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SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

DNA methylation and transcriptomic profiling of intestinal epithelial cells in children with Inflammatory Bowel Disease treated with Infliximab: preliminary results.

Settore scientifico-disciplinare: **MED/38 PEDIATRIA GENERALE E SPECIALISTICA**

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INTRODUCTION

Inflammatory bowel disease: an umbrella definition for different diseases

Inflammatory bowel diseases (IBD) are a heterogeneous group of diseases that affect the digestive tract. The pediatric onset of IBD account for 8-15% of the cases.(1-4)

Historically, IBD have been subdivided into Crohn's disease (CD) and ulcerative colitis (UC) and IBD-unclassified (IBD-U) even if this classification fails to capture the variety of phenotypes encountered in daily clinical practice.

CD can affect any part of the gastrointestinal tract and involve the full thickness of the bowel wall leading to complications such as strictures, abscesses, or fistulas, while UC is typically limited to the colon and restricted to the mucosal layer. The term IBD-U identifies those patients with colonic disease who have clinical or endoscopic findings that are atypical for UC.

IBD is more common in industrialized countries and has a North-South and West-East epidemiological gradient. In fact, the incidence and prevalence of pediatric-onset IBD are highest in Northern Europe and North America and lowest in Southern Europe, Asia and the Middle East. Most of the studies report a constant increase in the incidence of pediatric IBD.(2-4)

In the context of Western countries, Italy occupies an intermediate position (Figure 1).(2,5) The minimal incidence of pediatric IBD, measured between 2009 and 2018, ranged from 1.59 to 2.04 per 100,000 inhabitants under 18 years of age, with a slight predominance of UC over CD.(6) These figures represent a two-fold increase in incidence compared to the period between 1996 and 2003.(7)

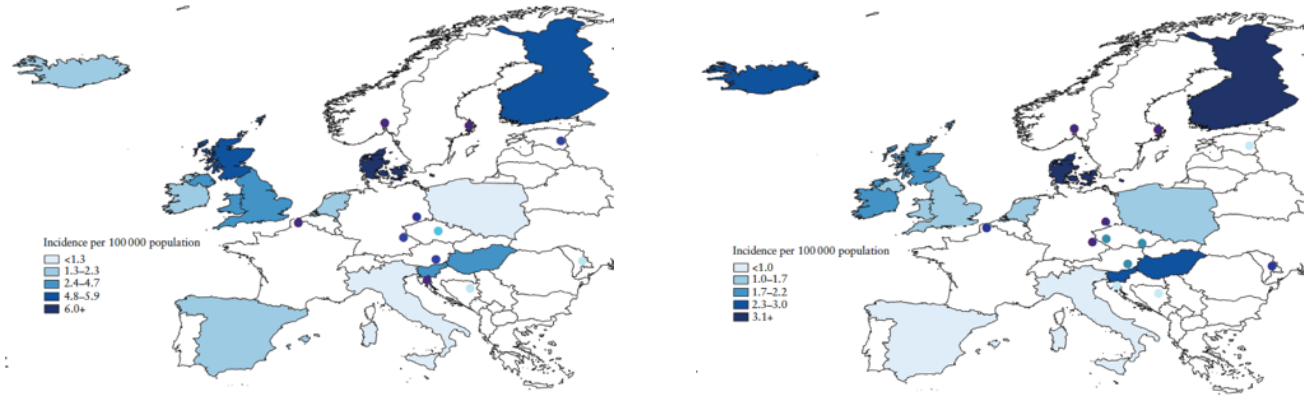


Figure 1. Incidence of CD (on the left) and UC (on the right). (modified from reference 3)

Data on Italy are based on studies conducted between 1987 and 2003

IBD pathogenesis: a complex interplay between genetics and environment

IBD pathogenesis remains largely unknown. A dysregulated immune response involving both innate and adaptative immunity, resulting from a complex interaction between genetic susceptibility and environmental triggers, is supposed to stand as the central driver of IBD pathogenesis.(8) Meta-analysis of genome-wide association studies (GWAS) identified over 230 disease loci linked to polygenic IBD, highlight the importance of host–microbe interactions, autophagy and specific inflammatory signaling pathways in IBD pathogenesis.(8,9) However, the contribution of common variants identified with GWAS can explain only a fraction of the expected heritability in IBD.(10,11)

Assuming the human genome to be highly stable even over centuries, the major increase in the incidences of IBD in recent decades could be seen as further evidence in support for a key role of environmental factors in IBD pathogenesis.

Many environmental risk factors for IBD have been studied all of which are linked to the Westernization of lifestyle, including cigarette smoking, obesity, physical inactivity, and diets high in saturated fats, refined carbohydrates, red and processed meats, and low in fruits, vegetables, and fiber.(12) These environmental factors have been shown to promote

gut dysbiosis, reducing microbiome diversity or introducing non-commensal microorganism. They can also compromise the integrity of the epithelial barrier and trigger an altered immune response.(13)

Epigenetic modifications have been advocated as the mediator between exposure to these environmental factors and heritable changes in cellular phenotypes.(14)

Prolonged or repeat exposure to certain environmental factors may lead to the development of stable epigenetic changes, which predispose to an individual to the development IBD.(15)

Epigenetics in the pathogenesis of IBD

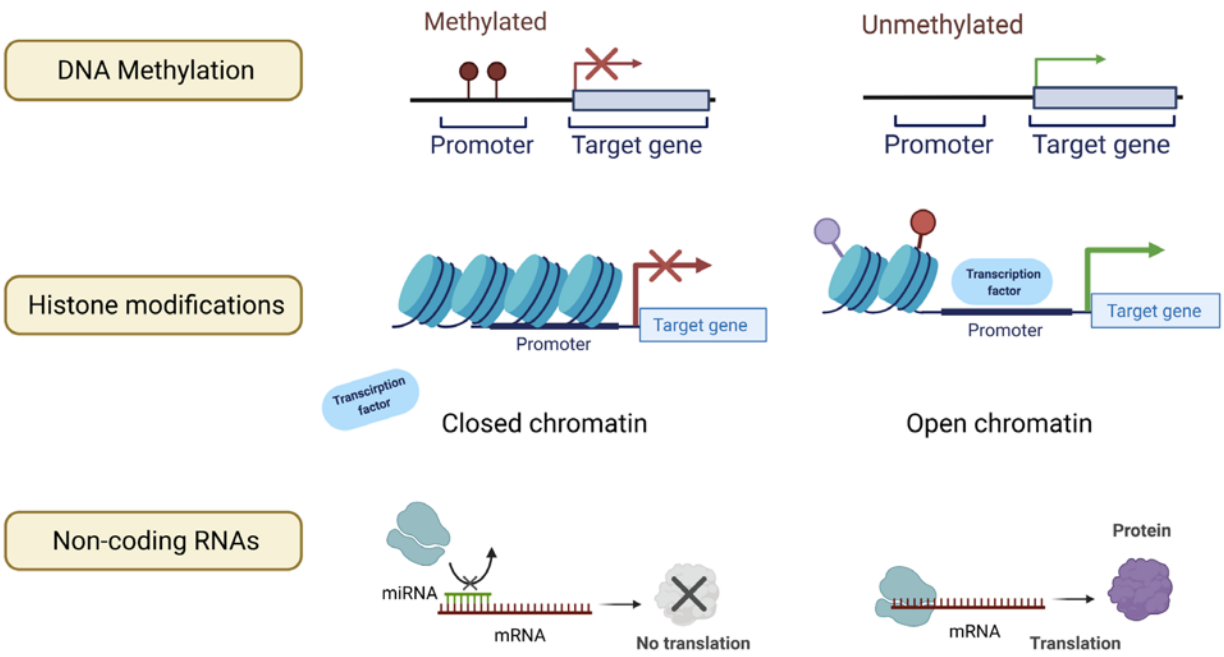
In contrast to genetics, which focuses on heritable changes in gene expression or function due to direct alterations in the genetic code (such as point mutations, insertions, deletions, and translocations), epigenetics refers to the study of heritable changes in phenotype—such as gene expression—that are not caused by alterations in the underlying DNA sequence.

Thus, epigenetic modifications regulate gene expression without altering the underlying DNA sequence, enabling for dynamic responses to environmental stimuli.

These mechanisms play a key role in several fundamental biological processes, including cellular differentiation, cell-type- specific gene transcription and cellular function, and immunological memory and are involved both in health and in a wide range of diseases, including IBD.(16)

The three main type of epigenetic modifications are: DNA methylation, histone modifications, and non-coding RNA expression (Figure 2).

Figure 2. Epigenetic mechanisms.(Adapted from reference 17)



DNA Methylation

DNA methylation is the most extensively studied epigenetic mechanism. It refers to the covalent modification of the DNA by the addition of a methyl group onto the 5' position of cytosine residues, resulting in the formation of 5-methylcytosine. This typically occurs at CpG sites, where a cytosine is immediately followed by a guanine nucleotide, and is catalyzed by enzymes called DNA methyltransferases.(18)

In the human genome, most CpGs are methylated, except in CpG islands which are regions, often located within promoter areas, that have a high concentration of CpG motifs. Generally, hypermethylation of a promoter region leads to reduced gene expression, while hypomethylation can increase expression. However, methylation can also occur within gene bodies, where it may have more complex effects, sometimes even leading to gene activation.

DNA Methylation and IBD pathogenesis

Epigenetic changes, influenced by external triggers such as diet, infection, and the microbiome, resulting in dysregulated immune response, epithelial barrier function, and microbial defense have been advocated in the pathogenesis of IBD.(19)

Studies have been conducted on intestinal epithelial cells (IEC) and on peripheral blood mononuclear cells (PBMCs).

