

Original Article

# Diagnostic Yield of Next-generation Sequencing in Very Early-onset Inflammatory Bowel Diseases: A Multicentre Study

Fabienne Charbit-Henrion,<sup>a,b,c,d</sup> Marianna Parlato,<sup>a,b,d</sup> Sylvain Hanein,<sup>e</sup> Rémi Duclaux-Loras,<sup>a,b,d</sup> Jan Nowak,<sup>a,b,d,f</sup> Bernadette Begue,<sup>a,b,d</sup> Sabine Rakotobe,<sup>a,b,d</sup> Julie Bruneau,<sup>b,g</sup> Cécile Fourrage,<sup>b,h</sup> Olivier Alibeu,<sup>b,i</sup> Frédéric Rieux-Laucat,<sup>b,j</sup> Eva Lévy,<sup>b,j</sup> Marie-Claude Stolzenberg,<sup>b,j</sup> Fabienne Mazerolles,<sup>b,j</sup> Sylvain Latour,<sup>b,k</sup> Christelle Lenoir,<sup>b,k</sup> Alain Fischer,<sup>b,l,m</sup> Capucine Picard,<sup>b,k,n</sup> Marina Aloi,<sup>d,o</sup> Jorge Amil Dias,<sup>d,p</sup> Mongi Ben Hariz,<sup>d,q</sup> Anne Bourrier,<sup>r</sup> Christian Breuer,<sup>d,s</sup> Anne Breton,<sup>d,t</sup> Jiri Bronski,<sup>d,u</sup> Stephan Buderus,<sup>d,v</sup> Mara Cananzi,<sup>d,w</sup> Stéphanie Coopman,<sup>d,x</sup> Clara Crémilleux,<sup>d,y</sup> Alain Dabadie,<sup>d,z</sup> Clémentine Dumant-Forest,<sup>d,aa</sup> Odul Egritas Gurkan,<sup>d,bb</sup> Alexandre Fabre,<sup>d,cc</sup> Aude Fischer,<sup>d,dd</sup> Marta German Diaz,<sup>d,ee</sup> Yago Gonzalez-Lama,<sup>ff</sup> Olivier Goulet,<sup>b,c,d</sup> Graziella Guariso,<sup>d,gg</sup> Neslihan Gurcan,<sup>d,bb</sup> Matjaz Homan,<sup>d,hh</sup> Jean-Pierre Hugot,<sup>d,ii</sup> Eric Jeziorski,<sup>d,jj</sup> Evi Karanika,<sup>d,kk</sup> Alain Lachaux,<sup>d,ll</sup> Peter Lewindon,<sup>d,mm</sup> Rosa Lima,<sup>d,p</sup> Fernando Magro,<sup>nn</sup> Janos Major,<sup>d,oo</sup> Georgia Malamut,<sup>a,b,pp</sup> Emmanuel Mas,<sup>d,t</sup> Istvan Mattyus,<sup>d,qq</sup> Luisa M. Mearin,<sup>d,rr</sup> Jan Melek,<sup>d,ss</sup> Victor Manuel Navas-Lopez,<sup>d,tt</sup> Anders Paerregaard,<sup>d,uu</sup> Cecile Pelatan,<sup>d,vv</sup> Bénédicte Pigneur,<sup>a,b,c,d</sup> Isabel Pinto Pais,<sup>d,ww</sup> Julie Rebeuh,<sup>d,xx</sup> Claudio Romano,<sup>d,yy</sup> Nadia Siala,<sup>d,zz</sup> Caterina Strisciuglio,<sup>d,aaa</sup> Michela Tempia-Caliera,<sup>d,bbb</sup> Patrick Tounian,<sup>d,ccc</sup> Dan Turner,<sup>d,ddd</sup> Vaidotas Urbonas,<sup>d,eee</sup> Stéphanie Willot,<sup>d,fff</sup> Frank M. Ruemmele,<sup>a,b,c,d\*</sup> Nadine Cerf-Bensussan<sup>a,b,d,\*</sup>

<sup>a</sup>INSERM, UMR1163, Laboratory of Intestinal Immunity, and Imagine Institute, Paris, France <sup>b</sup>Université Paris Descartes-Sorbonne Paris Cité, Paris, France <sup>c</sup>Paediatric Gastroenterology, Hepatology and Nutrition Unit, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>d</sup>GENIUS Group [GENetically ImmUne-mediated enteropathieS] from the European Society for Paediatric Gastroenterology, Hepatology and Nutrition [ESPGHAN] <sup>e</sup>INSERM, UMR 1163 Translational Genetic, and Imagine Institute, Paris, France <sup>f</sup>Department of Paediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poznan, Poland <sup>g</sup>Pathology Department, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>h</sup>Bioinformatics Platform, Imagine Institute Paris, France <sup>i</sup>Genomic Platform, Imagine Institute, Paris, France <sup>j</sup>INSERM, UMR1163, Immunogenetics of Paediatric Autoimmunity, and Imagine Institute, Paris, France <sup>k</sup>INSERM, UMR1163, Lymphocyte activation and EBV susceptibility, and Imagine Institute, Paris France <sup>l</sup>Collège de France, Médecine expérimentale, Paris, France <sup>m</sup>INSERM UMR 1163 and Imagine Institute, Paris, France <sup>n</sup>Investigation Centre for Immunodeficiency, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris and Imagine Institute,

