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PRECISION MEDICINE IN PEDIATRIC INFLAMMATORY BOWEL DISEASE: AN OMICS AND NON-OMICS DATA-DRIVEN PATIENT-CENTERED TRANSLATIONAL RESEARCH APPROACH

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I am a part of all that I have met;
Yet all experience is an arch wherethro'
Gleams that untravell'd world whose margin fades
For ever and forever when I move.
How dull it is to pause, to make an end,
To rust unburnish'd, not to shine in use!
As tho' to breathe were life! Life piled on life
Were all too little, and of one to me
Little remains: but every hour is saved
From that eternal silence, something more,
A bringer of new things; and vile it were
For some three suns to store and hoard myself,
And this gray spirit yearning in desire
To follow knowledge like a sinking star,
Beyond the utmost bound of human thought.

—Alfred Tennyson, *Ulysses*

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LIST OF ABBREVIATIONS

AAF	Alternative allele frequency
ANCA	Anti-neutrophil cytoplasmic antibodies
APDS	Activated PI3Kδ syndrome
APS	Autoimmune polyendocrine syndrome
AR	Autosomic recessive
ARDS	Acute respiratory distress syndrome
ASCA	Anti-*Saccharomyces cerevisiae* antibodies
AUC	Area under the curve
BAM	Binary alignment map
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Crohn's disease
CH	Compound heterozygous
CK18	Cytokeratin 18
CLE	Confocal laser endomicroscopy
CLIA	Chemiluminescent immunoassay
COVID-19	Coronavirus Disease 2019
CP	Capping protein
CRP	C reactive protein
DAPI	4',6-diamidino-2-phenylindole
DWI	Diffusion-weighted imaging
EBV	Epstein-Barr virus
EEN	Exclusive enteral nutrition
EGDS	Esophagogastroduodenoscopy
EHR	Electronic health record
EOIBD	Early-onset inflammatory bowel disease
ERAD	Endoplasmic-reticulum-associated protein degradation
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
GC	Guanine-cytosine

GPP	Gastrointestinal pathogen panel
HPLC	High-performance liquid chromatography
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
IBDU	Inflammatory bowel disease unclassified
IOIBD	Infantile-onset inflammatory bowel disease
IP	Intestinal permeability
IQR	Interquartile range
LMR	Lactulose to mannitol ratio
LoF	Loss-of-function
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAF	Minor Allele Frequency
MIS-C	Multisystem inflammatory syndrome in children
MRE	Magnetic resonance enterography
MW	Molecular weight
NAAT	Nucleic acid amplification test
NGS	Next-generation sequencing
NLH	Nodular lymphoid hyperplasia
P/I	Phorbol 12-myristate 13-acetate and ionomycin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Triton
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH	Pleckstrin-homology
PIBD	Pediatric inflammatory bowel disease
PID	Primary immunodeficiency
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl-methylsulfonyl fluoride
PRR	Proline-rich region
RBD	Receptor-binding domain
RDT	Rapid diagnostic test
ROC	Receiver operating characteristic

RT	Room temperature
RWD	Real world data
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIPT	Sugar intestinal permeability test
TCR	T cell receptor
TEER	Transepithelial electrical resistance
Th	T helper cells
TJ	Tight junction
TNF	Tumor necrosis factor
Treg	T regulatory cells
UC	Ulcerative colitis
VCF	Variant Call Format
VEOIBD	Very early-onset inflammatory bowel disease
WB	Western blot
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild type

Introduction

Research territory, research niche, and research purpose

Research territory

Pediatric inflammatory bowel disease

Inflammatory bowel disease (IBD) is a spectrum of complex, multifactorial immune disorders characterized by chronic relapsing intestinal inflammation.[1,2] In patients with IBD prolonged, untreated gut inflammation can cause, over time, progressive irreversible structural and functional damage to the gastrointestinal tract.[1]

The proposed etiopathogenesis IBD is multifactorial. It is hypothesized that chronic intestinal inflammation results from a dysregulated gut mucosal immune response to environmental triggers and to gut microbiota in genetically susceptible individuals.[2]

IBD encompasses a continuum of overlapping phenotypes.[3] The diagnosis of ulcerative colitis (UC) and Crohn's disease (CD) are applied to differentiate the two major subtypes of IBD.[4,5] While UC affects the colon and is characterized by inflammation of the mucosal layer, CD can involve any segment of the gastrointestinal tract from the oral cavity to the anus and is distinguished by transmural inflammation. The term IBD unclassified (IBDU) is used when the inflammation is limited to the colon, with features that make the differentiation between UC and CD uncertain even after a complete workup.[6] Nonetheless, current classification tools are suboptimal to capture the disease heterogeneity of IBD.[3]

IBD may present at any age, but peak incidence is in teenagers and young adults. Overall, IBD develops during childhood or adolescence in 20-25% of patients.[4,7] The increasing understanding of age-specific characteristics of IBD has led to the introduction of different subgroups of IBD according to the age at diagnosis: neonatal IBD (onset within first 28 days of age), infantile (and toddler) onset IBD (IOIBD; less than 2 years of onset), very early-onset IBD (VEOIBD; less than 6 years of age IBD-onset), early-onset IBD (EOIBD; A1a Paris Classification; less than 10 years of age-onset), pediatric IBD (PIBD; A1a and A1b Paris Classification; less than 17 years of age-onset) and adult-onset IBD (17 years and older; A2 and A3 Paris Classification).[7,8]

From a clinical point of view, pediatric-onset IBD differs from adult-onset disease in several aspects. Patients with pediatric-onset IBD often present a delay in growth and puberty and are more likely to have extensive disease, to progress over time, to receive a diagnosis of IBDU, and to develop extraintestinal manifestations. Moreover, these patients tend to have more severe disease and are more likely to develop complicated disease, according to some studies, but true prospective data are lacking.[3]

From an etiological point of view, the relative contribution of genetic factors seems to be inversely related to the age of onset of IBD. While conventional IBD exhibits a polygenic architecture, with a large number of common genetic variants (>1% allelic frequency in the general population) contribute to disease susceptibility, Mendelian or monogenic forms of IBD are caused by rare high penetrance variants in a single gene. Monogenic disorders presenting with IBD-like intestinal inflammation are more highly represented in patients presenting with VEOIBD as compared with IBD diagnosed at an older age.[7–14] A recent study identified rare variants in genes previously linked to monogenic IBD in 3% of patients with PIBD, that is 7.8% of subjects in the subgroup of VEOIBD, in contrast to 2.3% of children diagnosed with IBD after 6 years of age.[15]

To date, more than 75 genes associated to monogenic disorders presenting with IBD-like intestinal inflammation have been identified and this number is constantly increasing. Monogenic defects are involved in intestinal immune-epithelial homeostasis and include many primary immunodeficiencies, as well as intestinal epithelial cell defects. Despite mimicking IBD, many of these disorders are presumably a different entity along the IBD heterogeneity axis.[7,8,15]

Next-generation sequencing (NGS) technologies, including whole exome sequencing (WES) and whole genome sequencing (WGS) have been increasingly used in the research and in the clinical setting to screen for monogenic disorders associated with IBD and to identify novel causal genetic variants once the known disease-causing ones have been ruled out.[7,8]

Genetics cannot explain the drastic increase in the incidence of IBD reported worldwide,[16,17] which indicates an influence of the environmental exposures associated with Western lifestyle, urbanization, and industrialization on the risk of IBD in genetically susceptible individuals.[17] While newly industrialized countries are currently in the "Acceleration in Incidence stage", during which incidence rises and prevalence is still relatively low, Western regions are in the "Compounding Prevalence stage", during which incidence is stable, but prevalence is rising steeply.[16]

Since 2000 the incidence of pediatric CD across Europe has varied from 0.3 to 10 per 100,000 population, while the incidence of pediatric UC has ranged from 0 to 9.5 per 100,000. Prevalence reported for pediatric CD ranged from 8.2 to approximately 60 per 100 000 and, for UC, from 8.3 to approximately 30.[18] While most studies reported stable or decreasing incidence of IBD in the Western world across all ages, many researchers have found that the incidence of IBD in pediatric age has increased in the Western world over the last two decades.[18–21] This increase in incidence mainly affects young children, particularly under 6 years of age. In Canada and northern Europe VEOIBD represents approximately 25% of all PIBD and the fastest growing group of newly diagnosed patients of any age.[14,19,20,22–26]

A true rise in the incidence of a disease must be the result of exposure to environmental determinants.[16]

Furthermore, epidemiological studies have shown that the onset of IBD does not seem to be driven by ancestry or ethnicity, but rather by the environment. In fact, children of individuals who immigrated from developing countries with a low IBD prevalence to the Western world assume the same risk of IBD as the base population.[27]

Finally, the immune divergence between genetically identical twins during ageing is consistent with a major role for non-heritable factors, although some epigenetic changes could also contribute. Moreover, immunological parameters become more variable with age, suggesting a cumulative influence of environmental exposure, including the many different microbes encountered during lifetime.[28]

Translational research and precision medicine and in IBD

Translational medicine is a rapidly evolving area of biomedical research that aims to facilitate the transfer of scientific discoveries from research into clinical practice.[29]

Traditional, "forward" translational medicine purpose is to improve human health using a "benchtop-to-bedside" approach, through identification of therapeutic targets, discovery and optimization of drugs, and development of new biomarkers. On the other hand, reverse translation, also called "bedside-to-benchtop" research, begins with real-life patients, and works backward to uncover the mechanistic basis for clinical observations, to achieve a deeper understanding of the disease.[30,31]

Despite the advances made in recent years in the knowledge of IBD pathogenesis, translating this understanding into personalized clinical decisions remain an unmet need. As the determinants of disease outcome and treatment response are largely unknown, IBD management still employs "a one-size-fits-all approach" designed for the "average patient".[32,33]

Recently, there has been an increasing drive towards personalized or precision medicine across all fields.[33] Precision medicine refers to the tailoring of medical treatment to the individual characteristics patients and relies on the ability to classify individuals into subpopulations that differ in disease biology, response to treatment, or prognosis.[34] In complex diseases, combinations of various multi-omics datasets through multi-layered analyses could benefit both the individual, by delivering better, more personalized care, and the population health, by accurately identifying and stratifying population cohorts.[35]

The aim of precision medicine in IBD is to deliver truly individualized care so that the entire patient journey from diagnosis to treatment is based on the specific biology underlying IBD in the individual patient.[36]

IBD pathogenesis is multifactorial, therefore a systems biology approach aiming to integrate biological omics and non-omics datasets through bioinformatic tools and artificial intelligence-based systems could help to resolve the disease etiopathology and define its endotypes, to establish new biomarkers, and to develop the best interventional strategies.[37]

The rapid technological development that has characterized biomedical research in the last decades has led to a significant increase in data availability.[29]

As for omics data, the development of high throughput technologies has led to the increasing availability of various omics platforms, including genomics, transcriptomics, proteomics, microbiomics, metabolomics, lipidomics, and others.[32,33,37]

As for non-omics data, the growing number of variables on which the clinical decisional process relies require to be collected, categorized, and analyzed using appropriate informatic tools. Alongside traditional sources of data, such as electronic health record (EHR) archiving systems, there is great interest in real world data (RWD), harvested from real life contexts, and in deep phenotypic characterization in research cohort settings through recall of participants, to address specific hypothesis and understand disease endotypes.[29,38]

Reclassifying IBD molecularly in combination with revised phenotyping should be one of the key priorities in the future and should lead to tailored treatment algorithm for every individual suffering from IBD.[3]

Research niche

Forecasting models predict that Western countries would reach a prevalence value of 1% by 2030, highlighting a significant impending challenge for health-care systems.[16,27]

Moreover, IBD is an extraordinarily complex disease; it affects a wide diverse population and has variable and unpredictable responses to any type of intervention. Regardless of the drugs used, approximately one-third of patients are primary non-responders to initial treatment, and half of patients lose response over time, indicating that a therapeutic ceiling has been reached.[32]

The disease heterogeneity within IBD, particularly according to age of onset, is suggestive of differences in the relative contributions of genetics, host immune system, environmental factors, and gut microbiota composition, giving rise to different pathways that ultimately lead to diverging phenotypes.[14]

To address the increasing disease burden of IBD, to face its high heterogeneity, and to break its current therapeutic ceiling novel, multifaceted innovations in health-care delivery will be required.

Among them, a precision medicine approach holds the most promise.

A proposed model for addressing the knowledge gap in IBD in order to unravel its biological complexity and to define personalized treatment options is to implement an integrative analysis of all types of omics and non-omics datasets generated from many patients at different time points. With a systems biology approach, heterogenous population of patients with IBD could be profiled by multi-omics techniques to be used alongside accurate and extensive clinical phenotyping data to generate signatures in sizeable subgroups of phenotypically homogeneous subjects. These disease signatures could pave the ways towards personalized IBD treatment, based on the underlying disease biology of the patient, to ensure that he receives the therapies that are most suitable for him.[29,32,33,37]

This unbiased and comprehensive strategy will help to develop personalized interventional strategies in IBD patients, to uncover disease-specific pathways, and to discover biomarkers that can characterize IBD in a subtype-specific manner and can be non-invasive, highly specific, reliable, and easy to assess in routine practices.[37]

The final objective will be to obtain the most detailed characterization of each patient identifying genetic and molecular singularities through omics technologies to enable delivery of truly individualized IBD care.

Research purpose

The general objective of this PhD project was to develop new tools or combining existing ones to stratify patients based on their disease risk, likely treatment response, or prognosis and to individualize pediatric IBD care, allowing precise diagnosis, monitoring and treatment.

In particular, the two main aims were:

1. To identify, validate, and characterize new variants of known or novel candidate genes implicated in pediatric IBD pathogenesis.
2. To establish diagnostic-therapeutic clinical pathways and biomarkers that could help to manage pediatric IBD patients.

The PhD project revolved around the collection and analysis of phenotypic and genotypic data from pediatric IBD patients from our tertiary referral center, the University Hospital of Padova. In particular, the main activities carried out included:

- Integration of phenotypic and genotypic data from pediatric IBD patients, particularly those with VEOIBD or EOIBD who underwent WES for suspected or proven monogenic IBD, to individualize patient care and to discover relevant and actionable pathological pathways of IBD.
- Retrospective analysis of clinical, laboratory and radiological features of pediatric IBD, and subsequent patient stratification to improve patient care, such as to predict disease progression and treatment response. Anonymized data from IBD patients followed at our hospital were also included in the national pediatric IBD disease register and used to participate to multicenter retrospective studies coordinated by SIGENP (Italian Society of Pediatric Gastroenterology Hepatology and Nutrition).

This PhD thesis consists of a compendium of four separate, already published or publishable, scientific papers. The four parts are each free standing, in the sense that each can be read and understood independently. Each individual sub-project contributes somehow to the individual profiling of IBD patients, to match them to the most appropriate management based on the likelihood of response. All the papers contribute to the purpose of this work, that is to tailor medical care to the individual characteristics of each IBD patient.

Aim 1

To identify, validate, and characterize new variants of known or novel candidate genes implicated in pediatric IBD pathogenesis

PART 1.1

Study of the role of variants of *CARMIL2* gene in the susceptibility to pediatric IBD

Bosa L, Batura V, Colavito D, Fiedler K, Gaio P, Guo C, Li Q, Marzollo A, Mescoli C, Nambu R, Pan J, Perilongo G, Warner N, Zhang S, Kotlarz D, Klein C, Snapper SB, Walters TD, Leon A, Griffiths AM, Cananzi M, Muise AM. Novel CARMIL2 loss-of-function variants are associated with pediatric inflammatory bowel disease. Sci Rep. 2021 Mar 15;11(1):5945. doi: 10.1038/s41598-021-85399-9. PMID: 33723309; PMCID: PMC7960730.

Abstract

Background

CARMIL2 is required for CD28-mediated co-stimulation of NF-κB signaling in T cells and its deficiency has been associated with primary immunodeficiency and, recently, very early-onset inflammatory bowel disease (VEOIBD). Monogenic IBD are Mendelian disorders caused by rare genetic variants with a large effect on gene function that alter intestinal immune homeostasis. The objectives of this study were to: 1) Identify pediatric patients with *CARMIL2* variants and report their phenotype; 2) Confirm the pathogenicity such variants; 3) Establish a pragmatic model of patient-centered reverse translation in monogenic IBD research.

Methods

Whole exome sequencing (WES) was performed in the index case, who presented at our hospital with IBD-like pancolitis. Segregation analysis was conducted in his family. Subsequently, a screening for *CARMIL2* variants was performed interrogating WES data from a large cohort of pediatric IBD patients enrolled at SickKids, Toronto. Bioinformatic analysis pipeline was applied, and resulting *CARMIL2* variants were prioritized using population, computational, and functional data. Pathogenicity of filtered variants was investigated with immunostaining of patients' intestinal biopsies, analysis of expression of *CARMIL2* variants in transfected cells with Western Blot (WB) and immunofluorescence staining, and NF-κB luciferase reporter assay.

Results

Novel biallelic *CARMIL2* variants were identified in three patients presenting with pediatric-onset IBD and in one patient with autoimmune polyendocrine syndrome (APS). None manifested overt clinical signs of immunodeficiency before their diagnosis. The first patient presented with very early onset IBD (VEOIBD). His brother was found homozygous for the same *CARMIL2* null variant and diagnosed with APS. Two other IBD patients were found homozygous for a nonsense and a missense *CARMIL2* variant, respectively, and they both experienced a complicated postoperative course marked by severe infections. Immunostaining of bowel biopsies showed reduced CARMIL2 expression in all the three patients with IBD. WB and immunofluorescence of transfected cells revealed an altered expression pattern of the missense variant, which was also associated with reduced NF-κB promoter activation.

Conclusions

Our work expands the genotypic and phenotypic spectrum of CARMIL2 deficiency, which can present with either IBD or APS, aside from classic immunodeficiency manifestations. *CARMIL2* gene should be included in the diagnostic work-up of patients with suspected monogenic IBD. This collaborative research project represented an example of patient-centered forward and reverse translation medicine and has led to the creation of a pragmatic model of translational research in monogenic IBD.

Background

CARMIL2

Capping Protein Regulator and Myosin 1 Linker 2 or *CARMIL2* gene (also known as RGD Motif, Leucine Rich Repeats, Tropomodulin Domain And Proline-Rich Containing or RLTPR) is a gene located on chromosome 16 that encodes a member of the CARMIL (capping protein, Arp2/3, myosin-I linker) family of proteins.

This protein, known as CARMIL2, is a cell membrane-cytoskeleton-associated protein expressed in many cell types, including bone marrow and lymphoid tissue, endocrine glands, and the gastrointestinal tract (**Figure 1**).

CARMIL2 regulates actin polymerization at the barbed end of actin filaments. By preventing activity of F-actin heterodimeric capping protein (CP), CARMIL2 generates uncapped barbed ends and, in so doing, enhances actin polymerization. This process is important to regulate a variety of cell functions related to membrane-associated actin assembly and signaling, such as formation of cell protrusion, cell polarity, lamellipodial assembly, membrane ruffling, macropinosome formation, cell migration, and invadopodia formation during wound healing.[39–43]

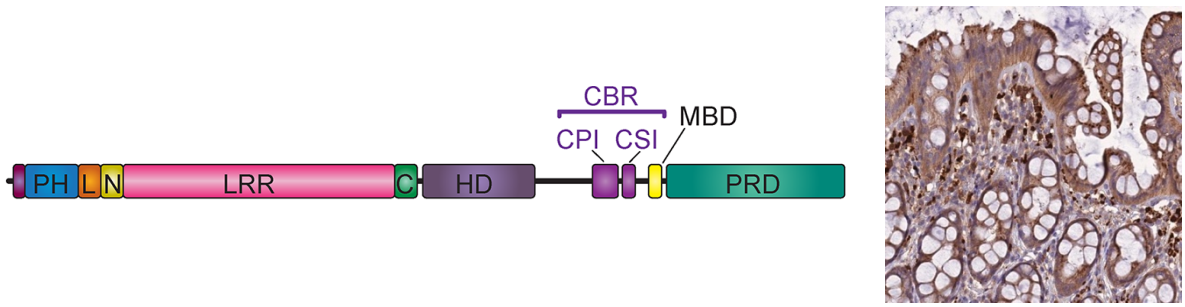


Figure 1. *CARMIL family protein domain architecture and immunohistochemical staining for CARMIL2 of healthy human rectum (Sigma, HPA041402), that shows moderate cytoplasmic positivity in glandular cells.*[43] *PH: Pleckstrin-Homology domain; L: Linker domain; N: N-cap; LRR: Leucine-Rich Repeat domain; C: C-cap sequence; HD: Helical Dimerization domain; CBR: Capping Protein-Binding Region; CPI: Capping Protein Interaction motif; CSI: CARMIL-Specific Interaction motif; MBD: Membrane-Binding Domain; PRD: Proline-Rich Domain.*

Independently of its actin-uncapping function, CARMIL2 is required for CD28-mediated co-stimulation of NF-ϰB signaling in T cells, which is important for naive T cells activation, maturation into T memory cells, and differentiation into T helper (Th) and T regulatory (Treg) cells. CARMIL2 has been shown to act as a scaffold, bridging CD28 to the CARD11/CARMA1 cytosolic adaptor and to the NF-ϰB signaling pathway (**Figure 2**). Finally, CARMIL2 has a role in antigen-receptor signaling in B cells, leading to NF-ϰB activation after B cell receptor (BCR), but not CD40, ligation.[44]

Interestingly, the actin-uncapping function of CARMIL2 has been proven dispensable for CD28 co-stimulation in both mouse and human. In contrast, the leucine-rich repeat (LRR) domain, the noncanonical pleckstrin-homology (PH) domain, and the proline-rich region (PRR) are mandatory for the task.

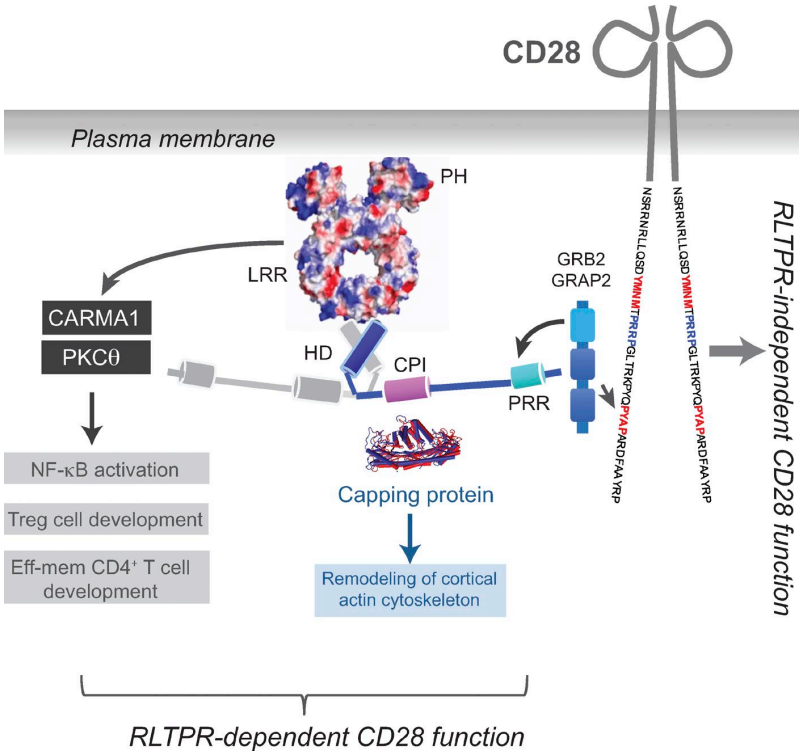


***Figure 2**. The function of CARMIL2/RLTPR in T cells. CARMIL2 is required for coupling CD28 to PKC-θ and CARMA-1, two cytosolic effectors of the CD28 signaling pathway. CARMIL2 exists as constitutive dimer. Its Capping Protein Interaction (CPI) motif is constitutively associated with a capping protein, conferring it actin-uncapping properties. As a result, CARMIL2 molecules are thought to remodel actin cytoskeleton upon T cell activation.*[45]

Experimental models have shown that complete absence of RLTPR had a strong impact on Treg cells and effector memory CD4+ T cell development. On the contrary, in mouse model RLTPR did not play any detectable role in BCR-mediated signaling and T cell-independent B cell responses.[44,45]

CARMIL2 and immunodeficiency

CARMIL2 variants have been implicated in human disease.

CARMIL2 deficiency was initially associated with an autosomal recessive primary immunologic disorder, called Immunodeficiency-58 (IMD58, MIM: 618131), characterized by recurrent and/or chronic bacterial, viral, and fungal infections, cutaneous manifestations including eczematous dermatitis, and disseminated Epstein-Barr virus (EBV)-associated smooth muscle tumors. Immunologic analysis showed defective T-cell function with decreased Treg and deficient CD3/CD28 co-stimulation responses in both CD4+ and CD8+ T cells. B-cell function could also be impaired.[44,46–48]

Wang et al.[44] reported 6 patients from 3 unrelated families with skin and pulmonary allergy and a variety of bacterial and fungal infectious diseases, including invasive tuberculosis and mucocutaneous candidiasis.

Sorte et al.[46] described 4 patients from 3 Norwegian families with skin phenotype of warts, molluscum contagiosum and dermatitis since early childhood and various other immunological features.

Schober at al.[47] reported 4 patients with EBV+ disseminated smooth muscle tumors, skin manifestations, recurrent infections, and chronic diarrhea.

Alazami et al.[48] described 7 patients from 3 unrelated consanguineous multiplex families with dermatitis, esophagitis, and recurrent skin and chest infections with evidence of combined immunodeficiency; one patient had severe colitis with crypt abscesses.

CARMIL2 and VEOIBD

Two recent papers[49,50] have reported for the first time patients with CARMIL2 deficiency presenting with VEOIBD or with an IBD-like inflammatory gastrointestinal disorder.

Magg et al.[49] reported 5 patients from 3 unrelated kindred with IBD-like phenotypes and suspected underlying primary immunodeficiency (PID). WES revealed 3 novel *CARMIL2* loss-of-function (LoF) mutations. T cells immunoblotting showed abrogated CARMIL2 protein expression. Immunophenotyping of peripheral blood mononuclear cells (PBMC) indicated reduction of regulatory and effector memory T cells and impaired B cell class switching, while T

cell proliferation and activation assays confirmed defective responses to CD28 co-stimulation, consistent with CARMIL2 deficiency. Moreover, RNAscope analysis on intestinal biopsies showed reduced frequency of CD4+FOXP3+ cells.

Kurolap et al.[50] described a boy with severe infantile-onset colitis and eosinophilic gastrointestinal disease, with a homozygous missense variant of *CARMIL2*. Interestingly, there was no evidence of recurrent or severe infections. CARMIL2 protein levels were reduced in patient's cells compared with controls. Immunological studies revealed reduced Treg and impaired *in vitro* Treg generation and CD4+ T cell proliferation. The patient was, therefore, the first reported case of CARMIL2 deficiency with intestinal inflammation but without clinical signs of immunodeficiency.

Objectives of the study

The objective of this project was to better understand the role of the variants of the gene *CARMIL2* in the susceptibility to pediatric IBD, to expand the phenotype spectrum of CARMIL2 deficiency, and to develop a collaborative reverse translation approach for the diagnosis and the study of monogenic IBD.

Three specific objectives were established:

1. To identify pediatric patients with *CARMIL2* variants and to report their phenotype.
2. To confirm the pathogenicity of newly discovered *CARMIL2* variants.
3. To establish a pragmatic model of patient-centered reverse translation in monogenic IBD research.

Methods

Helsinki guidelines

All human experiments followed the Helsinki Guidelines. Informed consent was obtained from the participants parents and the study had local ethics board approval at The Hospital for Sick Children (SickKids), Toronto, Canada (Research Ethics Board: REB1000024905).

Patients

Patient 1 and his brother, Patient 2, were recruited from the Pediatric Gastroenterology, Hepatology and Liver Transplantation Unit at the Pediatric Clinic of the University Hospital of Padova.

Patient 3 and Patient 4 were discovered through screening of a large cohort of pediatric IBD patients recruited at SickKids as previously described.[15] Children (age <18) undergoing evaluation for IBD were enrolled over a 13-year period (2003-2015). Diagnosis of IBD was made according to the recommendations of the Porto criteria.[51] Patients with known primary immunodeficiency, chromosomal abnormalities, syndromic disease, or diagnosed with other forms of monogenic intestinal disease were excluded. After obtaining the caregivers' consent, clinical data were recorded, and biological samples were processed and stored in a biobank. WES analyses of blood samples were thus carried out as detailed below.

For Patient 1 and Patient 2, the phenotype review was carried out at the University Hospital of Padova using EHR software Galileo (NoemaLife, Dedalus).

For the SickKids cohort, following variant prioritization,[52] each patient deemed to have pathogenetic biallelic *CARMIL2* variants was reversed phenotyped, using clinical data from databases and EHR systems.

For all IBD patients the phenotype was classified according to the pediatric modification of the Montreal classification, also known as Paris classification.[4]

Whole exome sequencing

For Patient 1 and 2, WES and segregation analysis were performed. After obtaining informed consent, genomic DNA was extracted and purified from a sample of whole blood. DNA was prepared for sequencing with the kit SureSelectXT All Exon V5 kit (Agilent). Exome sequencing was performed with NGS technology on the Illumina HiSeq 2500 platform, using paired-end 100

bp read. DNA sequences were mapped and analyzed using as reference genome assembly GRCh38 (Genome Reference Consortium Human Build 38).

For the SickKids cohort, banked genomic DNA isolated from whole blood collected using a Qiagen Puregene Blood Core Kit was processed for exome capture using the NimbleGen VCRome 2.1 design. Captured libraries were sequenced on the Illumina HiSeq 2500 platform using paired-end 75 bp reads at the Regeneron Genetics Center (RGC), Tarrytown, NY, USA. Exome sequencing coverage was 30X or greater for >85% of the bases targeted.

Bioinformatic analysis

For Patient 1, a bioinformatic analysis of exons and close intronic portions of 79 genes associated with IBD, hyperinflammatory and autoinflammatory disorders, immunoregulation disorders, phagocytosis defects and neutropenia, T and B lymphocytes defects, apoptosis defects, and epithelial barrier defects was performed (**Table 1**). Genetic variants were analyzed and compared with several population databases (Exome Aggregation Consortium [ExAC], Genome Aggregation Database [gnomAD], 1000 Genomes database, Exome Variant Server [EVS]) and genetic clinical databases (OMIM, ClinVar, HGMD, GWAS, PGKB, Cosmic). Every variant was also evaluated *in silico* for its possible effects on protein structure or function (using Polyphen2, SIFT, VAAST Variant Prioritizer [VVP], Combined Annotation Dependent Depletion [CADD], Mutation Assessor, Mutation Taster) and for evolutionary conservation (phyloP score).