Early studies in patients with UC showed different degrees of methylation in the colonic mucosa of patients with inactive disease compared to those with active disease and in patients with dysplasia.(20-22) A positive relation between methylation of p16INK4a (a cyclin-dependent kinase inhibitor), E-cadherin (a specific calcium ion-dependent cell adhesion molecule), Estrogen receptor (ER), MYOD, GSPG2 and p16 gene exon 1 and inflammation, as well as neoplastic progression was observed.(23,24)

Alterations of the MDR1 (multidrug resistance 1) gene, which encodes for the P-glycoprotein (P-gp) that functions as a transmembrane efflux pump thus influencing disposition and response of many drugs, are taught to contribute to the pathogenesis of UC.(25) Hypermethylation of MDR1 has been shown to correlate with chronic continuously active colitis, extensive colitis, younger age of disease onset (<20 years old), higher number of hospitalizations, and severe disease phenotype.(26)

Higher methylation of the Protease-activated receptor (PAR2), which is a member of the large family of 7-transmembrane-region receptors that couple to guanine nucleotide-binding proteins, in rectal mucosa has been shown to differentiate extensive colitis from proctitis and to correlate with steroid-dependent or steroid-refractory colitis.(27)

Hypermethylation of DAPK (Death-associated protein kinase), which is the translational product of a tumor suppressor gene, was found to be associated to the inflammation of mucosa in UC patients and a gradient from lower to higher methylation was similar to the gradient from mild to severe inflammation. Moreover, in unmethylated mucosa DAPK

protein expression was proportional to the severity of inflammation, probably representing a protective role of DAPK during chronic inflammation in UC.(28)

Several data exist also on the role of DNA methylation in CD pathogenesis. Changes in seven CpG loci have been associated with both diseases with 25 CpG sites linked specifically to CD and 13 to UC.(29) Cooke et al. further demonstrated differential DNA methylation in genes involved in IBD pathogenesis, such as DOK2, Tap1, CD28, and ICAM3.(30) Notably, increased methylation of CDH1, which encodes E-cadherin, was observed, a gene previously linked to UC inflammation. Some genes were methylated in both UC and CD, while others were CD-specific. Additionally, no significant DNA methylation changes were found in non-inflamed tissue from CD patients compared to controls.(31)

IBD treatment: different treatments for different phenotypes

IBD has a chronic, relapsing course with a heterogeneous natural history. While some patients experience a mild progression, others may develop complications, such as strictures or fistulas, that require surgery and frequent hospitalizations.(32,33)

Since there is no known definitive cure for IBD, its chronic and intermittent nature necessitates long-term treatment, often involving a combination of medications to induce and maintain clinical, endoscopic, and histological remission, while also ensuring adequate growth in children. The ultimate goal of IBD treatment is to change the disease's natural course, preventing inflammation from causing intestinal and systemic damage, avoiding complications that require surgery, and improving the patient's quality of life.(34) Treatment decisions are based on factors such as disease severity, location, the presence of associated conditions, and the patient's response to previous therapies.(35)

In CD, the first-line drugs for inducing remission are corticosteroids and exclusive enteral nutrition. Steroids have a very rapid symptomatic effect but do not heal the mucosa and

are often associated with significant side effects. Budesonide, a steroid with high first-pass hepatic metabolism and minimal systemic effects, is useful in cases with ileocolonic involvement.

Polymeric nutritional therapy, less frequently semi-elemental or elemental, when administered exclusively for a period of 6-8 weeks, can promote mucosal healing and support the nutritional status and growth of the child. Relapses occur upon discontinuation of treatment in 50-80% of cases.(35) New nutritional schemes based on partial enteral nutrition and specific exclusion diet, the most commonly used known as Crohn's disease exclusion diet, have recently shown comparable efficacy to exclusive enteral nutrition and better acceptability.(36)

The treatment of UC can be limited to topical steroid or salicylate formulations in cases with more distal localization or may require, in extended forms, systemic salicylates, often combined with short courses of oral steroids. Maintenance of remission is generally ensured by low doses of salicylates.(37)

Azathioprine or its metabolite 6-mercaptopurine are used as maintenance therapy for remission, starting immediately in cases with incomplete response to induction therapy or after the first relapse in both CD and UC. In CD patients, methotrexate has been shown to be a valid alternative in cases intolerant to azathioprine.(35)

Unfortunately, a significant proportion of patients are resistant to first- and second-line therapies or experience side effects that require discontinuation, and are then treated with biologic drugs. Moreover, a certain percentage of patients, from the moment of diagnosis, exhibit severe characteristics that place them at risk for a complicated disease course, for whom immediate initiation of biologic therapy is recommended.(35,37) The only biological drugs approved for treating pediatric IBD are two monoclonal antibodies against tumor necrosis factor–α: infliximab and adalimumab.

The need for surgery represents an indicator of the severity of IBD. Up to 35% of patients with CD undergo surgery within the first 5 years, while about one-fifth of children with UC require colectomy within the first 10 years of disease.(38,39)

In CD, surgery is reserved for complicated forms (fistulas/strictures) or for highly localized inflammatory forms (e.g., terminal ileum). Surgery in the form of colectomy is definitively curative in UC, but it is still a very invasive procedure with potential consequences. Moreover, the impact of a long-term stom on the psychological weel being of a child or adolescent needs to be taken into account and should, where possiible, be addressed timely with the young person and their family, allowing for preparation to be as adequate as it can be possible.

Colectomy becomes necessary in severe acute forms (PUCAI > 65) that are not controlled by intravenous steroid therapy, cyclosporine, or infliximab, or if the disease is not controlled by maximal maintenance therapy and is affected by the side effects of the drugs used.

Infliximab: a key biologic therapy in IBD treatment

Infliximab (IFX), a chimeric antibody to TNF, is a highly effective biologic in IBD.(38,39) Despite its efficacy, 20–40% of patients do not respond to treatment or lose response (LOR) (40-42) and 10% of patients experience infusion reactions leading to treatment discontinuation.(43,44) Low-serum IFX concentrations and the development of antibodies to IFX (ATI) are two major factors affecting IFX efficacy and durability.(45) Several observations have linked IFX concentrations at trough, before the following infusion, with IFX durability. In one prospective pediatric cohort study IFX concentrations at the time of entering into maintenance, at week 14, above 3, 4, and 7 μg/mL had a predictive value for persistent remission at week 54 of 64%, 76% and 100% respectively.(46) Similarly, several clinical observations linked the development of ATI to LOR and adverse reactions to IFX.

(43) Standard dose IFX is administered at 5 mg/kg given in a 0, 2, and 6 weeks induction regimen followed by a maintenance regimen with infusions every 8 weeks. This standard dosing is extrapolated from adult studies. However, IFX has a highly variable pharmacokinetic and pharmacodynamics that is dependent on body weight, disease extent, levels of inflammation and the presence of ATI.(47,48) In children with IBD all these factors often result in suboptimal drug exposure.(49) Different strategies have been evaluated to optimize IFX durability and safety. The use of a concomitant immunomodulator results in lower frequency of ATI, higher IFX concentrations and better outcomes.(50-52) However, this strategy is less desirable in pediatrics due to the risk of increased toxicity. Therapeutic drug monitoring (TDM) has been used in clinical practice in patients with IBD who lose response to treatment. This, so called, “Reactive” TDM strategy has proven more cost-effective compared with empiric dose escalation.(53)

An alternative strategy, known as “Proactive” TDM, consists of introducing TDM while the patient is clinically stable and adjusting IFX dosing based on serum concentrations at trough, before the following infusion, with the goal of achieving and maintaining IFX concentrations within a therapeutic window.(54,55) Preliminary observations suggest that a Proactive TDM, to target concentration of IFX within 5-10mcg/ml (up to > 12mcg/ml in perianal CD) during maintenance therapy, improve cost-effectiveness and safety of biologic therapy.(56,57) A recent observation in pediatric patients have shown that dosing decisions based on an IFX concentration at week 10 (4 weeks before the first maintenance dose) could reduce ATI formation, enhance IFX durability, and negate the need for combination therapy(58). Similarly, median IFX concentration levels > 18 mcg/ml at week 6 were found to be strongly associated with improved early outcome and optimal IFX concentration (> 5mcg/ml) at the end of induction.(59) Overall these observations suggest that in pediatric IBD, IFX dosing based on individual serum drug concentrations

introduced as early as the induction phase can guide the timing and dose of the first maintenance infusion and may thereafter improve IFX durability, efficacy and safety.

A step forward in treatment personalization will be the ability to predict treatment response to select the best drug for the individual patient's biology whilst individualizing dosing. DNA methylation and transcriptomic profiling of IEC from children with IBD have shown distinct alterations that are retained in organoid culture and correlate with disease outcome.(58) These profiles might offer the possibility to predict response to treatment and clarify the molecular mechanisms driving treatment response and failure. A clinical trial is the optimal setting to test the predictive value of epigenetic profiling as detailed data on clinical and follow-up variable as well as drug exposure are available.

New treatments for pediatric IBD

In recent years, a better understanding of the pathogenetic mechanisms of IBD, along with the need to offer therapeutic alternatives to patients who are unresponsive to anti-TNF biologics, has led to the development of new drugs. These include anti-integrin antibodies that inhibit leukocyte migration (vedolizumab), anti-IL12/IL23 antibodies (ustekinumab), anti-IL23 antibodies (risankizumab, guselkumab, and mirikizumab), JAK inhibitors (tofacitinib, upadacitinib), and S1P receptor modulators (ozanimod, etrasimod). While these new treatments are commonly used in adults, they have not yet been licensed for children thus limiting their availability.(60)

Epigenetic based biomarkers able to successfully predict disease severey and disease response to specific drugs, would be particularly helpful to estabilish the ideal sequencing for these new advance therapies.

AIMS OF THE STUDY

The study aims at evaluating DNA methylation and transcriptomic profiling of IEC from children with IBD treated with infliximab to identify genetic and epigenetic patterns that predict response or failure to IFX.