Paris, France <sup>o</sup>Sapienza University of Rome, Paediatric Gastroenterology and Liver Unit, Department of Pediatrics, Rome, Italy <sup>p</sup>Department of Paediatrics, Centro Hospitalar São João, Porto, Portugal <sup>q</sup>Department of Paediatrics, Hôpital La Marsa, Tunisia <sup>r</sup>Department of Gastroenterology, Hôpital St Antoine, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>s</sup>Department of Paediatrics, Universitätsklinikum Hamburg, Hamburg, Germany <sup>t</sup>Department of Paediatrics, Gastroenterology, Hepatology, Nutrition, and Diabetes, Centre Hospitalier Universitaire de Toulouse, Toulouse, France <sup>u</sup>University Hospital Motol, Prague, Czech Republic <sup>v</sup>St. Marien Hospital, Bonn, Germany <sup>w</sup>Unit of Paediatric Hepatology, Department of Woman and Child Health, University Hospital of Padova, Padova, Italy <sup>x</sup>Division of Gastroenterology, Hepatology and Nutrition, Department of Paediatrics, Jeanne De Flandre Children's Hospital, Lille University Faculty of Medicine, Lille, France <sup>y</sup>Department of Paediatrics, Centre Hospitalo-Universitaire de St-Etienne, St-Etienne, France <sup>z</sup>Service de médecine de l'enfant et de l'adolescent, Hôpital Sud – Centre Hospitalo-Universitaire de Rennes, Rennes, France <sup>aa</sup>Department of Paediatrics, Centre Hospitalo-Universitaire Charles Nicolle, Rouen, France <sup>bb</sup>Paediatric Gastroenterology, Hepatology and Nutrition, Gazi University, Ankara, Turkey <sup>cc</sup>Department of Paediatrics, Assistance publique Hôpitaux de Marseille, Hôpital de la Timone, Marseille, France <sup>dd</sup>Department of Paediatrics, Centre Hospitalo-Universitaire Sud Réunion, St Pierre, France <sup>ee</sup>Unit of Paediatric Nutrition, Hospital Universitario 12 de Octubre, Madrid, Spain <sup>ff</sup>Inflammatory Bowel Disease Unit, Hospital Universitario Puerta de Hierro–Majadahonda, Madrid, Spain <sup>gg</sup>University of Padua, Italy <sup>hh</sup>Department of Gastroenterology, Hepatology and Nutrition, University Children's Hospital, Ljubljana, Slovenia <sup>ii</sup>Departments of Paediatric Digestive and Respiratory Diseases, Hôpital Robert-Debré, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>jj</sup>Department of Paediatrics, Infectious diseases and Immunology, Centre Hospitalo-Universitaire de Montpellier, Montpellier, France <sup>kk</sup>Department of Paediatrics, University General Hospital of Thessaloniki, Thessaloniki, Greece <sup>ll</sup>Department of Paediatric Gastroenterology, Hepatology and Nutrition, Centre de Nutrition parentérale à domicile, Hôpital Femme-Mère-Enfant CHU de Lyon HCL - GH Est, Bron, France <sup>mm</sup>Department of Gastroenterology and Hepatology, Lady Cilento Children's Hospital and the Faculty of Medicine and Biomedical Sciences, TUniversity of Queensland, Brisbane, Australia <sup>nn</sup>Gastroenterology Department, Hospital de São João, Institute of Pharmacology and Therapeutics Faculty of Medicine and MedInUP - Centre for Drug Discovery and Innovative Medicines, University of Porto, Porto, Portugal <sup>oo</sup>MRE Bethesda Gyermekórháza; Department of Pediatrics, Budapest, Hungary <sup>pp</sup>Department of Gastroenterology, Hôpital Européen Georges Pompidou, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>qq</sup>Semmelweis University; Department of Paediatrics, Budapest, Hungary <sup>rr</sup>Leiden University Medical Centre, Department of Paediatrics, Leiden, The Netherlands <sup>ss</sup>University Hospital, Hradec Kralove, Czech Republic <sup>tt</sup>Hospital Regional Universitario de Málaga, Departamento de Pediatría, Malaga, Spain <sup>uu</sup>Hvidovre University Hospital, Department of Paediatrics, Copenhagen, Denmark <sup>vv</sup>Department of Paediatrics, Centre Hospitalier du Mans, Le Mans, France <sup>ww</sup>Centro Hospitalar Gaia Espinho, Department of Paediatrics, Vila Nova de Gaia, Portugal <sup>xx</sup>Department of Paediatrics, Centre Hospitalo-Universitaire de Strasbourg, Strasbourg, France <sup>yy</sup>Department of Paediatrics, Hospital of Messina, University of Messina, Messina, Italy <sup>zz</sup>Department of Paediatrics, Hôpital Mongi Slim, La Marsa, Tunisia <sup>aaa</sup>Department of Woman, Child and General and Specialized Surgery, Second University of Naples, Naples, Italy <sup>bbb</sup>Department of Paediatrics, FMH Pédiatrie et FA Gastroentérologie et hépatologie, Clinique des Grangettes, Geneva, Switzerland <sup>ccc</sup>Department of Paediatric Nutrition and Gastroenterology, Hôpital Armand Trousseau, Assistance Publique-Hôpitaux de Paris, Paris, France <sup>ddd</sup>Shaare Zedek Medical Centre, Jerusalem, Israel <sup>eee</sup>Department of Paediatric Gastroenterology, Vilnius University Clinic for Children's Diseases, Vilnius, Lithuania <sup>fff</sup>Department of Paediatrics, Centre hospitalier régional universitaire, Hôpital Clocheville, Tours, France

\*Co-senior authorship.

Corresponding author: Nadine Cerf-Bensussan, Laboratory of Intestinal Immunity, Institut IMAGINE-INSERM 1163, Université Paris Descartes-Sorbonne Paris Cité. 24, boulevard du Montparnasse. 75015 Paris, France. Tel: 33-[0]1-42-75-42-88; Email: [nadine.cerf-bensussan@inserm.fr](mailto:nadine.cerf-bensussan@inserm.fr)

## Abstract

**Background and Aims:** An expanding number of monogenic defects have been identified as causative of severe forms of very early-onset inflammatory bowel diseases [VEO-IBD]. The present study aimed at defining how next-generation sequencing [NGS] methods can be used to improve identification of known molecular diagnosis and to adapt treatment.

**Methods:** A total of 207 children were recruited in 45 paediatric centres through an international collaborative network [ESPGHAN GENIUS working group] with a clinical presentation of severe VEO-IBD [ $n = 185$ ] or an anamnesis suggestive of a monogenic disorder [ $n = 22$ ]. Patients were divided at inclusion into three phenotypic subsets: predominantly small bowel inflammation, colitis

with perianal lesions, and colitis only. Methods to obtain molecular diagnosis included functional tests followed by specific Sanger sequencing, custom-made targeted NGS, and in selected cases whole exome sequencing [WES] of parents-child trios. Genetic findings were validated clinically and/or functionally.

**Results:** Molecular diagnosis was achieved in 66/207 children [32%]: 61% with small bowel inflammation, 39% with colitis and perianal lesions, and 18% with colitis only. Targeted NGS pinpointed gene mutations causative of atypical presentations, and identified large exonic copy number variations previously missed by WES.

**Conclusions:** Our results lead us to propose an optimised diagnostic strategy to identify known monogenic causes of severe IBD.

**Key Words:** Genetics and molecular epidemiology; paediatrics; VEO-IBD; monogenic disorders; TNGS

## 1. Introduction

Very early-onset inflammatory bowel diseases [VEO-IBD] are defined by the development of chronic intestinal inflammation before the age of 6 years. A subgroup of patients suffer from a rapid and severe disease evolution, which raises difficult diagnostic and therapeutic issues.<sup>1</sup> In the latter patients, disease-causing mutations are identified in an increasing number of genes expressed in haematopoietic immune cells, in epithelial cells, or in both. Early molecular diagnosis is crucial in order to reduce mortality and morbidity, notably by defining whether haematopoietic stem cell transplantation [HSCT] is indicated.