For the SickKids cohort, WES data from 2307 participants (1005 index patients and 1302 parents and siblings) were analyzed using the FORGE (Finding of Rare Disease Genes) pipeline. Raw sequencing reads were aligned to human reference genome (GRCh38/hg38) using BWA-mem (Burrows-Wheeler Aligner, ver. 0.7.12), followed by indel realignment using Genome Analysis Toolkit (GATK, ver. 3.5). Five variant callers (GATK HaplotypeCaller ver. 3.5, Vardict ver. 1.4.6, Varscan ver. 2.3.9, Samtools ver. 1.3, and Freebayes ver. 1.0.0) were run on the Binary Alignment Map (BAM) files of each family to produce family based Variant Call Format (VCF) files when at least 2 of the 5 agreed on a called variant. Inheritance modeling on family level VCF files was performed using the GEMINI tool to query for rare (Minor Allele Frequency [MAF] <0.01) protein coding variants that fit autosomal recessive, compound heterozygous, *de novo*, autosomal dominant, and X-linked inheritance models filters. VarSeq software (Golden Helix) was used to import, filter, and do inheritance modeling on the variants from each trio. Common variants (MAF >0.01), defined using publicly available variant databases (ExAC frequency database [ver. 0.3], 1000 Genomes database [phase 1], and the NHLBI Exome Sequencing Project V2 Exome Variant Frequencies), were filtered out. Variants were further classified according to

whether they were deemed to be coding. Non-synonymous and unclassified variants were then scored using the database for non-synonymous functional predictions (dbNSFP 2.8), filtering out variants with CADD score <10 or no other damaging score (Polyphen2, SIFT, LRT, Mutation Assessor, Mutation Taster, Functional Analysis through Hidden Markov Models [FATHMM], Protein Variation Effect Analyzer [PROVEAN]). Variants were also evaluated *in silico* for protein domains in which they were predicted to be located (using Uniprot database and Simple Modular Architecture Research Tool or SMART database) and for evolutionary conservation of corresponding amino acid (based on Aminode webtool, available at http://www.aminode.org).[53]

Interpretation of all detected *CARMIL2* variants was performed according to the American College of Medical Genetics guidelines.[54]

Biological Category	Genes
Hyper- & Auto- inflammatory disorders	*MEFV, MVK, NLRC4, NLRP12, PLCG2, SH2D1A*
Immunoregulation disorders	*AIRE, FOXP3, HPS1, HPS4, HPS6, IL10, IL10RA, IL10RB, IL2RA, STAT1, STXBP2, XIAP*
Phagocyte defects	*CYBA, CYBB, G6PC3, ITGB2, NCF2, NCF4, SLC37A4*
T- and B-cell defects	*ADA, AICDA, BTK, CD3G, CD40LG, COG6, CTLA4, DCLRE1C, DKC1, DOCK8, ICOS, IL21, IL2RG, LCK, LIG4, LRBA, NFKBIA, PIK3R1, RAG1, RAG2, RTEL1, TNFRSF13B, WAS, ZAP70*
Apoptosis defects	*CASP8, MASP2, TRIM22*
Epithelial barrier defects	*ADAM17, COL7A1, EGFR, EPCAM, FERMT1, IKBKG, TGFBR1, TGFBR2,*
Others	*ABCB1, ANKZF1, ARPC1B, ATG16L1, CD19, DUOX2, GUCY2C, IFIH1, IL23R, IL6, IL7R, IRF5, IRGM, NFAT5, NOD2, NOX1, RLTPR, TTC37, TTC7A*

Table 1. *Exome-based genetic testing performed for Patient 1.*

Sanger sequencing

Sanger sequencing was performed to confirm the mutations identified with WES.

For Patient 1, Patient 2 and their family, primers were designed to select and amplify through polymerase chain reaction (PCR) the region containing the reported mutation within the genomic DNA. PCR products were then sequenced using cycle sequencing Big Dye Terminator ver. 3.1 (Applied Biosystems) and ABI 3100 Avant automated capillary electrophoresis sequencer (Applied Biosystems).

For candidate patients identified within the SickKids pediatric IBD cohort, sequencing was carried out at The Centre for Applied Genomics (TCAG, The Hospital for Sick Children, Toronto,

Canada), that provides high-quality capillary-based fluorescent sequencing on dual ABI 3730XL instruments. Different sets of primers were used to troubleshoot sequencing reactions.

Immunofluorescence histochemical staining of bowel biopsies

Bowel samples were fixed in neutral buffered formalin without methanol and embedded in paraffin using routine protocols (formalin-fixed paraffin-embedded, FFPE).

Tissue samples, including normal control and IBD control, were retrieved from the Unit of Anatomical Pathology, Medicine Department, University Hospital of Padova and from the Division of Pathology, The Hospital for Sick Children, Toronto. Only FFPE with well-preserved tissue architecture were chosen, to avoid false negative staining.

As negative control and disease control, a non-IBD patient sample and an unrelated IBD patient sample were used, respectively.

Immunofluorescent histochemical staining was performed on sigmoid FFPE sections as previously described.[55] Briefly, paraffin-embedded sections were deparaffinized using xylene and rehydrated with different percentages of ethanol. Antigen retrieval was achieved with high-pressure cooking in EDTA-borax buffer made with 1 mM EDTA, 10 mM borax, 10 mM boric acid and 0.001% ProClin 300 (Supelco) at pH 8.5. To block non-specific staining, the slides were incubated for 1 h at room temperature (RT) in 4% bovine serum albumin (BSA) and 20% donkey serum in phosphate-buffered saline (PBS).

Slides were incubated with primary antibodies, including anti-CARMIL2 antibody produced in rabbit (Sigma, HPA041402) and anti-cytokeratin 18 mouse monoclonal antibody (Abcam), overnight at 4°C. On the following day, stained slides were washed 3 times for 5 min with PBS. Secondary antibodies, namely Rhodamine Red-X (RRX) Affin- iPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories) and Fluorescein (FITC) AffiniPure F(ab')2 Fragment Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories), were incubated at RT in darkness for 2 h, then slides were washed 3 times for 10 min in darkness. RedDot2 Far-Red Nuclear Stain (Biotium) was used for nuclear counterstaining at a dilution of 1:200. Finally, sections were mounted overnight with Vectashield antifade mounting medium (Vector Laboratories).

Immunostained slides were imaged using a Leica confocal laser scanning microscope (Leica, TCS-SP8) and LAS-AF software (Leica Microsystems). Image processing, including color resolution, color separation, and merging of fields, was done using Adobe Photoshop CS5 software (Adobe Systems Incorporated).

Plasmids

Plasmid containing an insert with human *CARMIL2* sequence with 3 tandem FLAG epitopes (DYKDDDDK) on N-terminus was a gift from John Cooper (Washington University, St. Louis, Addgene plasmid #118740).[41]

Mutations of *CARMIL2* were generated using site-directed mutagenesis by ACGT Corp. (Toronto, Canada).

Plasmid were expanded through transformation and harvest from *Escherichia coli* DH10B bacteria as per standard protocols and purified using EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic, BS614) and PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Thermo FisherScientific, K210007).

Positive control plasmid FLAG-TRAF6 was provided by Muise laboratory (SickKids).

For luciferase reporter assay, NF-κB luciferase reporter plasmid and pRL-TK (*Renilla luciferase*) reporter plasmid were obtained from Muise laboratory.

Cell culture and transfection

HEK293T cells and HCT116 cells were provided by Muise laboratory and maintained in DMEM (Wisent Inc.) containing 10% of heat-inactivated fetal bovine serum (FBS) and antibiotic-antimycotics at 37°C in 5% CO_2. Jurkat cells (provided by Muise laboratory) were cultivated in RPMI (Wisent Inc) containing 10% of heat-inactivated FBS and antibiotic-antimycotics at 37°C in 5% CO_2.

For western blot, HEK293T cells were grown in a Falcon 6-well plate (Corning) to reach 60-70% confluence and transiently transfected with 1 μg of construct DNA (3xFLAG-CARMIL2 wild type [WT] or mutation, or FLAG-TRAF6 as positive control) per each well using PolyJet (SignaGen Laboratories) transfection reagent, according to the manufacturer's instructions. Cells were collected for lysis and protein analysis 48 h after transfection.

For immunofluorescence, HEK293T cells and HCT116 cells were transiently transfected for 48 h as described above using 0.5 μg of construct DNA (3xFLAG-CARMIL2 WT or mutation) per well. Jurkat cells were transfected through nucleofection using Nucleofector II system (Amaxa), according to the manufacturer's instructions. After Jurkat cells number and viability had been assessed (Countess II Automated Cell Counter, Thermo Fisher Scientific), approximately $1x10^6$ cells were isolated and nucleofected with 2 ug of construct DNA (3xFLAG-CARMIL2 WT or mutation) selecting program X-005.

For luciferase reporter assay, HEK293T cells were seeded on a Falcon 24-well plate and transfected the following day at 60-70% confluency using PolyJet (SignaGen Laboratories) transfection reagent, according to the manufacturer's instructions. Each well was transfected with 250 ng of NF-κB luciferase reporter plasmid, 250 ng of pRL-TK reporter plasmid to normalize for transfection efficiency, and 500 ng of either construct DNA (3xFLAG-CARMIL2 WT or mutation) or empty control plasmid, to ensure that each transfection employed the same amount of total DNA.

Western blotting

Cells were lysed for 20 min in RIPA buffer (Sigma) supplemented with 1 mM phenyl-methylsulfonyl fluoride (PMSF), 500 uM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na3VO4), 1:500 Protease Inhibitor Cocktail (Sigma-Aldrich, P2714), and 1% Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, P0044). Each sample was sonicated with 5 pulses at 30% amplitude (Q125 Sonicator, Qsonica) and centrifuged 20 min at 4°C (Eppendorf Centrifuge 5430 R, Eppendorf).

Protein concentration in lysate was measured using the Bradford assay and a BSA protein standard in water (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad; Epoch Microplate Spectrophotometer, BioTek). Samples were resuspended in 1xSDS (sodium dodecyl sulfate) protein sample buffer (40% glycerol, 240 mM Tris/HCl, 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol) and a volume corresponding to 40 ug of protein per sample was loaded onto a 4-20% gradient gel (Mini-PROTEAN TGX Gels, Bio-Rad).

Gel electrophoresis was performed (Mini-PROTEAN Tetra Vertical Electrophoresis Cell and PowerPac HC High-Current Power Supply, Bio-Rad) in running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3) for approximately 1 h at 200 V. Sample was then transferred to a nitrocellulose membrane (Amersham Protran, GE Healthcare) using a semi-dry blotting process (Trans-Blot Turbo Transfer System, Bio-Rad) in transfer buffer (25 mM Tris base, 192 mM glycine, 0.00375% SDS, 20% (v/v) methanol, pH 8.3) for 12 min at 25 V and RT. Membrane was blocked using 5% skim milk in PBST (PBS with Triton X-100 0.05%) for 1 hour at RT.

The immunoblot was then incubated with appropriate primary and secondary antibodies diluted in blocking buffer, overnight at 4°C and for 1 hour at RT, respectively. Primary antibodies included monoclonal anti-FLAG M2 antibody produced in mouse (Sigma-Aldrich, F3165), anti-CARMIL2 antibody produced in rabbit (Sigma, HPA041402), and monoclonal anti-β-Actin antibody produced in mouse (Sigma-Aldrich, A5441). Secondary antibodies included Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, 115-035-146),

Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, 115-035-003), and Peroxidase IgG Fraction Monoclonal Mouse Anti- Rabbit IgG light chain specific (Jackson ImmunoResearch Laboratories, 211-032-171). After incubation with each antibody, membranes were washed 3 times with PBST for 5-10 min.

Blots were imaged using chemiluminescent horseradish peroxidase detection reagent (Immobilon Forte Western HRP substrate, MilliporeSigma or Clarity Max Western ECL Substrate, Bio-Rad) and imaged through chemiluminescence detection (Chemi-Doc MP Imaging System and Image Lab software, Bio-Rad). To re-examine the same protein sample with different antibodies, stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, 21059) was added for 10 min. Membrane was then washed in PBST for 3 times and re-blocked.

In total, the experiment was repeated 3 times.

An online tool (available at https://www.bioinformatics.org/sms/prot_mw.html) was used to calculate the predicted molecular weight (MW) of each CARMIL2 variant, taking into account the size of the protein tag (3xFLAG).

Immunofluorescence staining of transfected cells

For immunofluorescence, HEK293T, HCT116, and Jurkat cells were cultured as described above and seeded at low density on cover slips (Fisherbrand) coated with poly-d-lysine (Sigma) onto Falcon 24-well plates (Corning). HEK293T and HCT116 cells were transfected 1 hour after seeding, as previously explicated and left for 48 hours. Jurkat cells were seeded 48 hours after being nucleofected as described earlier. To promote their attachment to cover slips, they were centrifuged inside the plate 2 minutes at 1000 rpm (Centrifuge 5810 R, Eppendorf) and let settle 30 minutes at 37°C.

After 48 h, cells where fixed in methanol-free 4% paraformaldehyde (PFA) for 30 min, then washed three times with PBS. For selective plasma membrane labeling, cells were incubated for 10 minutes at RT with Wheat Germ Agglutinin, Alexa Fluor 647 Conjugate (Invitrogen, Thermo Fisher Scientific, W32466) diluted (1:200) in PBS, then washed two times with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 10% goat serum in PBS for 30 min at RT.

Cells were incubated with the primary antibody, monoclonal ANTI-FLAG M2 antibody produced in mouse (Sigma, F1804) diluted in 10% goat serum PBS (1:200) for 1 hour at RT. After washing 3 times with PBS-Tween (0.05%), cells were incubated with Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 Conjugate (Invitrogen, Thermo Fisher Scientific, A-11031) diluted in 10% goat serum PBS (1:200) for 45 min at RT, then washed again

3 times. 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS (1:2500) was used for nuclear staining, while actin was assessed via phalloidin staining (ActinGreen 488 Ready- Probes reagent, according to the manufacturer's instructions); they were incubated together 10 min at RT.

Cover slips were then washed with PBS and mounted onto glass slides (Fisherbrand) with mounting medium (Dako Fluorescent Mounting Medium, Agilent, S3023).

Slides were imaged using a quorum spinning disk confocal microscope (Olympus IX81) set at 63X objective magnification (oil imaging medium). Images were analyzed and deconvolved using Volocity 6.3 software (Perkin Elmer). For each condition, at least 3 images were acquired.

Luciferase reporter assay

For luciferase reporter assay, HEK293T cells were cultured and transfected for 24 hours as described above. Approximately 6 hours before harvesting (18 hours after transfection), cells were left unstimulated or stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (P/I, 1 μM/1 μg/mL), as previously described.[56] PMA can activate protein kinase C, while ionomycin, a calcium ionophore, activates the Ca^{2+}-calcineurin-NFAT signaling pathway. Stimulation with these compounds can bypass the T cell membrane receptor (TCR) complex, which is absent in HEK293T cells and leads to activation of several intracellular signaling pathways, including NF-κB.[57]

Approximately 24 hours after transfection, NF-κB activation was analyzed measuring the Firefly luciferase activity. Immediately after, *Renilla luciferase* activity was read as internal control. Reagents from Dual-Luciferase Reporter Assay System kit (Promega) were used. Samples were dispensed into a white opaque 96-well microplate.

Luminescence measurements were performed using a Varioskan LUX multimode microplate reader Type 3020 (Thermo Scientific), controlled by SkanIt Software for Microplate Readers 6.0.1 (Thermo Scientific), keeping default settings and selecting a measurement time of 1000 ms.

A single experiment included identical transfections in triplicate for each condition. The experiment was repeated five times.

Statistical analysis

Statistical analysis and graphs were generated using GraphPad Prism Ver. 8.2.1 (GraphPad Software). Statistical analysis of luciferase assay data consisted of 2-way ANOVA followed by post-hoc Tukey test to compare across conditions. Statistical significance was assessed using a significance level of 0.05.

Results

Identification of patients with biallelic CARMIL2 variant

In total, 4 patients from 3 unrelated families were identified with homozygous variants of *CARMIL2* (**Figure 3**).

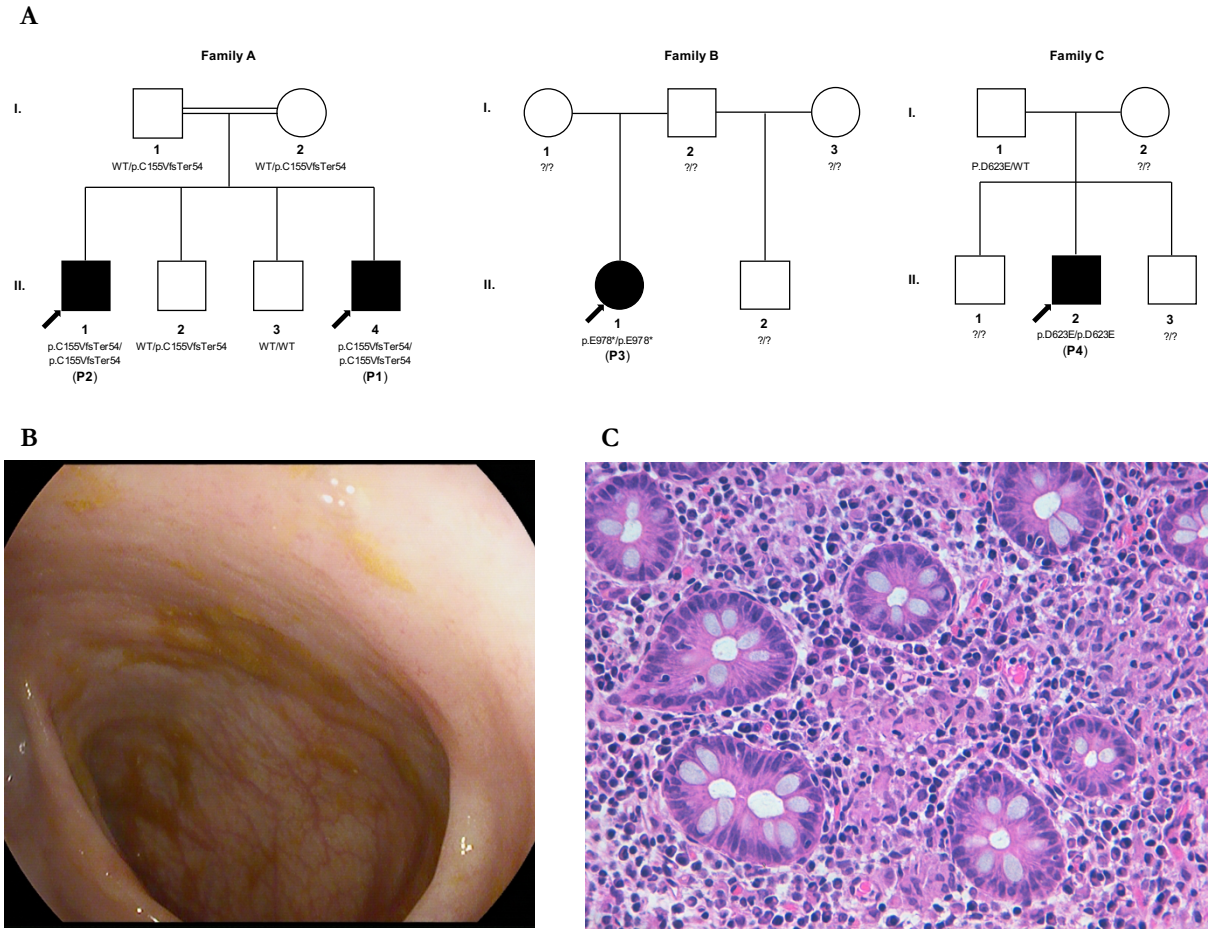


Figure 3. *Novel biallelic CARMIL2 variants identified in four patients from three families. (**A**) Family pedigree and segregation analysis of the three kindreds. As for Family A, both parents and their second son, who had myoclonic epilepsy and attention deficit hyperactivity disorder, were heterozygous. (**B**) Colonoscopy image of Patient 1, showing only a mild patchy loss of vascular pattern throughout the colon. (**C**) Histopathologic findings in a colonic biopsy sample from Patient 1, showing epithelioid granulomas in the lamina propria (Hematoxylin-Eosin X 400).*

Patient 1 was identified through WES, performed during the evaluation for suspected monogenic IBD at the University Hospital of Padova.

Patient 2, the eldest brother of Patient 1, was recognized by family segregation analysis and then further sequenced by WES.

Patient 3 and 4 were identified by screening for biallelic *CARMIL2* variants from WES data of a large cohort of 1005 pediatric IBD patients enrolled at SickKids, Toronto. Application of the bioinformatic analysis pipeline led to the identification of five candidate patients (**Table 2**).

Candidate patients	Age at Diagnosis	Gender	Inheritance model	Nucleotide position	Exon	Nucleotide change	Amino acid change	Predicted Domain	CADD	Damaging score	Max AAF	ExAC allele frequency	N heterozygous in ExAC	N homozygous in ExAC
A	11.4	F	AR	67,653,066	29	G>T	E978*	PRR	41	-	0	0	0	0
B	11.5	M	AR	67,653,021	30	G>T	A936S	PRR	16.7	0/7	0	0	0	0
C	15.4	M	AR	67,649,569	21	C>A	D623E	LRR	26.4	5/7	0.0007	0.0002	26	0
D	?	M	AR	67,651,827	25	A>T	D857V	HD	25.8	4/7	0.0143	0.0059	638	6
E	12	F	CH	67,651,827	25	A>T	D857V	HD	25.8	4/8	0.0143	0.0059	638	6
				67,652,329-67,652,333	27	AAG >-	K937del	PRR	-	-	0	0.0136	0	0

Table 2. *Candidate patients with biallelic CARMIL2 variants from SickKids cohort. Grey background indicates the prioritized variants. Inheritance model is autosomal recessive (AR), when the patient has two copies of the same variant or compound heterozygous (CH), when the two variants differ. The predicted protein domain to which the substituted amino acid belongs is based on the current (incomplete) knowledge of CARMIL2 structure, based on SMART (Simple Modular Architecture Research Tool) database and Uniprot (HD: Helical Dimerization domain; LRR: Leucine-Rich Repeat; PRD: Proline-Rich Domain). CADD (Combined Annotation Dependent Depletion) score predicts the deleteriousness of single nucleotide variants and insertion/deletions variants in the human genome by integrating multiple annotations, including conservation and functional information, into one value. The "Damaging score" field synthetizes the result of seven different algorithms that predict the potential pathogenicity of a variant: SIFT Pred, Polyphen2 HDIV/HVAR, LRT Pred, Mutation Taster Pred, FATHMM Pred, PROVEAN Pred, MetaLR Pred). Max AAF is the maximum alternative allele frequency (AAF) of a variant in the databases considered. The number of heterozygous and homozygous in ExAC reflect how many heterozygous or homozygous individuals for the considered variant are present within that database.*

To prioritize the *CARMIL2* variants resulting from the pipeline, the American College of Medical Genetics 2015 guidelines[54] were considered. *CARMIL2* variant p.D857V, found in two patients, was not prioritized, given the presence of several homozygous individuals in the ExAC database and the not low enough maximum alternative allele frequency (AAF) reported in the considered databases. *CARMIL2* variant p.A936S was also excluded, even if CADD was rather significant, since other prediction algorithms considered the variant non-deleterious. When *in silico* predictions disagree, computational and predictive data should not be used in classifying a variant.[54]

Therefore, *a priori* data led to prioritization of p.E978* and p.D623E *CARMIL2* variants (see below).

Clinical features of patients with biallelic CARMIL2 variants

Patient 1 was born in Italy to consanguineous (first cousins) healthy Moroccan parents, fourth-born of four male sons. At 3.25 years he was diagnosed with colonic CD, phenotype A1aL2B1G1 according to the Paris classification (**Figure 3** and **Table 3**).[4] Symptoms started when he was 2.6 years old. The patient was induced with prednisone, and after clinical remission he was maintained with azathioprine. Follow-up endoscopy performed after 18 months revealed histologic persistent mild total colonic inflammation in biopsies while in clinical remission. At the age of 5 years, the patient developed *Streptococcus pneumoniae* pneumonia complicated by sepsis, despite being vaccinated against pneumococcal disease. Past medical history included eczema in the first months of life. Anti-thyroglobulin antibodies and anti-thyroid peroxidase antibodies were significantly raised, while thyroid function tests and thyroid ultrasound were unremarkable.

Patient 2 was the eldest brother of Patient 1 (**Table 3**). The boy was diagnosed with Addison's disease at 12 years of age, and shortly after with Hashimoto's thyroiditis. No other endocrinopathies were detected. He suffered from difficult-to-treat eczema from a young age. He had no candidiasis, but at the age of 17 he was clinically diagnosed with extended *Tinea corporis* skin infection. Laboratory tests were significant for negative anti-adrenal antibodies and positive anti-thyroid autoantibodies. Plasma assay for very long-chain fatty acids was normal, thereby excluding peroxisomal disorders. Abdomen magnetic resonance imaging revealed bilateral adrenal hypoplasia. Sanger sequencing of the *AIRE* gene was non informative. Thus, Patient 2 was diagnosed with autoimmune polyendocrine syndrome type 2 (APS-2), or Schmidt syndrome, whose distinctive feature is Addison's disease, associated with at least one among autoimmune thyroid disease and type 1A diabetes mellitus. There was neither history of significant or recurrent infectious episodes, nor evidence of bowel disease and fecal calprotectin was normal.

	Patient 1	Patient 2	Patient 3	Patient 4
Demographics				
Age at diagnosis	2 y	12 y	11 y	15 y
Gender	Male	Male	Female	Male
Consanguinity	Yes	Yes	No	Unknown
Parents origin (ethnicity/country)	Arab-Berber/Moroccan	Arab-Berber/Moroccan	Indigenous Canadian (mother), Israeli-Polish (father)	Unknown
Gastrointestinal disease				
Family history of IBD	None	None	None	None
IBD (Paris classification of IBD)	CD (A1aL2B1G1)	None	UC (A1bE4S1)	UC (A1bE4S1)
Symptoms at onset	Chronic diarrhea, +/- blood and mucus, +/- abdominal pain, failure to thrive		Abdominal pain, bloody diarrhea, weight loss	Abdominal pain, bloody diarrhea, fever, weight loss
EGD/IC	Normal/Mild patchy loss of vascular pattern		Gastric erythema and superficial erosion of duodenal cap/ Pancolitis with normal terminal ileum	Hyperemic esophagus, small ulcers and inflammation of stomach/Severe pancolitis with backwash ileitis
Pathology (upper/lower gastrointestinal tract)	Altered villous profile and atrophy, mild lymphomonocytic inflammation and epithelioid microgranulomas in duodenal bulb/Crypt distortion, mild lymphomonocytic inflammation, Paneth cell metaplasia and epithelioid granulomas of the colon		Mild increase of duodenal lamina propria mononuclear cells, moderate antritis with focal acute activity, moderate chronic body gastritis/Mild-moderately active IBD of entire colon, focal superficial acute terminal ileitis	Normal duodenum, mild chronic non-atrophic gastritis, mild esophagitis/ Chronic colitis with mild to moderate acute activity throughout all biopsies
Surgery	None		Colectomy, ileostomy, J pouch	Colectomy, ileostomy, J pouch
Other clinical features				
Infections	*Streptococcus pneumoniae* pneumonia complicated by sepsis	*Tinea corporis*, prolonged upper respiratory tract infections	Intestinal leak with intraabdominal sepsis, pouchitis, pneumonia	Hepatic abscesses, *C. difficile* infection, abdominal abscess with enterocutaneous fistula
Endocrine disorders	None	Addison's disease, Hashimoto thyroiditis	None	None
Skin	Eczema (first months)	Eczema	None	None
Others	Neuropsychiatric disorder (developmental delay and behavioral disorders)	Depressive disorder	Reactive airway disease	Unknown
Autoantibodies	Anti-thyroid Abs; negative anti-Harmonin antibodies	Anti-thyroid Abs, LAC	ANCA	ANCA

Table 3. *Demographic and clinical features of CARMIL2-deficient patients. EGD esophagogastroduodenoscopy, IBD inflammatory bowel disease, IC ileocolonoscopy, LAC Lupus anticoagulant.*

Immunological work-up of Patient 1 and 2 revealed a marked increase in the proportion of naïve T and B cells, with a reduction in memory T and B cells and marginal zone B cells. Patient 1, but not Patient 2 had an increase in the number of double negative T cells (CD3 + CD4-CD8-TCRαβ+) (**Table 4**). Both patients had negative anti-EBV IgG and IgM and EBV-DNA on peripheral blood. Both patients had low titers of specific antibody against tetanus and diphtheria toxoids.

Parameter (unit)	Patient 1	Patient 2
Age	4 years	17 years
IgG (g/L)	6.69 (5.52-11.98)	11.89 (6.2-14.2)
IgA (g/L)	1.53 (0.54-1.99)	2.44 (0.50-3.00)
IgM (g/L)	0.78 (0.40-1.45)	1.39 (0.50-1.70)
Lymphocyte number (x 10^9/L)	7.07	3.24
CD3+ (%)	58 (60-78)	82 (63-80)
CD4+CD3+ (%)	38 (31-47)	47 (33-52)
CD8+CD3+ (%)	17 (16-27)	27 (19-29)
CD19+ (%)	32 (13-29)	15 (12-21)
CD16+CD56+(%)	9 (5-16)	3 (4-16)
CD4+CD45RA+ (% of CD4+ Ly)	92 (61.8-85.0) ↑↑	86 (40.9-65.7) ↑↑
CD4+CD45RO+ (% of CD4+ Ly)	8 (14.8-37.2) ↓	14 (25.1-52.1) ↓
CD3+CD25+ (%)	1 (0.9-4.7)	2 (3.8-7.8)
CD3+CD4-CD8-TCRαβ+ (% of CD3+ Ly)	3.7 (<2.5) ↑	0.55 (<2.5)
Naive B IgD+CD27- (% of CD19+ Ly)	97 (59.7-88.4) ↑↑	92 (61.6-87.4) ↑↑
Marginal zone B IgD+CD27+ (% of CD19+ Ly)	0.86 (3.1-17.4) ↓	0.7 (2.6-13.4)
Memory B IgD-CD27+ (% of CD19+ Ly)	1.7 (2.9-17.4) ↓	2.8 (4.0-21.2) ↓
Transitional B cells IgM+ CD38+ (% of CD19+ Ly)	6.4 (2-30)	20 (0.7-24)
Plasmoblasts IgM- CD38++ (% of CD19+ Ly)	0.34 (0.1-4)	0.65 (0.7-6)
Dihydrorhodamine test	Normal	NA

Table 4. *Immunological work-up of Patient 1 and 2.*

Patient 3 was a female diagnosed with UC, type pancolitis (A1bE4S1), at the age of 11 years old (**Table 3**). Remission was achieved after induction with prednisone, and maintained first with sulfasalazine, then with azathioprine. After relapse, she failed infliximab and at the age of 17 years underwent colectomy with loop ileostomy and J pouch. Her post-operative course was complicated by intestinal leakage causing intraabdominal sepsis, stricture at pouch anastomosis and pouchitis. Past medical history included reactive airway disease and an episode of pneumonia. Before IBD onset she had no other significant history of infections or immune-related diseases. She had no familial history of IBD.

Patient 4 was a male diagnosed with UC, type pancolitis (A1bE4S1), at the age of 15 years (**Table 3**). The patient was induced with IV methylprednisolone, became steroid dependent first, then steroid refractory; azathioprine was thus introduced. He developed azathioprine-induced pancreatitis with pseudocysts, hepatic abscesses, and *C. difficile* infection. Drain insertion was complicated by peri-sigmoid abscess, colonic-peripancreatic enterocutaneous fistula and toxic megacolon. At the age of 16 the patient underwent subtotal colectomy and ileostomy, then J pouch surgery. Past medical history was unremarkable. Particularly, he had no previous history of infections, or immune-related diseases. He had no familial history of IBD.

Genetic analysis of patients with biallelic CARMIL2 variant

As mentioned above, all four patients were found to have novel biallelic variants of *CARMIL2* (**Figure 4** and **Table 5**).

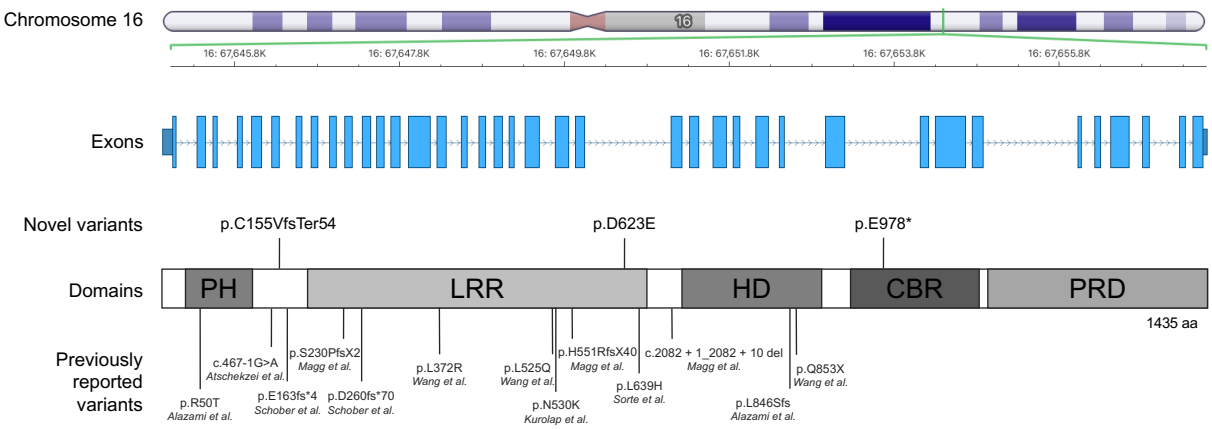


Figure 4. *CARMIL2 gene and protein view with newly identified and previously published variants. Schematic representation of the intron-exon structure of the CARMIL2 gene, which is located on Chromosome 16. The three novel variants reported in this study are labelled above the schematic illustration of the protein domains of CARMIL2, along with the previously described variants below. PH Pleckstrin-Homology domain, LRR Leucine- Rich Repeat domain, HD Helical Dimerization domain, CBR Capping Protein-Binding Region, PRD Proline-Rich Domain.*

Patient 1 and 2 had a homozygous nonsense variant p.Cys155ValfsTer54 of *CARMIL2*, located on Exon 6 (**Table 5**). This frameshift mutation is caused by a single nucleotide (cytosine) deletion resulting in a non-synonymous substitution of cysteine (C) with valine (V) and premature termination of translation after 54 codons (predicted number of amino acids: 207, while wild type CARMIL2 consists of 1435 amino acids).