Since the study is still ongoing, this manuscript reports the preliminary results obtained from May 2022 to September 2024.

METHODS

Patients enrollment

Intestinal biopsies, from which IEC were derived, were obtained from patients enrolled in a multicenter, randomized, controlled, open-label study designed to evaluate the effect of proactive monitoring of infliximab in pediatric inflammatory bowel disease (EPIC Study).

The Study involved six Italian referral centers for pediatric IBD: the Institute for Maternal and Child Health IRCCS “Burlo Garofolo”, Trieste a coordinating center, the “La Sapienza” University of Rome, the Maggiore "CA Pizzardi" Hospital of Bologna, the IRCCS “Giannina Gaslini” of Genoa, the Meyer Children's Hospital, the “Ca Foncello” Hospital of Treviso, the University of Messina as collaborator centers.

The inclusion and exclusion criteria of the EPIC Study are as follows.

Inclusion criteria:

1. Anti-TNF naive children and adolescents, 6-17 years, with a diagnosis of IBD confirmed by a prior endoscopic biopsy that is consistent with the diagnosis.

2. Indication to start anti-TNF therapy in accordance with current pediatric guidelines for the treatment of pediatric IBD including: severe growth delay, no-response after induction with EEN or corticosteroids, extensive disease or deep colonic ulcers, stricturing or penetrating disease, perianal disease.

3. Active inflammation supported by CRP > 5mg/L and /or FC > 150 µg/g before the 1st IFX dose.

Indication to start anti-TNF therapy in children with IBD as per ECCO-ESPGHAN guidelines include: severe growth delay, non response after induction with exclusive enteral nutrition or corticosteroids, extensive disease or deep colonic ulcers, stricturing or penetrating disease

Exclusion criteria:

1. Consent withdrawal.

2. Stenosing or penetrating disease requiring surgery, abdominal abscess, symptomatic stricture.

3. Abdominal surgery within the previous 6 months.

4. Acute severe UC attack defined by a PUCAI score >65.

5. Infective contraindication to IFX treatment including positive tuberculin skin test or Quantiferon-TB test, recent opportunistic infection, infection with hepatitis B (HBV), C (HCV), human immunodeficiency virus (HIV).

6. Previous exposure to anti-TNF.

7. Exposure to concomitant prohibited medications including other biologics (including but not limited to ustekinumab, vedolizumab, abatacept, anakinra..), thalidomide, investigational drugs.

8. Pregnancy or lactation.

Patients treatment

Patients received infliximab IV at 5mg/kg at week 0, 2 and 6. At week 6, patients were randomized and assigned to receive two different IFX dosing and monitoring strategies.

In the intervention group, IFX infusion were based on IFX concentrations determined before every IFX infusion, starting from week 6, with the goal of achieving and maintaining IFX concentrations at trough > 5 µg/ml. IFX optimization was performed by shortening the interval between infusions, while maintaining a fixed dose of 5mg/kg. IFX dose increase was performed a second step in case of persistent IFX subtherapeutic concentration after interval shortening treatment intensification.

In the control group patients received IFX infusions according to the licensed dosing schedule. The first maintenance infusion was administered at week 14 and subsequently

every- 8 weeks at 5mg/kg. In this group IFX levels were notified upon investigator's request in case of non-response or LOR to guide treatment intensification

All patients were followed for 54 weeks or until IFX discontinuation

Biopsy collection

Intestinal biopsies were collected during endoscopic procedure at baseline (T0) and at week 54 (T1) or earlier in case of IFX discontinuation.

Three biopsy samples were obtained from terminal ileum and two from sigmoid colon in patients with CD. Three biopsy samples are obtained from sigmoid colon in patients with UC. (Figure 3). Biopsy samples were frozen at -80°C and centralized to the coordinator center (IRCCS Burlo Garofolo).

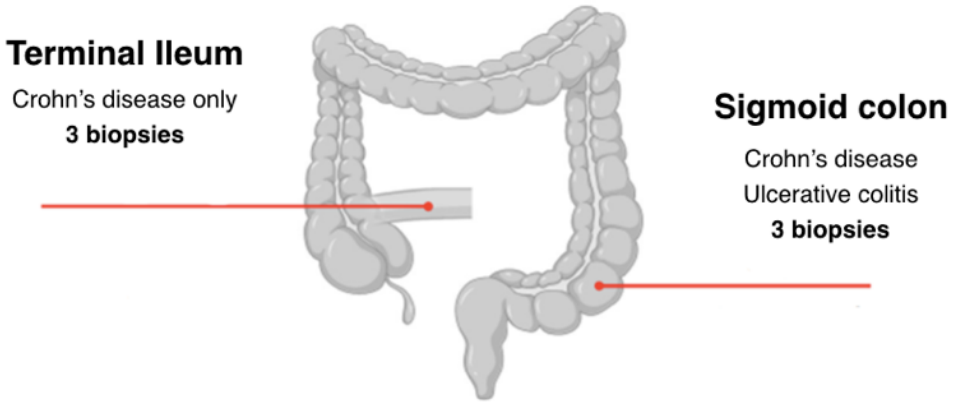


Figure 3. Site of biopsies for patients with CD and UC.

Isolation of intestinal epithelial cells

IEC were highly purified from other cell types by using a well-established protocol.(58) This protocol involved a first enzymatic digestion of the tissue samples and a filtration and separation of epithelial cells by the use of magnetic columns (MACS® Columns).

In detail, intestinal biopsies were first rapidly thawed at 37°C, using the thermoblock and then processed. All biopsies were initially washed twice with Hank's Balanced Salt solution (Sigma-Aldrich) to remove mucus, and then incubated with two enzymes, Liberase (Roche) and Ialuronidase (Merck), at 37°C on a shaker at 750 rpm for 45 minutes. After digestion, the cell suspension were passed through a 40 μm filter, centrifuged at 500 xg for 7 minutes at 10 ºC and then the pellet was resuspended with 300 μl of MACS Running Buffer (Miltenyi Biotec) and 20 μl of DNAse (Roche). Then 100 μl of FcR Blocking Reagent to block Fc receptor and 100 μl of anti- Epithelial Cell Adhesion Molecule (anti-CD326/anti-EpCAM) magnetic microbeads (Miltenyi Biotec) to bind the epithelial cells expressing the adhesion molecule CD326 on the surface were added. The suspension was incubated at 4ºC for 30 minutes. After incubation, MACS Running Buffer was added up to a volume of 7 ml and the suspension was centrifuged at 300 xg for 10 minutes at 10°C. The pellet was resuspended with 600 μl of MACS Running Buffer and then the separation of epithelial cells (EpCAM+) was performed by using the magnetic separation system (autoMACS, Miltenyi Biotec). The column was washed three times with MACS Running Buffer, in order to elute all non-epithelial cells (EpCAM-) and collect the epithelial cells (EpCAM +).

The epithelial cells fraction (EpCAM +) was centrifuged at 500 xg for 7 minutes at 10°C and resuspended with 350 μl of RLT Lysis Buffer (Qiagen) modified with β-Mercaptoethanol 10 μl/ml (Sigma). The suspension was then passed through homogenizer columns (QIAshredder Spin Column) and centrifuged at maximum speed for 2 minutes at room temperature. Finally, the lysate was stored at -80°C until DNA extraction.

DNA extraction and quantification

The DNA was extracted form epithelial cells purified by intestinal biopsies.

The AllPrep DNA/RNA Mini kit (Qiagen) was used to extract DNA from intestinal epithelial cells. The cell lysate was initially thawed and then several centrifugations were performed using the AllPrepDNA columns. The first centrifugation at 11,000 xg for 30 seconds was needed to hold the DNA on the column and elute the RNA. After moving the column on a new 2 ml tube, DNA was washed with two buffers followed by centrifugations. Finally, 50μL of elution buffer were added. After a brief-incubation at room temperature the column was centrifuged for 1 minute at 11,000 xg. The extracted DNA was stored at -20°C until further analysis.

DNA of intestinal epithelial cells was quickly quantified with NanoDrop ND-1000 Spectrophotometer which provides DNA quantification by measuring absorbance values at 260 nm and also samples purity by measuring the ratios A260/280 and A260/230.

Methylation Analysis

Whole genome DNA methylation was studied in IEC. 500 ng to 1 μg of DNA of each sample was bisulfite-converted using EZ DNA Methylation™ Kit (Zymo Research). Bisulfite conversion was based on three steps: DNA denaturation, conversion, desulphonation. The protocol provided for the addition of 5 μl of M-Dilution Buffer to the DNA sample and water up to the volume of 50 μl and then the incubation at 37°C for 15 minutes. The sample with the addition of 100 μl of CT Conversion Reagent was incubated in a thermocycler at 95°C for 30 seconds and at 50°C for 60 minutes for 16 cycles. The day after the sample was loaded on the column (Zymo-Spin™ IC Column) containing the M-Binding Buffer and centrifuged at full speed. After a first wash using the M-Wash Buffer, the desulphonation phase was carried out adding to the column the M-Desulphonation Buffer. Finally, the

elution of the DNA was performed adding 10 μl of M-Elution Buffer and centrifugation at full speed for 30 seconds.

Each DNA sample bisulfite-converted was screened using Illumina HumanMethylationEPIC BeadChips. First, the DNA was denatured and then isothermally amplified in an overnight step. The amplified product was fragmented in an enzymatic process and then precipitated with isopropanol. The precipitated DNA was resuspended on the BeadChips and then incubated to allow the hybridization on the BeadChips. After washing, the captured DNA on the BeadChip was used as a template for the single-base extension that incorporates detectable nucleotides. At the end, the Illumina HiScan or iScan System was used to scan the BeadChip and determines the methylation level of the CpG sites. Signal intensity of each CpG was expressed as beta value, a measure of methylation degree at each locus (from 0=unmethylated to 1=fully methylated).