Molecular diagnosis in VEO-IBD patients is classically based on precise phenotyping followed by selected functional tests and Sanger sequencing of candidate genes.<sup>2,3</sup> Onset of severe bloody diarrhoea and perianal lesions a few weeks after birth suggests the existence of mutations inactivating the interleukin 10 [IL-10] receptor or IL10,<sup>4-7</sup> whereas exudative diarrhoea in the context of autoimmunity—and notably type I diabetes—is highly suggestive of IPEX syndrome [Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked] due to mutations in Forkhead box P3 [FOXP3] gene.<sup>8</sup>

However, such approach to molecular diagnosis has limitations given the increasing number of genes in which mutations have been identified and given recurrent evidence of lack of strict phenotype-genotype correlations.<sup>9</sup> Furthermore, in patients with overlapping clinical presentations, different pathophysiological defects require distinct therapeutic approaches.<sup>10</sup> Therefore, precise molecular diagnosis is a prerequisite for offering these patients access to optimal care with the best possible outcome.

These issues stress the need to establish time- and cost-effective clinical diagnostic protocols.<sup>3,11</sup> Next-generation DNA sequencing [NGS] methods allow efficient genotype-based diagnosis. Whole genome [WGS] and whole exome [WES] sequencing provide unbiased approaches, but they remain relatively expensive, and can raise difficult challenges for analysis and interpretation, which limits their use for diagnostic purposes. Targeted NGS [TNGS]—also called targeted gene panel sequencing—emerges as a powerful tool for fast and simple screening of genes of interest. Since 2014, TNGS was proven to be valuable in the identification of primary immunodeficiencies, notably in patients with atypical presentations.<sup>9,12,13</sup> More recently, TNGS could identify causative mutations in small cohorts of VEO-IBD patients.<sup>1,14</sup>

The present study aimed at defining how next-generation sequencing [NGS] methods can be used to improve identification of known molecular defects and to adapt treatment accordingly. Based on our results, we propose an optimised diagnostic strategy to identify known monogenic causes of severe IBD depending on the phenotype of the patients.

## 2. Methods

### 2.1. Patients

A working group from ESPGHAN [European Society for Paediatric GastroEnterology, Hepatology and Nutrition] called GENIUS [GENetically and/or ImmUne-mediated enteropathieS] was created in 2009 in order to improve diagnosis of monogenic intestinal disorders and to optimise patients' care. GENIUS [www.genius-group.org] gathers paediatric gastroenterologists from 45 centres [see Appendix in [Supplementary data](#), available at *ECCO-JCC* online].

Inclusion criteria were chronic diarrhoea developed before the age of 6 years, with severe disease course requiring immunosuppressive treatments (including steroids, azathioprine, or anti-tumour necrosis factor [TNF]), surgery [partial or subtotal colectomy], and/or parenteral nutrition [185 patients]. Additional patients were included despite disease onset after the age of 6 years in the presence of a familial history suggestive of Mendelian inheritance [nine patients with affected siblings] or of a disease course refractory to treatment [13 patients]. Overall, 42 patients were from consanguineous families [20%]. Based on the clinical phenotype, patients were divided at inclusion into three subsets: 1] predominantly inflammatory small bowel inflammation with massive lymphocytic infiltrate and villous atrophy of the small bowel associated with various signs of autoimmunity; 2] inflammatory colitis associated with severe perianal lesions such as fistula or abscess; 3] severe isolated colitis. Patients were included in each centre after obtaining informed written consent for functional and genetic studies from a parent or legal guardian. The study adhered to the tenets of the revised Declaration of Helsinki and was approved by an institutional review board [CPE Ile de France II, 2014.01.04 AF].

### 2.2. DNA preparation

Genomic DNA was extracted from peripheral blood mononuclear cells [PBMC] isolated by Ficoll HyPaque Plus [GE Healthcare, Velizy-Villacoublay, France] from umbilical cord tissue [patient 25] or from whole blood lysed by BD lysis buffer [BD Biosciences, Le Pont de Claix, France] using the QIAamp<sup>®</sup> DNA Blood Mini Kit [Qiagen, Courtaboeuf, France].

### 2.3. Whole exome and targeted next-generation sequencing

A custom-made TNGS panel was designed and gathered 66 genes in which mutations can cause VEO-IBD or congenital diarrhoeal disorders [[Supplementary Table 1](#), available as *Supplementary data*, available at *ECCO-JCC* online]. The choice of VEO-IBD associated

genes was mostly guided by the list suggested by Uhlig in 2014<sup>11</sup> and Canani *et al.* in 2015.<sup>15</sup> Some genes of common variable immunodeficiency were included as well, as they can lead to severe intestinal inflammation.<sup>16–22</sup> Genes of severe combined immunodeficiencies, in which diarrhoea is usual but with a different clinical context, and genes associated with diseases with very specific and primarily extra-intestinal symptoms such as bullous skin diseases or albinism, were excluded as affected patients are unlikely to be diagnosed in paediatric gastroenterology centres.

Genomic DNA libraries were generated from DNA [3 µg for WES and 1 µg for TNGS] sheared with a Covaris S2 Ultrasonicator using SureSelect<sup>XT</sup> Library PrepKit [Agilent, Garches, France] on the Genomic Platform at the Imagine Institute. Capture by hybridisation was performed using either Agilent Sure Select All Exon V5 [Agilent, Les Ulis, France] for WES or biotinylated complementary 120-bp RNA baits designed with SureSelect SureDesign software [*H. sapiens*, hg19, GRCh37, February 2009] that cover the 1130 regions of interest [ROI] encompassing all exons of the 66 selected genes.

