitis 14(Supplement_1):S345-S346, January 2020

5. Todaro F, Tamassia N, Pinelli M, Moratto D, Dotta L, Grassi A, Consonni F, Giacomelli M,
Lionetti P, Gardiman E, Cassatella MA, Gambineri E, Canani RB, Badolato R.
Multisystem autoimmune disease caused by increased STAT3 phosphorylation and

dysregulated gene expression. Haematologica. 2019 Jul;104(7):e322-e325. doi: 10.3324/haematol.2018.202374. Epub 2019 May 9.