Bioinformatics and statistical analysis

Raw methylation data were analysed using RStudio with R version 4.2.1 (2022-06-23) and the package ChAMP (Chip Analysis Methylation Pipeline). The champ.load function was used to load and filter the dataset. Probes were filtered based on p-value detection, chromosomal location, presence of single nucleotide polymorphisms (SNP) in the probe sequence and multiple hybridization. The quality control of the dataset verified if the dataset can be used for downstream analysis and it was performed using the champ.QC function. On Illumina BeadChips, probes (called type-I and type-II) have different distributions due to their different biological characteristics and their hybridization chemistry. For this reasons, normalization was performed using champ.norm function. After normalization, champ.SVD function was used to perform a Singular Value Decomposition analysis, a powerful tool for assessing the number and the nature of the

significant components of variation in a dataset. The effect of these variables (e.g beadchip of origin of the samples) was removed using the ComBat function.

First, Principal Component Analysis (PCA) was performed on the methylation β-values of the 1,000 most variable probes on the correct dataset by ggplot2 function. PCA is a descriptive statistical technique used to analyse and reduce the size of large datasets, increasing interpretability but at the same time minimizing information loss.

Differentially methylated regions (DMRs) were then calculated using champ.DMR function and the Bumphunter algorithm. The result of Bumphunter algorithm is a data frame containing all detected DMRs, with their corresponding CpGs and genes. A plot for each DMR was also generated by this algorithm. DMRs were extended segments of the genome that show a quantitative alteration in DNA methylation levels between two groups. The DMRs were considered statistically significant when the p-value is inferior to 0.05.

RESULTS

Patients' Baseline Characteristics

At the time of data retrieval, 55 patients were enrolled in the EPIC study. Five patients dropped out, 31 patients completed the study, and 19 are still ongoing.

IEC were availability from 25 patients, although not all had IEC collected at both T0 and at T1

Patients' characteristics at baseline are reported in Table 1.

The distribution of IEC availability by disease phenotype, biopsy site, and time point is shown in Table 2.

	Crohn's disease	Ulcerative colitis	Total
Number of patients	16	9	25
Male **Female**	9 7	5 4	14 11
PCDAI **PUCAI**	21.3 (19.4-34.4)	33.7 (20-47.5)	21.3 (19.4-34.4) 33.7 (20-47.5)
Disease localisation **L1** **L3** **E2** **E3** **E4**	 2 14	 2 1 6	

Table 1. Patients' Characteristics

PCDAI: Pediatric Crohn's Disease Activity Index; PUCAI: Pediatric Ulcerative Colitis Activity Index; L1: terminal ileum; L3: ileo-colonic; E2: distal colitis, E3: extensive colitis, E4: pancolitis

	T0 and T1	only T0	only T1	Total
Crohn **- ileum** **- sigmoid colon**	 6 7	 0 2	 5 6	 11 15
Ulcerative colitis	2	4	3	9

Table 2. IEC availability according to disease phenotype, site and time of biopsy.

T0: baseline endoscopy; T1: 52-week endoscopy.

Among the 25 patients with IEC available, 12 (4 UC, 8 CD) were randomized to the epTDM group and 13 (5 UC, 8 CD) in the SOC group.

At the end of the study, only one patient with CD had mild clinical activity, while the rest were in clinical remission. The median number of infliximab infusions was 11 (IQR 9-13) and the median pro/kg dose was 5.1 mg/Kg (IQR 4.9-8.4 mg/kg).

Colonic IEC in patients with UC compared to patients with CD

Comparing colon IEC from patients with CD with those from patients with UC, several genes resulted differentially methylated at T0.

Figure 4 shows the findings of the PCA.

The hypermethylated genes in patients with CD in comparison with patients with UC included: THBS1, LOC100130872, LOC100130872-SPON2, CRYZ, TYW3, NUDT12, SERPINA5, CIITA.

The hypomethylated genes in patients with CD in comparison with patients with UC included: MIR886, HOXB8, GATA2, HLA-DPB2, HSF4, FBXL8, UNC45A, HOXA4, HOXB4.

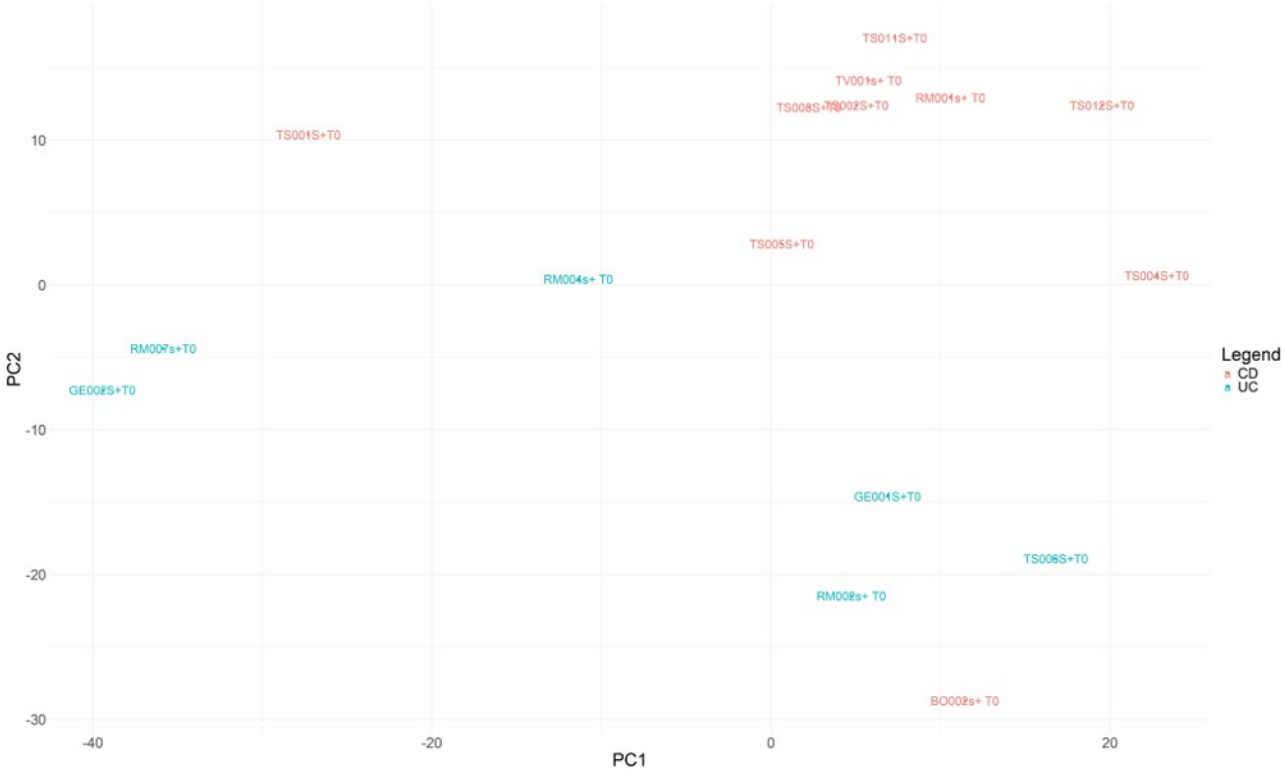


Figure 4. PCA of whole genome methylation data of DNA from IEC of patients with UC and patients with CD shows the different clusterization between the two groups.

Colonic IEC in patients with UC at T0 compared to T1

Thirty DMRs were found in colonic IEC of patients with UC comparing T0 with T1. These DMRs include twenty-eight differentially methylated genes.

Figure 5 shows the findings of the PCA.

Twenty-two genes were found hypomethylated at T0 in comparison with T1, including: THBS1, SUB1, HOXB8, IL20RA, CCDC88B, DNAH26, CD81, ACOT7, CIITA, RAB17, KAZALD1, ZBTB22, LTBP1, TAP1, RNASE1, TM4SF19, DUSP6, STAU2-AS1, STAU2, SOD3, RUFY1.

NDEx Integrated Query analysis showed that 3 hypomethylated genes at T0 in comparison with T1 (THBS1, TAPBP, TAP1) are involved in antigen processing and presentation pathway. However, this pathway is not statistically significant (p value= 0,077).

Six genes were found hypermethylated at T0 in comparison with T1, including: ATP10A, ZNF382, ZNF529, REC8, LOC14566, ZNF154. These genes are not associated with any common pathway.