Targeted exons or ROI were pulled out with magnetic streptavidin beads, polymerase chain reaction [PCR]-amplified using indexing primers and sequenced on an Illumina HiSeq2500 HT system [Illumina, San Diego, USA]. Data analysis was performed with Paris Descartes University/Imagine Institute's Bioinformatics core facilities. Paired-end sequences were mapped on the human genome reference [NCBI build37/hg19 version] using the Burrows-Wheeler Aligner. Downstream processing was carried out with the Genome Analysis Toolkit [GATK], SAMtools,<sup>23</sup> and Picard, according to documented best practices [http://www.broadinstitute.org/gatk/guide/topic?name=best-practices]. Variant calls were made with the GATK Unified Genotyper based on the 72nd version of ENSEMBL database. Genome variations were defined using the in-house software PolyQuery for WES or PolyDiag for TNGS, which filters out irrelevant and common polymorphisms based on frequencies extracted from public databases: US National Center for Biotechnology Information database of single nucleotide polymorphisms [SNP] [dbSNP<sup>24</sup>], 1000 genomes,<sup>25</sup> Exome Variant Server [EVS, http://evs.gs.washington.edu/EVS/], and Exome Aggregation Consortium [ExAC, http://exac.broadinstitute.org].<sup>26</sup> Consequences of mutations on protein function were predicted using three algorithms: Polyphen2 [http://genetics.bwh.harvard.edu/pph2/],<sup>27</sup> SIFT [Sorting Intolerant From Tolerant, J. Craig Venter Institute],<sup>28</sup> and Mutation Taster [www.mutationtaster.org].<sup>29</sup> Mutations were then ranked on the basis of the predicted impact of each variant by combined annotation-dependent depletion [CADD],<sup>30</sup> and compared with the mutation significance cut-off [MSC], a gene-level specific cut-off for CADD scores [http://pec630.rockefeller.edu:8080/MSC].<sup>31</sup>

#### 2.4. Quality control of TNGS

DNA samples from all patients were sequenced between August 2015 and April 2016 in three consecutive runs. Mean coverage of the panel was 682 reads, and varied depending on the number of samples sequenced by run with medians of 826, 629, and 593 reads in runs 1 [29 samples], 2 [51 samples], and 3 [90 samples], respectively. ROI with the smallest coverage were the first exons of *DGAT1* [50 to 200 reads] and *MALT1* [10 to 100 reads], likely due to their high GC content which impairs capture, and exon 7 of *ITCH* [20 to 200 reads] and exon 1 of *APOB* [20 to 160 reads], which both contain repeated regions. Overall, all ROI were well covered, including *NCF1*, *IKBK*, and *NEUROG3*, known to be difficult to capture. Due to the presence of pseudogenes, careful consideration was, however, required to attribute variants to *IKBK* and *NCF1*.

The sensitivity of TNGS was assessed by comparing against Sanger sequencing in a validation sample of 12 patients with established molecular diagnoses. All mutations were identified by TNGS, including a complex compound heterozygous defect in *IL10RB* [VS12] consisting of a large deletion of exon 3 on the maternal allele and a duplication of exon 6 on the paternal allele [Charbit-Henrion *et al.*, in submission].

#### 2.5. Gene validation

All mutations identified by WES or TNGS, and not previously described as disease-causing in the literature, were validated as follows.

Novel mutations in *SKIV2L* and *TTC37* were confirmed by reassessment of the clinical phenotype. The patients presented the canonical signs of syndromic diarrhoea within a tricho-hepato-enteric syndrome<sup>32</sup>: congenital diarrhoea [4/4], hair abnormalities with trichorrhexis nodosa [4/4], intrauterine growth retardation [3/4], oligohydramnios [2/4], facial dysmorphism [2/4], and immune defect [3/4]. Novel mutations in *MYO5B* and *EPCAM* were confirmed by histology (haematoxylin and eosin [HE]), periodic acid-Schiff [PAS] staining for *MYO5B* and immunohistochemistry [EPCAM staining] performed on formalin-fixed intestinal biopsies. EPCAM was revealed with clone 323/A3 [Acris Antibodies GmbH, Montluçon, France] as described.<sup>33</sup> Novel *FOXP3* mutations were validated by demonstrating a reduced frequency of CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>+</sup> FOXP3<sup>+</sup> regulatory T cells or abnormal suppressive function.<sup>34,35</sup> IL-10 receptor functional test was performed as described.<sup>6</sup> Novel *XIAP* mutations were authenticated by showing impaired IL-8 production by PBMC stimulated with N-acetyl-muramyl-M-alanyl-D-isoglutamine hydrate [Muramyl-dipeptide/MDP, Sigma, St. Quentin Fallavier, France] or lipopolysaccharide [LPS, Sigma] as described previously,<sup>36</sup> and protein expression was assessed by *XIAP* intracellular staining performed on 2 × 10<sup>6</sup> fixed-permeabilised PBMC using the Intraprep Permeabilization Reagent kit [Beckman Coulter, Villepinte, France] and incubated with anti-*XIAP* antibody [clone 28/hILP, BD] or isotype control antibody [mouse IgG1, Sony Biotechnologies, Weybridge, UK] and with secondary PE-goat anti-mouse IgG1 antibody. For extracellular staining, cells were incubated with anti-CD3-Bv510, anti-CD14-Pe/Cy7, anti-CD11c-APC, anti-CD86-Bv421 [Sony Biotechnologies], and anti-CD19-Bv711 [BD Biosciences]. *LRBA* and *NCF1* mutations were confirmed by absent protein expression on western blot performed on cell lysates according to standard protocols. Proteins were detected with monoclonal antibodies against human *NCF1* [sc-14015 phox-p47, Santa Cruz Biotechnology, Dallas, USA], *LRBA* [clone HPA023597, Sigma], horseradish peroxidase-linked secondary antibodies or horseradish peroxidase-conjugated GAPDH rabbit antibody [Ozyme, Montigny-le-Bretonneux, France], and ECL Prime Western Blotting Detection reagent [GE Healthcare].

### 3. Results

#### 3.1. Description of the cohort

Between August 2009 and August 2015, 207 consecutive patients with severe chronic diarrhoea were recruited; 144 patients had started their disease before the age of 2 years [70%] and 22 patients after the age of 6 years [Table 1]. As described in Methods, and shown in Table 2, patients were divided into three groups based on phenotype: 51 patients with small bowel inflammation and autoimmune features; 33 with colitis and perianal lesions; and 123 with colitis only. As shown in Figure 1, 160 patients benefited from functional tests

**Table 1.** Diagnosis yield according to age at disease onset.

	Small bowel inflammation	Colitis + perianal	Colitis only	Total
Whole cohort				
Number of patients	51	33	123	207
Number of molecular diagnoses	31	13	22	66
	61%	39%	18%	32%
With disease onset before age 2 years				
Number of patients	45	22	77	144
Number of molecular diagnoses	30	12	17	59
	67%	55%	22%	41%
With disease onset after age 6 years				
Number of patients	2 <sup>a</sup>	7 <sup>b</sup>	13 <sup>c</sup>	22
Number of molecular diagnoses	1	1	2	4
	50%	14%	15%	18%

<sup>a</sup>Two patients from two multiplex families.

<sup>b</sup>Four patients from four multiplex families and three patients with refractory disease.