Variant p.C155VfsTer54 is not reported in literature and no data are available on allele frequency on reference databases (ExAC, gnomAD, 1000 Genomes database, EVS) (**Table 5**). Segregation analysis showed that both the parents and the second brother were heterozygous carriers of the variant. On the contrary, the fourth brother was homozygous for WT *CARMIL2*.

Patient 3 had a homozygous nonsense *CARMIL2* variant p.Glu978*, located on exon 29 of *CARMIL2* (**Table 5**) resulting in stop-gain nucleotide substitution and premature termination codon. CARMIL2 variant p.E978* is not reported in literature and is absent from population databases. Patient 3 was recruited as a "singleton", so parental DNA was not available for variant segregation analysis. Patient 4 had a homozygous missense variant p.Asp623Glu, located on exon 21 of *CARMIL2* (**Table 5**). It has not been reported in literature, and its frequency is extremely low (<0.001) in population databases.

	Patient 1	Patient 2	Patient 3	Patient 4
Chromosome position[a]	Chr16:67,646,513		Chr16:67,653,066	Chr16:67,649,569
cDNA change (GenBank: NM_001013838.3)	c.462delC		c.2932G>T	c.1869C>A
Amino acid change[b] (GenPept: NP_001013860.1)	p.Cys155ValfsTer54		p.Glu978*	p.Asp623Glu
Exon number	6		29	21
Predicted domain	None		PRD	LRR
***In silico* evaluation**				
CADD	Unknown		41	26.4
SIFT Pred	Unknown		Unknown	Damaging
Polyphen2 Pred	Unknown		Unknown	Probably damaging
LRT	Unknown		Neutral	Deleterious
Mutation Assessor	Unknown		Unknown	Medium (2.645)
Mutation Taster	Disease causing		Disease causing	Disease causing
FATHMM	Unknown		Unknown	Tolerated
PROVEAN	Unknown		Unknown	Damaging
Population databases				
Maximum AAF[c]	0		0	0.0007
ExAC allele frequency	None		None	0.0002
N of heterozygous in ExAC	None		None	26
N of homozygous in ExAC	None		None	None
gnomAD allele frequency	None		None	0.0002499
gnomAD allele count	None		None	38
N of homozygous in gnomAD	None		None	None
1000 Genomes	None		None	None
EVS	None		None	0.0003

Table 5. *Genetic features of CARMIL2-deficient patients. AAF alternative allele frequency, AR autosomal recessive, CADD Combined Annotation Dependent Depletion, EVS Exome Variant Server, ExAC Exome Aggregation Consortium, FATHMM Functional Analysis through Hidden Markov Models, gnomAD Genome Aggregation Database, LRR Leucine-Rich Repeat domain, LRT likelihood ratio test, PRD Proline-Rich Domain, PROVEAN Protein Variation Effect Analyzer. [a]According to human genome assembly GRCh38. [b]According to NCBI reference sequence NP_001013860.1. [c]Represents the maximum AAF of a variant in the databases considered.*

Variant p.D623E is predicted to be deleterious by most of the algorithms examined. Moreover, the aspartic acid at position 623 is located in a LRR domain of CARMIL2, is part of an evolutionarily constrained region (ECR) and is highly conserved throughout evolution (**Figure 5** and **Table 6**). The patient was recruited as a "duo", and only the unaffected father's DNA was available for allele segregation analysis. The father was a heterozygous carrier for the same variant.

Sanger sequencing confirmed the presence of the biallelic variant p.C155VfsTer54 in Patient 1 and 2 but failed for both the variants identified in Patient 3 (p.E978*) and Patient 4 (p.D623E), due to a guanine-cytosine-rich template (**Figure 6**), despite troubleshooting and optimization strategies, including primer substitution and use of different buffers and annealing temperatures. However, careful reexamination of raw WES reads showed high coverage support for homozygous mutant calls in both candidates. Furthermore, since LoF is the pathogenic mechanism of CARMIL2 deficiency and tissue immunostaining proved reduced protein expression in both patients, confirmation by sequencing was deemed unnecessary.[58]

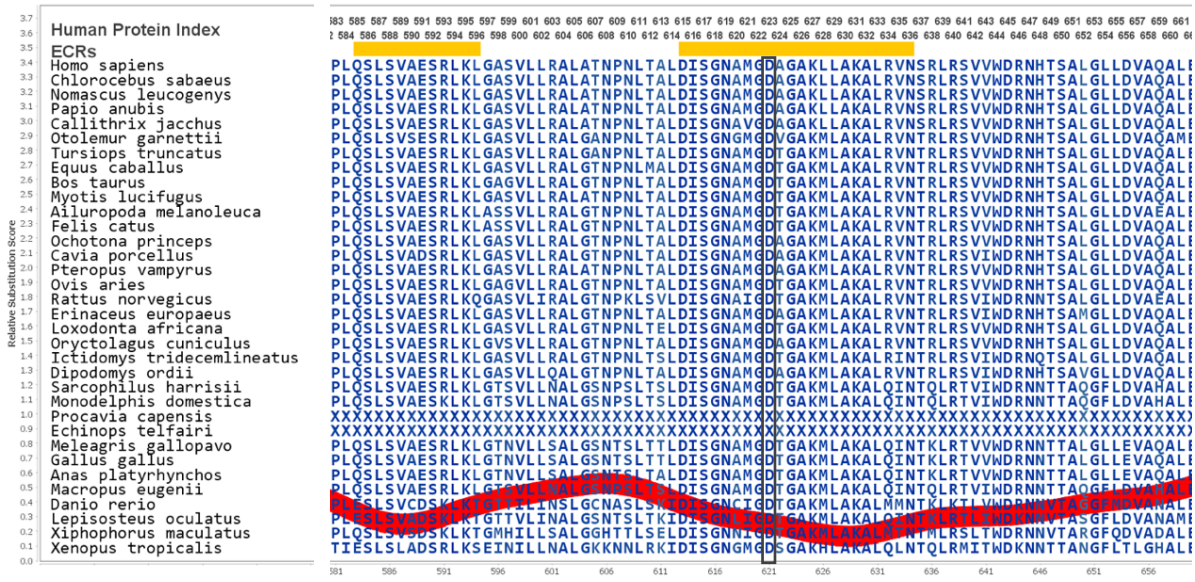


Figure 5. *Comparison of the amino acid sequences of protein homologs belonging to Homo sapiens and different vertebrate species in correspondence to residue 623. The image was generated with Aminode, a webtool (available at http://www.aminode.org) that can be used for the rapid inference of Evolutionarily constrained regions (ECRs), that often correspond to critical sites for the protein.*[53] *The red line represents the relative rate of amino acid substitution throughout evolution, calculated at each position. The yellow bars are Local minima, or ECR, regions with low substitution rates. Bluer shade indicates more conserved amino acids, greener shade less conserved residues. Therefore, aspartic acid (D) in position 623 is highly conserved throughout evolution and is positioned inside an ECR.*

												623									
CARMIL2	*Homo sapiens*	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	L	L	A	K	A	L
	Chlorocebus sabaeus	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	L	L	A	K	A	L
	Nomascus leucogenys	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	L	L	A	K	A	L
	Papio anubis	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	L	L	A	K	A	L
	Callithrix jacchus	A	L	D	I	S	G	N	A	V	G	**D**	A	G	A	K	L	L	A	K	A	L
	Otolemur garnettii	A	L	D	I	S	G	N	G	M	G	**D**	V	G	A	K	M	L	A	K	A	L
	Tursiops truncatus	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Equus caballus	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Bos taurus	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Myotis lucifugus	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Ailuropoda melanoleuca	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Felis catus	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Ochotona princeps	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Cavia porcellus	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Pteropus vampyrus	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Ovis aries	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Rattus norvegicus	V	L	D	I	S	G	N	A	I	G	**D**	T	G	A	K	M	L	A	K	A	L
	Erinaceus europaeus	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Loxodonta africana	E	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Oryctolagus cuniculus	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Ictidomys tridecemlineatus	S	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Dipodomys ordii	A	L	D	I	S	G	N	A	M	G	**D**	A	G	A	K	M	L	A	K	A	L
	Sarcophilus harrisii	S	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Monodelphis domestica	S	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Meleagris gallopavo	T	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Gallus gallus	T	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Anas platyrhynchos	A	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Macropus eugenii	S	L	D	I	S	G	N	A	M	G	**D**	T	G	A	K	M	L	A	K	A	L
	Danio rerio	K	I	D	I	S	G	N	C	I	G	**D**	T	G	A	K	M	L	A	K	A	L
	Lepisosteus oculatus	K	I	D	I	S	G	N	L	I	G	**D**	T	G	A	K	M	L	A	K	A	L
	Xiphophorus maculatus	E	L	D	I	S	G	N	N	I	G	**D**	T	G	A	K	M	L	A	K	A	L
	Xenopus tropicalis	K	I	D	I	S	G	N	G	M	G	**D**	S	G	A	K	H	L	A	K	A	L

CARMIL1	***Homo sapiens***	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Pan troglodytes	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Nomascus leucogenys	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Callithrix jacchus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Otolemur garnettii	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Equus caballus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Cavia porcellus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Loxodonta africana	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Papio anubis	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Chlorocebus sabaeus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Canis familiaris	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Monodelphis domestica	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Ictidomys tridecemlineatus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Ailuropoda melanoleuca	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Rattus norvegicus	K	V	D	I	S	G	N	S	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Mus musculus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Felis catus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Ochotona princeps	K	V	D	I	S	G	N	A	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Tursiops truncatus	T	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Bos taurus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Ovis aries	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Echinops telfairi	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Microcebus murinus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Anolis carolinensis	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Meleagris gallopavo	K	V	D	I	S	G	N	A	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Ficedula albicollis	K	V	D	I	S	G	N	A	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Gallus gallus	K	V	D	I	S	G	N	A	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Pelodiscus sinensis	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Xenopus tropicalis	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Latimeria chalumnae	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Dipodomys ordii	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Choloepus hoffmanni	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Erinaceus europaeus	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
	Poecilia formosa	K	L	D	I	S	G	N	S	M	G	**D**	M	G	A	K	I	L	A	K	A	L
	Xiphophorus maculatus	K	L	D	I	S	G	N	S	M	G	**D**	M	G	A	K	I	L	A	K	A	L
	Macropus eugenii	K	V	D	I	S	G	N	G	M	G	**D**	M	G	A	K	M	L	A	K	A	L
CARMIL3	*Homo sapiens*	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Pongo abelii	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Papio anubis	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Chlorocebus sabaeus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Callithrix jacchus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Ictidomys tridecemlineatus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Otolemur garnettii	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Bos taurus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Sus scrofa	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Canis familiaris	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Mustela putorius furo	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Felis catus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Rattus norvegicus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Mus musculus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Myotis lucifugus	K	V	D	L	N	G	N	G	M	E	**D**	I	G	A	K	M	L	S	N	A	L
	Oryctolagus cuniculus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Tursiops truncatus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Loxodonta africana	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Cavia porcellus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Ailuropoda melanoleuca	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Ovis aries	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Sarcophilus harrisii	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Ochotona princeps	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Microcebus murinus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Pteropus vampyrus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Dipodomys ordii	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Echinops telfairi	K	V	D	L	S	G	N	S	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Macropus eugenii	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
	Erinaceus europaeus	K	V	D	L	S	G	N	G	M	E	**D**	I	G	A	K	M	L	S	K	A	L
Invertebrate CARMIL	XP_021699954.1_LRRC16A_Mosquito	V	L	D	I	T	G	N	L	M	G	**D**	I	G	A	R	L	L	A	K	A	L
	NP_001260788.1_Fruit_Fly	K	L	D	I	S	G	N	F	M	G	**D**	V	G	A	R	L	L	A	K	A	L
	XP_006568383.2_LRRC16A_Bee	I	L	D	I	S	G	N	Q	I	G	**D**	P	G	A	R	L	L	A	K	A	L

Table 6. *Multiple sequence alignments of CARMIL-family proteins from diverse organisms containing the highly conserved aspartic acid residue 623 (sequence numbering is for human CARMIL2).*

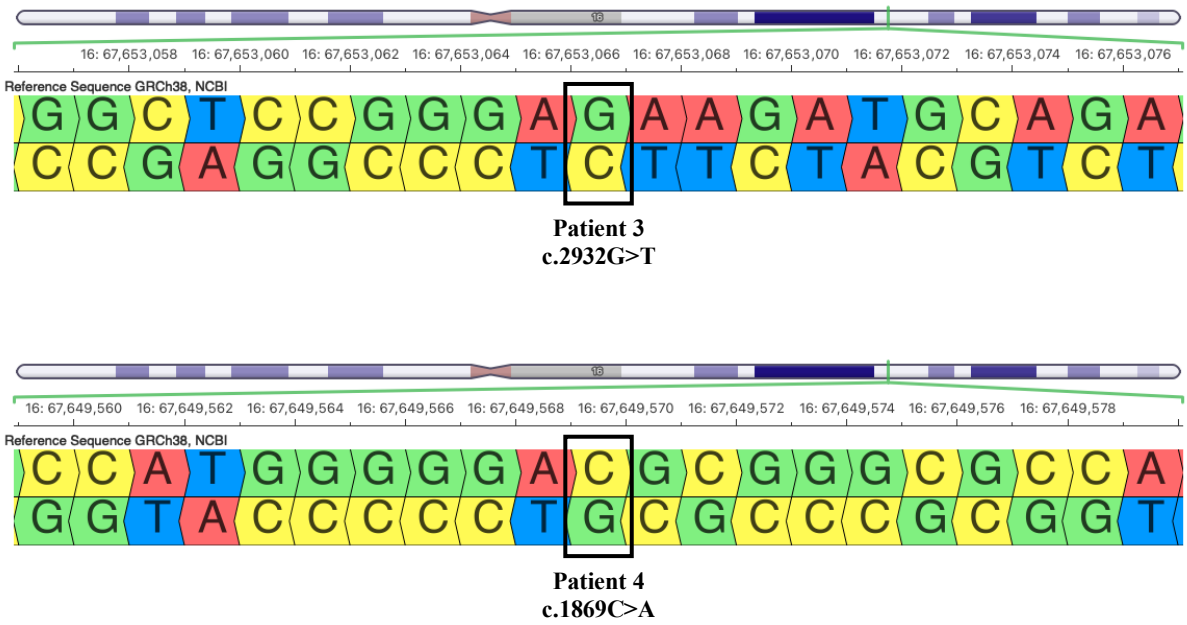


Figure 6. *Reference nucleotide sequence of CARMIL2 gene centered at position Chr16:67,653,066 (**A**) and Chr16:67,649,569 (**B**), where the variants of Patient 3 and 4 are located, respectively.*

Immunostaining of bowel biopsies in biallelic CARMIL2 variant carriers with IBD

Immunostaining on sigmoid FFPE sections was performed to assess the expression levels of CARMIL2 in the bowel of the three patients affected by IBD (Patient 1, 3 and 4; **Figure 7**).

In both normal and IBD control sigmoid sections CARMIL2 was strongly expressed in the enterocytes of mucosal layer and stromal cells. In line with The Human Protein Atlas, CARMIL2 was mainly localized in cell cytoplasm, but some nuclear expression was also observed in blood cells.[50,59]

In the two patients with homozygous protein-truncating variants (p.C155VfsTer54 and p.E978*), CARMIL2 protein expression was nearly absent or remarkably down-regulated in both glandular layer and stromal area. In Patient 4, carrying the homozygous missense variant p.D623E, CARMIL2 expression in the mucosal layer was significantly weaker in comparison to controls, while infiltrated blood cells located in the stromal area showed focally positive staining. This range of staining intensity in different cell types is consistent with previously published immunohistochemistry images, showing stronger staining in lymphocytes than in intestinal epithelium.[50]

Cytokeratin 18 (CK18) staining pattern in all samples was as expected based on previous reports,[60] indicating a preserved tissue architecture.

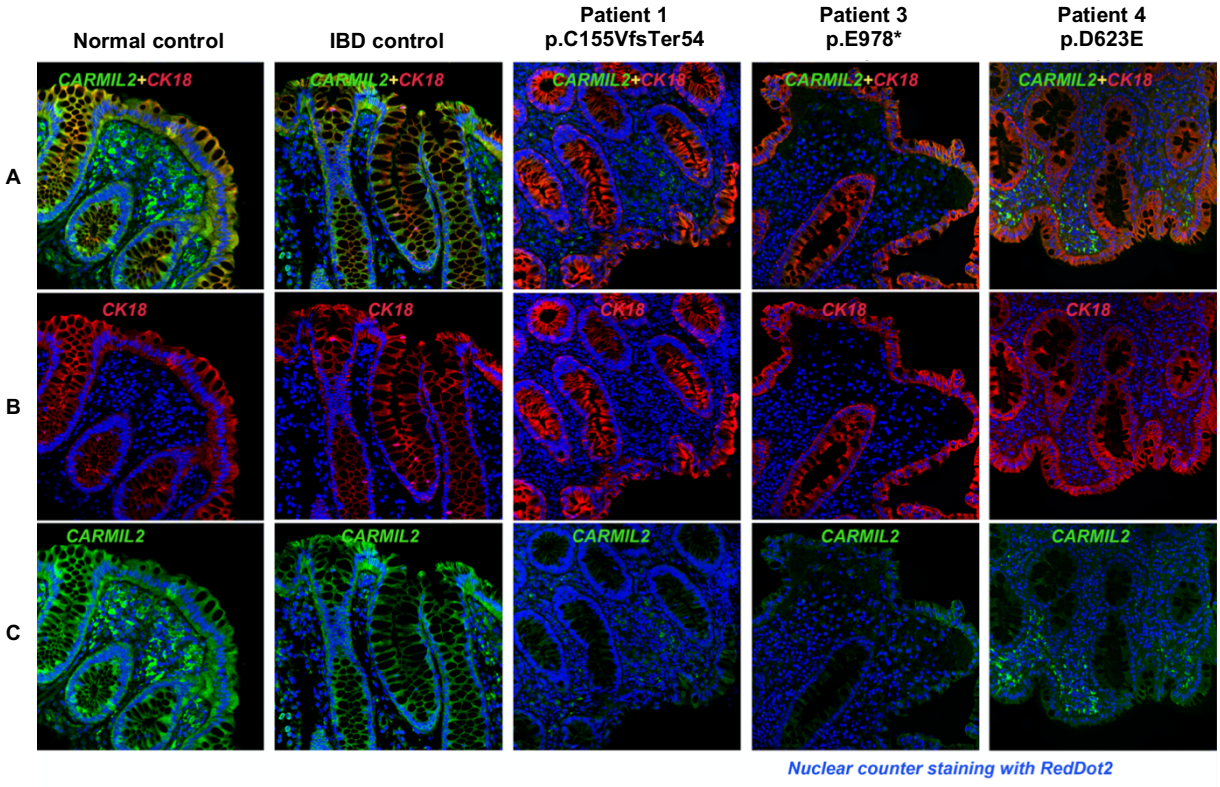


Figure 7. *Dual immunofluorescence staining for CARMIL2 protein and CK18 on sigmoid FFPE sections of healthy control, IBD control, and three biallelic CARMIL2 variant carriers diagnosed with IBD. DAPI counterstaining was used to visualize nuclei. (**A**) Composite image, where green staining indicates CARMIL2, red represents cytokeratin 18 (CK18), a marker for single layer epithelial cells, and blue marks nuclear DAPI stain. In Patient 1 and 3, carriers of the two protein-truncating variants (respectively p.C155VfsTer54 and p.E978*), CARMIL2 signal was almost absent in sigmoid sections. In Patient 4, carrier of missense variant p.D623E, immunofluorescence staining was weaker than the controls particularly in the mucosal layer. (**B**) Single-label immunofluorescence for CK18 (red). (**C**) Single-label immunofluorescence for CARMIL2 (green).*

Functional validation of CARMIL2 variants

Next, we investigated the impact of the novel *CARMIL2* variants on protein expression and cellular localization using cellular model systems.

Western blotting was used to analyze the expression of 3xFLAG tagged CARMIL2 variants in HEK293Tcells (**Figure 8A**).

Thus, plasmids encoding either WT or variants of *CARMIL2*, tagged with 3xFLAG at the N-terminus, were transfected in HEK293T cells. The cells were collected 48 hours post transfection and WB analysis of whole cell lysate was performed.

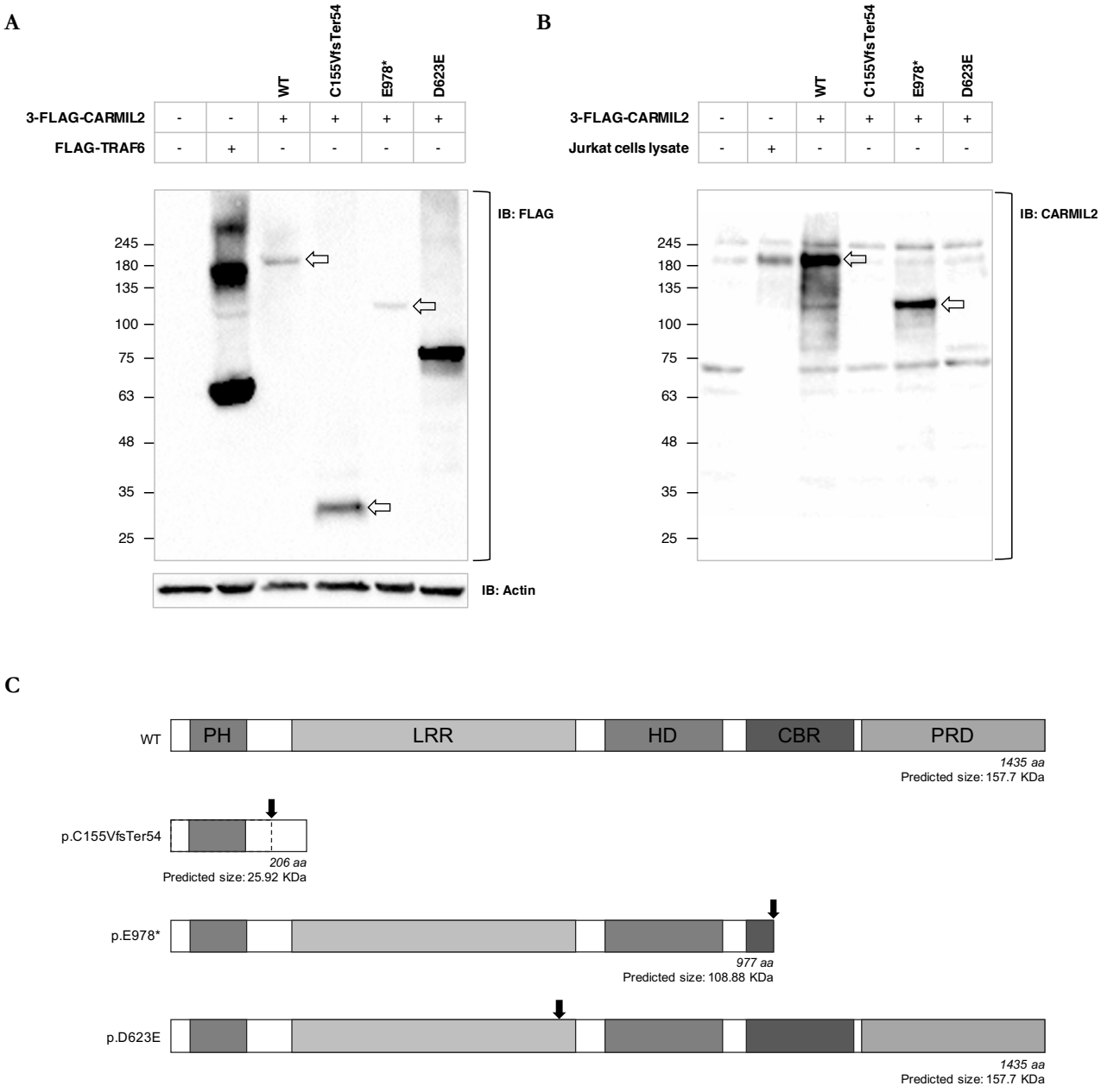


Figure 8. *Western blot analysis of CARMIL2 (WT and variants) protein expression. (**A**) Immunoblotting with anti-FLAG antibody. FLAG-TRAF6 served as positive control, β-actin as loading control. (**B**) Immunoblotting with anti-CARMIL2 antibody. Jurkat cells lysate was used as positive control. An unspecific band of ~70 KDa appeared only in HEK293T cells lysate. Upper bands correspond to endogenous CARMIL2 and, possibly, a form of post-translational protein modification (e.g., phosphorylation). (**C**) The expected molecular weight (MW) of CARMIL2 WT and variants (considering the 3xFLAG tag) is shown for comparison. Online tool https://www.bioinformatics.org/sms/prot_mw.html was used to calculate the predicted MW. The black arrow points to the position of the mutation inside the protein. The observed band (~180 kDa) of WT CARMIL2 does not correspond to the predicted protein size (~155 kDa); this is consistent with what previously reported and with The Human Protein Atlas (available from www.proteinatlas.org).*[47,59]

Immunoblotting with anti-FLAG antibody detected bands corresponding to the predicted molecular weight (MW) both for CARMIL2 WT and for the protein-truncating variants, p.C155VfsTer54 and p.E978*. On the other hand, the MW of p.D623E variant was ~80 kDa, much lower than the WT, the only difference being the substitution of a single amino acid residue from aspartic acid (D, MW 133 Da) to glutamic acid (E, MW 147 Da). This strongly suggested proteolysis of the p.D623E variant to a lower MW species (some of them retaining the 3xFLAG tag). From a quantitative point of view, p.C155VfsTer54 and especially D623E bands appeared to be more intense than WT and p.E978* bands; loading control (actin) bands indicated equal loading of samples across all the wells.

Immunoblotting with anti-CARMIL2 antibody (**Figure 8B**) failed to recognize both p.C155VfsTer54 and p.D623E variants. Instead, WT and p.E978* CARMIL2 bands corresponded to those detected by anti-FLAG antibody.

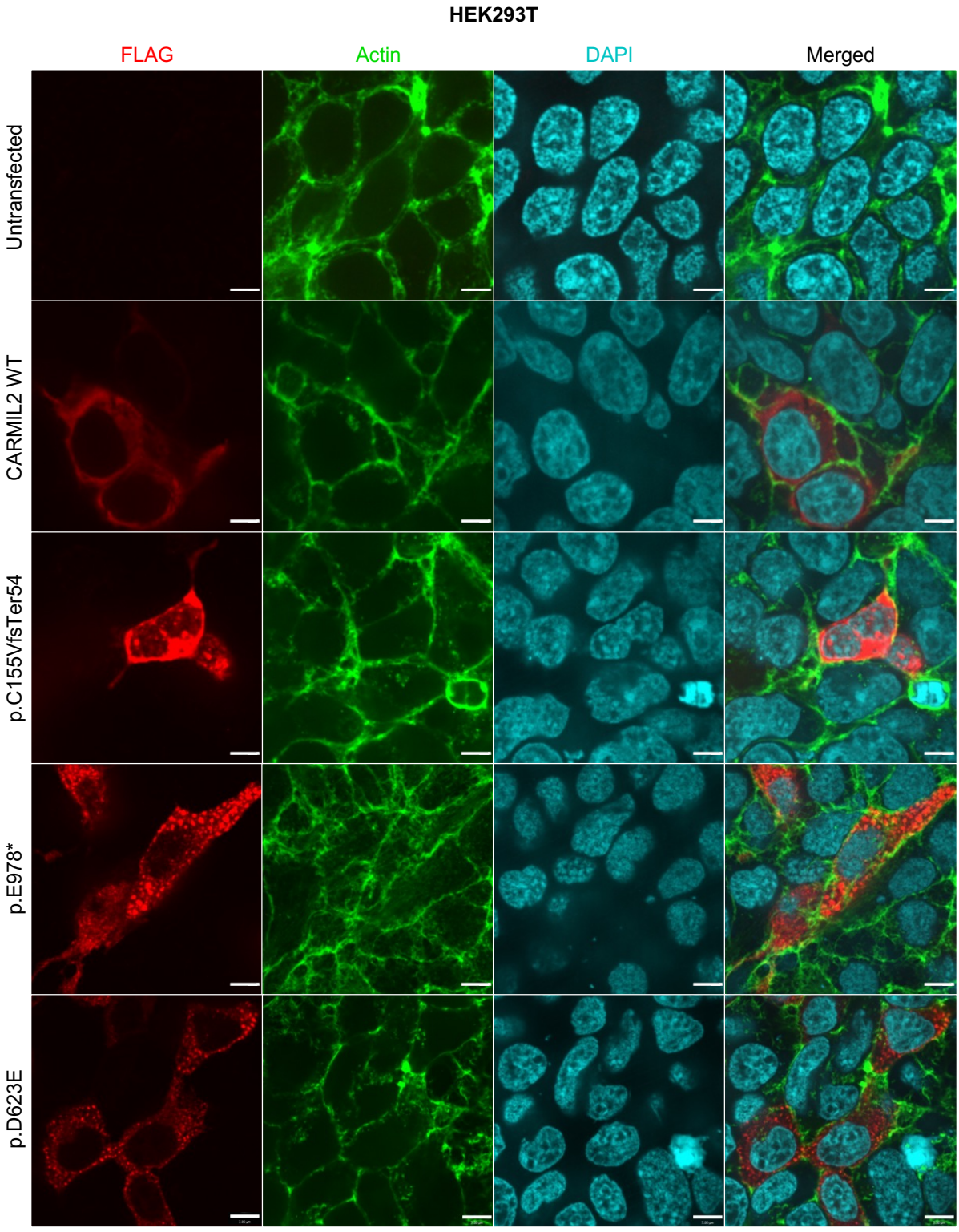
A biological model of CARMIL2 expression in the cell was then established by transfecting different cell lines, specifically HEK293T cells (chosen for the ease of transfection; **Figure 9A**), HCT116 cells (selected for being a colon cancer cell line, thus closer to intestinal cells; **Figure 9B**), and Jurkat cells (chosen for being a T lymphocyte cell line, as CARMIL2 deficiency affects T cells; **Figure 9C**) with plasmids encoding either WT or variants of 3xFLAG-CARMIL2 and performing immunofluorescence staining.

Immunofluorescence imaging showed that the expression pattern of CARMIL2 WT was diffuse across the cytoplasm in all cell strains, in line with the existing knowledge of the protein, as reported in The Human Protein Atlas.[59]

Similarly, CARMIL2 variant p.C155VfsTer54 exhibited a cytoplasmic expression, albeit less homogeneous. Conversely, CARMIL2 variants p.E978* and particularly p.D623E signal appeared as puncta structures throughout the cytoplasm of HEK293T cells, with a more granular pattern of expression. For variant p.D623E the puncta staining was consistent in HCT116 cells. Therefore, this missense variant appeared to form aggregates when expressed in two different cell lines. Immunostaining of Jurkat cells didn't give any information about protein localization in the cell, given the high nucleo-cytoplasmic ratio of this immortalized T lymphocyte cell line, but from a quantitative point of view it showed that expression of p.D623E variant was the highest among all *CARMIL2* alleles, as in the case of WB.

Overall, different lines of evidence suggest that variant p.D623E is susceptible to mislocalization inside the cell and proteolysis, thus behaving as a null or non-functional allele.