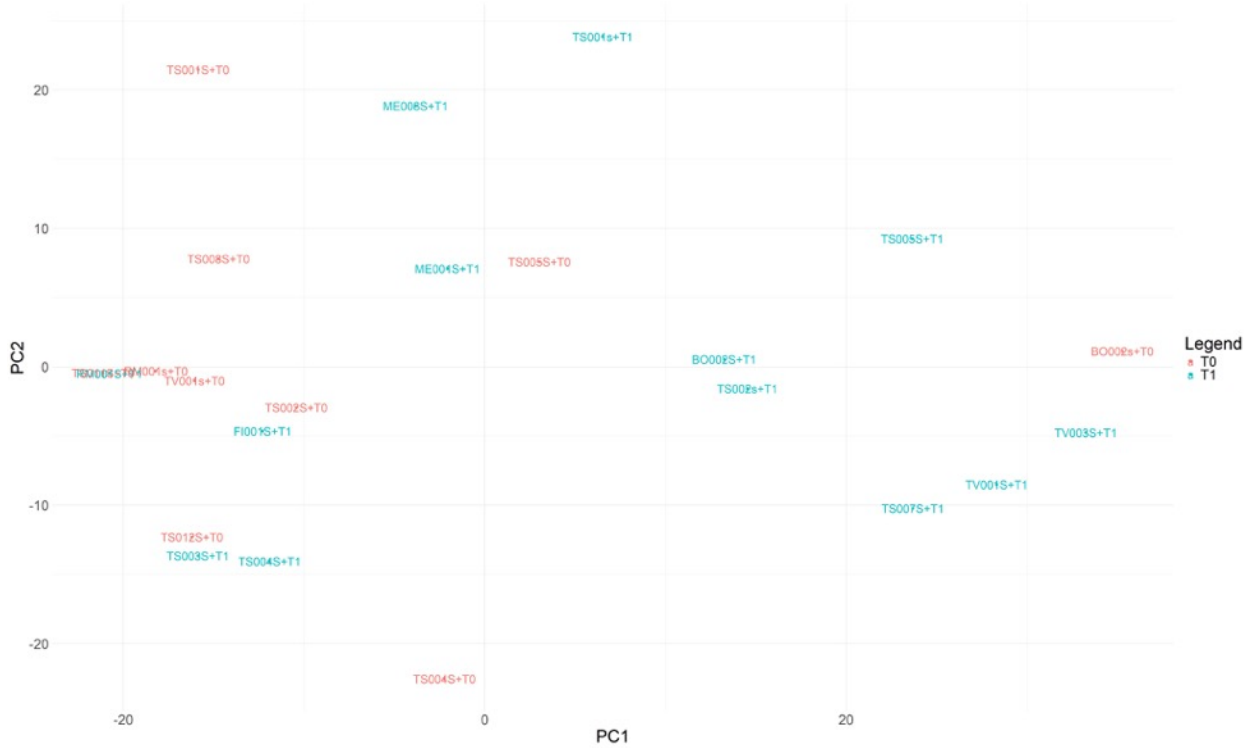


Figure 5. PCA of whole genome methylation data of DNA from IEC of patients with UC at T0 and T1.

Colonic IEC in patients with CD at T0 compared to T1

Forty-two DMRs were found in colonic IEC of patients with CD comparing T0 with T1. These DMRs include forty-six differentially methylated genes.

Figure 6 shows the findings of the PCA.

Seventeen genes were found hypomethylated at T0 in comparison with T1, including: MIR886, GATA2, HOXD12,MEIS1,EVX2,RARB,RNF39,RPL14,HOXD10,INTS5, EVX1, SRPRB, FGFR2, OSGIN2, HOXB4, HOXB8, HOXD4..

Twenty-nine genes were found hypermethylated at T0 in comparison with T1, including:PRAC,PRAC1,HOXA2,GRIK2 ,LOC100130872, LOC100130872-SPON2, HOXA5, CRYZ, TYW3, SFRP2, KRBOX1-AS1,KCNAB2,C22orf45,UPB1,LOC339524,LINC01140, NUDT12, GLIPR1L2, UNC5C, NT5E, TXNRD1, MIR548F5, MAB21L1, LRRC14B, DEF6, USP4, C3orf62, C17orf93, PRAC2. . No statistically significant pathways associated to these genes were found.

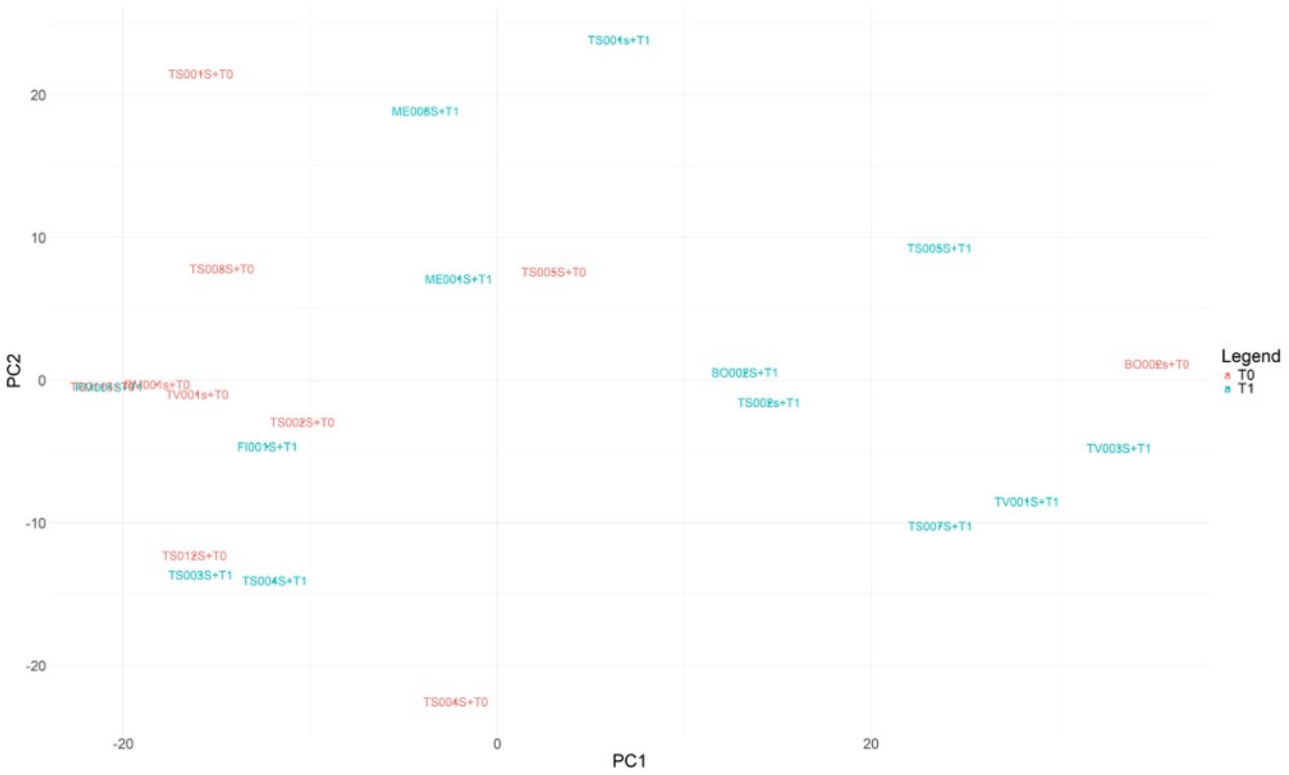


Figure 6. PCA of whole genome methylation data of DNA from colonic IEC of patients with CD at T0 and T1.

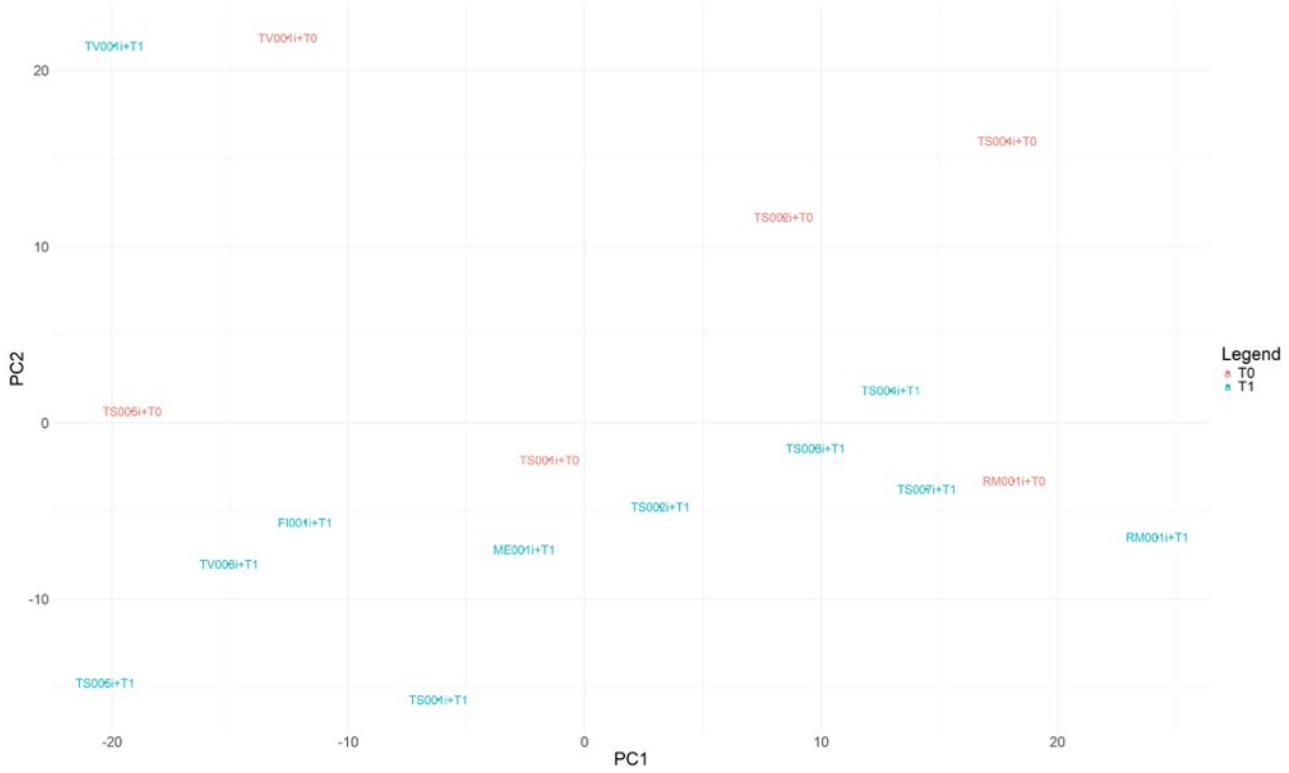
Ileal IEC in patients with CD at T0 compared to T1

One-thousand-three-hundreds-nineteen DMRs were found in ileal IEC of patients with CD comparing T0 with T1. These DMRs include one-thousand-seven-hundreds-sixty differentially methylated genes.

Figure 7 shows the findings of the PCA.