<sup>c</sup>Three patients from three multiplex families and 10 patients with refractory disease.

that were orientated by the clinical presentation: numbers and phenotype of regulatory T cells were determined in patients with small bowel inflammation, and signalling downstream IL-10 receptor or XIAP was assessed in patients with colitis with or without perianal lesions. In 32 patients with abnormal results, Sanger sequencing was used to confirm mutations in *FOXP3*, *IL2RA*, *IL10RA*, *IL10RB*, or *XIAP*. Between 2013 and 2015, WES was performed on DNA trios [DNA from child and both parents] in 51 cases with normal functional tests and very severe phenotypes. A known monogenic disorder was identified in 10 patients [20%]. In 2015, TNGS was introduced and performed in all patients previously investigated but left undiagnosed and also systematically in the newly included patients [ $n = 49$ ]. TNGS identified causative mutations in 24/167 patients [14%]. As expected, diagnosis yield varied depending on whether TNGS was used to complete previous diagnostic approaches or as the first method. Thus, TNGS reached eight molecular diagnoses [10%] in the 77 functionally tested patients who had not benefited from WES [because DNA was not available from both parents:  $n = 45$ ; or because of less severe phenotypes]. However, when used as first screening [ $n = 49$ ], diagnosis yield of TNGS was much higher, with 13 molecular defects identified [26.5%]. Importantly, in our study, TNGS had a superior diagnostic yield compared with WES. Indeed, it allowed diagnosis in three patients who had been already investigated by WES but harboured copy number variations hardly detectable by this method [one girl with large *LRBA* deletion, two siblings with large *NCF1* deletion]. Overall, a known monogenic disorder was identified in 66/207 patients [32%].

### 3.2. Yield of molecular diagnoses according to the clinical phenotype

By combining selected functional tests and next-generation sequencing, approximately 30% of all the patients included in the present cohort received a molecular diagnosis. Yet, as mentioned above, clinical presentation differed between patients and functional tests were

**Table 2.** Phenotype based genetic characterisation of VEO-IBD patients.

	Small bowel inflammation	Colitis + perianal	Colitis only
Number of patients	51	33	123
% of boys	78%	72%	58%
Number of diagnosed patients	31	13	22
% of molecular diagnoses	61%	39%	18%
% of diagnoses done by			
Functional tests	55%	92%	14%
WES	19.5%	0%	18%
TNGS	26%	8%	68%
	<i>FOXP3</i> [19]	<i>IL10RA</i> [3]	<i>EPCAM</i> [1]
	<i>IL2RA</i> [2]	<i>IL10RB</i> [7]	<i>ICOS</i> [1]
	<i>LRBA</i> [5]	<i>XIAP</i> [3]	<i>MYO5B</i> [2]
	<i>MALT1</i> [2]		<i>NCF1</i> [2]
	<i>MYO5B</i> [1]		<i>NLRC4</i> [1]
	<i>NEUROG3</i> [1]		<i>SI</i> [1]
	<i>STAT3</i> [1]		<i>SKIV2L</i> [3]
			<i>STAT1</i> [1]
			<i>TTC37</i> [1]
			<i>TTC7A</i> [2]
			<i>XIAP</i> [7]

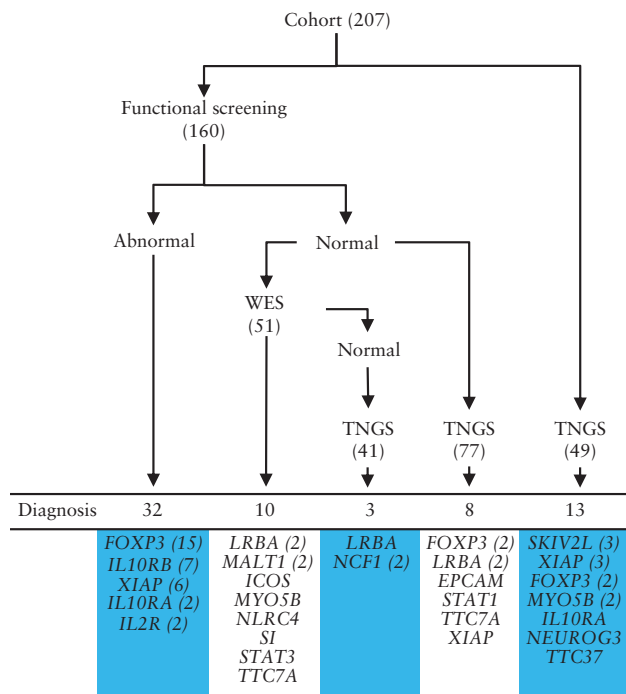
List of genes and number of patients for each gene in brackets.

WES, whole exome sequencing; TNGS, targeted next-generation sequencing.

only available to validate a few gene defects. To optimise diagnostic work-up, we then analysed whether the frequency of identified molecular defects differed between the three groups of patients and, within each group of patients, when and how functional testing and TNGS had been most useful for diagnosis [Table 2].

Among 51 patients [37 boys, 72.5%] who presented with small bowel inflammation, a molecular diagnosis was obtained in 31/51 patients [61%]. In 19/31 patients, number or phenotype of Tregs were abnormal. In 17 of these 19 patients, Sanger sequencing identified mutations in *FOXP3* [ $n = 15$ ] and in *IL2RA* [ $n = 2$ ]. Other mutations were identified either by WES in six patients or TNGS in eight patients. Mutations identified by WES were: a homozygous *MALT1* mutation in two siblings with decreased number of Tregs,<sup>34</sup> a homozygous *LRBA* mutation in two other siblings,<sup>37</sup> a *de novo* gain-of-function mutation of *STAT3* in one girl, and a homozygous *MYO5B* mutation in one boy in whom marked intestinal inflammation had erroneously led to ascribe the severe diarrhoea to an immune-mediated process. Mutations identified by TNGS included *FOXP3* mutations in four boys, for three of whom only DNA was available and in one case normal number and phenotype of regulatory T cells but with abnormal suppressive function; a homozygous *NEUROG3* mutation in one boy with chronic diarrhoea since the first months of life and type 1 diabetes since the age of one<sup>38</sup>; compound heterozygous *LRBA* mutations in three patients, one boy with two missense mutation, and two siblings with one missense mutation and a large deletion [from exon 3 to exon 48]. As indicated above, this large deletion in *LRBA* had been missed by WES performed before TNGS.

The group with colitis and perianal lesions included 33 patients of whom 73% were boys. Among the 33 patients, 13 [40%] were diagnosed with mutations in *IL10RA* [ $n = 4$ ], *IL10RB* [ $n = 6$ ], or *XIAP* [ $n = 3$ ]. Based on their phenotype highly suggestive of defective



**Figure 1.** Cohort screening for gene identification. Number of diagnoses obtained by each method. Number of patients for each gene defect is reported in brackets. WES: whole exome sequencing; TNGS: targeted next-generation sequencing.