A


HEK293T
FLAG
Actin
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CARMIL2 WT
p.C155VfsTer54
p.E978*
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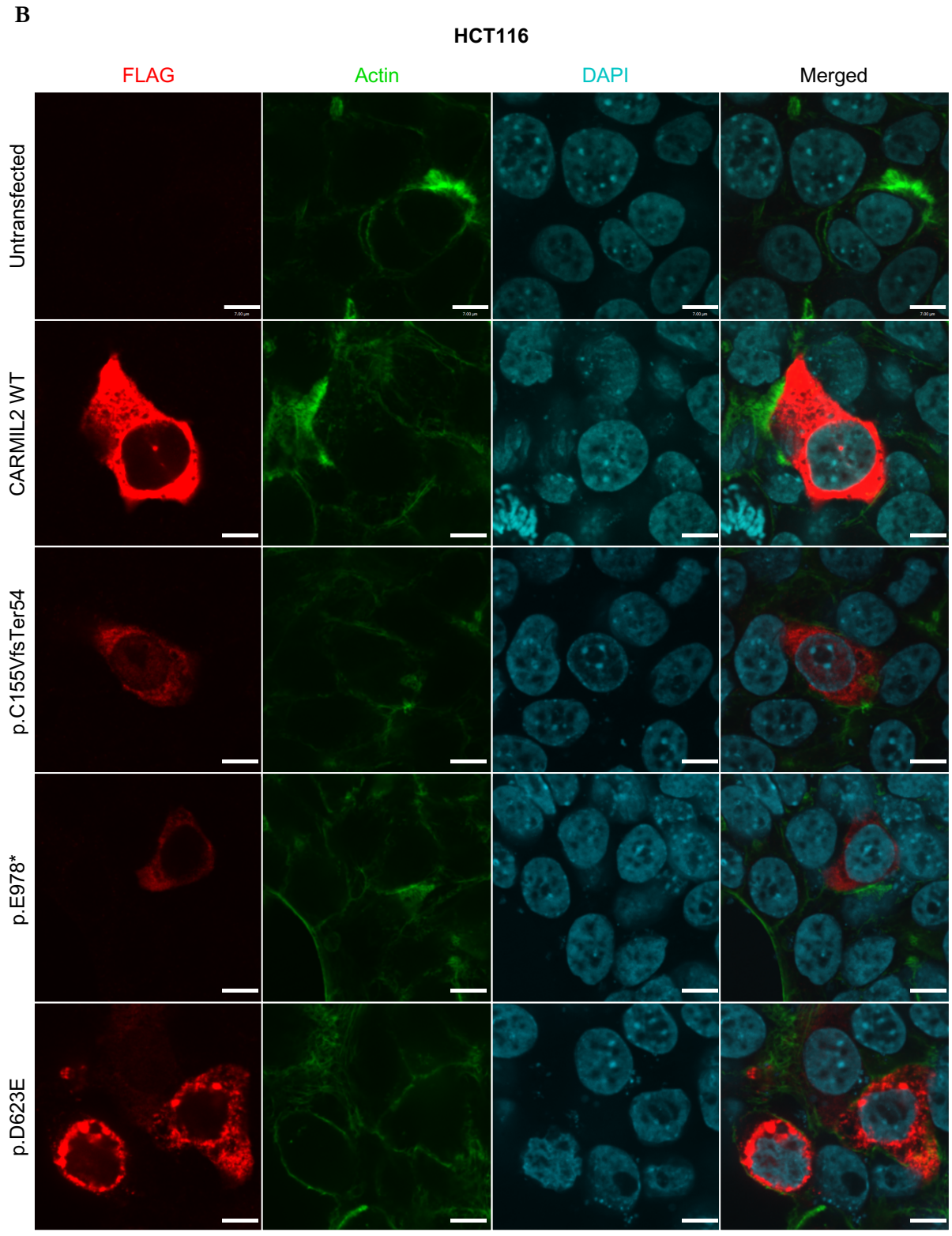
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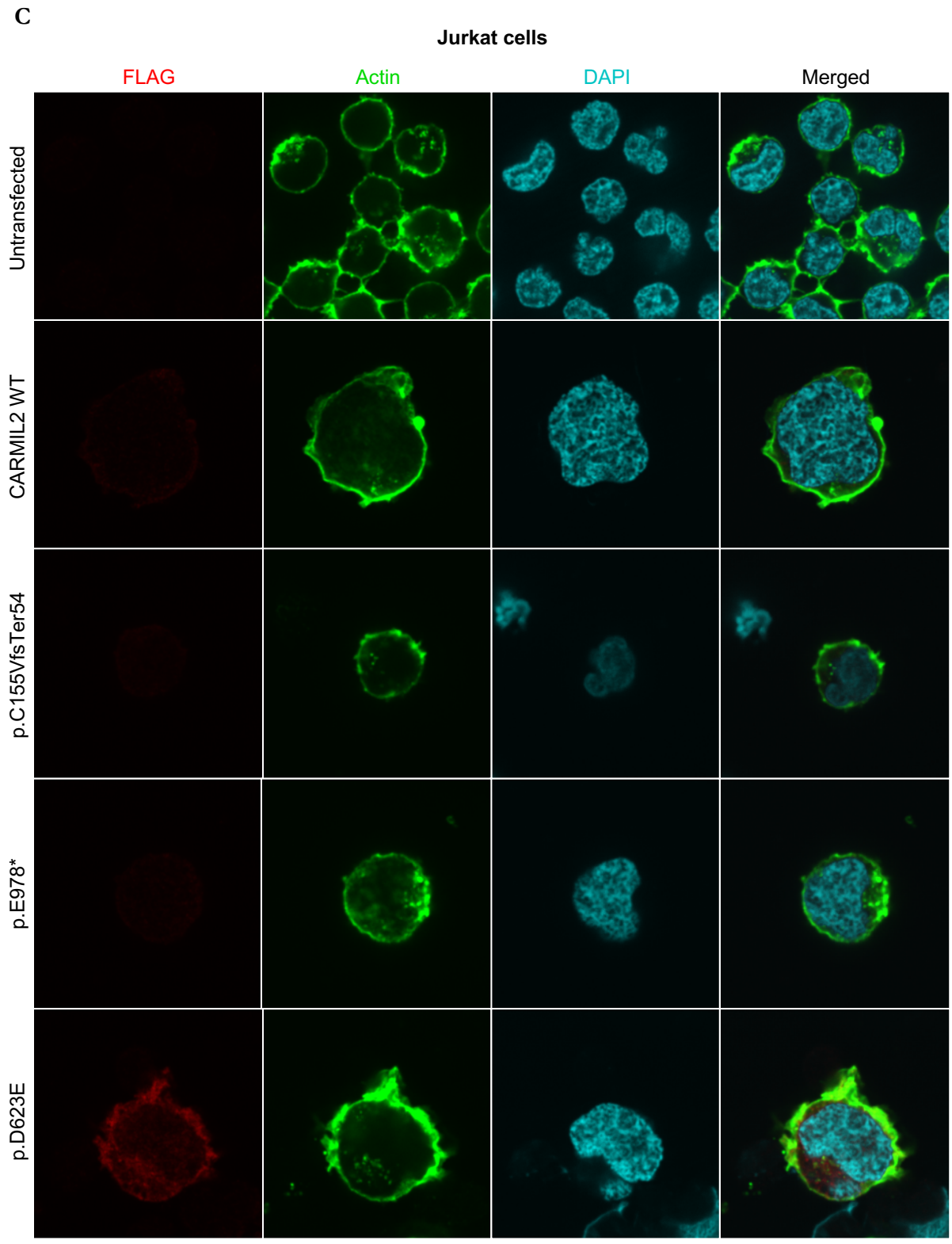


Figure 9. *Immunofluorescence staining of HEK293T cells (**A**), HCT116 cells (**B**), and Jurkat cells (**C**) transfected with wild type CARMIL2 or the indicated variants. The first three columns represent the immunofluorescence images of FLAG tagged CARMIL2 (red), actin (green) and nuclei (light blue). The fourth column is a composite image. Scale bar: 7 µm.*

Finally, a Luciferase reporter assay was performed to functional assess *CARMIL2* variants (**Figure 10**).

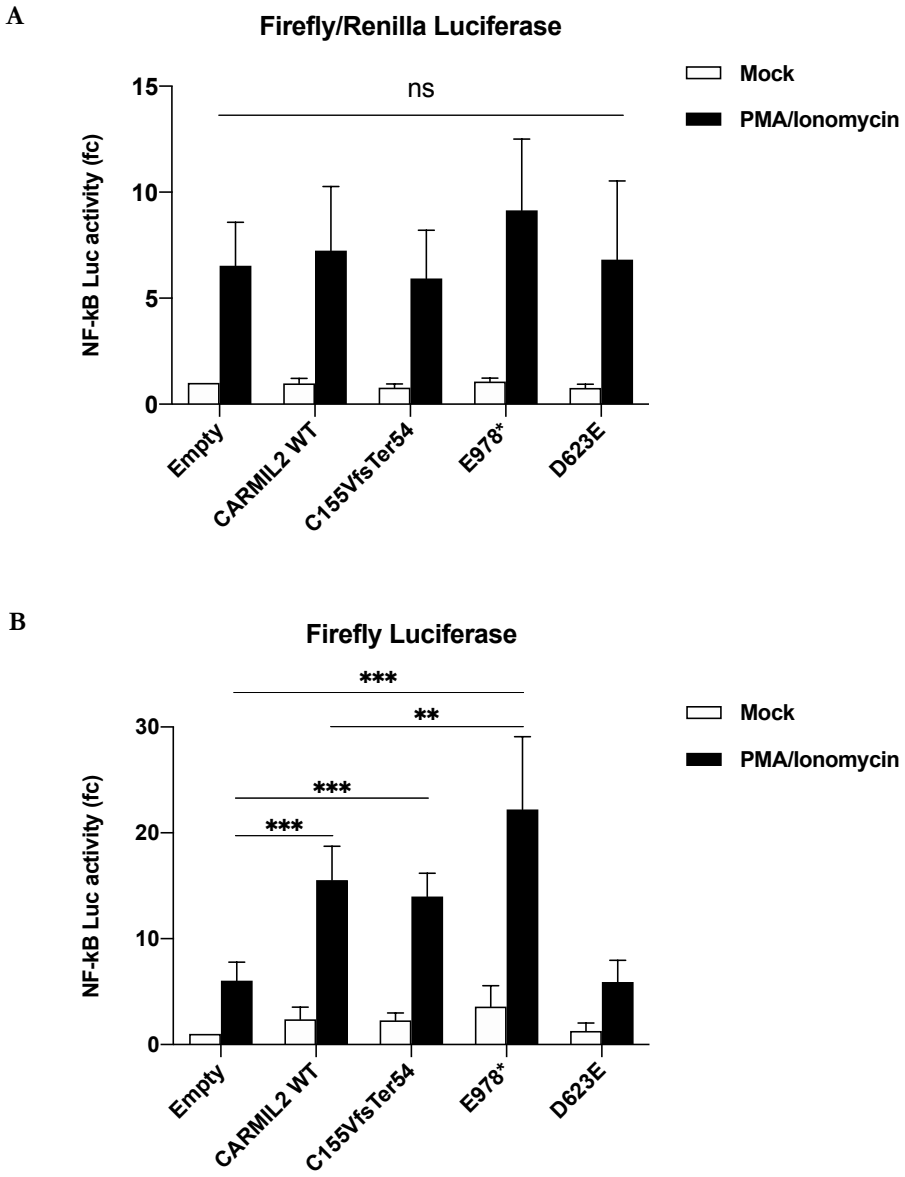


Figure 10. *Luciferase reporter assay to measure NF-κB activation in HEK293T cells transiently expressing CARMIL2 constructs and P/I stimulated. (**A**) Dual-Luciferase reporter assay. After normalizing by the activity of Renilla luciferase, activation of NF-kB was not significantly with both WT and variants of CARMIL2. (**B**) Luciferase reporter assay. Without normalizing, NF-κB activation is significantly higher (***) than empty vector for both CARMIL2 WT and truncating variants; E978* activation is significantly higher (**) than WT. Cells transfected with variant D623E showed reduced NF-κB promoter activation after stimulation, non-significantly higher than empty vector. Data are representative of five independent experiments with triplicate samples (ns: not significant, P >0.05; *: significant, P ≤0.05; **: very significant, P ≤0.01; ***: very significant, P ≤0.001after Tukey's multiple comparisons test).*

Being known that CARMIL2 stimulates NF-κB activation in T cells, it was hypothesized that CARMIL2 variants were incapable of doing so. To test this idea, plasmids expressing 3xFLAG-tagged *CARMIL2* WT or variants were transiently transfected, along with NF-κB-luciferase and TK-*Renilla* reporter plasmids, into HEK293T cells, then stimulated with PMA and ionomycin (P/I).

Luciferase activity was then analyzed as a measurement of NF-κB activation (**Figure 10A**). After luciferase reporter activity was normalized by the activity of a second luciferase, *Renilla*, used as internal control, NF-κB reporter gene activation resulted enhanced in P/I stimulated cells, but non significantly different between empty vector and any *CARMIL2* construct, or between different *CARMIL2* alleles. Thus, it seemed that co-transfecting *CARMIL2* did not lead to an additional increase in NF-κB reporter gene expression.

However, re-analysis of raw luminescence data revealed that *Renilla* luciferase activity was consistently different between constructs, roughly following the trend of Firefly luciferase activity. Given the unreliability of pRL-TK plasmid as internal control, statistical analysis was repeated only considering Firefly luciferase activity (**Figure 10B**). This time, there was a significative difference in NF-κB activation between empty vector and WT *CARMIL2*. In addition, unexpectedly, both protein-truncating variants promoted NF-κB activation after P/I stimulation to a level similar (p.C155VfsTer54) or significantly higher (p.E978*) than that of WT. Conversely, cells transfected with missense variant p.D623E showed significantly reduced NF-κB promoter activation after P/I stimulation compared to all the other constructs, and no significative difference from cells transfected with empty vector.

To compensate for the absence of internal control and correct for possible transfection imbalance, the experiment was repeated five times with triplicate samples.

Establishment of a pragmatic model of patient-centered reverse translation in monogenic IBD research

The first specific aim of this project, to identify pediatric patients with *CARMIL2* variants from a cohort of IBD patients and report their phenotype, exemplified a bedside-to-benchtop approach, hence reverse translational research. The second specific aim, to confirm the pathogenicity of such variants, aspired to be the first step of a benchtop-to-bedside approach, or conventional (forward) translational medicine, whose ultimate purpose is precision medicine.

Therefore, within CARMIL2 collaborative research the two approaches complemented one another, representing an example of patient-centered forward and reverse translation. This

has led to the standardization of such concept and to the creation of a pragmatic model of translational research in monogenic IBD (**Figure 11**).

According to this model, all patients with suspected monogenic IBD should undergo a full work-up that include WES. For some of those patients, WES is enough to diagnose one of the known forms of monogenic IBD. As for the other subjects, a part of them is affected by monogenic IBD, but NGS is not sufficient to establish conclusively a culprit gene. In this respect, several assays, such as immunohistochemistry or functional studies may help to establish the pathogenicity of a candidate variant/gene. This bedside-to-benchtop approach, the lower half of the cycle, starts with the comprehensive characterization of the patients, sometimes identifying unique genotypes or phenotypes of IBD, but eventually contributes to a deeper understanding of the disease, that affects many other individuals.

Reverse translational research prompts the generation of hypotheses about disease mechanisms and drug targets, thereby contributing to the upper half of the cycle, the traditional (forward) translational research. This benchtop-to-bedside approach is particularly useful to identify new therapeutic targets, hence driving precision medicine strategies.[30]

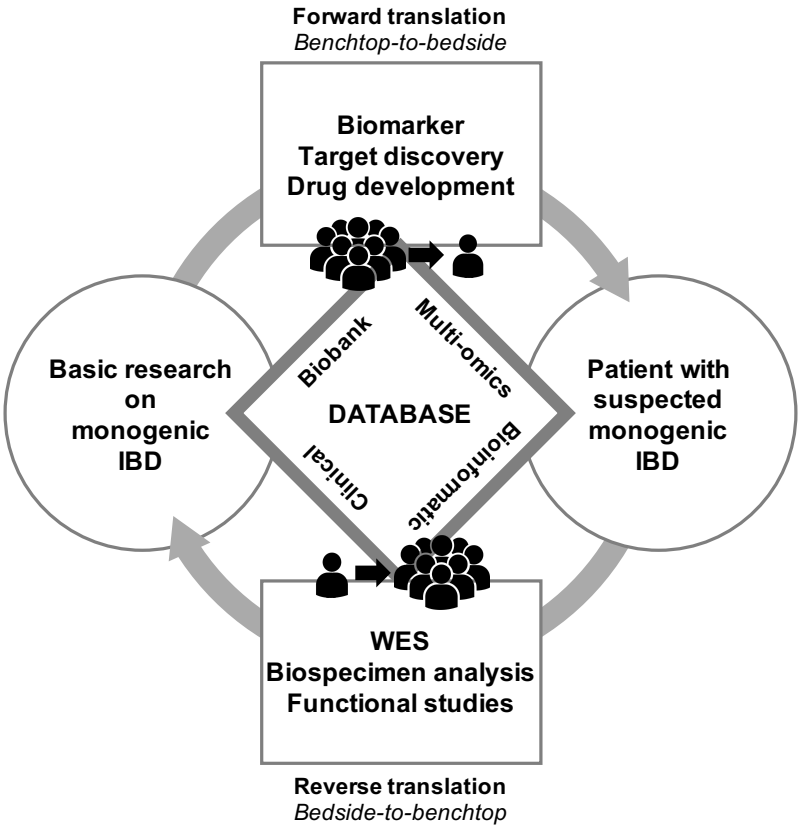


Figure 11. *Proposed model of translational research in monogenic IBD.*

Conclusions

CARMIL2 deficiency can manifest with isolated IBD

Our study confirms that CARMIL2 deficiency can manifest only with isolated IBD.

Unlike more recent studies[49,50] reporting a very early IBD onset (6 out of 6 patients), two of our patients shared a later age of onset of IBD symptoms, namely at 11 and 15 years of age. Although the majority of studies on monogenic IBD have focused on the very early onset population, running the risk of selection bias, recent findings have pointed out that a genetic disorder should be considered in all patients with pediatric onset IBD.[15] Resistance to conventional lines of treatment and complicated disease course, similar to what was reported in Patients 3 and 4, should always prompt the execution of WES, in line with existing recommendations.[7,8]

None of our patients presented overt clinical signs of immunodeficiency before the diagnosis of IBD. Patient 1 developed bacterial sepsis after the initiation of immunosuppression (i.e., azathioprine). Notably, one patient reported in the study of Magg et al.[49] died due to septic complications at the age of 4 years while on treatment with azathioprine.

Patient 3 and 4 experienced severe infectious complications after surgery. The need for a surgical treatment appears to be a common feature of pediatric IBD associated to CARMIL2-deficiency, since it occurred in the majority of the patients reported so far (6 out of 9 patients, including our cases) and was frequently due to a failure of medical treatment (3 out of 6 patients, including our cases).[49,50] A recent study found that progression to surgery can be itself an indicator of monogenic etiology among pediatric IBD patients.[15] There is no consensus with respect to surgical timing, indications, and strategies in monogenic IBDs, including CARMIL2 deficiency.[61]

Our results suggest that CARMIL2-deficient IBD patients harbor a significant risk of life-threatening immunosuppression-and surgery-related infections. A treatment option for several genetic immunodeficiencies underlying IBD-like phenotypes is hematopoietic stem cell transplantation (HSCT).[7,8] However, it could be inappropriate or even harmful if an epithelial barrier defect coexists.[23] Herein, we confirm that CARMIL2 protein is expressed in gastrointestinal epithelium, but it remains to be clarified if this is relevant to IBD pathogenesis in deficient patients.

Overall, *CARMIL2* gene should be included in the diagnostic work-up of patients with suspected monogenic IBD regardless of the age at disease onset and of the presence of overt clinical signs of immune deficiency. In fact, the identification of CARMIL2 deficiency has the potential to influence treatment choice and might improve disease prognosis, especially by means of prevention and prompt recognition of infectious complications.

CARMIL2 deficiency can manifest with autoimmune polyendocrine syndrome and should be considered an IPEX-like syndrome

This is the first report showing the association between CARMIL2 deficiency and autoimmune endocrinopathies. Autoimmune polyendocrine syndromes (APS) are characterized by functional impairment of multiple endocrine glands due to loss of immune tolerance toward them in genetically susceptible hosts.

Much like IBDs, most APSs result from polygenic predisposition, although they rarely present as part of a broader syndrome with an underlying monogenic etiology. Monogenic APSs are caused by mutations in genes involved in maintenance of central (e.g., *AIRE*) or peripheral (e.g., *FOXP3*, *CTLA4*, *LRBA*, *STAT1*, *STAT3*, *STAT5b*, *ITCH*, *BACH2*) immune tolerance, leading to aberrant Treg function or activation of self-reactive effector T cells.[62–67]

Several lines of evidence support CARMIL2 as a candidate gene for APS-2.

First, the significance of rare LoF variants of CARMIL2 in human disease has already been established, consistently with a recessive disease model.[44] This is confirmed by the modest LOUEF (loss-of-function observed/expected upper bound fraction) score (0.552 for CARMIL2, indicating a degree of intolerance to LoF variants) shown using gnomAD (Genome Aggregation Database) data.[68]

Secondly, biallelic *CARMIL2* LoF variants fully segregate with an immune dysregulation disorder in multiple independent families with autoantibodies and absence of antigen-specific antibodies to bacterial vaccines.[44,69] This is consistent with the known B cell phenotype of human CARMIL2 deficiency. Additionally, skin manifestations have been described in the majority of CARMIL2-deficient patients, and eczema is part of other monogenic APSs.[44–50,62,69]

Thirdly, the function of CARMIL2 is consistent with the known pathogenesis of APS, being expressed in immune cells and endocrine glands and being essential for development of regulatory T cells and for Th17 differentiation, similarly to other genes mutated in monogenic APS.[45,59,62–65,70]

Moreover, *CARMIL2* interactome include *DOCK8*, that is mutated in patients presenting with autoimmune disorders, eczema and compromised Treg function.[45,71]

Notably, CARMIL2-deficient mice and humans did not develop any obvious organ-specific autoimmune disorder, despite a reduction in Tregs.[45,70] This might depend on the coincident defect in effector T cells, also determined by a lack of CD28 co-stimulation.[45] Furthermore, CARMIL2-deficient mice did not display any intrinsic B cell defect.[44,45,70] Therefore, knockout mice might not be a suitable model for studying human CARMIL2 deficiency.

Remarkably, enteropathy and/or IBD-like intestinal inflammation, autoimmune endocrinopathy, and skin manifestations are common in IPEX and other syndromes presenting with IPEX-like features, caused by monogenetic defects affecting Treg function.[7,8,62,72] Hence, CARMIL2 deficiency should be added to the increasing group of IPEX-like syndromes, as already proposed.[50]

Genotypic heterogeneity of CARMIL2 and pathogenicity of newly discovered CARMIL2 variants

Alongside phenotypic differences, CARMIL2 deficiency is characterized by marked genotypic heterogeneity.

The known variants associated with CARMIL2 deficiency have different impact on the protein (e.g., nonsense, frameshift, missense, etc.) and they are spread along the gene (**Figure 4**). The only consistency seems to be the lack of mutations in the C-terminus portion of the gene. One could speculate that mutations occurring in the C-terminus, close to end of the coding sequence, may preserve some level of protein function. Therefore, failure to produce the expected phenotype would lead to missed diagnosis. The lack of obvious genotypic-phenotypic correlation, as well as the inter- and intrafamilial clinical heterogeneity, even among carriers of the same *CARMIL2* variant, seems to point towards a contribution of additional environmental, genetic, or epigenetic-modifying factors in determining the clinical manifestations of CARMIL2 deficiency.

Through our work we were able to discover three *CARMIL2* variants previously not reported in literature: null variants p.C155VfsTer54 (frameshift) and p.E978* (nonsense) and missense variant p.D623E.

Null variants can be deemed to disrupt gene function, but with some caution: they must represent a known mechanism of pathogenicity for the disease, according to the established inheritance pattern.[54] However, if the variant occurs close to the 3' terminus, nonsense-mediated decay may not happen, and a truncated protein could be expressed. This is especially true when the truncating variant is located downstream of the most 3' truncating variant established as pathogenic in the literature, as in the case of the very distal p.E978* variant of *CARMIL2*.

Missense variants, such as p.D623E, can be interpreted using various *in silico* algorithms that assess the predicted impact of a missense change. Bioinformatic tools can predict the damaging effects of mutations, but might overestimate them, therefore they shouldn't be used as the sole source of evidence to assume pathogenicity.[54] Hence the importance of experimental validation to demonstrate the pathogenicity of newly found variants, i.e., through demonstration of the absence of the gene product or functional assays.

Different lines of evidence from the experiments we performed showed that p.D623E variant behaves as a non-functional allele, i.e., a mutant copy of the gene that results in complete lack of the associated gene product or, as in this case, a product that does not function properly.

Particularly, immunofluorescence of transfected cells resulted in a granular pattern. Endoplasmic-reticulum-associated protein degradation (ERAD) of p.D623E protein variant might explain this finding. In fact, the residue 623 of CARMIL2 belongs to an LRR domain, a structural motif with a horseshoe shape, with an interior parallel beta sheet, an exterior array of helices, and a hydrophobic core containing many leucine residues.[73] An amino acid substitution in this region may lead to the exposure of normally hidden hydrophobic patches, usually buried inside the protein to keep the lowest energy state. Exposed patches might lead to protein aggregation, or they could be recognized as a substrate by ERAD, a cellular pathway that targets misfolded or mutated proteins, which are retained inside the endoplasmic reticulum (ER) and targeted for ubiquitination and subsequent proteolytic degradation.[74,75] ER retention and impaired trafficking could explain the staining pattern observed in transfected cells, the proteolysis the lower than predicted MW. Protein overexpression might make the process more obvious by engulfing the cell. A way to confirm this hypothesis would be to examine co-localization with an ER marker, such as anti-Calnexin antibody. Another, non-exclusive possibility is that substitution from aspartic acid to glutamic acid leads the protein to be recognized by a glutamic-acid-specific protease.

Luciferase reporter assay showed that variant p.D623E failed to activate NF-κB signaling, unlike other *CARMIL2* constructs and with no significant difference in comparison with the empty vector. However, the assay had some limitations. First, it was an overexpression experiment, which is not always a good model; in fact, protein overexpression can cause cellular defects itself.[76] Secondly, statistical significance was observed only when considering absolute Firefly luciferase activity but not when normalizing by *Renilla* luciferase activity. An internal control, such as *Renilla*, is used to normalize the test reporter data for transfection variability from well-to-well. Thanks to its constitutively active promoter, it's reporter activity should correlate only with the amount of DNA transfected and not be modulated by other experimental factors. However, several conditions that alter the basal constitutive expression of *Renilla* have been identified previously, including PMA treatment and activation of protein kinase C pathway, which is activated downstream of CARMIL2.[77] This could explain the observed fluctuation in *Renilla* luciferase activity. Therefore, pRL-TK plasmid cannot be considered a valid internal control, and an alternative way of normalization should probably have been used.

Finally, quite surprisingly, expression of protein-truncating variants p.C155VfsTer54 and p.E978* potentiated P/I-induced NF-κB activation, as much as WT CARMIL2 (p.C155VfsTer54)

or more (p.E978*). One could speculate that the N-terminal portions of the truncated proteins may retain some ability to stimulate the CARD11/CARMA1 complex. However, this could hardly happen *in vivo*. In fact, it can be assumed that protein-truncating variants p.C155VfsTer54 and p.E978* are targeted by nonsense-mediated mRNA decay, a quality-control mechanism that destroys mRNAs with premature termination codons to prevent the synthesis of potentially harmful truncated proteins.[78] However, this couldn't be definitely proven for variant p.C155VfsTer54 by immunostaining, because the anti-CARMIL2 antibody employed recognizes an immunogen sequence located between amino acids 693 and 777 of CARMIL2, which is absent in variant p.C155VfsTer54 (but present in variant p.E978*, as the WB demonstrated).

To conclude, according to criteria of the American College of Medical Genetics 2015 recommendations,[54] variants p.C155VfsTer54 and p.E978* of *CARMIL2* can be classified as pathogenic. In fact, positive criteria are (one very strong criterion plus one strong criterion are sufficient to confirm pathogenicity):

- Very strong evidence: they are null variants.
- Strong evidence: immunostaining supports a damaging effect on gene product since protein expression is essentially absent for both variants.
- Moderate evidence: both variants are absent from population databases.

According to the same recommendations, variant p.D623E as likely pathogenic. Positive criteria are (one strong and one moderate are sufficient):

- Strong evidence: immunostaining and *in vitro* studies (WB, immunofluorescence, luciferase reporter assay) support a damaging effect on the gene product.
- Moderate evidence: the variant is absent from population databases.
- Supporting evidence: computational data support a deleterious effect and amino acid is evolutionary conserved.

Limitations of the study

Our study had some limitations.

First, Sanger sequencing failed to confirm the presence of homozygous *CARMIL2* variants in Patient 3 and 4, despite repeated attempts. This was probably due to a guanine-cytosine (GC)-rich template. DNA with high GC-content is more stable, but notoriously more difficult to denature and very challenging to sequence, because each GC base pair is held together by three hydrogen bonds (while AT and AU base pairs are held together by two hydrogen bonds). However, there has been a lot of debate regarding the necessity of confirming variants detected by NGS with Sanger sequencing.[79] A recent paper[58] found that most Sanger validations performed were

themselves incorrect, leading to false negative results, because a second round of sequencing after primers optimization validated the variants found via NGS.

Second, overexpression experiments might have been unreliable for protein-truncating variants p.C155VfsTer54 and p.E978*. In fact, it can be assumed that *in vivo* these variants are targeted by nonsense-mediated mRNA decay, as stated above. For this reason, the more intense immunofluorescence signal of p.C155VfsTer54 compared to WT CARMIL2 in cultured cells probably does not reflect what happens *in vivo* and could have been determined by higher transfection efficiency due to a shorter peptide.

Toward a pragmatic model of patient-centered forward and reverse translation in monogenic IBD research

Basic or "benchtop" research has mostly a hypothesis-generating approach, while "bedside" clinical research is strongly data-driven and inductive. Translational research, both forward (conventional) and reverse, represents the bridge that connect clinical and basic research.

Forward translational "from benchtop to bedside" has limited success if there is not a reverse path. Reverse translational research ensures feedback "from bedside to bench", identifies patients that demand laboratory investigation and establishes connections between laboratory findings and clinical observations.

Big data, and particularly omics sciences have allowed translational research to come full circle, with a reciprocal strategy that pairs hypothesis-generating and data-driven approaches. Genomics is the most striking example of this. Pre-genomics approach to the understanding of genotype-phenotype relationship was "function first": the quest for genes that provided a previously known function. Post-genomics approach is "gene first": it starts with virtually all genes (e.g., with WES) and then looks for a culprit.[80] Big data, combining clinical records, biobanks, multi-omics databases (genomics, transcriptomics, proteomics, metabolomics, etc.), and bioinformatic tools make available vast amounts of information and manages to combine observational with experimental data.[81]

The aim of this proposed model of research in monogenic IBD is to close the loop of translation: observational data from clinical practice and clinical research, offering a large sample and broad coverage of patient variables, should stimulate new hypotheses to direct basic research on IBD mechanisms. Reverse translational research, then, solves clinical questions using basic research techniques, toward the ultimate goal: precision medicine. When started, this paradigm should become a continuous, cyclical process, back and forth between clinical and basic research.

Closing remarks

In summary, the phenotypic spectrum of CARMIL2 deficiency is broader than previously known, ranging from severe immunodeficiency to IBD and organ-specific autoimmunity.

CARMIL2 gene should be part of the diagnostic evaluation of patients with suspected monogenic IBD, even in the absence of obvious signs of immunodeficiency. Genetic diagnosis may be vital in monogenic IBD, to guide specific treatment, prevent surgery or unnecessary therapies, anticipate complications, and help genetic counseling.[7,8,23,82,83]

Moreover, CARMIL2 deficiency can present exclusively with APS.

A possible explanation of such heterogeneity is that the type and the severity of clinical manifestations may derive from the direction and the entity of the imbalance between regulatory T cells and effector T cells in the individual patient. This is consistent with the fact that immune dysregulation due to genetic defects can cause both immunodeficiency and autoimmunity.[84]

Our study proposed a model to standardize patient-centered translation in monogenic IBD research. Future directions include further research with a view to closing the loop of translation by finding a therapeutic target for precision medicine. Further studies are needed to better define IBD immunopathogenesis in CARMIL2 deficiency and the potential therapeutic utility of HSCT.