One-thousand-seven hundreds- fifty-six genes were found hypermethylated at T0 in comparison with T1. The 100 gene with most staistically significant differences in methylation include: BAT3, FLOT1, IER3, BLCAP, FLJ45983, GATA3, PTEN, ATM, NPAT, STK19, DOM3Z, MRPS18B, DAXX, TRIM26, AKAP12, LYPLAL1, MCCC1, ATP5J, GABPA, MIR191, DALRD3, NDUFAF3, MIR425, NUDT6, SPATA5, SLC39A7, RXRB, TLL1, RPAIN, NUP88, ZNRD1, NCRNA00171, SNORD48, C6orf48, NAALAD2, BAT2, WDR46, PFDN6, MIR6834, RGL2, UGP2, PRKCSH, CCDC151, MSH5, HOXA11-AS, HOXA11, HOXA11AS, TMEM87A, GANC, UTP15, ANKRA2, ISM1, ZFP36L2, LOC100129726, LINC01126, KIAA0586, TIMM9, KIAA1712, FBXO8, CEP44, C3orf58, WDPCP, C2orf86, MDH1, BDNF, DDAH2, BRUNOL4, FAM174A, DHX16, HNRNPH1, TMEM106A, NBR1, DARS2, CENPL, DNAJC28, AARS, DDX19B, RG9MTD2, TRMT10A, MTTP, MDC1, TAF9, RAD17, KIF9, ALKBH5, RAB2B, TOX4, TROVE2, UCHL5, PEX13, PUS10, KCTD9, CDCA2, KIF20A, BRD8, TTI2, C8orf41, NGLY1, OXSM, TBX2,

The complete list of the genes hypermethylated at T0 in comparison with T1 is reported in appendix 1.



Many biological pathway were associated to these including most of these genes NADH dehydrogenase complex assembly, mitochondrial respiratory chain complex I assembly, granulocyte migration, humoral immune response mediated by circulating immunoglobulin, mitochondrial respiratory chain complex assembly, complement activation, B cell receptor signaling pathway, phagocytosis, engulfment, cell killing, and T cell receptor signaling pathway.

Four genes were found hypomethylated at T0 in comparison with T1, including: NOS1AP, TMEM232, MIR445HG, HOXA2. No biological pathway associated to these genes was found.

Figure 7. PCA of whole genome methylation data of DNA from ileal IEC of patients with CD at T0 and T1.

DISCUSSION

This study reports the interim analysis of IEC methylation profiles in children with IBD treated with infliximab.

To date, few studies have investigated the different methylation patterns in immune cells or intestinal epithelial cells (IECs) in children with IBD in relation to specific treatments. (61,62) We chose to focus on IECs because they serve as the primary barrier between the gut lumen and underlying tissues. Disruptions in IEC integrity can lead to increased intestinal permeability, microbial translocation, and the subsequent activation of inflammatory responses. Thus, methylation changes in IECs can directly affect their barrier function, modulate inflammatory processes, and influence tissue repair mechanisms.

Although our data are very preliminary, it is possible to make some considerations.

The first key finding is that IEC extracted from sigmoid colon biopsies of CD and UC patients exhibit distinct methylation profiles.

This contrasts with some previous studies that instead reported many similarities between the methylation profiles of colonic IEC from UC or CD patients. McDermott et al. reported that 1443 (97%) of UC-associated DMPs were also differentially methylated in CD, while only 38 UC DMPs (3%) were unique to UC. In contrast, 1753 (55%) CD DMPs were unique to CD. The $\Delta\beta$ values for CD-associated DMPs were strongly correlated ($r = 0.99$, $p < 0.0001$) with UC-associated differences at the same probes compared to healthy controls.(63)

Similarly, Howell et al. showed that CD-specific DNA methylation and gene-expression changes were present in ileal IEC compared to UC patients and controls, whereas molecular changes in the colonic epithelium showed substantial overlap between CD and UC, reflecting a common IBD signature.(61)

At present, we cannot explain why our results differ from those of these two studies, although the discrepancy may be attributed to the relatively small sample size in our study.

The second finding is that differences in methylation patterns were detected before and after infliximab treatment.

A study on whole blood samples from patients with CD treated with infliximab showed widespread differences in DNA methylation induced by anti-TNF drug treatment and show that baseline DNA methylation profiles can predict anti-TNF drug concentration at week 14. Between baseline and week 14, authors identified 4,999 differentially methylated probes (DMPs), annotated to 2,376 genes, following anti-TNF treatment. Pathway analysis revealed 108 significant gene ontology terms, many related to immune system processes and responses. Epigenome-wide association (EWAS) analysis identified 323 DMPs annotated to 210 genes at baseline associated with higher anti-TNF drug concentrations at week 14. Notably, 125 DMPs showed shared associations with other common traits, including body mass index (23.2%), CRP (11.5%), smoking (7.4%), alcohol consumption (7.1%), and IBD type (6.8%). An EWAS of primary non-response to anti-TNF identified 20 DMPs associated with both anti-TNF drug concentration and primary non-response, with a strong correlation (Spearman's rho = -0.94, $p < 0.001$).(62)

Moreover, in a blood-based multi-omics study in two prospective IBD patient cohorts, 85,728 and 58,347 DMPs were identified in remitters and non-remitters, respectively. In patients achieving remission at week 14, a preponderance of hypermethylated DMPs was observed, constituting around 70% (30,132) at 2 weeks and 60% (43,478) of the DMPs at 6 weeks. Cellular deconvolution analysis showed that major parts of these DNAm signatures originated from granulocytes, B cells, CD4+ T cells, and monocytes. In total, 357 differentially methylated regions (DMRs) were found in remitters, while 1,163 DMRs were observed in non-remitters. The majority of these DMRs were located in enhancer regions (348 in remitters and 1,147 in non-remitters). These regions overlapped with binding sites for several transcription factors, including IRF4, BATF, MEF2C, and MEF2A for hypermethylated regions, and CEBPD and STAT3 for hypomethylated regions.(64)

In a differnet study conducted on rectal IEC from patients with UC, many DMPs and DMRs in non-remitting patients were transiently observed only at week 2, while many DMPs in remitting patients were stably regulated at both week 2 and week 6, suggesting that cell type-specific epigenetic changes occurring in UC correlate with disease severity and outcomes.(65)

Further evidence also comes from studies on other inflammatory conditions. In patients with rheumatoid arthirtis (RA), treatment with anti-TNF was associated with significant longitudinal peripheral blood methylation changes in biological RA-related pathways. Over one hundred biological functions were modified by therapy, with methylation levels changing systematically towards a signature similar to that of healthy controls. Differences in the methylation profile of T cell activation and differentiation, GTPase-mediated signaling, and actin filament organization pathways were associated with the clinical response. Cell type deconvolution analysis identified CpG sites in CD4 +T, NK, neutrophils and monocytes that were significantly associated with the response to TNFi.(66)

So far, we have not been able to conduct a comparative analysis between those who responded to therapy and those who did not, as all patients, except for one, were in clinical remission at the time of the second evaluation. Furthermore, we have not yet assessed whether there are differences between the patients included in the two arms of the EPIC study, nor have we been able to determine whether therapy modulation might influence the methylation profiles.

Looking at the possible pathways related to the genes that have been identified as differentially methylated after the treatment with IFX, those that have been suggested involved the immune system and signal transduction.

These pathways have already been implicated in either IBD pathogenesis or IEC function. (61) However, given the complexity of molecular mechanisms, it is not yet possible to identify specific pathways in greater detail.

This study has several limitations. First, as it is an interim evaluation, the data are partial and incomplete. The sample size is very small, limiting the power to detect significant differences between the groups and the possibility to conduct subanalysis. Additionally, the availability of biopsies and IEC for each patient is not homogeneous and for some patients biopsies were not collected at both T0 and T1 because the endoscopy was not performed. As a result, the analysis was based on a comparison of available samples at T0 and T1, rather than individual pre- and post-treatment evaluations.

Thawing biopsies as opposed to using fresh tissue straight after sampling from endoscopy could have affected the purification of IEC. However, thawing was necessary to centralize the samples and preliminar tests showed similar results on IECs from fresh and thawed biopsies.

The specific regions of genes that are differentially methylated, and their significance in terms of gene silencing or activation, have not been explored. Gene expression studies have also not been conducted, meaning we do not yet know whether the observed differences in methylation correlate with changes in protein expression or IEC function. Finally, without a control group, we cannot determine whether the differences observed between T0 and T1 are specific to infliximab therapy or are simply due to changes in inflammation levels.

FUTURE PERSPECTIVES

The study will continue over the next few months, with a primary focus on collecting new samples for IEC isolation from additional patients. Efforts will be made to optimize sample collection by ensuring that both T0 and T1 samples are obtained from the same patient. Furthermore, the analysis will continue to explore the biological implications of the observed methylation differences and assess the potential for using these findings in prognostic or therapeutic applications.

A separate cohort of patients will be enrolled to validate the findings.

Finally, evaluating the methylation profiles of IECs from patients treated with other therapies (e.g., nutritional therapies, immunomodulators, or non-anti-TNF biologics) and comparing them with the findings of the present study could help clarify whether the differences in methylation profiles are primarily associated with infliximab treatment or the disease status (inflammation/remission).

CONCLUSIONS

The preliminary results of this study suggest that IEC methylation profiles are altered by IFX therapy. Continuing the study may help confirm these initial findings and provide more detailed insights into the role of methylation in treatment response, as well as clarify the molecular mechanisms underlying treatment efficacy and failure.

REFERENCES

1. Vernier-Massouille G, Balde M, Salleron J, et al. Natural history of pediatric Crohn's disease: a population-based cohort study. Gastroenterology. 2008;135:1106–1113.

2. Kuenzig ME, Fung SG, Marderfeld L, et al. Twenty-first Century Trends in the Global Epidemiology of Pediatric-Onset Inflammatory Bowel Disease: Systematic Review. Gastroenterology. 2022;162:1147-1159.e4.