IL-10 or XIAP signalling, all patients except one benefited from functional tests.<sup>6,36</sup> Overall, diagnosis was oriented in 12 patients by functional tests, which showed either lack of responsiveness of peripheral monocytes to the inhibitory effect of exogenous IL-10 [ $n = 9$ ] or lack of stimulation of the NOD2-XIAP pathway by muramyl-dipeptide [ $n = 3$ ]. Sanger sequencing confirmed mutations in *IL10RA* [ $n = 3$ ], *IL10RB* [ $n = 6$ ], or *XIAP* [ $n = 3$ ]. In one patient for whom only DNA was available, TNGS identified a homozygous *IL10RA* mutation.

The group of patients with only colitis comprised 123 patients of whom 59% were male. As defined above, they displayed neither autoimmune symptoms nor perianal lesions. Only 22 of them reached a molecular diagnosis [18%]. Three boys [3/22, 13.5%] were diagnosed with *XIAP* mutations after abnormal functional screening and targeted Sanger sequencing of *XIAP*. WES allowed diagnosis in four additional patients [4/22, 18%] harbouring mutations in four different genes: *NLR4*, *SI*, *TTC7A*, and *ICOS*. However, most molecular diagnoses in this group [15/22, 68%] were obtained by TNGS. TNGS pinpointed mutations in *EPCAM* [ $n = 1$ ], *MYO5B* [ $n = 2$ ], *NCF1* [in two siblings with a large homozygous deletion which was missed by WES], *SKIV2L* [ $n = 3$ ], *STAT1* [ $n = 1$ ], *TTC37* [ $n = 1$ ], *TTC7A* [ $n = 1$ ], and *XIAP* in four patients: in three boys for whom only DNA was available and in a 17-year-old girl with severe and refractory VEO-IBD since the age of 6 months. Because of X-linked inheritance, *XIAP* deficiency had not been considered. Following identification of a premature stop codon by TNGS, abnormal signalling downstream XIAP and reduced protein expression in 50% of PBMC were demonstrated. The same mutation was found in her sister, who had also developed very severe IBD. Of note, in three additional patients, rare variants identified by TNGS were ruled out, either because they were incompatible with the clinical phenotype [a rare heterozygous variant in *PLCG2* was highly unlikely to be causative since the patient did

not display any auto-inflammatory symptoms] or through functional testing [two variants in *MYO5B* in a patient with normal PAS staining of duodenal sections; two rare variants in *IL21R* but normal STAT3 phosphorylation of PBMC in response to IL-21].

### 3.3. Yield of molecular diagnoses depending on age at disease onset

As mentioned above, diagnostic yield on the whole cohort was 32%; 70% of the patients started their disease before the age of two years [144/207, Table 1]. Among the latter patients, diagnostic yield was slightly higher [41%]. Further analysis shows that, in patients with small bowel inflammation or colitis with perianal lesions, the vast majority of molecular diagnoses were identified in children with disease onset before the age of two years. In patients included despite later age at disease onset [ $n = 22$ ], a monogenic defect was identified in four cases [18%].

## 4. Discussion

An expanding number of monogenic defects have been identified as causative of severe forms of VEO-IBD. These diseases are individually rare but, collectively, they raise difficult diagnostic and therapeutic issues due to their severity and considerable genetic heterogeneity. Paediatric gastroenterologists from ESPGHAN created the GENIUS working group in 2009 with the goals of fostering clinical knowledge of these diseases and facilitating development of therapeutic strategies based on precise molecular diagnosis. The resulting cooperation between 45 paediatric centres allowed us to comprehensively investigate the potential monogenic causes of severe VEO-IBD and to propose guidelines for the diagnosis of known molecular defects.

The 207 patients described in this study represent the largest European cohort to date. Overall, 32% of patients obtained a molecular diagnosis. Diagnostic yield, however, varied when patients were stratified by their phenotypes. Molecular diagnosis was obtained in two-thirds of children presenting with small bowel inflammation suggestive of 'IPEX' syndrome, and in almost half of patients having colitis with severe perianal lesions. In contrast, diagnosis yield in the 'Colitis only' subset was relatively low [less than 20%]. These differences remain to be explained. A possible hypothesis is that not all cases of early-onset colitis have a monogenic cause, notably in patients with disease onset beyond the first 2 years of life and in absence of familial history.

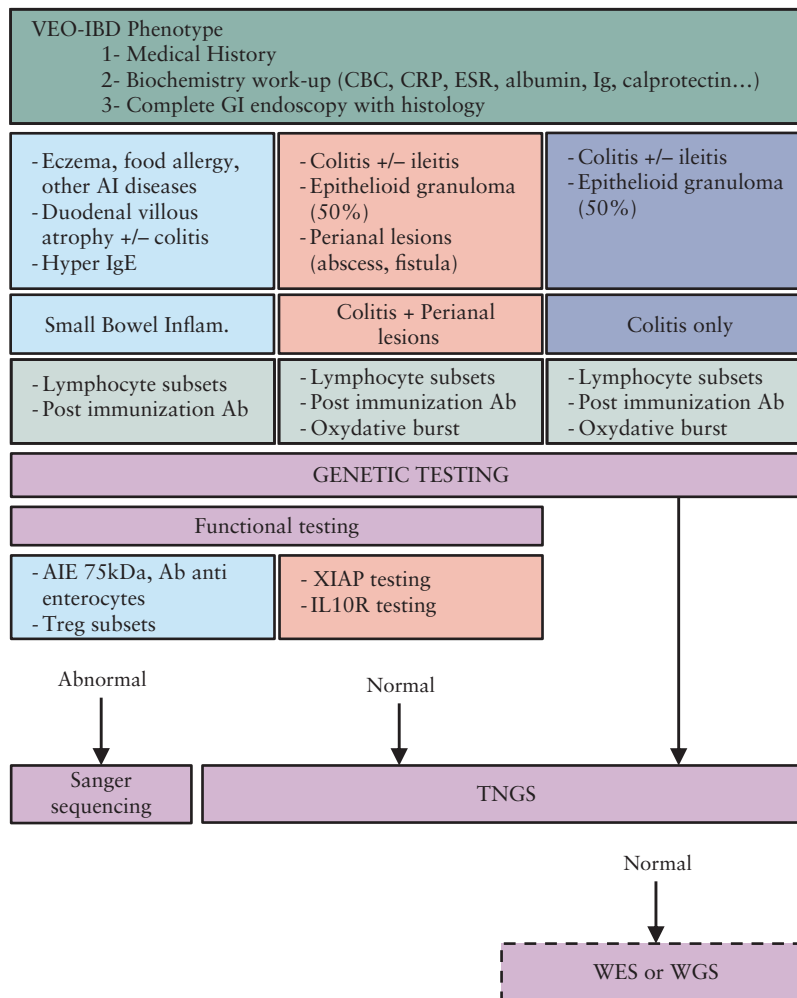
In our settings, Sanger sequencing orientated by abnormal functional tests, WES and TNGS presented comparable diagnostic yields. The first method allowed molecular diagnosis in 32 out of 160 tested patients [20%]; WES identified a known monogenic disorder in 10 patients out of 51 [20%]. It may seem that TNGS was less efficient, with a global diagnostic yield of only 14% [24/167]. Nonetheless, one needs to analyse separately patients who benefited from TNGS as a first screening test [13 molecular diagnoses out of 49 patients, 26.5%] and patients who were functionally tested [eight molecular diagnoses out of 77 patients, 10%]. Altogether our findings confirm and extend results obtained in two previous studies in smaller cohorts of VEO-IBD patients, in which TNGS could identify causative mutations in 4/25<sup>1</sup> and in 5/71 patients,<sup>14</sup> respectively. Furthermore, in patients with higher genetic heterogeneity such as those with isolated colitis, TNGS is more efficient [68% of monogenic defects were identified by TNGS in this group] as it can reveal diagnosis in patients with atypical clinical presentation, as exemplified in the present study in one girl with *XIAP* deficiency.