This project provides a good example of the translational approach in the context of IBD research and attempts to standardize it, in a visual way too. It also contributes to add another dimension to the translational research paradigm: the worldwide collaboration between countries, centers, and researchers committed to the pursuit of target discovery in IBD, to address its increasing burden. By doing this, the big data revolution can reach its full potential, increasing knowledge, broadening horizons, and saving lives.

Aim 2

To establish diagnostic-therapeutic clinical pathways and biomarkers that could help to manage pediatric IBD patients

PART 2.1

Evaluation of sugar intestinal permeability test as a diagnostic and prognostic tool in pediatric IBD

Bosa L, Faggian G, Basso D, Frigo AC, Muraca M, Gaio P, Cananzi M. Sugar intestinal permeability test correlates with pediatric Crohn's disease phenotype and activity and predicts the development of a stricturing behavior. Manuscript in preparation.

Abstract

Background

Increasing evidence supports the central role of intestinal barrier dysfunction in the pathogenesis of inflammatory bowel disease (IBD). Sugar intestinal permeability test (SIPT) is a useful, non-invasive method to assess intestinal permeability (IP). Few studies have evaluated the clinical implication of impaired IP and the potential role of SIPT in pediatric IBD.

The objectives of this study were: 1) To evaluate the degree of IP impairment in pediatric IBD patients at diagnosis and within different IBD subtypes; 2) To evaluate the relationship between IP and other markers of Crohn's disease (CD) activity, in order to assess the potential role of SIPT in CD follow-up; 3) To evaluate the prognostic implication of impaired IP in the development of CD complications and in the need for surgery.

Methods

A monocentric, retrospective, observational study was performed. We included children with an established diagnosis of IBD who underwent at least one SIPT with lactulose and mannitol between January 2010 and July 2021, either at IBD diagnosis or during follow-up. SIPT results were compared to clinical, radiological, endoscopic, and histologic disease parameters, inflammatory indexes, growth parameters and disease location.

Results

Objective 1) 84 children (M 52%, median age 12 years) underwent SIPT at IBD diagnosis, 55 with CD, 18 with Ulcerative Colitis (UC), and 11 with IBD unclassified (IBDU). An alteration of IP was observed in 87.3% of CD, in 38.9% of UC and in 63.6% of IBDU patients. The lactulose/mannitol ratio (LMR) was significantly higher in children with CD compared to those with UC (p 0.0004). The LMR showed a good accuracy in discriminating between children with CD and other IBD subtypes (AUC 0.75; 95% CI: 0.65-0.87), the most accurate cut-off of LMR being 0.05 (67% sensitivity, 79% specificity).

Objective 2) 136 SIPT were performed in 71 CD patients (M 50.7%, median age 13 years), either at diagnosis or during follow-up. LMR positively correlated with markers of CD activity: PCDAI clinical score, CRP, ESR, fecal calprotectin, and SES-CD endoscopic activity score ($p < 0.0001$). LMR was significantly higher in patients with histologic signs of active CD compared to those with histologic remission. LMR showed a good accuracy in identifying patients with histologic CD

activity (AUC 0.76; 95% CI: 0.65-0.88). A LMR cut-off of 0.03 resulted as the most accurate in identifying patients with active CD (72% sensitivity, 68% sensibility).
Objective 3) The outcome of 53 CD patients (M 56.6%, median age 12 years) who underwent SIPT at CD diagnosis was assessed (median length of follow-up 5.2 years): 8 patients required abdominal surgery, 11 developed perianal disease, 15 a stricturing phenotype, and 5 a penetrating disease. An increased urinary excretion of lactulose at diagnosis predicted the development of a stricturing phenotype in the logistic regression analysis (p 0,02; OR 2,25 95% CI: 1,09-4,66).

Conclusions

Alterations of IP are highly prevalent in children with IBD. SIPT proved to be a useful non-invasive tool to discriminate CD among the other IBD subtypes at diagnosis, to detect luminal inflammation during follow-up, and to predict the risk of developing a stricturing phenotype over time. Further studies are needed to confirm these results prospectively in a broader cohort of children diagnosed with IBD.

Background

Intestinal barrier and intestinal permeability

The intestinal barrier, intended as a functional entity, is a complex structure that separates the internal milieu of the gut from the luminal environment. It consists of a physical barrier, that includes the vascular endothelium, the epithelial cell lining, and the mucus layer (composed of a gel formed by the interaction of mucins, trefoil peptides and surfactant lipids), and of a chemical barrier, made of digestive secretions, antimicrobial peptides, and other cell products. Moreover, intestinal microbiota, immune elements, and motility also contribute to the intestinal barrier. Such barrier allows the absorption of nutrients and the exchange of molecules with the environment, while preventing the loss of water and electrolytes and the entry of antigens and microorganisms into the body. Thus, it carries out two apparently contrasting functions: it permits the peaceful coexistence with intestinal symbionts, which are necessary for our organism, without eliciting chronic inflammation, while providing a measured defensive and inflammatory response to pathogens.[85–87]

Intestinal permeability (IP) refers to a functional feature of the intestinal barrier, measurable by analyzing flux rates of defined molecules (such as electrolytes or sugars) of different MW across the intestinal wall as a whole (*in vivo* situations) or across wall components (*ex vivo* experimental models).[85,86] Therefore, normal IP can be defined as a stable permeability state found in healthy subjects with no inflammation, intoxication, or altered intestinal functions, while impaired IP is a non-transient change in permeability, leading to a loss of intestinal homeostasis, functional impairment, and disease.[85,86]

Both endogenous (e.g., psychological stress, intestinal inflammation, gut hypoperfusion) and exogenous factors (e.g., diet, viral infections, drugs, alcohol, toxins) can increase IP. An abnormally increased IP, also referred to as "leaky gut", allows the entrance of food antigens, commensal or pathogenic bacteria, and bacterial components into the lamina propria and then into the systemic circulation, possibly causing inflammation.[88]

Assessment of intestinal permeability

There are four different pathways through which luminal products can cross the intestinal epithelium, depending on their physico-chemical properties, such as size and hydrophobicity:

1. Transcellular route, used by small hydrophilic and lipophilic compounds.

2. Paracellular route, regulated by tight junction (TJ) proteins and used by ions, water, and larger hydrophilic compounds (from ~400-600 Da to 10-20 kDa) that cannot cross transcellularly.
3. Transcellular active transport, involving membrane transporters and channels, used by nutrients (sugars, amino acids, and vitamins).
4. Transcellular endocytosis, via vesicles, used by larger peptides, bacterial components, or whole bacteria.

The paracellular and the transcellular endocytic routes are probably the most implicated in diseases pathophysiology.

Most studies evaluate the IP of the paracellular pathway, even though the transcellular endocytic pathway is potentially more relevant in several disorders. In fact, in contrast to common belief, the endocytic route, and not the paracellular one is the mechanism used to cross the intestinal epithelium by whole bacteria (bacterial translocation) and bacterial components, such as endotoxins (lipopolysaccharide, LPS).[88]

The assessment of IP can take place *ex vivo* or *in vivo*, through direct or indirect methods.

The *ex vivo* approaches require intestinal epithelial sources, such as cell cultures grown in membranes or tissue from endoscopic biopsies or resection specimens. The Ussing chamber technique allows the measurement of transepithelial electrical resistance (TEER), which reflects the resistance of the epithelium to the current flow, i.e., the crossing of ions from the luminal to the basolateral side. An increase in intestinal epithelium paracellular permeability to ions results in a gain in conductance. The Ussing chambers can also be used to assess the transcellular pathway by adding probes of large MW or whole bacteria that are known to cross the epithelium using through exocytosis.

Another *ex vivo* approach is the histological one, that aims to quantify changes in tight junction proteins in tissue samples.[88]

As for the *in vivo* approaches, almost all direct methods assess paracellular IP through measurement of the urinary excretion of orally ingested probes, metabolically inert, which should be absorbed exclusively through the paracellular route and then freely filtered at the glomerulus and not reabsorbed in the tubule. Such probes include PEG, ^{51}Cr-EDTA, and, especially, sugars, such as sucrose, sucralose, lactulose, mannitol, and rhamnose.

Among sugar intestinal permeability test (SIPT), the most used parameter is the urinary ratio of lactulose to mannitol (LMR), which evaluates the differential urinary excretion of the disaccharide lactulose and the sugar alcohol mannitol. The two sugars have different absorption routes: while lactulose crosses the intestinal barrier in a highly restricted manner via the paracellular

route, at the base of the villi, mannitol, smaller in size, moves freely across the intestinal mucosa via the transcellular route, through aqueous pores at the tip of the villi. Under physiological conditions, mannitol is much more absorbed than lactulose. In the case of pathological alteration of the small intestine, when the integrity of intestinal mucosa is lost, changes in sugars uptake can occur. Lactulose absorption could increase since it can cross more easily the epithelium trough intercellular leaks. On the contrary, if the intestinal damage causes a reduction of the absorptive surface, mannitol uptake is reduced.[89] The combination of the two effects (excessive permeability and mucosal damage) is represented by the LMR. The use of a ratio corrects for individual non-mucosal confounders, such as variances in absorption kinetics through the gastrointestinal tract (e.g., gastric emptying, intestinal transit time) and differences in renal excretion, being both sugars affected in the same manner.[90] Since both lactulose and mannitol are degraded by the colonic microbiota, they are used to evaluate small intestinal, but not colonic permeability.[88]

Other *in vivo* direct methods to measure IP include confocal laser endomicroscopy (CLE), that measures the leakage of fluorescein into the bowel lumen after intravenous administration, and laser mucosal impedance testing.[88]

The main indirect methods to assess mucosal integrity are blood or urinary biomarkers, based on the detection of molecules that normally resides in the gut lumen or are components of the intestinal barrier, which can be interpreted, with some limitations, as signs of impaired function or damage of the barrier itself.[88]

Intestinal permeability and IBD

Several gastrointestinal and non-gastrointestinal diseases have been found to be associated with an alteration of the intestinal barrier and increased IP.[85–88]

In particular, increasing evidence suggests that IP might be critical in IBD pathogenesis, to the point that some authors consider IBD as an impaired barrier disease.[87] Many studies have demonstrated an increased IP in IBD patients, with different findings depending on the markers used for the assessment.

Using orally administered ^{99m}Tc-DTPA, IP was increased in patients with CD and UC, with both active and inactive disease, and the degree of intestinal inflammation was associated with the measure of permeability.[91]

Similarly, the permeability to oral ^{51}Cr-EDTA was increased in patients with active IBD, especially with colonic disease.[92]

As for first-degree healthy relatives of IBD patients, an increase in IP was found using PEG-400 in CD patients' families,[93] but not confirmed in other studies, not even when LMR or other sugars were used.[94–98]

Studies also showed that increased IP could resolve after IBD treatment. The lactulose/rhamnose ratio improved after exclusive enteral nutrition (EEN) was given as induction therapy in pediatric patients with active small bowel CD.[99] Moreover, anti-TNF-α therapy normalized permeability to ^{51}Cr-EDTA in patients with refractory CD.[100]

As for IBD prognosis, some studies demonstrated that an altered IP in CD patients in clinical remission predicts an increased risk of early relapse.[101]

Finally, the serum markers of altered intestinal barrier associated with bacterial translocation were found to be increased in patients with both active and inactive CD, but only in active UC patients.[102]

Remarkably, CD patients seem to have both a dysregulation of the paracellular route in inflamed colon and small bowel epithelia and an increased transcellular permeability to protein antigens and whole bacteria in non-inflamed ileal regions.[88,102]

Objectives of the study

SIPT is a useful, non-invasive, non-expensive, *in vivo* method to assess IP in a reproducible way.[86,87] However, only a few studies have evaluated the clinical implication of impaired IP and the potential diagnostic role of SIPT in IBD, especially in pediatric patients.

Therefore, the specific objectives of this study were:

1. To evaluate the degree of IP impairment in pediatric IBD patients at diagnosis and within different IBD subtypes (CD, UC, IBDU).
2. To evaluate the relationship between IP and other markers of CD activity, in order to assess the potential role of SIPT in CD follow-up.
3. To evaluate the prognostic implication of impaired IP in the development of CD complications (perianal disease, fistulas, and strictures) and in the need for surgery.

Methods

Study design and population

This was a monocentric, retrospective, non-profit, observational study, conducted on pediatric patients (aged 0-18 years old) with a diagnosis of IBD followed at the Department of Women's and Children's Health of the University Hospital of Padova, a tertiary referral pediatric hospital and a regional center for PIBD in the North-East of Italy.

The results of all SIPT's performed in pediatric subjects affected by IBD from January 2010 to July 2021 were collected and analyzed.

Inclusion criteria were:

- Age less than 18 years.
- Diagnosis of IBD based on established clinical, radiological, endoscopic, and histological criteria.
- Availability of at least one SIPT performed at IBD diagnosis and/or during IBD follow-up.

Exclusion criteria were:

- Prior colectomy.
- Pre-existing ileostomy.
- Time interval superior to 4 months between SIPT and other IBD investigations (i.e., magnetic resonance enterography [MRE] and/or endoscopy).
- Administration of steroids or other pharmacological treatments (e.g., infliximab) that could significantly modify IBD course between SIPT execution and other IBD investigations (i.e., MRE and/or endoscopy).
- Ongoing gastrointestinal infection.

Sugar intestinal permeability test

IP was assessed by measuring the 6-hours urinary excretion of a disaccharide, lactulose, and a of sugar alcohol, mannitol.

After fasting for at least 6 hours, patients were requested to empty their bladder and then to drink a solution containing 6.67 gr of lactulose (10 mL of lactulose sirup 66.7%) and 3 g of

mannitol dissolved in 50 mL (younger than 10 years) or 100 mL (older than 10 years) of water. The sugars were prepared by the hospital pharmacy. Urine was collected for the next 6 hours in a plastic container containing 1 mL of chlorhexidine (Clorexifarm® 20%), added to prevent possible bacterial degradation of the sugars. The total volume of the urine was measured, and a 10 mL urine sample was stored at -20°C until assayed. During the test the patients were kept nil per os, except for a glass of water.

Sugars were measured in the urine sample using High-Performance Liquid Chromatography (HPLC), allowing the simultaneous determination of urinary lactulose and mannitol by cation-exchange chromatography and evaporative light-scattering detection. Samples were purified by solid phase extraction on a C18 silica cartridge and subsequent addition of an anion-exchange resin.[103]

The percentage of recovery of each sugar in the urine and the lactulose/mannitol ratio (LMR) were calculated as follows:

- Percentage of sugar recovery = {[Total urine volume (dL) over 6 hours x sugar concentration (mg/dL) in urine sample] / Amount of sugar ingested (mg)} x 100.
- Lactulose to mannitol ratio (LMR) = Percentage lactulose recovery / Percentage mannitol recovery.

The upper limit of normal of the LMR provided by the laboratory was 0.03, based on data from the adult population.

IBD assessment

Disease activity of IBD patients was determined using Pediatric Crohn's Disease Activity Index (PCDAI)[104] for CD patients and Pediatric Ulcerative Colitis Activity Index (PUCAI)[105] for UC and IBDU patients, calculated immediately before (1-7 days) other IBD investigations (MRE and endoscopy) were performed.

As for laboratory findings, blood testing was performed in all patients 1 to 7 days before endoscopy and/or imaging. Blood tests considered included full blood count, serum inflammatory markers (c-reactive protein, or CRP and erythrocyte sedimentation rate, or ESR), serum albumin, ASCA (anti-*Saccharomyces cerevisiae* antibodies), and ANCA (anti-neutrophil cytoplasmic antibodies). Fecal calprotectin was also determined. Normal laboratory values were as follows: CRP <6.0 mg/L; ESR 2-38 mm/h; ANCA anti-proteinase III <1.9 KU/L; ANCA anti-MPO <6.9 KU/L; ASCA IgA <10 KU/L; ASCA IgG <10 KU/L; fecal calprotectin <50 ug/g.

The work-up for all patients included an endoscopic examination (esophagogastroduodenoscopy, or EGDS, and ileocolonoscopy) with biopsies, to confirm the

diagnosis of IBD and to assess disease extent and severity. Bowel preparation was done according to standard protocols, in line with international guidelines.[106] Endoscopies were performed in deep sedation by an experienced pediatric endoscopist or gastroenterologist. The endoscopic activity of IBD was quantified by two independent endoscopists during or after the exam using existing scores: the Simple Endoscopic Activity Score in Crohn's Disease (SES-CD)[107] for CD patients and the Mayo endoscopic score[108] for UC and IBDU patients. Disease localization and behavior were classified using the Paris classification of pediatric IBD.[4] Bowel specimens were fixed in formalin, processed, and evaluated for histological analysis by a pathologist expert in pediatric IBD.

MREs were performed using a 1.5-T magnetic resonance unit by trained technicians. To ensure optimal intestinal distension, after adequate bowel cleansing and a 6-hour fast, the patients were instructed to drink an oral contrast agent (either a PEG-based or a mannitol-based preparation, that have been shown to achieve comparable distension quality and side effect profiles)[109] 45-60 minutes before the examination. Moreover, a body weight-based dose of hyoscine butylbromide (10-20 mg) was administered intravenously before starting the examination, followed by a gadolinium chelate contrast agent (Dotarem®, 0.1 mmol/kg). Each series of images included breath-hold axial and coronal fat-suppressed T2-weighted sequences, axial and coronal steady-state free precession sequences, and coronal fat-suppressed T1-weighted 3-D sequences acquired before and during arterial, venous, and delayed phases. Since 2017 balanced turbo-field echo and diffusion-weighted imaging (DWI) 3b sequences were also included.

The radiologic disease activity of IBD was quantified using the Simplified Magnetic Resonance Index of Activity (MaRIAs).[110] The evaluated MRE items included mural thickening (wall thickness ≥3 mm), mural edema (high signal intensity on T2 sequences with fat saturation, compared with normal-appearing loops, or, alternatively, DWI restriction), fat stranding (loss of the normal sharp interface between intestinal wall and mesentery, with edema/fluid in the perienteric fat), and mucosal ulcerations (depressions beyond the mucosal surface), analyzed as binary categorical variables (absent/present) for each of the six considered intestinal segments (ileum, ascending colon, transverse colon, descending and sigmoid colon, and rectum). The MaRIAs was then calculated for each segment as follows:

$$\text{MaRIAs} = (1 \times \text{thickness} > 3\text{ mm}) + (1 \times \text{edema}) + (1 \times \text{fat stranding}) + (2 \times \text{ulcers})$$

The global MaRIAs resulted from the sum of each intestinal segment's index, while colonic MaRIAs sub-score was considered as the highest score among all analyzed colon segments.

As for bowel ultrasound, a bowel wall thickness of 3 mm or more was considered abnormal, due to its positive correlation with small bowel localization of IBD.[111]

Statistical analysis

Data were collected in a password-protected Excel database. The statistical analysis was performed employing GraphPad Prism Ver. 9.1.1 (GraphPad Software).

Quantitative variables were expressed as medians with interquartile range (IQR) and means ± standard deviation (SD), as appropriate. Qualitative variables were expressed as absolute values and percentages (to the first decimal place). The normal distribution of quantitative variables was established using the Q-Q plot and the Shapiro-Wilk test. Differences between groups were assessed using the Mann-Whitney test for paired data and with the Kruskall-Wallis test when comparing more than two groups. Differences between qualitative variables were evaluated using the Chi-square test or the Fisher's exact test. Correlations between quantitative variables were performed using the Spearman rank test. The strength of relationship between variables was determined using univariate logistic regression. P values of less than 0.05 (5%) were considered significant.

To measure the diagnostic accuracy of SIPT and its optimal cut-off value, a receiver operating characteristic (ROC) curve analysis was performed and the area under the curve (AUC) was calculated. Thus, the best cut-off value was identified using three different approaches: the Youden index method (the cut-point that maximizing the Youden function, which is the difference between true positive rate and false positive rate), the point closest-to-corner in the ROC plane method (the cut-point that minimize the Euclidean distance between the ROC curve and the point), and the Liu method (the cut-point that maximize the product of sensitivity and specificity).

Results

Intestinal permeability at IBD diagnosis

84 pediatric patients who underwent SIPT at the diagnosis of IBD were retrospectively recruited.

Among them, 55 (66.5%) were diagnosed with CD, 18 (21.4%) with UC, and 11 (13.1%) with IBDU.

Age and sex distribution were not significantly different among the three IBD subtypes (**Table 1 and 2**).

Pediatric IBD patients	
Number of patients	84
Sex, n (%)	
M	44 (52.4)
F	40 (47.6)
Age	
Patients number (%)	
<6 y	6 (7.1)
6-9 y	12 (14.3)
10-16 y	66 (78.6)
Median (range)	12 y (9 mo-16 y)
Diagnosis, n (%)	
CD	55 (66.5)
UC	18 (21.4)
IBDU	11 (13.1)
Follow-up, median (range)	4.4 (0.1-9.4)
CD	5.2 (0.1-9.4)
UC	2.9 (0.3-9.3)
IBDU	2.8 (0.4-8.3)

Table 1. *Demographic characteristics of the study population at IBD diagnosis.*

The LMR at IBD diagnosis (**Figure 1**) ranged from 0.003 to 0.556 (median 0.053) and resulted pathological (i.e., ≥0.03) in 61 patients (72.6%), of which 48 had CD (87.3% of the patients diagnosed with CD), 7 had UC (38.9% of the patients diagnosed with UC), and 7 IBDU (63.6% of patients the diagnosed with IBDU).

LMR was significantly higher in patients who received a diagnosis of CD compared to UC patients (p 0.0004). LMR values of patients diagnosed with IBDU was not significantly different from those found in patients who received a diagnosis of CD or UC.

	CD		UC		IBDU	
Patients	55		18		11	
IBD location, n (%)	L1 (distal ileum +/- cecum) L2 (colon) L3 (ileum + colon) L4a (proximal to Treitz) L4b (distal to Treitz)	14 (25.5) 13 (23.6) 28 (50.9) 12 (21.8) 3 (5.5)	E1 (proctitis) E2 (left-sided colitis) E3 (extensive colitis) E4 (pancolitis)	0 (0.0) 8 (44.4) 2 (11.1) 8 (44.4)	E1 (proctitis) E2 (left-sided colitis) E3 (extensive colitis) E4 (pancolitis)	1 (9.1) 0 (0.0) 1 (9.1) 8 (72.7)
IBD phenotype, n (%)	B1 (inflammatory) B2 (stricturing) B3 (penetrating) B2B3 (stricturing + penetrating) P1 (perianal disease)	39 (70.9) 12 (21.8) 2 (3.6) 2 (3.6) 8 (14.5)	S0 S1	16 (88.9) 2 (11.1)	S0 S1	11 (100) 0 (0)
Growth delay, n (%)	G0 G1	42 (76.4) 13 (23.6)	G0 G1	18(100) 0 (0.0)	G0 G1	9 (81.8) 2 (18.2)
Disease activity	PCDAI, n (%) 0-10 (inactive) 11-30 (mild) >30 (moderate/severe) Median, range	 9 (16.4) 27 (49.1) 19 (34.5) 25 (0-57.5)	PUCAI, n (%) 0-9 (inactive) 10-34 (mild) 35-64 (moderate) ≥65 (severe) Median, range	 1 (5.5) 3 (16.7) 12 (66.7) 2 (11.1) 45 (0-70)	PUCAI, n (%) 0-9 (inactive) 10-34 (mild) 35-64 (moderate) ≥65 (severe) Median, range	 2 (18.2) 7 (63.6) 2 (18.2) 0 (0.0) 22.5 (5-35)
Endoscopic activity	SES-CD, n (%) Inactive (0-2) Mild (3-6) Moderate (7-15) Severe (≥16) Median (range)	 2 (3.6) 11 (20) 27 (49.1) 15 (27.3) 11 (0-27)	MAYO score, n (%) Score 0 Score 1 Score 2 Score 3 Median (range)	 1 (5.6) 2 (11.1) 13 (72.2) 2 (11.1) 1 (0-3)	MAYO score, n (%) Score 0 Score 1 Score 2 Score 3 Median (range)	 0 (0.0) 1 (9.1) 9 (81.8) 1 (9.1) 2 (1-3)
Radiologic activity	Global MaRIAs Median (IQR) Average (SD) MaRIAs ileum, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range) MaRIAs colon, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range)	 2.0 (0.5-4.0) 3.03 (3.73) 17 (33.3) 12 (23.5) 12 (23.5) 6 (11.7) 4 (7.7) 0 (0.0) 1 (0-4) 29 (56.9) 9 (17.6) 9 (17.6) 3 (5.9) 1 (2.0) 0 (0.0) 0 (0-4)	Global MaRIAs Median (IQR) Average (SD) MaRIAs ileum, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range) MaRIAs colon, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range)	 0.5 (0.0-1.25) 1.25 (1.91) 8 (66.7) 2 (16.7) 1 (8.3) 1 (8.3) 0 (0.0) 0 (0.0) 0 (0-3) 8 (66.7) 4 (33.3) 0 (0.0) 0 (0.0) 0 (0.0) 0 (0.0) 0 (0-1)	Global MaRIAs Median (IQR) Average (SD) MaRIAs ileum, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range) MaRIAs colon, n (%) Score 0 Score 1 Score 2 Score 3 Score 4 Score 5 Median (range)	 0 (0-3) 1.44 (2.30) 8 (88.9) 0 (0.0) 1 (11.1) 0 (0.0) 0 (0.0) 0 (0.0) 0 (0-2) 6 (66.7) 1 (11.1) 2 (22.2) 0 (0.0) 0 (0.0) 0 (0.0) 0 (0-2)
ESR, mm/h	Median (range)	39 (6-120)	Median (range)	19 (2-92)	Median (range)	30 (2-120)
CRP, mg/L	Median (range)	9.6 (<2.9-150.0)	Median (range)	2.9 (<2.9-7.7)	Median (range)	2.9 (<2.9-57.0)
Fecal calprotectin, n (%)	<50 ug/g 50-300 ug/g 300-2100 ug/g >2100 ug/g	0 (0.0) 3 (5.8) 14 (26.9) 35 (67.3)	<50 ug/g 50-300 ug/g 300-2100 ug/g >2100 ug/g	0 (0.0) 1 (7.1) 5 (35.7) 8 (57.1)	<50 ug/g 50-300 ug/g 300-2100 ug/g >2100 ug/g	0 (0.0) 0 (0.0) 4 (40.0) 6 (60.0)
Serological markers, n (%)	ASCA+ ANCA+	31 (56.3) 9 (16.4)	ASCA+ ANCA+	1 (5.6) 9 (50.4)	ASCA+ ANCA+	1(9.1) 3 (27.3)

Table 2. *Clinical characteristics of the study population at IBD diagnosis.*

Lactulose urinary excretion ranged from 0.023% to 20.68% (median 0.57%) and, similarly to LMR, was significantly higher in CD patients compared to UC patients (p 0.01). Mannitol excretion ranged from 2.646% to 38.66% (median 13.71%) and was not significantly different between IBD subtypes (**Table 3**).

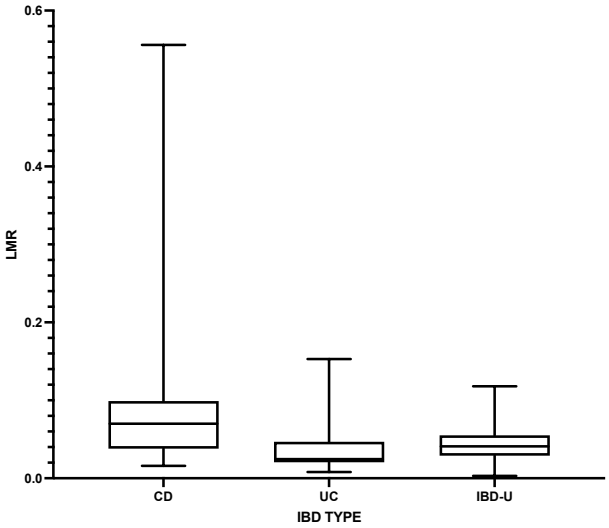


Figure 1. *LMR at the diagnosis of different IBD subtypes: CD, UC, IBDU.*

	All IBD	CD	UC	IBDU
Lactulose excretion %				
Median (range)	0.57 (0.023-20.68)	0.741 (0.117-20.68)	0.42 (0.06-1.75)	0.399 (0.023-1.71)
Mannitol excretion %				
Median (range)	13.71 (2.646-38.66)	13.99 (2.646-38.66)	14.05 (4.88-35.16)	13.07 (2.82-21.2)
LMR				
Median (range)	0.053 (0.003-0.556)	0.07 (0.016-0.556)	0.025 (0.008-0.153)	0.041 (0.003-0.118)
LMR <0.03, n (%)	23 (27.4)	7 (12.7)	11 (61.1)	4 (36.4)
LMR ≥0.03, n (%)	61 (72.6)	48 (87.3)	7 (38.9)	7 (63.6)

Table 3. *Sugar intestinal permeability test at the diagnosis of IBD in the study population.*

To test the ability of SIPT to discriminate CD from the other IBD subtypes at IBD diagnosis, we performed a ROC analysis for LMR (**Figure 2**), that revealed a moderately good accuracy (AUC 0.75; 95% CI: 0.65-0.87).

The best cut-off value of LMR to differentiate CD from non-CD patients was 0.05 (the same value with all three methods: Youden, point closest-to-corner, and Liu), with a sensitivity of 67% and a specificity of 79%.

We also performed a ROC analysis (**Figure 3**) to test the ability of LMR to distinguish between CD and UC patients at diagnosis, thus excluding IBDU patients from the analysis, assuming that they might eventually be reclassified as CD during follow-up. We found a better accuracy of LMR in discriminating CD patients from UC patients (AUC 0.79; 95% CI: 0.66-0.92).

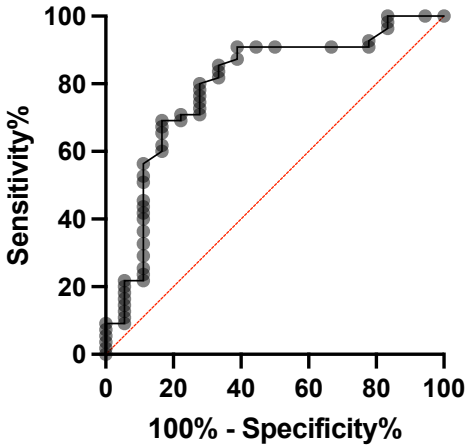


Figure 2. *ROC curve of LMR in distinguishing CD from other IBD subtypes.*

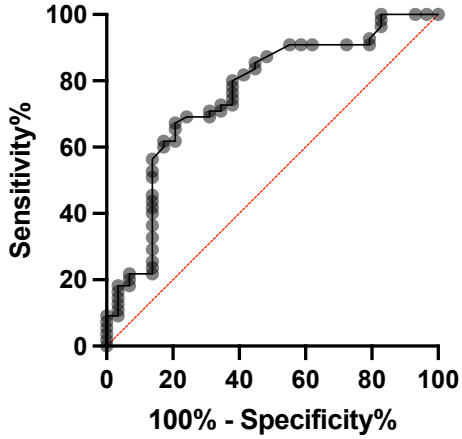


Figure 3. *ROC curve of LMR in distinguishing CD from other IBD subtypes.*

The best LMR cut-off value was again 0.05 (Youden index and Liu method were concordant, while the point closest-to-corner method was discordant), with a sensitivity of 69% and a specificity of 83%.

15 patients (17.9%) presented growth delay at the diagnosis of IBD. LMR levels were significantly higher (**Figure 4**) in patients with growth delay compared to those with normal growth (p 0.03). Furthermore, the mannitol urinary levels were significantly lower in the group of patients with growth delay (p 0.02).

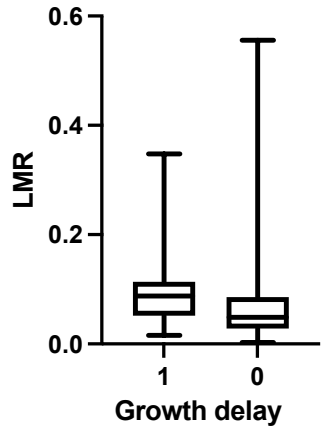


Figure 4. *LMR for growth delay.*

In the CD group, no significant differences in the urinary sugar levels were found between patients with different disease phenotype according to the Paris classification.

In the CD group, the PCDAI ranged from 0 to 57.5 (median 25) and was significantly higher (**Figure 5**) in patients with a LMR ≥0.03 (p 0.02). Moreover, a positive correlation (**Figure 6**) was found between the PCDAI and the LMR value (Spearman correlation coefficient 0.41, p 0.002).