3. Ng SC, Shi HY, Hamidi N, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet. 2017;390:2769-2778.

4. Khan R, Kuenzig ME, Benchimol EI. Epidemiology of Pediatric Inflammatory Bowel Disease. Gastroenterol Clin North Am. 2023;52:483-496.

5. Roberts SE, Thorne K, Thapar N, et al. A Systematic Review and Meta-analysis of Paediatric Inflammatory Bowel Disease Incidence and Prevalence Across Europe. J Crohns Colitis. 2020;14:1119-1148.

6. Alvisi P, Labriola F, Scarallo L, et al. Epidemiological trends of pediatric IBD in Italy: A 10-year analysis of the Italian society of pediatric gastroenterology, hepatology and nutrition registry. Dig Liver Dis. 2022 Apr;54(4):469-476.

7. Castro M, Papadatou B, Baldassare M, et al. Inflammatory bowel disease in children and adolescents in Italy: data from the pediatric national IBD register (1996-2003). Inflamm Bowel Dis. 2008;14:1246-52.

8. de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. Nat Rev Gastroenterol Hepatol. 2016;13:13-27.

9. Jostins L, Ripke S, Weersma RK, et al. Host–microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119–24.

10. Liu JZ, van Sommeren S, Huang H, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet 2015;47:979–86.

11. de Lange KM, Moutsianas L, Lee JC, et al. Genomewide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. Nat Genet. 2017;49:256–61.

12. Uhlig HH, Schwerd T, Koletzko S, et al. The diagnostic approach to monogenic very early onset inflammatory bowel disease. Vol. 147, Gastroenterology. 2014. p. 990–1007.

13. Carreras-Torres R, Ibáñez-Sanz G, Obón-Santacana M, et al. Identifying environmental risk factors for inflammatory bowel diseases: a Mendelian randomization study. Sci Rep. 2020;10:19273.

14. Halfvarson J, Brislawn CJ, Lamendella R, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol. 2017;2:17004.

15. Jenke AC, Zilbauer M. Epigenetics in inflammatory bowel disease. Curr Opin Gastroenterol 2012; 28:577–84.

16. Zilbauer M, Zellos A, Heuschkel R. Epigenetics in paediatric gastroenterology, hepatology, and nutrition: present trends and future perspectives. J Pediatr Gastroenterol Nutr 2016;6 2:521–9.

17. Natasha G, Zilbauer M. Epigenetics in IBD: a conceptual framework for disease pathogenesis. Frontline Gastroenterol. 2022;13(e1):e22-e27.

18. Hornschuh M, Wirthgen E, Wolfien M, et al. The role of epigenetic modifications for the pathogenesis of Crohn's disease. Clin Epigenetics 2021;13:108.

19. Kraiczy, J., Nayak, K., Ross, A. et al. Assessing DNA methylation in the developing human intestinal epithelium: potential link to inflammatory bowel disease. Mucosal Immunol 2016, 9, 647–658.

20. Gloria L, Cravo M, Pinto A, et al. DNA hypomethylation and proliferative activity are increased in the rectal mucosa of patients with long-standing ulcerative colitis. Cancer 1996;78:2300-2306.

21. Hsieh CJ, Klump B, Holzmann K, et al. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. Cancer Res 1998;58:3942-3945.

22. Saito S, Kato J, Hiraoka S, et al. DNA methylation of colon mucosa in ulcerative colitis patients: correlation with inflammatory status.Inflamm Bowel Dis 2011;17:1955-1965

23. Azarschab P, Porschen R, Gregor M, et al. Epigenetic control of the E-cadherin gene (CDH1) by CpG methylation in colectomy samples of patients with ulcerative colitis. Genes Chromosomes Cancer 2002; 35:121-126.

24. Issa JP, Ahuja N, Toyota M, et al. Accelerated age-related CpG island methylation in ulcerative colitis. Cancer Res 2001;61:3573- 3577

25. Annese V, Valvano MR, Palmieri O, et al. Multidrug resistance 1 gene in inflammatory bowel disease: a meta-analysis. World J Gastroenterol. 2006;12:3636-44.

26. Tahara T, Shibata T, Nakamura M, et al. Effect of MDR1 gene promoter methylation in patients with ulcerative colitis. Int J Mol Med 2009;23:521-527.

27. Tahara T, Shibata T, Nakamura M, et al. Promoter methylation of protease-activated receptor (PAR2) is associated with severe clinical phenotypes of ulcerative colitis (UC). Clin Exp Med 2009;9:125-130

28. Kuester D, Guenther T, Biesold S, et al. Aberrant methylation of DAPK in long-standing ulcerative colitis and ulcerative colitisassociated carcinoma. Pathol Res Pract 2010;206:616-624.

29. Lin Z, Hegarty JP, Cappel JA, et al. Identification of diseaseassociated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. Clin Genet 2011;80:59-67.

30. Cooke J, Zhang H, Greger L, et al. Mucosal genome-wide methylation changes in inflammatory bowel disease. Inflamm Bowel Dis 2012;18:2128-2137.

31. Karatzas PS, Gazouli M, Safioleas M, Mantzaris GJ. DNA methylation changes in inflammatory bowel disease. Ann Gastroenterol. 2014;27:125-132

32. Torres J, Mehandru S, Colombel JF, et al. Crohn's disease.Lancet.2017;389:1741-1755.

33. Ungaro R, Mehandru S, Allen PB, et al. Ulcerative colitis. Lancet. 2017;389:1756-1770.

34. Turner D, Ricciuto A, Lewis A, et al. STRIDE-II: An Update on the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) Initiative of the International Organization for the Study of IBD (IOIBD): Determining Therapeutic Goals for Treat-to-Target strategies in IBD. Gastroenterology. 2021;160:1570-1583.

35.Van Rheenen PF, Aloi M, Assa A, et al The Medical Management of Paediatric Crohn's Disease: an ECCO-ESPGHAN Guideline Update. J Crohns Colitis. 2021, 15:171–194

36. Levine A, Wine E, Assa A, et al. Crohn's Disease Exclusion Diet Plus Partial Enteral Nutrition Induces Sustained Remission in a Randomized Controlled Trial. Gastroenterology. 2019 Aug;157(2):440-450.e8.

37. Turner D, Ruemmele FM, Orlanski-Meyer E, et al. Management of Paediatric Ulcerative Colitis, Part 2: Acute Severe Colitis-An Evidence-based Consensus Guideline From the European Crohn's and Colitis Organization and the European Society of Paediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr. 2018;67:292-310.

38. Malaty HM, Abraham BP, Mehta S, et al. The natural history of ulcerative colitis in a pediatric population: a follow-up population-based cohort study. Clinical and Experimental Gastroenterology 2013:6 77– 83

39. Turunen P, AshornM, Auvinen A, et al. Long-term health outcomes in pediatric inflammatory bowel disease: a population-based study. Inflamm Bowel Dis. 2009;15:56-62.

40. Hyams J, Crandall W, Kugathasan S, et al; REACH Study Group. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. Gastroenterology. 2007;132:863–873

41. Hanauer SB, Feagan BG, Lichtenstein GR, et al; ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet. 2002;359:1541–1549

42. Ben-Horin S, Chowers Y. Review article: loss of response to anti-TNF treatments in Crohn's disease. Aliment Pharmacol Ther 2011; 33: 987–95.

43. O'Meara S, Nanda KS, Moss AC. Antibodies to infliximab and risk of infusion reactions in patients with inflammatory bowel disease: a systematic review and meta-analysis. Inflamm Bowel Dis. 2014;20:1–6.

44. Sprakes MB, Ford AC, Warren L, Greer D, Hamlin J. Efficacy, tolerability, and predictors of response to infliximab therapy for Crohn's disease: a large single centre experience. J Crohns Colitis 2012; 6: 143–53.

45. Kennedy NA, Heap GA, Green HD, et al. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study. Lancet Gastroenterol Hepatol. 2019;4:341-353.

46. Singh N, Rosenthal CJ, Melmed GY, et al. Early infliximab trough levels are associated with persistent remission in pediatric patients with inflammatory bowel disease. Inflamm Bowel Dis. 2014;20:1708–1713.

47. Lega S, Bramuzzo M, Dubinsky MC. Therapeutic Drug Monitoring in Pediatric IBD: Current Application and Future Perspectives. Curr Med Chem. 2018;25:2840-2854.

48. Lee LY, Sanderson JD, Irving PM. Anti-infliximab antibodies in inflammatory bowel disease: prevalence, infusion reactions, immunosuppression and response, a meta-analysis. Eur J Gastroenterol Hepatol. 2012;24:1078–1085

49. Winter DA, Joosse ME, de Wildt SN, et al. Pharmacokinetics, Pharmacodynamics, and Immunogenicity of Infliximab in Pediatric Inflammatory Bowel Disease: A Systematic Review and Revised Dosing Considerations. J Pediatr Gastroenterol Nutr. 2020;70:763-776.

50. Vermeire S, Noman M, Van Assche G, et al. Effectiveness of concomitant immunosuppressive therapy in suppressing the formation of antibodies to infliximab in Crohn's disease. Gut. 2007;56:1226–1231.

51. Colombel JF, Sandborn WJ, Reinisch W, et al; SONIC Study Group. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med. 2010;362:1383–1395.

52. Feagan BG, McDonald JW, Panaccione R, et al. Methotrexate in combination with infliximab is no more effective than infliximab alone in patients with Crohn's disease. Gastroenterology. 2014;146:681–688.e1

53. Steenholdt C, Brynskov J, Thomsen OØ, et al. Individualised therapy is more cost-effective than dose intensification in patients with Crohn's disease who lose response to anti-TNF treatment: a randomised, controlled trial. Gut. 2014;63:919-27.