Of note, a molecular diagnosis was identified in four out of 22 patients who had started their disease after 6 years. These four patients [with defects in *LRBA*, *NCF1*, and *XIAP*] would have remained undiagnosed with a cut-off for inclusion at 6 years. Given increasing evidence of *de novo* mutations, of incomplete penetrance, and of delayed disease onset in patients with mutations in notably *XIAP*, *CTLA4*, *LRBA*, *STAT3*, or *NLRCA*,<sup>17-22,36,37,39</sup> we thus recommend that access to TNGS screening should be considered not only in all severe VEO-IBD patients, but also in older children or teenagers with very severe intestinal diseases refractory to medical treatment. The modest cost per patient renders this option economically affordable and may prove instrumental to guide therapeutic strategy. This is exemplified in our cohort by a case of *XIAP* deficiency diagnosed in a young adult, who had already undergone colectomy, thus too late to propose treating the colitis by HSCT.<sup>40</sup>

Based on the retrospective analysis of this large multicentre cohort of severe VEO-IBD patients, we propose an algorithm to obtain a molecular diagnosis in an optimised manner [Figure 2]. It has to be validated prospectively in an independent cohort of patients. In addition to severe chronic diarrhoea starting early in life, some atypical clinical features may orientate towards specific monogenic defects [hair abnormalities, dysmorphia, autoimmunity, eczema, allergy, immune

deficiency, etc]. Combined with laboratory tests and endoscopic and histology findings, this precise phenotyping allows division of patients into three groups as described above. Since many monogenic forms of VEO-IBD are primary immunodeficiencies, standard immunological work-up is recommended [see Figure 2]. When pathognomonic symptom associations evoke a specific monogenic disorder, corresponding functional tests should be considered as they may quickly orientate molecular diagnosis within only a few days.

When functional tests fail to orientate toward a molecular defect, NGS methods need to be considered, given the genetic heterogeneity and important phenotype-genotype overlaps. TNGS, WES, and WGS are non-hypothesis driven assays that allow molecular diagnosis in patients with atypical clinical presentations. In a routine clinical setting, TNGS is the method to prefer as it is cost- and time-effective while providing optimal coverage of the genes of interest [see Supplementary Table 2, available as Supplementary data, available at ECCO-JCC online]. Regularly updating the panel of genes to be targeted upon identification of novel disease-causing genes should progressively increase sensitivity and diagnostic yield. WES could be performed as a second-line genetic assay when TNGS has failed to reveal a molecular diagnosis. WES—and furthermore WGS—will allow identification of novel causative genes, but they require an



**Figure 2.** Proposed algorithm for molecular diagnosis in VEO-IBD. VEO-IBD: very early-onset inflammatory bowel disease; CBC: cell blood count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; Ig: immunoglobulin; GI: gastro-intestinal; AI: autoimmune; Ab: antibodies; Treg: regulatory T cells; small bowel inflam: small bowel inflammation; TNGS: targeted next-generation sequencing; WES: whole exome sequencing; WGS: whole genome sequencing.

important investment in terms of cost, sequencing delay, analysis, and validation, which restricts their use to research protocols only. In our cohort, WES allowed us to ascribe VEO-IBD to biallelic mutations in *DUOX2*,<sup>41</sup> and more recently to biallelic mutations in intestinal alkaline phosphatase.<sup>42</sup> Furthermore, novel candidate genes are currently under validation in three distinct families.

In conclusion, development of accurate and cost-effective diagnosis tools to screen a putative monogenic VEO-IBD population is indispensable to obtain molecular diagnosis and optimise patients' care. Further genomic analyses including WES and, likely in the near future, WGS, should help in identifying novel candidate genes. Overall, genetic characterisation of VEO-IBD will help broaden knowledge of the phenotypic spectrum of inherited intestinal disorders. Identifying key genes for gut homeostasis may also help in designing new therapeutic targets for patients suffering from chronic intestinal diseases.

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## Conflict of Interest

The authors do not declare any conflict of interest.

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## Author Contributions

FCH, FR, and NCB designed the study; FCH and SH were in charge of designing the TNGS; OA and CF performed TNGS; FCH, MP, RDL, and JN analysed TNGS data; BB and SR extracted cells and DNA and performed ELISA experiments; JB performed histological analysis; FR, MCS, FM, and EL performed analysis of regulatory T cells and LRBA protein; SL and CL analysed XIAP expression; FCH, NCB, MP FR, AF, CP, FRL, and SL reviewed and analysed data; FCH, FR, MP, and NCB wrote the manuscript that was reviewed by all authors. Members of GENIUS group were in charge of the patients and acquired clinical data.

## Supplementary Data

Supplementary data to this article can be found at *ECCO-JCC* online.