In the UC and the IBDU groups, the PUCAI ranged from 0 to 70 (median 30). No correlations were found between urinary sugar levels and the PUCAI.

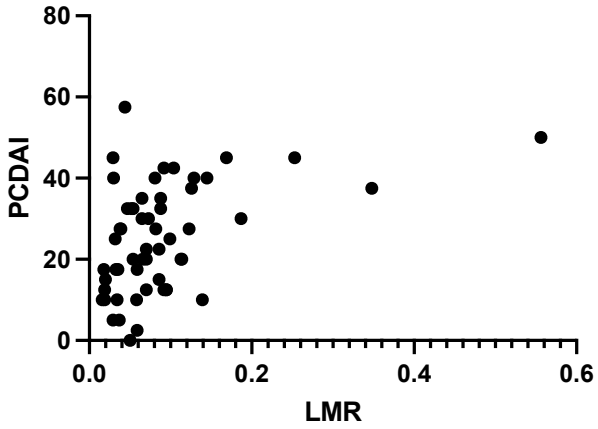


Figure 5. *PCDAI for LMR.*

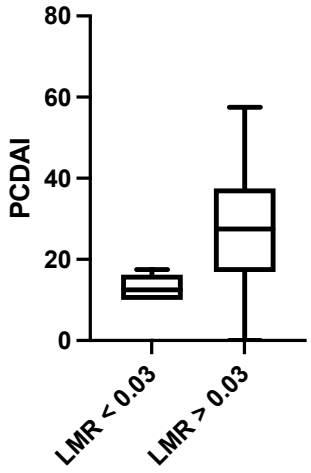
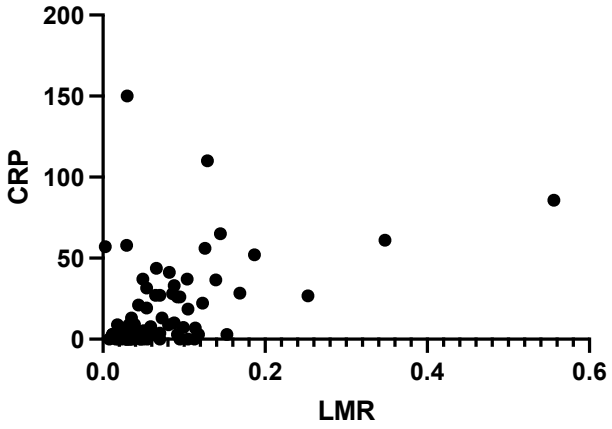


Figure 6. *Correlation between PCDAI and LMR.*

In the whole study population, CRP and ESR levels were significantly higher in CD patients compared to UC patients (p 0.0001 and 0.02 respectively). Both CRP and ESR showed a positive correlation with LMR (**Figure 7** and **Figure 8**) and lactulose urinary levels (CRP: Spearman correlation coefficient 0.47 and 0.35 with p<0.001 and 0.001 respectively for LMR and lactulose; ESR: Spearman correlation coefficient 0.47 and 0.34 with p<0.0001 and 0.002 respectively for LMR and lactulose).

Fecal calprotectin also showed a significant positive correlation with LMR and lactulose urinary levels (Spearman correlation coefficient 0.4 and 0.42 with p 0.0003 and 0.0001 respectively for LMR and lactulose).



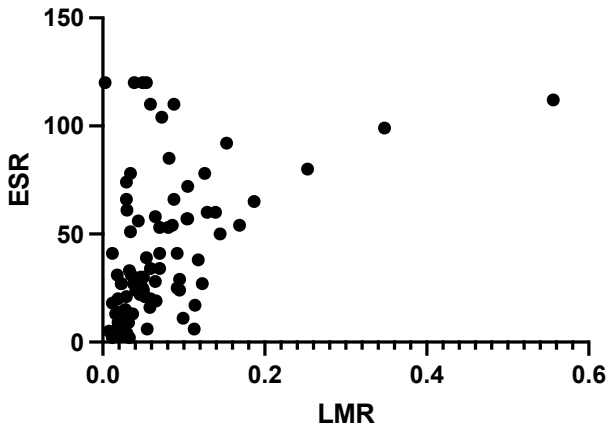


Figure 7*. Correlation between CRP and LMR*

Figure 8. *Correlation between ESR and LMR.*

As for serological markers, ASCA were significantly more positive in CD patients than in UC ones (p 0.0002). LMR (**Figure 9**) and lactulose urinary levels were significantly higher in patients with ASCA positivity (p 0.006 and 0.0002). ANCA were significantly more positive in UC patients than in CD ones (p 0.004). No differences in SPIT parameters were found between ANCA positive and ANCA negative patients.

As concerns endoscopic activity, lactulose urinary levels positively correlated with SES-CD in CD patients (Spearman correlation coefficient 0.33; p 0.02). No other correlation was found between endoscopic activity scores and SIPT parameters.

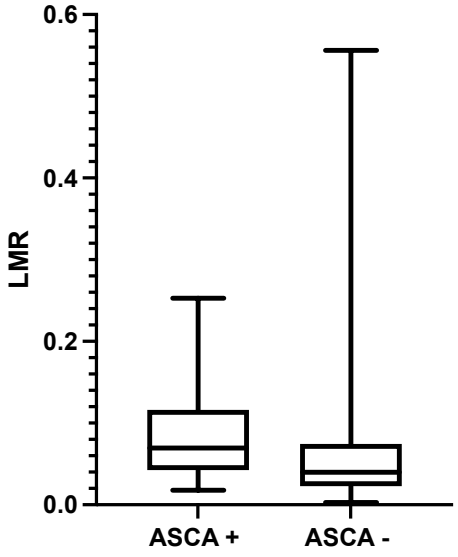


Figure 9. *LMR for ASCA.*

IBD localization and extent were assessed trough endoscopy (presence of macroscopic or microscopic inflammation) and radiologic examinations (characterization of intestinal wall through MRE or ultrasound). IBD localization for each gastrointestinal tract:

- Esophagus: 1/84 patients, 1/55 CD patients (1.2% of total IBD; 1.8% of total CD).
- Stomach: 13/84 patients, 13/55 CD patients (15.5% of total IBD; 23.6% of total CD).
- Duodenum: 10/84 patients, 10/55 CD patients (11.9% of total IBD; 18.2% of total CD).
- Small bowel, from the ligament of Treitz to the terminal ileum: 45/84 patients, 44/55 CD patients, and 1/18 UC patient (53.6% of total IBD; 80.0% of total CD; 5.6% of total UC).
- Large bowel, from the cecum to the rectum: 75/84 patients, 46/55 CD patients, 18/18 UC patients, and 11/11 IBDU patients (89.3% of total IBD; 83.6% of total CD; 100% of total UC and IBDU).

Patients with small bowel disease (**Figure 10**) had a significantly higher LMR (p 0.03). Urinary lactulose was significantly higher both in patients with small bowel disease (p 0.01) and in those with gastric disease (p 0.01). The correlation between esophagus localization of IBD and SIPT results could not be calculated because of the low sample size (only 1 patient).

Among patients diagnosed with CD diagnosis, no significant differences in SIPT parameters were found between patients with ileal, colonic, or ileo-colonic disease localization. CD patients with all these localizations had a significantly higher LMR compared to the UC population (p 0.03, 0.01, and 0.02 respectively), but no differences were found in comparison with the IBDU population.

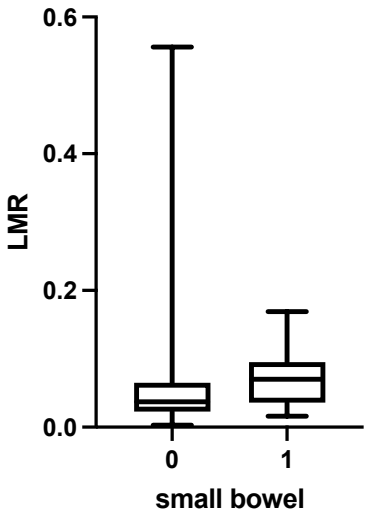


Figure 10. *LMR for small bowel disease.*

MRE was performed in 70 out of 84 patients. MaRIAs was not significantly different between CD, UC, and IBDU patients. None of the various MaRIAs scores (global, ileal, or colonic) correlated with SIPT parameters or was significantly different in patients with a an abnormal SIPT, i.e., LMR ≥0.03, compared to those with LMR <0.03.

Intestinal permeability in Crohn's disease assessment

For the second part of the study, SIPTs performed in pediatric CD patients who underwent a complete endoscopic and radiologic evaluation, either at diagnosis or during follow-up, were included (**Table 4**).

Pediatric CD patients	
SIPT, n	136
Tests performed at diagnosis, n (%)	55 (40.4)
Tests performed during follow-up, n (%)	81 (59.6)
Total population, n	71
Median number of tests per patient (range)	2 (1-6)
Sex, n (%)	
M	69 (50.7)
F	67 (49.3)
Age (years)	
Patient number (%)	
<6 y	6 (4.4)
6-9 y	16 (11.8)
>9 y	114 (83.8)
Median, (range)	13 (9 mo-18 y)
CD location, n (%)	
Esophagus	1 (0.7)
Stomach	17 (12.5)
Duodenum	14 (10.3)
Small bowel (from Treitz to ileum)	97 (71.3)
Large bowel (colon and rectum)	97 (71.3)
CD phenotype, n (%)	
B1 (inflammatory)	100 (73.5)
B2 (stricturing)	30 (22.1)
B3 (penetrating)	4 (2.9)
B2B3 (stricturing + penetrating)	2 (1.5)
P1 (perianal disease)	13 (9.6)
PCDAI, n (%)	
0-10 (inactive)	64 (47.1)
11-30 (mild)	49 (36.0)
>30 (moderate/severe)	23 (16.9)
Median (range)	12.5 (0-57.5)
ESR (mm/h)	
Median (range)	24 (2-120)
CRP (mg/L)	
Median (range)	8.9 (0.0-150)
Fecal calprotectin, n (%)	
<50 ug/g	8 (5.9)
50-300 ug/g	18 (13.2)
300-2100 ug/g	48 (35.3)
>2100 ug/g	45 (33.1)
Median (range), ug/g	519 (17->2100)
Serological markers	
ASCA+, n (%)	69 (50.7)
Median IgA titer, KU/L (range)	3.5 (0-100)
Median IgG titer, KU/L (range)	11.9 (0.5-144)
ANCA+, n (%)	18 (13.2)

SES-CD, n (%)	
Inactive (0-2)	30 (22.6)
Mild (3-6)	30 (22.6)
Moderate (7-15)	49 (36.8)
Severe (≥16)	24 (18.0)
Median (range)	8 (0-27)
MaRIAs	
Median (range)	2 (0-22)
Average (SD)	3.3 (3.88)
MaRIAs ileum, n (%)	
Score 0	33 (30.0)
Score 1	26 (23.6)
Score 2	38 (34.5)
Score 3	7 (6.4)
Score 4	5 (4.5)
Score 5	1 (0.9)
Median (range)	1 (0-5)
Average (SD)	1.35 (1.16)
MaRIAs colon, n (%)	
Score 0	59 (53.6)
Score 1	24 (21.8)
Score 2	18 (16.4)
Score 3	6 (5.5)
Score 4	2 (1.8)
Score 5	1 (0.9)
Median (range)	0 (0-5)
Average (SD)	0.83 (1.1)

Table 4. *Sugar permeability test in the study population at diagnosis of IBD.*

In total, 136 SIPTs were included, 55 (40.4%) performed at CD diagnosis and 81 (59.6%) performed during follow-up.

LMR ranged from 0.003 to 0.556 (median 0.04) and resulted pathological (i.e., ≥0.03) in 90 SIPTs (66.2%). Lactulose urinary excretion ranged from 0.045% to 20.68% (median 0.57%), while mannitol urinary excretion ranged from 2.646% to 38.66% (median 13.93%) (**Table 5**).

The PCDAI of the whole CD population ranged from 0 to 57.5 (median 12.5). The PCDAI was significantly higher in CD patients with a LMR ≥0.03 than those with a LMR <0,03 (p <0.0001). Moreover, a positive correlation (**Figure 11**) was observed between the PCDAI and the LMR and the lactulose urinary excretion (Spearman correlation coefficient respectively 0.62 and 0.47; p <0.0001).

SIPTs in CD patients	
Lactulose excretion %	
Median (range)	0.57 (0.045-20.68)
Mannitol excretion %	
Median (range)	13.93 (2.646-38.66)
LMR	
Median (range)	0.04 (0.003-0.556)
LMR <0.03, n (%)	46 (33.8)
LMR ≥0.03, n (%)	90 (66.2)

Table 5. *Sugar permeability test in the CD population.*

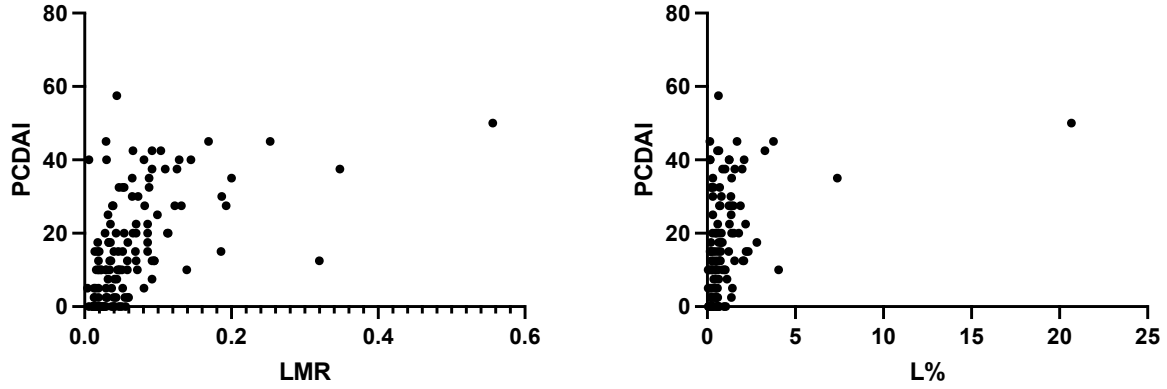


Figure 11. *Correlation between PCDAI and LMR and between PCDAI and urinary lactulose excretion (L%).*

As for laboratory tests, serum CRP ranged from <2.9 to 150 mg/L (median 8.9 mg/L) and ESR ranged from 2 to 120 mm/h (median 24 mm/h). Both LMR and lactulose urinary excretion (**Figure 12**) showed a significant positive correlation with ESR value (Spearman correlation coefficient 0.55 and 0.41 respectively, p <0.0001) and with CRP levels (Spearman correlation coefficient 0.58 and 0.45 respectively, p <0.0001).

Similarly, fecal calprotectin showed a significant positive correlation (**Figure 13**) with LMR and lactulose urinary excretion (Spearman correlation coefficient 0.65 and 0.57, respectively; p <0.0001).

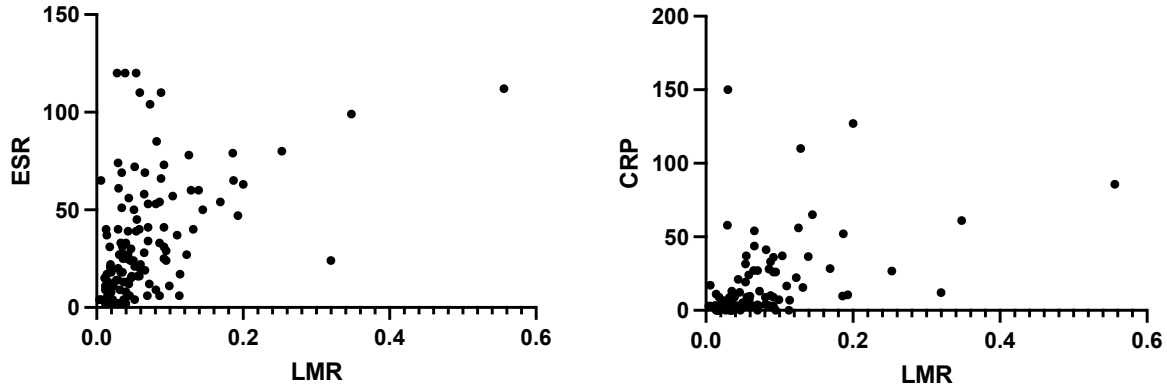


Figure 12. *Correlation between ESR and LMR and between CRP and LMR.*

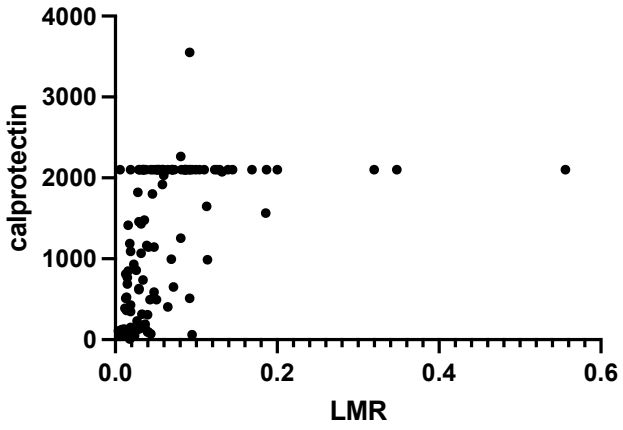


Figure 13. *Correlation between fecal calprotectin and LMR.*

ASCA were found positive in 69 CD patients (50.7%), while ANCA in 18 patients (13.2%). LMR levels were not significantly different in ASCA positive patients compared to ASCA negative ones. Lactulose urinary excretion was significantly higher in ASCA positive patients (p 0.02). No differences in SIPT results were found between ANCA positive and ANCA negative patients. No significant correlation was found between LMR and ASCA IgA and IgG levels. However, there was a significant positive correlation between lactulose urinary excretion and both IgA and IgG ASCA titers (Spearman correlation coefficient 0.19 for IgA and 0.23 for IgG; p 0.03 and 0.01 respectively).

Median SES-CD was 8 (range 0-27) and was indicative of inactive disease in 30/136 (22.6%) endoscopies, of mild disease in 30/136 (22.6%), of moderate disease in 49/136 (36.8%), and of severe disease in 24/136 (18.0%). LMR and lactulose urinary excretion positively correlated (**Figure 14**) with SES-CD score (Spearman correlation coefficient 0.45 and 0.42, respectively; p <0.0001).

As explained before, CD localization and extent were assessed trough endoscopy and radiologic examinations. CD localization for each gastrointestinal tract was as follow:

- Ileum: endoscopically active disease in 37/136 ileoscopies (27.2%), histologically active disease in 57/136 (41.9%). Ileum intubation during colonoscopy was not performed in 31/136 (22.8%).
- Colon: endoscopically active disease in 68/136 colonoscopies (50.0%), histologically active disease in 67/136 (41.9%).

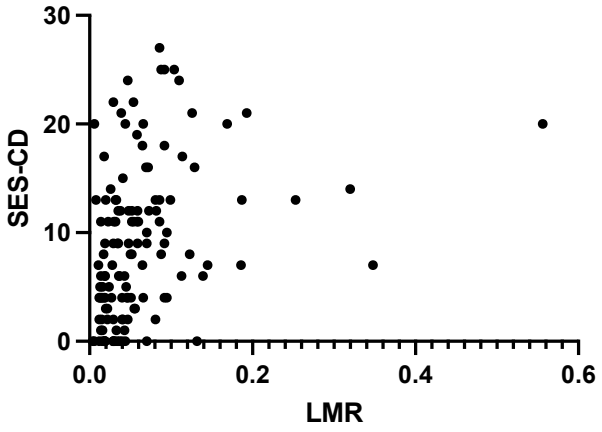


Figure 14. *Correlation between SES-CD and LMR.*

Endoscopic and histologic remission was found in 22/136 (16.2%) disease evaluations. LMR and lactulose urinary excretion were significantly higher in patients with endoscopic signs of active disease, either in the ileum or in the colon, than in those with endoscopic remission (p 0.0002). Besides, both LMR and lactulose urinary excretion were significantly higher (**Figure 15**) in patients with histologically active disease compared to those in deep remission (p <0.0001). On the contrary, urinary mannitol was not significantly different in patients with active CD compared to those with inactive CD.

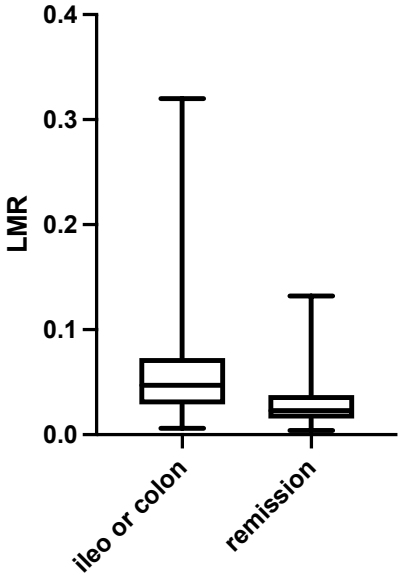


Figure 15. *LMR for active CD and CD remission.*

The ROC curve created to test the accuracy of LMR in discriminating between patients with active (ileal or colonic) CD and patients with deep, histological remission (**Figure 16**) evidenced a good accuracy (AUC 0.76; 95% CI: 0.65-0.88).

The optimal cut-off value of LMR (calculated with the three aforementioned methods) was 0.03, with a sensitivity of 72% and a specificity of 68%.

LMR and the lactulose urinary excretion were significantly higher (**Figure 17**) in patients with ileal endoscopic localization of CD, compared to those without ileal endoscopic involvement (either colonic CD or disease remission, p 0.004). Similarly, patients with histological CD activity showed higher LMR and lactulose urinary excretion (**Figure 18**) compared to those with normal ileal histology (p <0.0001). Mannitol urinary excretion was not different in patients with or without ileal CD (endoscopic or histologic involvement).

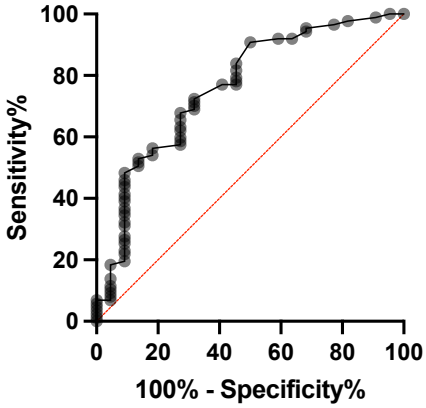


Figure 16. *ROC curve showing the performance of LMR in distinguishing between histologically active CD and remission.*

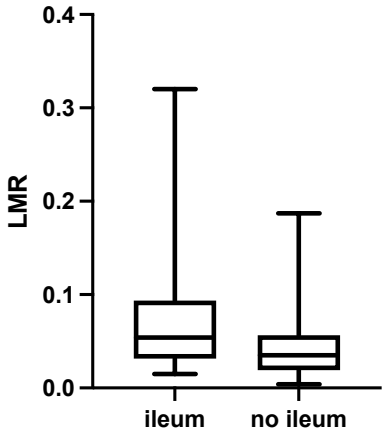


Figure 17. *LMR for endoscopically active ileal CD.*

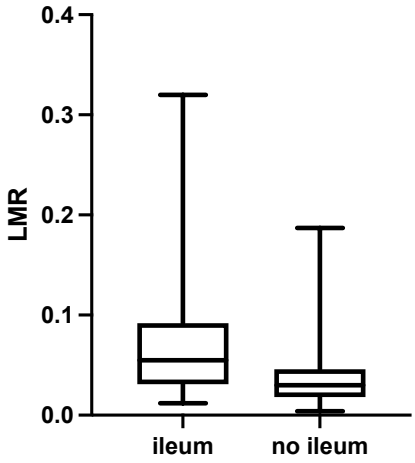


Figure 18. *LMR for histologically active ileal CD.*

MRE was performed in 111/137 CD evaluations. MaRIAs ranged from 0 to 22 (median 2, average 3.3 +/- 3.88). Ileal MaRIAs sub-score ranged from 0 to 5 (median 2, average 1.35 +/- 1.16), while colonic MaRIAs ranged from 0 to 5 (median 0, average 0.83 +/- 1.1). There was no significant correlation between MaRIAs scores and SIPT parameters. Moreover, there was not significant difference between MaRIAs scores of CD patients with LMR ≥0.03 and those with LMR <0.03.

SIPT results were not different in patients with different radiologic localization of CD (ileum or colon or both).

Intestinal permeability and Crohn's disease prognosis

Data on the clinical course of 53 CD patients were collected (**Table 6**). The median length of follow-up was 5.2 years (range 1 month to 9.4 years).

The disease course of CD was evaluated focusing on the development of complications.

Overall, CD phenotype was consistently inflammatory (B1) in 33 patients (62.3% of total CD), while 15 patients (28.3% of total CD) developed a stricturing phenotype (B2), 5 patients (9.4% of total CD) a penetrating phenotype (B3), and 2 patients (3.8% of total CD) a both structuring and penetrating phenotype (B2B3) at any point in the disease course. 11 patients (20.7% of total CD) developed perianal disease (P1).

8 patients (15.0% of total CD) underwent abdominal surgery: 6 of them received surgery for stenotic ileal disease (i.e., ileal resection with ileocolonic anastomosis), while in 2 cases a temporary ileostomy was needed for severe refractory colonic disease.

Followed-up pediatric CD patients	
Total population, n	53
Years of follow-up Median (range)	 5.2 (0.1-9.4)
Sex, n (%) M F	 30 (56.6) 23 (43.4)
Age (years) Patient number (%) <6 y 6-9 y >9 y Median, (range)	 4 (7.3) 8 (14.5) 43 (78.2) 12 (9 mo-16 y)
Disease course, n (%) B1 (inflammatory) B2 (stricturing) B3 (penetrating) B2B3 (stricturing + penetrating) P1 (perianal disease) Surgery	 33 (62.3) 15 (28.3) 5 (9.4) 2 (3.8) 11 (20.7) 8 (15.0)

Table 6. *Demographic characteristics and disease course of the followed-up CD population.*

A logistic regression analysis (**Table 7**) was performed to evaluate the ability of SIPT to predict the development of CD complications. A pathological LMR (≥0.03) did not predict the development of any of the CD complications. However, lactulose urinary excretion resulted a significant predictor of CD complications in general, and of the development of a stricturing phenotype in particular. Conversely, higher LMR values and mannitol urinary excretion were not predictive of any of the considered outcomes. A similar analysis was conducted to evaluate if ASCA could predict the development of CD complications. ASCA positivity and ASCA IgA titer were both found predictive of both CD complications in general and of a stricturing phenotype in particular. On the contrary, ASCA IgG titer was not predictive of any of the examined outcomes.

Outcomes	Risk factors	P value	OR (95% CI)
Complicated CD	L% ASCA positivity ASCA IgA	0.02 0.007 0.01	2.61 (1.16-5.91) 5.38 (1.57-18.45) 1.22 (1.04-1.43)
Surgery	L% ASCA positivity ASCA IgA	0.82 0.1 0.18	0.96 (0.68-1.36) 6.13 (0.7-53.95) 1.02 (0.99-1.04)
Perianal CD	L% ASCA positivity ASCA IgA	0.67 0.29 0.27	0.92 (0.61-1.38) 2.2 (0.51-9.48) 1.01 (0.99-1.04)
Stricturing phenotype	L% ASCA positivity ASCA IgA	0.03 0.022 0.016	2.25 (1.09-4.66) 5.22 (1.28-21.32) 1.06 (1.01-1.11)
Penetrating phenotype	L% ASCA positivity ASCA IgA	0.29 0.46 0.16	1.37 (0.77-2.46) 1.92 (0.34-10.96) 1.02 (0.99-1.04)

Table 7. *Logistic regression analysis of lactulose excretion (L%) and ASCA as predictors of CD complications.*

Conclusions

Intestinal permeability at IBD diagnosis

In our cohort, the prevalence of impaired IP (i.e., LMR ≥0.03) at IBD diagnosis was 72.6%, higher than previous studies, which reported an alteration of IP in 50-68% of adult IBD patients.[87,112,113] However, our study did not include a group of healthy controls for comparison.

As expected, the prevalence of altered IP was higher in CD patients (87.3%). This result is consistent with the fact that SIPT explores the permeability of small bowel, which is usually involved in CD.[114] However, IP was significantly higher in CD patients in general, compared to UC patients, independently from the presence of ileal or colonic disease localization. This could be due to different factors. The immunological and molecular pathogenesis of CD, that leads to transmural inflammation, is different from that of UC. First, the presence of systemic inflammation, associated to CD, might impair intestinal permeability to a greater extent than the local inflammation (i.e., limited to the bowel) which characterizes UC. Secondly, the longer preclinical phase of CD might lead to a prolonged intestinal damage and intestinal barrier impairment. Indeed, UC usually presents abruptly, implying a shorter preclinical period. The different duration of the preclinical phase between CD and UC has been suggested by different studies, which showed that serum autoantibodies are significantly higher in patients later diagnosed with CD, but not UC, many years before disease onset. Therefore, intestinal inflammation seems to be preceded by a stage when, in the absence of symptoms and bowel lesions, deregulated immune pathways, dysbiosis, gut barrier disruption, and other key pathogenic mechanisms are already in place, leading to the expansion of the inflammatory process that will culminate in intestinal inflammation, tissue injury, and, finally, overt symptoms. The duration of such preclinical phase is currently unknown, but it could possibly start many years before the diagnosis of CD, leading to a greater alteration of IP at the time of disease onset.[115]

Impaired IP was detected in 38.9% of UC patients and in 63.6% of IBDU patients at IBD diagnosis. Similarly, previous works found an alteration of IP in up to 43% of patients with UC.[116,117] On the contrary, other past studies focused on UC patients, although small in sample sizes, failed to demonstrate an increase in IP in this disorder.[96,118] As a confirmation of our findings, previous studies reported an increase of IP in first-degree relatives and/or non-related spouses of UC patients, supporting the hypothesis that genetic and/or environmental factors could cause epithelial barrier defects, contributing to UC pathogenesis and to altered IP in these subjects.[116]

ROC analysis showed a moderately good accuracy of LMR in distinguishing CD from UC. Based on our results, SIPT alteration can have a role in distinguishing between different subtypes at IBD diagnosis in patients with colonic disease.

The most accurate LMR cut-off to detect patients with CD at the diagnosis of IBD was 0.05, higher than the threshold commonly used to define an impairment of intestinal barrier (i.e., 0.03). Hence, this finding confirms the need for a higher cut-off of LMR to discriminate the moderately increased IP that could also be find in UC patients from the greater degree of alteration found in CD subjects.

However, further studies are needed to confirm the role of SIPT in IBD diagnosis and to validate the newly proposed LMR cut-off to discriminate CD subtype.

Intestinal permeability in Crohn's disease assessment

We found a positive correlation between several markers of CD activity, LMR, and lactulose urinary excretion. Specifically, SIPT parameters correlated with PCDAI and with systemic and intestinal inflammatory markers (ESR, CRP, and fecal calprotectin).

Previous studies have already reported a relationship between impaired intestinal permeability, clinical disease activity[117,119–121] and increased inflammatory markers.[122,123] Conversely, Chang et al.[124] found that neither ESR nor CRP were associated with the degree of alteration of IP measured with CLE.

Moreover, a positive correlation emerged between endoscopic activity, measured with SES-CD, and both LMR and urinary lactulose excretion. To the best of our knowledge, a similar correlation has not been previously reported in pediatric CD patients.

We have also found that LMR and urinary lactulose excretion were increased in patients with endoscopic or histologic ileal and/or colonic active disease compared to patients in remission. Contrarily, there were no differences in IP between patients with radiologic signs of ileal and/or colonic active disease and those with no radiologic findings. Furthermore, no correlation was found between MRE alteration, measured using MaRIAs, and SIPT parameters. This discrepancy suggests that SIPT is suitable for evaluating mucosal disease activity, rather than transmural inflammation. Thus, we conclude that LMR does not seem capable of replacing radiologic monitoring (ultrasound or MRE) during CD follow-up.

Overall, our data suggest that SIPT could be a useful non-invasive tool for CD follow-up. ROC curve analysis evidenced a moderately good accuracy (AUC 0.76) of LMR in detecting ileal or colonic disease activity in CD patients. Based on our results, we propose a LMR cut-off of 0.03

to identify CD patients with active ileal or colonic disease during CD follow-up. This LMR value has already been reported in many studies as the best threshold to define an alteration in IP.