54. Vaughn BP, Martinez-Vazquez M, Patwardhan VR, et al. Proactive therapeutic concentration monitoring of infliximab may improve outcomes for patients with inflammatory bowel disease: results from a pilot observational study. Inflamm Bowel Dis. 2014;20:1996–2003.

55. Vande Casteele N, Ferrante M, Assche G, et al. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. Gastroenterology. 2015;148:1320–1329.e3.

56. Vaughn BP, Sandborn WJ, Cheifetz AS. Biologic concentration testing in inflammatory bowel disease. Inflamm Bowel Dis. 2015;21:1435–1442.

57. Papamichael K, Chachu KA, Vajravelu R, et al. Improved long-term outcomes of patients with inflammatory bowel disease receiving proactive compared with reactive monitoring of serum concentrations of infliximab. Clin Gastroenterol Hepatol. 2017;15:1580–1588.

58. Lega S, Phan BL, Rosenthal CJ, et al. Proactively Optimized Infliximab Monotherapy Is as Effective as Combination Therapy in IBD. Inflamm Bowel Dis. 2019;25:134-141.

59. Clarkston K, Tsai YT, Jackson K, et al. Development of Infliximab Target Concentrations During Induction in Pediatric Crohn Disease Patients. J Pediatr Gastroenterol Nutr. 2019;69:68-74.

60. Lee RB, Gasparetto M. Novel pharmacological developments in the management of paediatric inflammatory bowel disease: Time for guideline update - A narrative review. J Paediatr Child Health. 2024;60:168-175

61. Howell KJ, et al. DNA Methylation and Transcription Patterns in Intestinal Epithelial Cells From Pediatric Patients With Inflammatory Bowel Diseases Differentiate Disease Subtypes and Associate With Outcome. Gastroenterology. 2018;154:585-598.

62. Lin S, Hannon E, Reppell M, et al. Whole Blood DNA Methylation Changes Are Associated with Anti-TNF Drug Concentration in Patients with Crohn's Disease. J Crohns Colitis. 2024;18:1190-1201.

63. McDermott E, Ryan EJ, Tosetto M, et al. DNA Methylation Profiling in Inflammatory Bowel Disease Provides New Insights into Disease Pathogenesis. J Crohns Colitis. 2016;10:77-86.

64. Mishra N, Aden K, Blase JI, et al. Longitudinal multi-omics analysis identifies early blood-based predictors of anti-TNF therapy response in inflammatory bowel disease. Genome Med. 2022;14:110.

65. Venkateswaran S, Somineni HK, Matthews JD, et al. Longitudinal DNA methylation profiling of the rectal mucosa identifies cell-specific signatures of disease status, severity and clinical outcomes in ulcerative colitis cell-specific DNA methylation signatures of UC. Clin Epigenetics. 2023;15:50.

66. Julià A, Gómez A, López-Lasanta M, et al. Longitudinal analysis of blood DNA methylation identifies mechanisms of response to tumor necrosis factor inhibitor therapy in rheumatoid arthritis. EBioMedicine. 2022;80:104053.

APPENDIX 1.

List of hypermethylated genes of Ileal IEC in patients with CD at T0 compared to T1**.**

BAT3, FLOT1, IER3, BLCAP, FLJ45983, GATA3, PTEN, ATM, NPAT, STK19, DOM3Z, MRPS18B, DAXX, TRIM26, AKAP12, LYPLAL1, MCCC1, ATP5J, GABPA, MIR191, DALRD3, NDUFAF3, MIR425, NUDT6, SPATA5, SLC39A7, RXRB, TLL1, RPAIN, NUP88, ZNRD1, NCRNA00171, SNORD48, C6orf48, NAALAD2, BAT2, WDR46, PFDN6, MIR6834, RGL2, UGP2, PRKCSH, CCDC151, MSH5, HOXA11-AS, HOXA11, HOXA11AS, TMEM87A, GANC, UTP15, ANKRA2, ISM1, ZFP36L2, LOC100129726, LINC01126, KIAA0586, TIMM9, KIAA1712, FBXO8, CEP44, C3orf58, WDPCP, C2orf86, MDH1, BDNF, DDAH2, BRUNOL4, FAM174A, DHX16, HNRNPH1, TMEM106A, NBR1, DARS2, CENPL, DNAJC28, AARS, DDX19B, RG9MTD2, TRMT10A, MTTP, MDC1, TAF9, RAD17, KIF9, ALKBH5, RAB2B, TOX4, TROVE2, UCHL5, PEX13, PUS10, KCTD9, CDCA2, KIF20A, BRD8, TTI2, C8orf41, NGLY1, OXSM, TBX2, WDR93, PEX11A, NNT, IFT122, RPS14, SLC25A46, ZNF566, LOC728752, EHMT2, SMC4, IFT80, EEF1A1, RPS27A, C2orf63, CLHC1, PPP1R7, PASK, GNB2L1, SNORD95, TRIM23, TRAPPC13, C5orf44, POLR1A, PTCD3, PFKM, SENP1, PTRH2, TMEM49, SFRS18, PNISR, ARL6IP1, LOC646851, FAM227A, CBY1, NAA20, LRIG3, MRPL46, MRPS11, PPPDE2, XRCC6, DESI1, AGPAT9, LMO3, SGOL1, MFSD8, C4orf29, CLSTN3, RBP5, SCAND3, DNAJC7, NKIRAS2, PHLDA2, SLC16A7, MOBKL3, MOB4, MOCS2, LOC257396, PPME1, C2CD3, OGFOD2, ABCB9, ZNF668, ZNF646, PLD6, C1orf66, ISG20L2, RRNAD1, CYP1A1, C6orf182, SESN1, CEP57L1, FAM134C, TUBG1, LUC7L3, TOPBP1, C20orf7, NDUFAF5, ESF1, FLJ31306, ARID4A, FAU, MRPL49, GATA5, VPS52, RPS18, CHFR, GSK3A, PPP3CB, PPP3CB-AS1, WDR65, MIR6733, EBNA1BP2, GPN3, FAM216A, SLC4A1AP, SUPT7L, CUTC, COX15, TTC31, CCDC142, GALR1, ERI2, LOC81691, CDC7, TMEM11, CRNKL1, C20orf26, MRPL18, TCP1, TADA3, ARPC4, NDUFA2, IK, SPPL2B, LSM7, ACAT1, ATF6B, C2orf60, C2orf47, TYW5, SNORD42B,

RPL23A, NDUFB2-AS1, LOC100134713, NDUFB2, RPRD2, HSPA2, FAM59A, UFM1, SLF1, C5orf36, ANKRD32, MDH2, STYXL1, CLCN3, CBR4, RAPGEF6, TUBE1, FAM229B, DPAGT1, MUTYH, FKBP10, P3H4, XPC, LSM3, OCIAD1, ATP6V1H, HIRIP3, INO80E, PSMA5, NCAPD2, MRPL51, PDCD11, USMG5, HADHA, HADHB, HERPUD2, TBCCD1, SYNRG, NUDCD2, HMMR, ZNF331, UBE4B, SGCE, ZNF140, PREPL, SLC2A4, TBC1D23, JARID2, GMPPA, PPP1R11, C11orf60, NUF2, BAT4, NELL2, TSSC4, CBX3, HNRNPA2B1, CEP97, HDLBP, SEPT2, YAE1D1, C7orf36, CHY1, THAP6, TTC27, EIF3I, C1orf91, TMEM234, KCNN2, MCM8, TRMT6, PPWD1, CENPK, NDC80, METTL4, DUS2L, DDX28, BRP44L, MPC1, PARP2, RPPH1, CDK14, CSNK1A1, NSMAF, TUBD1, RPS6KB1, MUDENG, EXOC5, AP5M1, HSPA13, TMTC3, CEP290, GMPR2, NEDD8, PPT2, ZMYND11, CDV3, SLC36A4, CRY1, C11orf57, PIH1D2, TMEM104, NAT9, RNPC3 , LOC101928436, LOC729082, TXNDC17, KIAA0753, GLT8D1, SPCS1, ETV1, TSPAN19, LRRIQ1, KIF23, PVRL3, GALNT3, HERC4, FOXRED1, SRPR, NUDT5, CDC123, WBP11, C12orf60, SPRY1, CREB3L4, SLC39A1, ETFDH, C4orf46, PSMC5, FTSJ3, CTDNEP1, C17orf81, DULLARD, TRNP1, CDKN1C, MTCH1, KRAS, PSMD9, HPD, UBE2I, DUSP6, RNF111, CCT3, TSACC, CDK7, TTC32, BTBD7, KIAA1409, AKAP8L, BAT1, NIT2, CHCHD6, ACTN3, ZDHHC24, NTNG1, PTPN12, ANKZF1, ATG9A, NRD1, SPC25, SFPQ, PYCR1, SMIM19, C8orf40, RNGTT, NUP153, MATN2, DCDC1, DNAJC24, C14orf119, ACIN1, SOCS2, HACL1, BTD, LEPRE1, C1orf50, ACAD10, BRAP, C10orf4, COPB2, LOC100507291, ECSIT, BRE-AS1, BRE, MEAF6, R3HDM1, ZRANB3, EDEM1, PRIM1, ILK, RRP8, CREB3L2, TOM1L2, LRRC48, OIP5, NUSAP1, FKBPL, CCHCR1, GTF2H4, SMIM7, TMEM38A, C19orf42, CYB561A3, TMEM138, CYBASC3, RAB1B, CCDC21, CEP85, TASP1, ILF3, SKIV2L, NELFE, RDBP, CAMK2B, UROS, BCCIP, NPC2, TINF2, DHRS1, C14orf21, FAM83G, SLC5A10, PSMC3IP, UBE3A, CD2BP2, CUGBP2, CELF2, NIPA2, PRMT2, PSMB4, PSMG2, CEP76, CEP120, MAP3K3, ARID5B, MRPS12, SARS2, TKFC, DAK, DDB1, OPA3, PTTG1IP, TBL2,

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