## References

- Kammermeier J, Drury S, James CT, *et al.* Targeted gene panel sequencing in children with very early onset inflammatory bowel disease – evaluation and prospective analysis. *J Med Genet* 2014;51:748–55.
- Picard C, Fischer A. Contribution of high-throughput DNA sequencing to the study of primary immunodeficiencies. *Eur J Immunol* 2014;44:2854–61.
- Uhlig HH. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. *Gut* 2013;62:1795–805.
- Glocker EO, Kotlarz D, Boztug K, *et al.* Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009;361:2033–45.
- Glocker EO, Frede N, Perro M, *et al.* Infant colitis – it's in the genes. *Lancet* 2010;376:1272.
- Begue B, Verdier J, Rieux-Laucat F, *et al.* Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol* 2011;106:1544–55.
- Pigneur B, Escher J, Elawad M, *et al.* Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the Genius Working Group. *Inflamm Bowel Dis* 2013;19:2820–8.
- Verbsky JW, Chatila TA. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked [IPEX] and IPEX-related disorders: an evolving web of heritable autoimmune diseases. *Curr Opin Pediatr* 2013;25:708–14.
- Stoddard JL, Niemela JE, Fleisher TA, Rosenzweig SD. Targeted NGS: a cost-effective approach to molecular diagnosis of PIDs. *Front Immunol* 2014;5:531.
- Lo B, Zhang K, Lu W, *et al.* AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. *Science* 2015;349:436–40.
- Uhlig HH, Schwerd T, Koletzko S, *et al.*; COLORS in IBD Study Group and NEOPICS. The diagnostic approach to monogenic very early onset inflammatory bowel disease. *Gastroenterology* 2014;147:990–1007.e3.
- Nijman IJ, van Montfrans JM, Hoogstraat M, *et al.* Targeted next-generation sequencing: a novel diagnostic tool for primary immunodeficiencies. *J Allergy Clin Immunol* 2014;133:529–34.
- Yu H, Zhang VW, Stray-Pedersen A, *et al.* Rapid molecular diagnostics of severe primary immunodeficiency determined by using targeted next-generation sequencing. *J Allergy Clin Immunol* 2016;138:1142–51.e2.
- Petersen BS, August D, Abt R, *et al.* Targeted gene panel sequencing for early-onset inflammatory bowel disease and chronic diarrhea. *Inflamm Bowel Dis* 2017;23:2109–20.
- Canani RB, Castaldo G, Bacchetta R, Martín MG, Goulet O. Congenital diarrhoeal disorders: advances in this evolving web of inherited enteropathies. *Nat Rev Gastroenterol Hepatol* 2015;12:293–302.
- Malamut G, Verkarre V, Suarez F, *et al.* The enteropathy associated with common variable immunodeficiency: the delineated frontiers with celiac disease. *Am J Gastroenterol* 2010;105:2262–75.
- Flanagan SE, Haapaniemi E, Russell MA, *et al.* Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. *Nat Genet* 2014;46:812–4.
- Haapaniemi EM, Kaustio M, Rajala HL, *et al.* Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. *Blood* 2015;125:639–48.
- Milner JD, Vogel TP, Forbes L, *et al.* Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood* 2015;125:591–9.
- Kuehn HS, Ouyang W, Lo B, *et al.* Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* 2014;345:1623–7.
- Schubert D, Bode C, Kenefeck R, *et al.* Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat Med* 2014;20:1410–6.
- Alangari A, Alsultan A, Adly N, *et al.* LPS-responsive beige-like anchor [LRBA] gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. *J Allergy Clin Immunol* 2012;130:481–8.e2.
- Li H, Handsaker B, Wysoker A, *et al.*; 1000 Genome Project Data Processing Subgroup. The sequence alignment/map format and SAMtools. *Bioinformatics* 2009;25:2078–9.
- Sherry ST, Ward MH, Kholodov M, *et al.* dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 2001;29:308–11.
- Auton A, Brooks LD, Durbin RM, *et al.*; 1000 Genomes Project Consortium. A global reference for human genetic variation. *Nature* 2015;526:68–74.
- Lek M, Karczewski KJ, Minikel EV, *et al.*; Exome Aggregation Consortium. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 2016;536:285–91.



27. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7:248–9.
28. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;4:1073–81.
29. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods* 2014;11:361–2.
30. Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014;46:310–5.
31. Itan Y, Shang L, Boisson B, et al. The mutation significance cutoff: gene-level thresholds for variant predictions. *Nat Methods* 2016;13:109–10.
32. Fabre A, Breton A, Coste ME, et al. Syndromic [phenotypic] diarrhoea of infancy/tricho-hepato-enteric syndrome. *Arch Dis Child* 2014;99:35–8.
33. Salomon J, Goulet O, Canioni D, et al. Genetic characterization of congenital tufting enteropathy: epcam associated phenotype and involvement of SPINT2 in the syndromic form. *Hum Genet* 2014;133:299–310.
34. Charbit-Henrion F, Jeverica AK, Bègue B, et al.; GENIUS Group. Deficiency in mucosa-associated lymphoid tissue lymphoma translocation 1: a novel cause of IPEX-like syndrome. *J Pediatr Gastroenterol Nutr* 2017;64:378–84.
35. Moes N, Rieux-Laucat F, Bègue B, et al. Reduced expression of FOXP3 and regulatory T-cell function in severe forms of early-onset autoimmune enteropathy. *Gastroenterology* 2010;139:770–8.
36. Aguilar C, Lenoir C, Lambert N, et al. Characterization of Crohn disease in X-linked inhibitor of apoptosis-deficient male patients and female symptomatic carriers. *J Allergy Clin Immunol* 2014;134:1131–41.e9.
37. Bakhtiar S, Ruemmele F, Charbit-Henrion F, et al. Atypical manifestation of LPS-responsive beige-like anchor deficiency syndrome as an autoimmune endocrine disorder without enteropathy and immunodeficiency. *Front Pediatr* 2016;4:98.
38. Germán-Díaz M, Rodríguez-Gil Y, Cruz-Rojo J, et al. A new case of congenital malabsorptive diarrhea and diabetes secondary to mutant neurogenin-3. *Pediatrics* 2017, Jul 19. doi: 10.1542/peds.2016-2210. [Epub ahead of print.]
39. Romberg N, Al Moussawi K, Nelson-Williams C, et al. Mutation of NLR4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet* 2014;46:1135–9.
40. Coelho R, Peixoto A, Amil-Dias J, et al. Refractory monogenic Crohn’s disease due to X-linked inhibitor of apoptosis deficiency. *Int J Colorectal Dis* 2016;31:1235–6.
41. Parlato M, Charbit-Henrion F, Hayes P, et al. First identification of biallelic inherited DUOX2 inactivating mutations as a cause of very early onset inflammatory bowel disease. *Gastroenterology* 2017;153:609–11.e3.
42. Parlato M, Charbit-Henrion F, Pan J, et al. Human ALPI deficiency causes inflammatory bowel disease and highlights a key mechanism of gut homeostasis. *EMBO Mol Med* 2018; 10. doi: 10.15252/emmm.201708483.