Further research will be needed to clarify the role of permeability test in pediatric CD follow-up, particularly to validate the performance of the proposed LMR cut-off in early detection of disease relapses.

Unlike lactulose, mannitol urinary excretion did not correlate with markers of disease activity. Thus, the pathological increase of LMR associated to active CD we found appears to be dependent on a higher excretion of lactulose, and not on a reduction in mannitol urinary levels. Previous studies focusing on SIPT in IBD patients reported similar findings.[98,101,112,119] Mannitol is a small molecule that can physiologically pass the intestinal epithelial barrier. However, in case of reduced absorptive surface, secondary to intestinal damage, mannitol absorption could be impaired.[89] In fact, previous studies have found a reduced mannitol urinary excretion in patients with celiac disease, possibly depending on a reduction of the bowel surface area because of villous atrophy.[89,125] It can be assumed that patients with active CD do not generally have significant atrophy of the intestinal mucosa: consequently, mannitol excretion is not altered. On the other hand, the bowel epithelial layer is damaged, thus determining an increase in IP and consequently an abnormal absorption of lactulose. Interestingly, mannitol urinary excretion was significantly reduced in the sub-group of patients with growth delay at IBD diagnosis, compared to those with regular stature and weight growth, suggesting that this subgroup of patients might indeed have a reduction of the absorbing surface, determining both an increase of IP and a state of malabsorption and thus impaired growth. Therefore, it might be relevant to consider not only the LMR, but also mannitol and lactulose excretion when interpreting the SIPT results.

Finally, LMR and urinary lactulose excretion were significantly higher in patients with ileal localization of CD compared to those without ileal disease. This result is in line with previous studies, which found higher LMR in patients with ileo-colonic disease compared to those with isolated colonic IBD,[112,126] thus confirming that the ileum is the intestinal segment best explored by SIPT.

Intestinal permeability and Crohn's disease prognosis

We investigated the potential role of SIPT in predicting the outcome of patients with CD, particularly the need for abdominal surgery, the development of a stricturing or penetrating phenotype, and the development of perianal disease. To the best our knowledge, there have never been similar studies in pediatric CD patients.

Our work showed that LMR was not a significant predictor of any of the explored outcomes. On the other hand, lactulose urinary excretion was found to be a predictor of the development of a stricturing phenotype. Additional studies, with larger numbers of patients, will be necessary to confirm the role of SIPT in predicting disease outcome.

Similarly, we found that ASCA positivity and ASCA IgA titer were predictive of the occurrence of a stricturing phenotype. These results confirm the known role of ASCA as predictor of CD complications.[127–129]

Limitations

One of the main limitations of our study was its retrospective design. However, it provided useful information for future prospective investigations, aiming to assess the role of SIPT in IBD diagnosis and in predicting its outcomes.

Other limiting factors were the non-homogeneous distribution of the length of follow-up of the CD population, ranging from a few months to almost ten years, and the limited number of patients included. Moreover, treatment was not considered. Studies with larger sample sizes and longer follow-ups will be needed to confirm the predictive potential of SIPT in CD management.

Closing remarks

This study confirms the high prevalence of altered intestinal permeability in patients with IBD, measured through sugars test. Particularly, LMR is significantly higher in patients with CD compared to UC. Thus, SIPT might be a useful diagnostic tool to discriminate between different subtypes of IBD.

There was a strong correlation between LMR and several markers of CD activity (PCDAI, ESR, CRP, fecal calprotectin, SES-CD), suggesting a potential complementary role of SIPT among non-invasive tests to monitor disease activity during follow-up. On the other hand, SIPT did not correlate with radiologic activity indexes of disease. Overall, our results confirm the relationship between IP and mucosal inflammation and suggest that the test may not be suitable to detect transmural inflammation.

Altogether, our data indicate that SIPT represents a useful complementary tool for the diagnosis and the follow-up of CD. However, we could not reach conclusive results on the potential role of the test in predicting the development of CD complications.

Broader prospective studies will be needed to prove the role of IP tests in CD diagnosis and follow-up and to determine their possible use in predicting disease outcomes.

PART 2.2

Multiplex PCR assay for the diagnosis of gastrointestinal infections in pediatric patients with active inflammatory bowel disease

Bosa L, Gaio P, Faggian G, Perilongo G, Besutti V, Cananzi M. Gastrointestinal infections detected by multiplex PCR stool testing are common in children with active inflammatory bowel disease. Manuscript in preparation.

Abstract

Background

Inflammatory bowel disease (IBD) patients are at increased risk of gastrointestinal infections, which can be indistinguishable from disease flares. Enteric pathogens can be detected during IBD exacerbations, but their etiologic role is often unclear. The BioFire FilmArray® GI Panel is a rapid, multiplex PCR assay that accurately detects 22 common enteric pathogens in stool samples. The objective of this study was to determine the rate and the clinical significance of gastrointestinal infections in children with active IBD.

Methods

Consecutive pediatric IBD patients tested with FilmArray® GI Panel from January 2018 to December 2021 were retrospectively included.

Results

73 FilmArray® tests from 40 IBD patients were included. 73% were negative, while 20/73 (27%) resulted positive for single (17/20) or multiple (3/20) enteric pathogens. Pathogenic *Escherichia coli* (n=12), toxigenic *Clostridioides difficile* (n=4), and norovirus (n=3) were the most detected pathogens. 18/20 subjects with a positive test were symptomatic, while 2/20 were in clinical remission, but had endoscopic inflammation. 17 patients with bacterial pathogens or *Cryptosporidium* received appropriate antimicrobials. Among the 15/17 symptomatic ones, 80% (12/15) clinically improved after antimicrobials, alone (n=9) or in combination with topical (n=1) or systemic (n=2) steroids. The other 3/15 patients with clinically active disease required surgery (n=2) or escalation of immunosuppressive therapy (n=1) despite adequate antibiotic therapy. All three of them tested positive for pathogenic *E. coli* strains.

Conclusions

Gastrointestinal infections are common in pediatric IBD patients with active disease. FilmArray® increases the detection of enteric pathogens, providing timely and sensitive results that might guide clinical management, possibly avoiding unnecessary escalation of immunosuppressive therapy.

Background

Gastrointestinal infections and IBD

The pathogenesis of IBD remains unclear, but it appears to involve a perturbation of intestinal immune homeostasis, largely driven by a complex bidirectional relationship between the mucosal immune system and the gut microbiome.[130]

The term dysbiosis refers to an alteration of the composition or of the function of the microbiota, particularly when harmful microbes overtake the beneficial ones, leading to a state of unbalance in the interactions between host and microbes.[131]

A state of dysbiosis has been observed during diseased conditions, including IBD, but whether it is a cause or a consequence of intestinal inflammation it has yet to be determined. Interestingly, several mouse models of colitis fail to develop inflammation under germ-free conditions. On the other hand, infections that cause gut inflammation, e.g., rotavirus, are employed to study bowel inflammation in experimental models. Taken together, these data suggest that chronic relapsing intestinal inflammation or its perpetuation in IBD are mostly driven by the gut microbes.[130]

Furthermore, patients with IBD appear to be at increased risk of gastrointestinal infections, due to alteration of gut mucosal immunity, loss of bowel mucosal integrity, modification in microbiota, and use of immunosuppressive agents.[132] However, clinical, laboratory, and endoscopic features of infectious gastroenteritis are often indistinguishable from those of an IBD exacerbation, posing a significant diagnostic challenge to the physician.

Several studies have investigated the involvement of enteric pathogens in flares of IBD, in which a microbe may be the sole etiology of symptoms, a complicating factor, or may coexist as a bystander, or be present as an asymptomatic colonizer.[133]

Using standard microbiological techniques, especially culture-dependent methods and *C. difficile* toxin test, only 10-15% of IBD patients experiencing disease symptoms has a detectable gastrointestinal tract infection. This low detection rate might depend on the limited sensitivity of conventional microbiological stool tests and on the narrow range of identifiable pathogens.[132,134] Moreover, traditional culture methods are limited by difficulties in identifying non-bacterial agents, high turnaround times, need for expertise in microscopy analysis, and influence of preanalytical factors, such as antibiotics.[133]

Recently, the advent of rapid, highly sensitive PCR-based stool testing has increased the number of infectious agents detected in individuals with signs of gastrointestinal infection.

Multiplex PCR panel assays, or Gastrointestinal Pathogen Panels (GPPs), allow to simultaneously identify multiple pathogens, including bacteria, viruses, and parasites, by detecting their nucleic acids in stool samples.[132,134] Consequently, rapid PCR-based stool tests have often replaced older culture-dependent methods for the identification of enteropathogens in clinical practice.

Moreover, the advent of this molecular biology techniques has reignited interest in the role of infectious agents in IBD relapses.

Recent studies have shown that multiplex PCR-based assays have increased the number of infectious agents detected in patients with active IBD, with a prevalence of enteric pathogens of more than 30% in subjects with IBD exacerbations, significantly higher than the prevalence found in subjects with quiescent IBD and in healthy controls.[132–135]

Moreover, a recent work[132] has found that treatment escalation occurred less frequently in patients presenting with active IBD and a positive stool multiplex PCR panel, and that flares complicated by infections were followed by a less aggressive clinical course compared to those without a detectable pathogen, even if the rates of surgery and hospitalization were similar.

However, the specificity of PCR-based tests for clinically significant infections might be low. Given the high sensitivity of this technology, past studies have reported a significant prevalence of positive tests even in asymptomatic subjects, due to carrier states, prolonged shedding of pathogens, or persistence of nonviable nucleic acids.[133] Therefore, understanding whether a positive multiplex PCR-based assay result represents a true infection remains a significant challenge.

Despite the possible involvement of enteric pathogens in flares of IBD, little is known about their role, and management strategies are lacking. Furthermore, there is still a need for data on the role of enteric pathogen in pediatric patients with IBD.

Objectives of the study

The objectives of this study were:

1. To determine the rate of gastrointestinal infections in pediatric patients with active IBD and their culprit pathogens using the BioFire® FilmArray® GI Panel.
2. To determine the clinical significance of enteric pathogens detection in pediatric patients with active IBD.

Methods

Study design and population

This was a single-center, retrospective cohort study conducted on pediatric IBD patients followed at the Department of Women's and Children's Health of the University Hospital of Padova, a tertiary referral center for PIBD in the North-East of Italy.

Inclusion criteria were:

- Age ≤18 years.
- Diagnosis of IBD (CD, UC, or IBDU).
- Having had at least one stool sample tested with BioFire® FilmArray® GI Panel as part of routine clinical care from January 2018 to December 2021.
- Evidence of active disease at the moment of the stool test, defined clinically (by a PCDAI[104] score ≥10 for CD, or a PUCAI score[105] ≥10 for UC and IBDU) and/or endoscopically (through histologic examination).

Exclusion criteria were:

- Antibiotic therapy in the 2 weeks prior to enrollment.
- Status post-colectomy or ileostomy placement.
- Missing clinical data.
- Multiple FilmArray® tests requested during the same IBD flare (only the first test was included in such cases). On the contrary, multiple FilmArray® assays, obtained from the same eligible subject, albeit in the context of different IBD exacerbations, were included.

Medical record review was performed on all patients. Baseline demographics, IBD subtype, medication use, and surgical history were collected. Conventional microbiological stool tests' results were recorded if available.

In symptomatic patients, duration and type of symptoms, antimicrobial therapy, and its outcome were recorded, together with the choice to escalate the immunosuppressive therapy or to proceed to surgery.

Data were entered into a password-protected Excel database and analyzed anonymously.

Gastrointestinal multiplex PCR stool testing

The BioFire® FilmArray® GI Panel (BioFire Diagnostics, Salt Lake City, UT) is a rapid, multiplex PCR assay capable of simultaneously detecting 22 common bacterial, viral, and parasitic enteric pathogens associated with gastroenteritis from one patient stool sample.

The following agents are included: *Campylobacter* (*C. jejuni*, *C. coli*, and *C. upsaliensis*), *Clostridioides* (*Clostridium*) *difficile* (toxin A/B), *Plesiomonas shigelloides*, *Salmonella*, *Yersinia enterocolitica*, *Vibrio* (*V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) lt/st, Shiga-like toxin-producing *E. coli* (STEC) stx1/stx2, *E. coli* O157, *Shigella*/Enteroinvasive *E. coli* (EIEC), *Cryptosporidium spp.*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia lamblia*, Adenovirus F40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, and Sapovirus (I, II, IV, and V).

Testing was performed according to the manufacturer's instructions on the BioFire® FilmArray® system, using 200 µl of stool re-suspended in Cary-Blair transport medium. The BioFire® FilmArray® pouch stores all the needed reagents in a freeze-dried format. During a test run, the device automatically extracts and purifies nucleic acids from the sample and performs a nested multiplex PCR.

The BioFire® System software automatically analyzes the results and generates a response for each target pathogen, using endpoint melting curve data.

Results are available in about one hour per run (i.e., per specimen).

Statistical analysis

Descriptive statistic was used to summarize the demographic and clinical characteristics of the study population. Data were synthesized as mean and SD, or median and IQR for quantitative variables, as numbers and percentages for categorical variables.

Excel software was used to create pie charts as appropriate.

Results

Prevalence of enteric pathogens in pediatric patients with active IBD

Our cohort included 125 pediatric patients with IBD who were followed-up at the Department of Women's and Children's Health of the University Hospital of Padova between January 2018 and December 2021.

In total, 73 FilmArray® assays performed in 40 IBD patients (18 females, 45%) were included in the study (**Figure 1** and **Table 1**). Median age at the test was 13 years (range 0-18 years, IQR 12-15 years). Of these patients, 21 (53%) had been diagnosed with CD, 11 (27%) with UC, and 8 (20%) with IBDU.

Overall, 20 FilmArray® tests out of 73 (27%) resulted positive (**Figure 2**). In 17/20 (85%) positive samples, only one enteric pathogen was detected, while in 3/20 (15%) multiple pathogens (i.e., two) were detected.

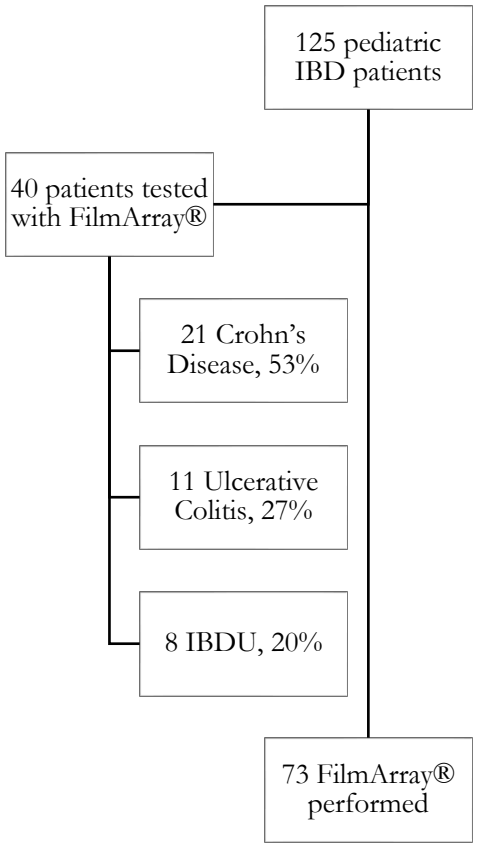


Figure 1. *Flow diagram of patient inclusion in the analysis.*

Pediatric IBD patients tested with FilmArray®	
Number of patients	40
Mean age at the test ± SD, years	12.3 ± 4.1
Sex, n (%) M F	 22 (55.0) 18 (45.0)
Ethnicity Caucasian Arab-Berber South Asian African Chinese	 34 2 2 1 1
Diagnosis, n (%) CD UC IBDU	 21 (53.0) 11 (27.0) 8 (20.0)

Table 1. *Demographic features and IBD diagnosis of the pediatric patients with IBD included in the study.*

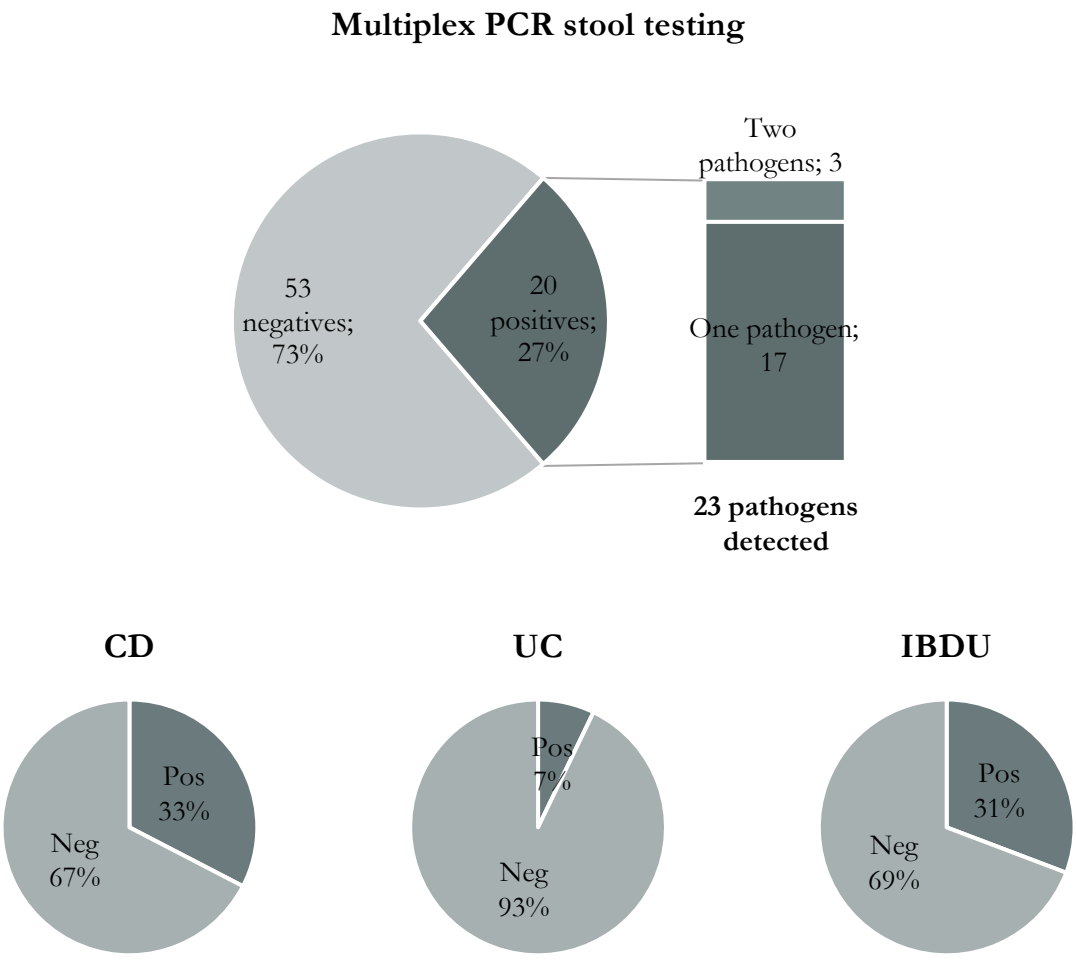


Figure 2. *FilmArray® tests results.*

In total, 23 target microorganisms were identified by multiplex PCR assays. EPEC (n=12), toxigenic *Clostridioides difficile* (n=4), norovirus (n=3), and STEC (n=2) were the most commonly detected enteric pathogens. *Campylobacter spp.*, *Salmonella spp.*, EAEC, *Cryptosporidium spp.*, and adenovirus were detected in one stool sample each (**Figure 3**).

Overall, 70% (16/23) of the pathogens was found in patients with CD, and 26% (6/23) in patients with IBDU. Only one pathogen out of 23 (4%) was detected in the UC subgroup.

As for comparison with standard microbiological techniques, stool culture was positive for group B *Salmonella* when *Salmonella spp.* was detected but resulted negative when *Campylobacter spp.* was found by FilmArray®.

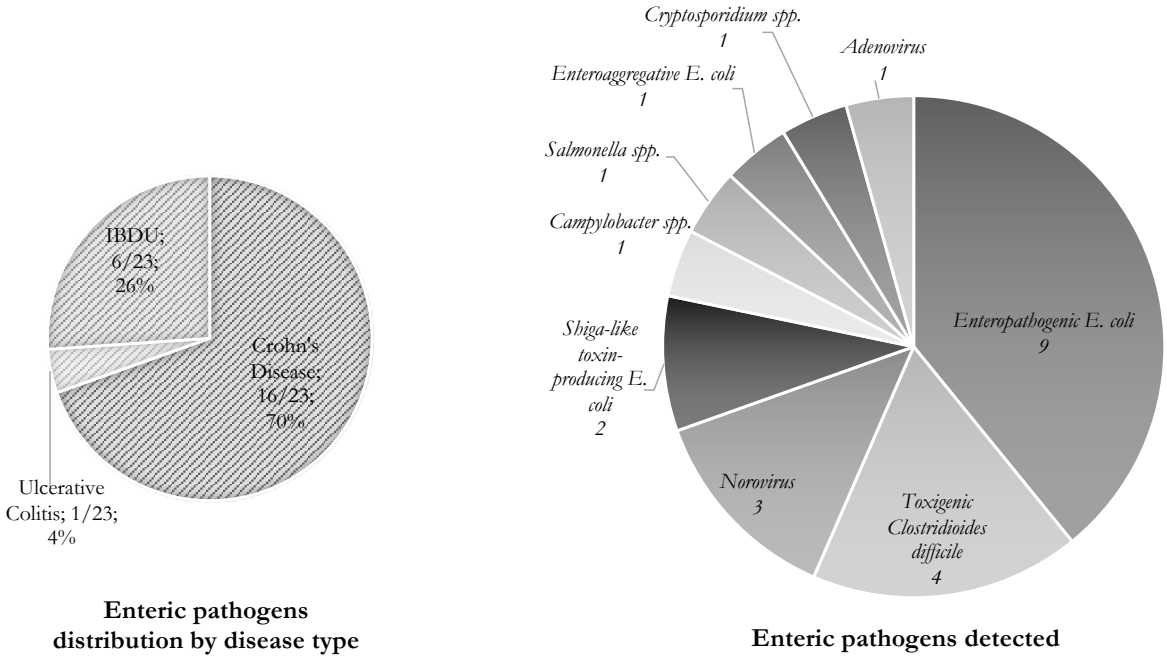


Figure 3. *Enteric pathogens detected by FilmArray® tests.*

Clinical significance of clinical significance of enteric pathogens detection in pediatric patients with active IBD

Of the 20 subjects with a positive FilmArray® test, 18 (90%) were symptomatic, while the remaining 2 (10%) were in clinical remission (i.e., PCDAI or PUCAI≥10), but had endoscopically active intestinal inflammation.

All the three patients with norovirus rapidly improved; two of them didn't receive antibiotic therapy, while the other was treated with metronidazole, since treatment had been started before the FilmArray® result was available. Adenovirus was found once, but in association with EPEC, thus the child was treated with ciprofloxacin, with partial benefit (i.e., disease activity improved, but he did not reach clinical remission).

All 17 patients whose stools resulted positive for bacterial pathogens or *Cryptosporidium spp.* received appropriate antimicrobial therapy, even the asymptomatic ones (2/17).

Among the 15/17 symptomatic ones, 80% (12/15) clinically improved after antimicrobials, alone (n=9) or in combination with IBD specific drugs (n=3). Specifically, two patients were treated with systemic steroids, while another with enemas (5-ASA in association with beclomethasone).

The single CD patient in whom *Cryptosporidium spp.* was detected was treated with paromomycin alone and underwent clinical remission.

The 3/15 patients with clinically active disease despite adequate antibiotic therapy required either surgery (n=2) or escalation of immunosuppressive therapy (n=1).

All three of them tested positive for pathogenic *E. coli* strains (EPEC, n=1; STEC, n=1; EAEC, n=1).

All the final test results were provided within one day.

Conclusions

Our study confirms the high burden of gastrointestinal infections in pediatric IBD patients with active disease. The application of the FilmArray® GI panel in children with IBD may allow to extend the range of detectable enteric pathogens in stool samples.

In our cohort, nearly one-third of subjects tested positive when utilizing a highly sensitive PCR-based assay. This result is in line with that a recent study[132] conducted in adults with IBD, which found that 31.1% of subjects with active intestinal inflammation had a positive stool GPP. Therefore, our finding further confirms that enteric pathogens might be important factors contributing to the disease course of IBD, particularly to flares. Our study also supports the hypothesis of some authors that intestinal infections are so frequent that they might explain, at least partially, the high placebo rates observed in IBD clinical trials. In fact, many patients entering clinical trials could have a self-limited gastrointestinal infection and return to their baseline symptoms after clearing the pathogen.[132]

On the other hand, an inflamed intestinal mucosa can itself increase the risk of acquiring infectious agents, making it difficult to establish whether a pathogen is the primitive cause of the symptoms or just a bystander. Moreover, since PCR-based assays are very sensitive for the presence of microbes, their specificity for clinically significant infections might be limited. Past studies[136,137] have found a high prevalence of positive tests in asymptomatic individuals, that could possibly be explained by a mixture of microbial colonization and false positives.

In our cohort, the rate of positive FilmArray® tests was significantly higher in patients with CD and IBDU, while only one enteric pathogen was detected in the UC subgroup. This finding could reflect, at least for CD patients, the peculiar tropism of some germs for the small intestine, which produces an increase in the turnover of intestinal epithelium and in the shedding of apoptotic cells into the stools.[132]

With regard to specific enteric pathogens, while past studies have largely demonstrated the role of *C. difficile* toward IBD exacerbations, less is known about the contribution of non-*C. difficile* pathogens, although it has been hypothesized that they can worsen the course of IBD in a similar manner.[1324,51] Interestingly, in our study patients with the worst outcome, i.e., surgery or escalation of immunosuppressive therapy, were positive for pathogenic *E. coli* strains. Nevertheless, the number of patients is small enough that the observed differences could easily be due to chance. Thus, further data are needed to ascertain the pathogenic role of different microbiological agents, especially *E. coli* strains.

Application of the FilmArray® GI panel may provide timely and sensitive results that might aid the physician in the clinical management of symptomatic children with IBD. In fact, detection of enteric pathogens during flares of IBD may help to avoid unnecessary escalation of immunosuppressive therapy, as shown in previous studies.[132]

Moreover, multiplex molecular-based methods could result in a decreased risk of surgery and hospitalization and in a reduction of health costs. However, this has not been clearly proven.[132] Therefore, further data are needed to evaluate the clinical impact of PCR-based stool tests.

Our study had several limitations. First, it did not include a control cohort of IBD patients in complete remission, that could have helped in defining which infectious agents are more likely to be clinically significant rather than colonizers. Secondly, it was a retrospective study, hence relationships are only associations and not causation. Moreover, there was no treatment protocol and management varied depending on treating physicians. Finally, our study did not attempt to assess the economic impact of FilmArray® assay.

However, our work has several strengths. To the best of our knowledge, it's one of the first works to analyze the use of multiplex molecular-based methods in pediatric patients diagnosed with IBD. Moreover, it was a real-life study evaluating patients in whom the gastroenterologist ordered FilmArray® as part of routine clinical care.

In conclusion, gastrointestinal infections in pediatric IBD patients with active disease are common and should be sought in disease flares. Advanced molecular tests might help the physicians to better direct treatment strategies and become an integral part of precision medicine in children with IBD.

PART 2.3

Impact of SARS-CoV-2 infection on IBD and vice-versa in pediatric patients

Bosa L †, Di Chiara C†, Gaio P, Cosma C, Padoan A, Cozzani S, Perilongo G, Plebani M, Giaquinto C, Donà D, Cananzi M. Protective SARS-CoV-2 Antibody Response in Children With Inflammatory Bowel Disease. Front Pediatr. 2022 Feb 10;10:815857. doi: 10.3389/fped.2022.815857. PMID: 35223697; PMCID: PMC8866952.

†Contributed equally

Abstract

Background

To date[#], there's no evidence of an increased risk of SARS-CoV-2 infection or more severe COVID-19 in patients with IBD. However, whether COVID-19 alters the clinical course of IBD or whether IBD treatment affects the immunological response to SARS-CoV-2 is still under investigation, especially in children. The aim of this study was to assess the serological response to SARS-CoV-2 in children with IBD, and to evaluate the impact of COVID-19 on the clinical course of IBD.

Methods

This prospective study enrolled children (0-18 years) followed-up at the University Hospital of Padova for IBD, who acquired a confirmed SARS-CoV-2 infection between 02/2020 and 02/2021. The anti-SARS-CoV-2 S-RBD IgG titer was evaluated at 3 months after infection and compared to that of a control group of healthy children matched for age, sex, and COVID-19 severity.

Results

Twelve children with IBD (M=5; median age 14 years) contracted COVID-19 during the study period. 11/12 patients were under immunomodulatory treatment (4/12 steroids; 6/12 azathioprine; 3/12 anti-TNFs; 2 vedolizumab; 1 ustekinumab). SARS-CoV-2 infection remained asymptomatic in 4/12 children and caused mild COVID-19 in the remaining 8. Mean anti-SARS-CoV-2 IgG S-RBD titer was similar between IBD patients and controls (27.3 ± 43.8 vs. 36.8 ± 35.3 kAU/L, p = ns). No children experienced IBD flares, nor required gastroenterological support during the infection period.

Conclusions

Children with IBD can mount a protective humoral response against SARS-CoV-2, which is comparable to that of their healthy peers regardless of ongoing immunomodulatory treatment. This study also supports the favorable course of PIBD during COVID-19 and vice-versa.

[#]*The work was submitted in November 2021; therefore, the results refer the first year of the COVID-19 pandemic, before vaccinations were available in pediatric age.*

Background

Coronavirus Disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread globally, evolving into a pandemic and a serious public health threat across the world.[138] When compared to adults, children and adolescents have a mild COVID-19 course with a good prognosis.[139] However, a small percentage of pediatric subjects experience life-threatening complications from SARS-CoV-2 infection either in the form of severe or critical COVID-19[140] or in the form of a severe hyperinflammatory condition, known as multisystem inflammatory syndrome in children (MIS-C).[141]

From the beginning of the SARS-CoV-2 emergency, special attention has been reserved to immunocompromised subjects, including patients treated for IBD.[1422] Despite concerns that patients with IBD could be more susceptible to COVID-19, there is to date no evidence of an increased risk of SARS-CoV-2 infection or development of more severe COVID-19 disease in this patient group compared to the general population, regardless of ongoing immunosuppressive therapy.[143–145] However, whether immunomodulatory treatment in IBD affects the degree and the duration of the antibody response to SARS-CoV-2 infection (and/or vaccination) is still under investigation, especially in children.[146,147] The induction of protective immunity to SARS-CoV-2 is critical to contain viral replication in individual subjects, suppress viral transmission across the population, and prevent the emergence of novel viral variants.[148,149]

Also, it remains to be determined whether COVID-19 affects the clinical course of IBD. The documented intestinal tropism of the virus, combined with the onset of gastrointestinal symptoms and the high fecal calprotectin levels observed in COVID-19 patients, has led to the hypothesis that SARS-CoV-2 infection could trigger bowel inflammation in subjects with IBD.[142,150,151] Unfortunately, the overlap between the clinical manifestations of active IBD and the gastrointestinal symptoms of COVID-19 make it challenging to prove or disprove the above speculation during the acute phase of COVID-19.[152]

In the attempt to investigate the consequences of SARS-CoV-2 infection in IBD and vice-versa, we performed a prospective study aiming to primarily assess the serological response to SARS-CoV-2 in a cohort of children with IBD and confirmed COVID-19 and, secondarily, to evaluate the impact of COVID-19 on the clinical course of PIBD.

Methods

Study design and population

A single-center, prospective, observational study was conducted on pediatric patients (aged 0-18 years old at February 2020) with a diagnosis of CD, UC, or IBDU followed at the Department of Women's and Children's Health of the University Hospital of Padova, a tertiary referral pediatric hospital and a regional center for PIBD in the North-East of Italy.

All the subjects of the cohort who acquired a SARS-CoV-2 infection from the February 21, 2020 (date of the first case of COVID-19 diagnosed in Italy) to February 28, 2021, were enrolled in the study after obtainment of the informed consent from the parents or legally authorized representatives.

Case definition

Patients were included in the study if satisfying the WHO definition of confirmed SARS-CoV-2 infection following either a positive Nucleic Acid Amplification Test (NAAT) or a positive SARS-CoV-2 antigen-rapid diagnostic test (RDT) and contact with a probable or confirmed COVID-19 case or cluster.[153]

Patients were also included in the study if satisfying the WHO criteria for probable SARS-CoV-2 infection (i.e., a patient who meets COVID-19 clinical criteria and is a contact of a probable or confirmed COVID-19 case or cluster) in adjunct to a positive serological test obtained after the resolution of symptoms.[153,154]

In confirmed COVID-19 cases, SARS-CoV-2 infection was dated either from the first positive diagnostic test (NAAT or RDT) or from the onset of symptoms. In serologically identified COVID-19 patients, SARS-CoV-2 infection was dated back to the onset of symptoms after the close contact with a probable or confirmed COVID-19 case or cluster.

The severity of COVID-19 was scored as mild, moderate, severe, or critical following the WHO classification based on clinical features, laboratory testing, and chest radiograph imaging (when available). Severe COVID-19 was defined by oxygen saturation <90% on room air or by signs of severe respiratory distress. Critical COVID-19 was defined by the criteria for acute respiratory distress syndrome (ARDS), sepsis, septic shock, or other conditions that would require the provision of life-sustaining therapies such as mechanical ventilation (invasive or non-invasive) or vasopressor therapy, or by a disease resulting in death.[155]

Data collection

PIBD patients with SARS-CoV-2 infection were actively identified by in-person or phone interviews with the parents, inquiring about SARS-CoV-2 testing, COVID-19 diagnosis, COVID-19 symptoms, close contacts with probable or confirmed COVID-19 cases, and quarantine assignments.

All patients experiencing the acute onset of gastroenterological symptoms in the context of IBD remission or a worsening of gastroenterological symptoms in comparison to baseline underwent a NAAT or an RDT. Moreover, a surveillance NAAT was performed in all PIBD patients at the time of biologic drug infusions or endoscopic check-ups, and in case of hospital admission or emergency department visits.

A standardized questionnaire was used to collect all data related to SARS-CoV-2 infection, including diagnostic modalities, date of diagnosis, source and duration of infection, clinical manifestations, duration of symptoms, clinical outcome, infection management, need for hospital admission or anti-viral treatment. The impact of COVID-19 on the course and the management of IBD was also investigated, inquiring each patient for disease activity before, at the time and after SARS-CoV-2 infection [remission was defined by a PCDAI[104] score <10 for CD, and a PUCAI score[105] <10 for UC and IBDU], fecal calprotectin levels before and after SARS-CoV-2 infection (a cut-off value of 250-300 μg/g was considered predictive of mucosal inflammation),[156] modifications of IBD treatment during the infection, and possible variations on scheduled hospital admissions, endoscopic procedures, or outpatient visits.

Demographic data (age, gender, ethnicity) and all the information concerning the patients' medical history, with particular regard to IBD [date of diagnosis, disease type,[6] extent, and disease behavior[4] and treatment], were obtained through clinical file revision.

Collected data were entered into a password-protected Excel database and analyzed anonymously.

At 3 months from the SARS-CoV-2 infection onset, all IBD patients received a gastroenterology and infectious diseases clinical evaluation along with a blood sample collection for SARS-CoV-2 serology testing.

Serological assay

The serological response to SARS-CoV-2 infection was evaluated in PIBD patients with confirmed COVID-19 at 3 months after infection employing a commercially available chemiluminescent immunoassay (CLIA) measuring the IgG antibody titer against the receptor-binding domain

(RBD) of the spike (S) protein in human serum (anti-SARS-CoV-2 S-RBD IgG; Snibe Diagnostics, New Industries Biomedical Engineering Co., Shenzhen, China).[139,140]

According to the manufacturer's instructions, all analyses were performed using a magnetic microbead separation technology (MAGLUMITM 2000 Plus, Snibe Diagnostics). Results were expressed in arbitrary units. The electrochemiluminescence signal from the reaction product was considered positive when ≥1.0 kAU/L in accordance with the cut-off recommended by the manufacturer.[157]

Control group and case-control study

The anti-SARS-CoV-2 S-RBD IgG titles of IBD patients in our cohort were matched 1:4 for age, sex, and COVID-19 severity with a control group of healthy children convalescent after SARS-CoV-2 infection. This control group was recruited from the COVID-19 Family Cluster Follow-up Outpatient Clinic (CovFC) program of the University Hospital of Padova, which included the measurement of anti-SARS-CoV-2 S-RBD IgG in healthy children (0-18 years old) at 3 months after SARS-CoV-2 infection.[158,159]

The Institutional Review Board approved the CovFC study protocol, and parents or legally authorized representatives of the children included in the control group provided written informed consent to use clinical data for research purposes.

Statistical analysis

Descriptive statistics were used to summarize the basic demographic and clinical characteristics of the study population. Data were synthesized as mean ± standard deviation or median and interquartile range for quantitative variables, as counts and percentages for categorical variables.

The normality of quantitative variables was checked with the Shapiro-Wilk test. Quantitative variables were compared across groups with independent t-test and categorical variables with $\chi 2$ or Fisher exact test, as appropriate. Significance was set at p-value <0.05.

Analyses were performed using SPSS 23.0 (IBM Corporation, Armonk, NY).

Results

Pediatric IBD patients with SARS-CoV-2 infection

Eighty-four pediatric patients with IBD (median age 14 years, range 1-18 years, IQR 12.25-17 years) were followed-up at the Department of Women's and Children's Health of the University Hospital of Padova between the 21st of February 2020 and the 28th of February 2021 (**Table 1**).

	Pediatric IBD patients	Pediatric IBD patients with a confirmed SARS-CoV-2 infection	Pediatric IBD patients without a confirmed SARS-CoV-2 infection
Number	84	12	72
Mean age ± SD, years	14.0 ± 3.5	15.4 ± 1.8	13.7 ± 3.6
Sex, n (%)			
M	40 (48.0)	5 (42.0)	35 (49.0)
F	44 (52.0)	7 (58.0)	37 (51.0)
Ethnicity			
Caucasian	73	10	63
Arab-Berber	7	2	5
African	2	0	2
Bengali	1	0	1
Chinese	1	0	1
Diagnosis, n (%)			
CD	49 (58.0)	9 (75.0)	40 (56.0)
UC	21 (25.0)	2 (17.0)	19 (26.0)
IBDU	14 (17.0)	1 (8.0)	13 (18.0)
IBD therapy			
5-ASA	31	1	30
AZA	46	6	40
MTX	2	0	1
Systemic steroids (any dosage)	15	4	11
Budesonide	1	1	0
Tacrolimus	3	0	3
MMF	1	0	1
Biologics			
IFX	19	1	18
ADA	14	2	12
UST	5	3	2
VDZ	4	2	2
Combination therapy			
IFX+AZA	14	1	13
ADA+AZA	8	0	8

Table 1. *Demographic features, IBD diagnosis and ongoing IBD treatment of the pediatric IBD patients followed at the Department of Women's and Children's Health of the University Hospital of Padova during the study period. 5-ASA: Mesalazine; ADA: Adalimumab; AZA: Azathioprine; CD: Crohn's disease; F: female; IBD; inflammatory bowel disease; IBDU: inflammatory bowel disease unclassified; IFX: Infliximab; M: male; MMF: Mycophenolate mofetil; MTX: Methotrexate; UC: Ulcerative Colitis; UST: Ustekinumab; VDZ: Vedolizumab.*

None of them received any anti-SARS-CoV-2 vaccination as, in Italy, no vaccine against COVID-19 was available for pediatric patients before March 2021.[160] Twelve children (M=5; median age 15 years, range 12-18 years, IQR 14-17 years) contracted a SARS-CoV-2 infection during the study period and were enrolled in the study. Nine out of these twelve children were affected by CD, one by UC and two by IBDU. At the time of SARS-CoV-2 infection, half of the patients (6/12) were on azathioprine treatment, and one-third (4/12) were on steroids. Three children were treated with anti-TNF agents (2 with adalimumab and one with infliximab), two with vedolizumab, and one with ustekinumab (**Table 2**). In all patients, the IBD treatment was continued without interruption for the entire duration of COVID-19.

IBD Case	1	2	3	4	5	6
Age at SARS-CoV-2 infection (years)	12	13	14	14	14	14
Sex	M	F	F	F	M	M
Ethnicity	Caucasian	Caucasian	Arab-Berber	Arab-Berber	Caucasian	Caucasian
IBD type	IBDU	CD	CD	CD	IBDU	CD
IBD therapy	None	UST, PDN	ADA, PEN	AZA, PEN	AZA, PDN	VDZ, PDN
IBD activity (PCDAI/PUCAI) before/after COVID-19	Remission (0) / Remission (0)	Remission (5) / Remission (7.5)	Mild (22.5) / Mild (22.5)	Remission (0) / Remission (7.5)	Remission (0) / Remission (0)	Mild (20) / Remission (0)
Fecal calprotectin before/after COVID-19 (ug/g)	>2100 / 1924	>2100 / 441	>2100 / 603	137 / <40	28 / 79	>2100 / 126
Relevant comorbidities	PSC, Hashimoto thyroiditis	PSC, NAFLD	Sacroiliitis	None	ASC/AIH	Arthritis, partial IgA deficiency
Reason for testing	Close contact	Close contact	Close contact	Close contact	COVID-19 symptoms	Close contact
Contact setting	Health care	Household	Household	Household	Community	Community
COVID-19 severity	Mild	Asymptomatic	Asymptomatic	Asymptomatic	Mild	Mild
COVID-19 symptoms	Fever, fatigue, headache, hypogeusia, sore throat, nausea	None	None	None	Headache, anosmia, ageusia, rhinitis, sore throat	Fatigue, cough
Viral clearance (days)*	9	16	11	11	10	33
COVID-19 outcome	Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery
SARS-CoV-2 S-RBD IgG (KuA/L)	145	20.03	4.7	36.5	21.4	3.505
Timing of serology (days)§	156	159	172	172	89	98
Impact on IBD management	Delayed endoscopy	Delayed visit and biologic drug administration	None	None	None	None

12	11	10	9	8	7
18	18	17	17	16	16
F	F	M	F	F	M
Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
CD	CD	CD	CD	RCU	CD
AZA	ADA, PEN	VDZ, PDN, MTX	AZA	IFX, AZA, 5-ASA	AZA
Remission (0)	Remission (0)	Remission (7.5)	Remission (0)	Remission (0)	Remission (0)
Remission (5)	Remission (0)	Remission (0)	Remission (0)	Remission (5)	Remission (0)
300	310	2075	28	>2100	474
NA	461	1117	249	>2100	162
None	None	HLA-B27 negative spondyloarthritis	None	None	None
Screening	COVID-19 symptoms	Close contact	Close contact	COVID-19 symptoms	Close contact
Unknown	Unknown	Household	Household	Household	Household
Asymptomatic	Mild	Mild	Mild	Mild	Mild
None	Fatigue, dysgeusia	Cough, rhinitis	Fever, cough, sore throat, abdominal pain, diarrhea	Fever, fatigue, headache, hyposmia, cough, rhinitis, diarrhea	Headache, ageusia, rhinitis
11	NA	27	NA	34	10
Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery
1.681	5.5	1.74	82.2	2.794	2.5
132	149	128	76	109	112
Delayed endoscopy	None	Delayed visit and biologic drug administration	None	Delayed visit and biologic drug administration	Delayed visit

Table 2. *Demographics and clinical characteristics of SARS-CoV-2 cases within our pediatric IBD cohort. Close contact (of a confirmed COVID-19 case) refers to high-risk exposure, according to the European Centre for Disease Prevention and Control (ECDC). *Viral clearance reflects the time from the first positive diagnostic test to the first negative test. §Timing of serology refers to the number of days elapsed from disease onset (the day when the symptoms started, or of the first positive nasopharyngeal swab in asymptomatic cases) to serological test. 5-ASA: Mesalazine; ADA: Adalimumab; AIH: autoimmune hepatitis; ASC: autoimmune sclerosing cholangitis; AZA: Azathioprine; CD: Crohn's disease; F: female; IBD; inflammatory bowel disease; IBDU: inflammatory bowel disease unclassified; IFX: Infliximab; M: male; MTX: methotrexate; N/A, not available; NAFLD: Nonalcoholic Fatty Liver Disease; PCDAI: Pediatric Crohn's Disease Activity Index; PDN: prednisone; PEN: partial enteral nutrition; PSC: primary sclerosing cholangitis; PUCAI: Pediatric Ulcerative Colitis Activity Index; UC: Ulcerative Colitis; UST: Ustekinumab; VDZ: Vedolizumab; WHO: World Health Organization.*

Clinical course of SARS-CoV-2 infection in children with IBD

The diagnosis of SARS-CoV-2 infection was based on a positive NAAT in eleven children (91.7%) and on a positive serological test in a single child who experienced fever during the cohabitation with a confirmed COVID-19 relative (8.3%).

Ten out of twelve patients (83.3%) contracted the infection after close contact with a COVID-19 subject, respectively in the household (*n* = 7), healthcare (n=1), or community (n=2) setting, while the source of infection was unknown in two patients (**Table 2**).

SARS-CoV-2 infection remained asymptomatic in four out of twelve children and caused a mild COVID-19 in the remaining eight. The most common complaints were constituted by fatigue, headache, and upper respiratory symptoms (cold, cough, sore throat). Gastroenterological symptoms occurred in three patients: nausea in one and acute diarrhea in two.Apart from the sporadic use of antipyretic or anti-inflammatory drugs, none of the symptomatic patients required anti-viral treatment or hospitalization for COVID-19, and all promptly recovered without sequelae after an average of 5.1 days (SD 3.7) from symptoms onset.

The average time from the first positive diagnostic test to the first negative test was 17.2 days (SD 10.1) (**Table 3**).

		Cases (n=12)		Controls (n=48)		p-value
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Age (years)		15.3 (2)	15 (14-17)	17.3 (9.5)	13 (10.3-25.3)	0.169
Male		5	41.7	20	41.7	1.000
		n	**%**	**n**	**%**	
COVID-19 WHO classification	**Asymptomatic**	4	33.3	15	31.3	1.000
	Mild	8	66.7	33	68.8	
	Moderate or more	0	0	0	0	
COVID-19 symptom duration (days)		5.1 (3.7)	3 (2.25-9.25)	5.2 (4.1)	5.2 (4.1)	0.972
Viral clearance time (days)		17.2 (10.1)	11 (10-28.5)	15.1 (5.8)	13 (10-18.5)	0.548
Collection time (days from infection)		129.3 (32.8)	130 (100.8-158.3)	129.5 (19.3)	133 (11-143.5)	0.985
IgG title (kAU/L)		27.3 (43.8)	5.1 (2.6-32.7)	31.7 (33)	24.5 (12.7-39.5)	0.700

Table 3. *Demographics, COVID-19 clinical and serological features of pediatric IBD patients vs. healthy children with SARS-CoV-2 infection.*

Serological response to SARS-CoV-2 infection in children with IBD in comparison to a control group of healthy children

The immunological response to SARS-CoV-2 infection of our cohort of twelve PIBD patients was compared to a control group of forty-eight healthy children convalescent after COVID-19.

The control group was similar according to age, sex, COVID-19 severity, duration of symptoms, and the time between the initial positive to the first negative diagnostic test (**Table 3**).

Each IBD patient was combined to 4 selected controls matched for age, sex, and COVID-19 severity.

SARS-CoV-2 serology was evaluated in both groups 3 months after infection (129 ± 31 days vs. 115 ± 21 days from infection in cases and controls, respectively; p=0.985; **Figure 1A** and **Table 3**). The mean anti-SARS-CoV-2 IgG S-RBD title was similar between IBD patients and healthy children (27.3 ± 43.8 kAU/L vs. 36.8 ± 35.3 kAU/L, p = 0.451; **Figure 1B** and **Table 3**).

Since cases with outlier levels of IgG were present (i.e., patients 1 and 9), outlier controls were also included. No clinical, demographic, and comorbidity differences were reported between outliers and other subjects.

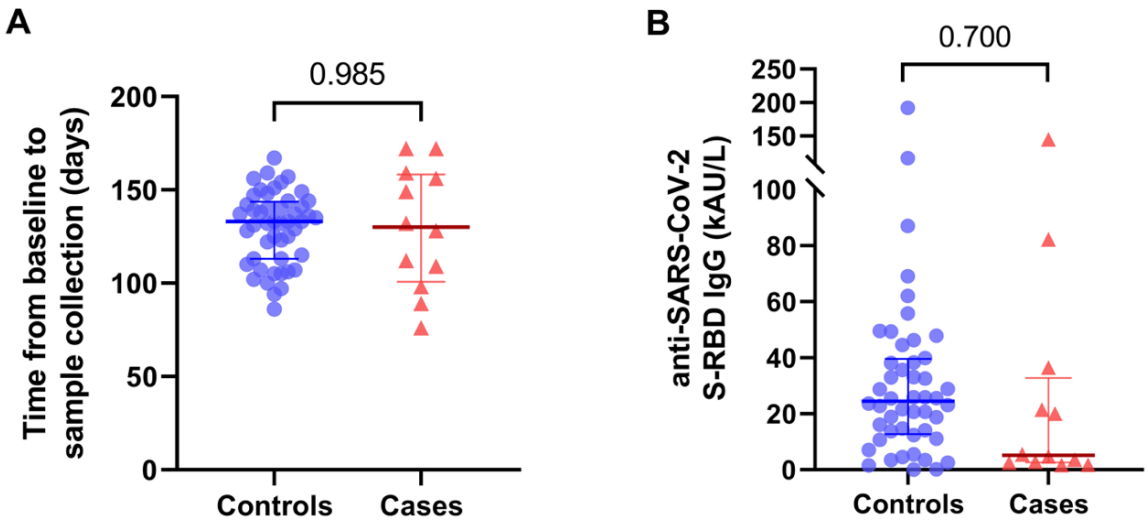


Figure 1. *(**A**) Time (days) from baseline to sera collection of controls and cases. (**B**) IgG S-RBD title (kAU/L) of controls and cases. Median and IQR are reported.*

Impact of SARS-CoV-2 infection on IBD clinical course and management

At the time of SARS-CoV-2 infection, IBD was in remission in ten out of twelve patients, and mild CD symptoms were present in two children. Of these two, one had a persistent mildly active CD after COVID-19 recovery, while all the other children were in clinical remission following infection.

No children experienced an IBD flare nor required gastroenterological support during the infection period. Fecal calprotectin, measured at 3 months after SARS-CoV-2 infection and compared to pre-COVID-19 levels, did not significantly worsen in any patient but remained stable or improved in six and four subjects, respectively.

No patient had the IBD treatment interrupted, but three patients experienced a delay in biologic drug infusions due to COVID-19 confinement measures.

Similarly, scheduled endoscopic reassessments and outpatient visits were postponed in two and four patients, respectively (**Table 2**).

Conclusions

Our prospective study shows that children with PIBD under treatment with immunomodulatory agents can mount a humoral immunological response against SARS-CoV-2 comparable to that of a control group of healthy subjects matched for age, sex, and COVID-19 severity.

Our data also provide further evidence for the overall benign course of COVID-19 in PIBD patients regardless of immunosuppressive therapy and show that SARS-CoV-2 infection does not negatively influence the course of IBD in children previously co-affected with COVID-19.

Specifically, in our cohort of children with IBD, we described the clinical course of COVID-19 in twelve children, most of whom were receiving an immunomodulatory treatment either as a monotherapy or a drug combination. In all cases, the ongoing IBD treatment was continued without interruption for the entire COVID-19 duration according to currently available recommendations.[161–163] All of our patients presented an asymptomatic infection or a mild COVID-19, and none required hospitalization. These results align with the most extensive study on PIBD (including 209 patients from the SECURE-IBD registry and the COVID-19 database of the Pediatric IBD Porto group), which reported a low risk of severe COVID-19 even in children receiving biologics and/or other immune-suppressive therapies.[145] Moreover, corticosteroid therapy, assumed by a third of our patients, was not associated with a worse COVID-19 outcome, differently from that reported in some adult IBD studies.[142,144,145,164–166] Gastrointestinal symptoms arose in 25% of the patients, similarly to that reported in the above-mentioned international cohort of children with IBD.[145] Diarrhea occurred in 17% of our patients, a percentage comparable to that of the other reported pediatric and adult IBD patients with COVID-19 (13 and 20%, respectively), but higher than that reported in the non-IBD population (4% and 7-10% in children and adults, respectively).[139,142,145,167]

In adult and pediatric immunocompetent subjects, the natural infection with SARS-CoV-2 initiates a humoral immune response that produces antibodies against specific viral antigens, such as the nucleocapsid (N) and the spike (S) protein.[156,159,168–170] Antibodies against the receptor-binding domain (RBD) within the S protein, which binds the cellular receptor for viral entry (ACE2), constitute the primary source of neutralizing antibodies (i.e., able to inhibit viral replication *in vitro*) and are considered a reliable measure to assess anti-viral immunity.[157,171,172] Indeed, the appearance of neutralizing antibodies is associated with viral clearance and their absence with an increased risk of fatal outcomes.[168,172] However, the kinetic of antibody response

shows large interindividual variability and varies significantly depending on multiple factors such as patient age, sex and immune status, COVID-19 severity, and testing system.[154,159,170,171,173–177]

In our cohort of PIBD patients, all children with COVID-19 developed anti-SARS-CoV-2 S-RBD antibodies. Moreover, independently from the ongoing IBD treatment, the titer of anti-spike antibodies resulted similar to a control population of healthy children matched for age, sex, and COVID-19 severity. These results are in agreement with the observation that immunocompromised adult IBD patients with COVID-19 can develop a serologic response comparable to that of age- matched healthy controls.[178] They are also in line with a recent study reporting a high rate of seroconversion (85%) in immunocompromised PIBD patients (n=12, median age 10 years, range 2-17 years) at a median time of 8 weeks after SARS-CoV-2 infection.[179] Our results differ, however, from the study of Dailey et al.[180] who reported lower titers of anti-SARS-CoV-2 S-RBD antibodies in pediatric and young adult IBD patients (n=44, median age 18 years, range 11-26 years) in comparison to a control group of non-IBD children and adults. The different outcome may be related to the diverse characteristics of control groups: in the study of Dailey's et al. control subjects consisted of non-IBD children hospitalized with SARS-CoV-2 infection and of non-IBD adults with mild to moderate COVID-19, while we compared PIBD patients 1:4 to healthy children matched for age, sex, and COVID-19 severity.

Contrarily to adult IBD reports,[146] but similarly to another PIBD study,[180] we did not observe different levels of anti-SARS-CoV-2 antibodies between children treated with anti-TNFs and children receiving other biologics or immunomodulatory drugs, probably due to the small size of our patient population. We also did not observe serological differences relative to COVID-19 severity since all patients in our cohort had a mild form of disease. Notably, children with high antibody titers (i.e., IgG outliers) were observed in both the IBD and the control group, likely reflecting immunological inter-individual variability.

In immunocompetent subjects, anti-SARS-CoV-2 (neutralizing) IgG antibodies are detectable for several months in most persons.[154,159,172] How long these antibodies persist after infection in exogenously immunocompromised subjects is still under investigation.[169] In our cohort of children with IBD, we detected anti-SARS-CoV-2 S-RBD IgG at 3 months after COVID-19, consistently with the observation of detectable anti-SARS-CoV-2 antibodies up to 2 months after infection in adult and pediatric IBD patients treated with biologics.[180,181] We did not investigate the kinetic of antibody response over time. Although future studies are needed to assess the persistence of anti-S RDB antibodies in the long term, the similar viral clearance time (i.e., time between the initial positive to the first negative diagnostic test) and the comparable titer of

neutralizing SARS-CoV-2 S-RBD antibodies allow hypothesizing a similar seroconversion time among children with IBD and healthy pediatric controls.[157]

To date, it remains uncertain whether SARS-CoV-2 infection affects the clinical course of IBD, especially in children. As for our cohort, none of our twelve COVID-19 patients experienced a disease flare or a worsening of disease activity after SARS-CoV-2 infection. Likewise, no significant elevation of fecal calprotectin was documented (neither in those patients experiencing gastrointestinal symptoms during the acute phase of COVID-19), but a significant improvement was observed in one-third of the patients. Moreover, none of the PIBD patients experiencing a disease flare during the study period tested positive for SARS-CoV-2. These results are in agreement with those of a single, recent, study reporting that no children with IBD (n= 44) suffered a disease flare following SARS-CoV-2 infection.[180]

Nevertheless, in our cohort of PIBD children, COVID-19 caused several forced delays in IBD management due to the containment measures required to avoid SARS-CoV-2 transmission by positive patients. Two children experienced a delay of planned biologic drug infusions, and another one in the initiation of biologic treatment. Similarly, scheduled endoscopic reassessment and outpatient visits were postponed in two and four patients, respectively. As previously reported,[182–184] the SARS-CoV-2 pandemic is strongly challenging the diagnostic and therapeutic management of IBD across the globe. Further studies will be needed to evaluate the long-term impact of the pandemic on the course of PIBD.

Our study has several limitations. First, the small number of PIBD patients with a confirmed SARS-CoV-2 infection, all adolescents between 12 and 18 years of age, hampers the possibility to draw definitive conclusions. Secondly, the therapeutic heterogeneity among enrolled patients does not allow to investigate the potential effect of pharmacological treatments on the serological response against SARS-CoV-2 or to perform any sub-group analysis. Lastly, the strict adherence to the WHO definition of confirmed SARS-CoV-2 infection may have resulted in the lack of recognition of asymptomatic COVID-19 patients and in an underestimation of eligible patients.

Despite being a small monocentric study, the present work has several strengths. To the best of our knowledge, this is one of the very few studies investigating the immunological response to SARS-CoV-2 and the impact of COVID-19 on IBD course in pediatric IBD patients.[179,180] Only subjects satisfying the WHO case definition of confirmed SARS-CoV-2 infection were enrolled in the study (both in the patient and in the control group). The serologic response to SARS-COV-2 infection was determined based on the titers of anti-S-RBD IgG, the primary source of neutralizing antibodies,[157,171,172] employing a high performant diagnostic test.[157] The control population of non-

IBD subjects was comparable to the group of IBD patients for demographic, COVID-19 characteristics, and timing of serological tests.

In conclusion, this prospective study shows that children with IBD can mount a protective humoral response against SARS-CoV-2 and further support for the overall favorable course of COVID-19 in PIBD (and vice-versa) regardless of ongoing immunomodulatory treatment. Further studies are needed to confirm these results in a broader population of children with IBD, to determine the longevity of humoral immunity over time, and to assess the serological response to COVID-19 vaccines in this patient group.

SELECTED PUBLICATIONS

IFIH1 loss-of-function variants contribute to very early-onset inflammatory bowel disease

Cananzi M, Wohler E, Marzollo A, Colavito D, You J, Jing H, Bresolin S, Gaio P, Martin R, Mescoli C, Bade S, Posey JE, Dalle Carbonare M, Tung W, Jhangiani SN, Bosa L, Zhang Y, Filho JS, Gabelli M, Kellermayer R, Kader HA, Oliva-Hemker M, Perilongo G, Lupski JR, Biffi A, Valle D, Leon A, de Macena Sobreira NL, Su HC, Guerrerio AL. IFIH1 loss-of-function variants contribute to very early-onset inflammatory bowel disease. Hum Genet. 2021 Sep;140(9):1299-1312. doi: 10.1007/s00439-021-02300-4. Epub 2021 Jun 29. PMID: 34185153; PMCID: PMC8423350.

Abstract

Genetic defects of innate immunity impairing intestinal bacterial sensing are linked to the development of Inflammatory Bowel Disease (IBD). Although much evidence supports a role of the intestinal virome in gut homeostasis, most studies focus on intestinal viral composition rather than on host intestinal viral sensitivity. To demonstrate the association between the development of Very Early Onset IBD (VEOIBD) and variants in the IFIH1 gene which encodes MDA5, a key cytosolic sensor for viral nucleic acids. Whole exome sequencing (WES) was performed in two independent cohorts of children with VEOIBD enrolled in Italy (n=18) and USA (n=24). Luciferase reporter assays were employed to assess MDA5 activity. An enrichment analysis was performed on IFIH1 comparing 42 VEOIBD probands with 1527 unrelated individuals without gastrointestinal or immunological issues. We identified rare, likely loss-of-function (LoF), IFIH1 variants in eight patients with VEOIBD from a combined cohort of 42 children. One subject, carrying a homozygous truncating variant resulting in complete LoF, experienced neonatal-onset, pan-gastrointestinal, IBD-like enteropathy plus multiple infectious episodes. The remaining seven subjects, affected by VEOIBD without immunodeficiency, were carriers of one LoF variant in IFIH1. Among these, two patients also carried a second hypomorphic variant, with partial function apparent when MDA5 was weakly stimulated. Furthermore, IFIH1 variants were significantly enriched in children with VEOIBD as compared to controls ($p = 0.007$). Complete and partial MDA5 deficiency is associated with VEOIBD with variable penetrance and expressivity, suggesting a role for impaired intestinal viral sensing in IBD pathogenesis.

Case Report: Intestinal Nodular Lymphoid Hyperplasia as First Manifestation of Activated PI3Kδ Syndrome Due to a Novel *PIK3CD* Variant

Marzollo A, Bresolin S, Colavito D, Cani A, Gaio P, Bosa L, Mescoli C, Rossini L, Barzaghi F, Perilongo G, Leon A, Biffi A, Cananzi M. Case Report: Intestinal Nodular Lymphoid Hyperplasia as First Manifestation of Activated PI3Kδ Syndrome Due to a Novel PIK3CD Variant. Front Pediatr. 2021 Oct 6;9:703056. doi: 10.3389/fped.2021.703056. PMID: 34692603; PMCID: PMC8528001.

Abstract

Nodular lymphoid hyperplasia (NLH) is a lymphoproliferative disease caused by non-clonal expansion of lymphoid cells in the gut mucosa. Little is known about the pathogenesis of NLH, which is often disregarded as an insignificant or para-physiologic phenomenon. We present the case of a girl with isolated diffuse NLH (extending from the stomach to the rectum) caused by activated PI3Kδ syndrome (APDS) due to the novel p.Glu525Gly variant in *PIK3CD*. The gain-of-function effect of the variant was confirmed by demonstration of over activation of the Akt/mTOR pathway in the patient's cells. APDS diagnosis led to treatment with sirolimus, which resulted in the complete remission of NLH and in the prevention of extra intestinal complications. In conclusion, we identify APDS as a novel cause of isolated NLH and suggest that patients with severe pan-enteric NLH should be screened for this disorder that may not be apparent on first-line immunological testing.

Induction of Remission With Exclusive Enteral Nutrition in Children With Crohn's Disease: Determinants of Higher Adherence and Response

Cuomo M, Carobbio A, Aloi M, Alvisi P, Banzato C, Bosa L, Bramuzzo M, Campanozzi A, Catassi G, D'Antiga L, Di Paola M, Felici E, Fioretti MT, Gatti S, Graziano F, Lega S, Lionetti P, Marseglia A, Martinelli M, Musto F, Sansotta N, Scarallo L, Zuin G, Norsa L. Induction of Remission With Exclusive Enteral Nutrition in Children With Crohn's Disease: Determinants of Higher Adherence and Response. Inflamm Bowel Dis. 2022 Oct 12:izac215. doi: 10.1093/ibd/izac215. Epub ahead of print. PMID: 36222487.

Abstract

Background: Exclusive enteral nutrition (EEN) is the first choice to induce remission and promote mucosal healing in pediatric Crohn's disease (CD). However, full adherence to EEN treatment may be problematic for children with CD.

Methods: The goal of the current multicenter retrospective study was to define predictive factors of nonadherence to treatment and nonremission at the end of induction treatment. Those data together were analyzed with the ultimate goal of trying to define an individualized induction treatment for children with CD.

Results: Three hundred seventy-six children with CD from 14 IBD pediatric referral centers were enrolled in the study. The rate of EEN adherence was 89%. Colonic involvement and fecal calprotectin >600 μg/g at diagnosis were found to be associated with a reduced EEN adherence. Exclusive enteral nutrition administered for 8 weeks was effective for inducing clinical remission in 67% of the total cohort. Factors determining lower remission rates were age >15 years and Pediatric Crohn's Disease Activity Index >50.

Conclusion: Although EEN is extremely effective in promoting disease remission, several patients' related factors may adversely impact EEN adherence and response. Personalized treatments should be proposed that weigh benefits and risks based on the patient's disease location, phenotype, and disease activity and aim to promote a rapid control of inflammation to reduce long-term bowel damage.

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