

Identification of Epigenetic Methylation Signatures With Clinical Value in Crohn's Disease

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INTRODUCTION: DNA methylation is an epigenetic mechanism that regulates gene expression and represents an important link between genotype, environment, and disease. It is a reversible and inheritable mechanism that could offer treatment targets. We aimed to assess the methylation changes on specific genes previously associated with Crohn's disease (CD) and to study their possible associations with the pathology.

METHODS: We included 103 participants and grouped them into 2 cohorts (a first [n = 31] and a second validation [n = 72] cohort), with active CD (aCD) and inactive CD (iCD) and healthy participants (CTR). DNA was obtained from the peripheral blood and analyzed by the Agena platform. The selected genes were catalase (CAT), α -defensin 5 (DEFA5), *FasR*, *FasL*, tumor necrosis factor (TNF), *TNFRSF1A*, *TNFRSF1B*, *PPA2*, *ABCB1*, *NOD2*, *PPAR γ* , and *PKC ζ* . We used the elastic net algorithm and R software.

RESULTS: We studied 240 CpGs. Sixteen CpGs showed differential methylation profiles among aCD, iCD, and CTR. We selected for validation those with the greatest differences: *DEFA5* CpG_11; CpG_13; *CAT* CpG_31.32; *TNF* CpG_4, CpG_12; and *ABCB1* CpG_21. Our results validated the genes *DEFA5* (methylation gain) and *TNF* (methylation loss) with *P* values < 0.001. In both cases, the methylation level was maintained and did not change with CD activity (aCD vs iCD). The subanalysis comparison between aCD and iCD showed significant differential methylation profiles in other CpGs: *TNF*, *FAS*, *ABCB1*, *CAT*, and *TNFRSF1B* genes.

DISCUSSION: The methylation status of *DEFA5* and *TNF* genes provides a signature biomarker that characterizes patients with CD and supports the possible implication of the environment and the immune system in CD pathogenesis.

SUPPLEMENTARY MATERIAL accompanies this paper at <http://links.lww.com/CTG/A104>, <http://links.lww.com/CTG/A105>, <http://links.lww.com/CTG/A106>, <http://links.lww.com/CTG/A107>

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INTRODUCTION

Inflammatory bowel disease (IBD) is a complicated and multifactorial disorder characterized by relapsing and remitting inflammation that can involve the entire gastrointestinal tract in the Crohn's disease (CD) form and that localizes exclusively in the colon in the ulcerative colitis (UC) form (1,2). IBD results from a complex interplay between genetic variation, intestinal microbiota, the host immune system, and environmental factors such as diet, drugs, breastfeeding, and smoking, although the exact cause of the disease remains unknown (3). Genetic studies, including candidate gene approaches, linkage mapping studies, and genome-wide association studies, have significantly advanced our

understanding of the importance of genetic susceptibility in IBD (4). Studies have shown that most risk genes leading to the onset of IBD are involved in the pathways of innate immunity rather than adaptive immunity (5). Genome-wide association studies have identified more than 240 IBD susceptibility gene loci. However, these known genetic variants only contribute to approximately 26% of CD and 19% of UC heritability, indicating a role for non-genetic factors in the disease etiology (a combination of genetic predisposition and environmental factors) (5–7). In particular, environmental factors must exert a decisive influence on the establishment and/or flare-ups of the disease. Epigenetic studies show how the gene expression can be regulated through

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mechanisms usually conditioned by the environment. Epigenetic modifications include DNA methylation, histone modifications, and small and long noncoding RNAs. DNA methylation is the most well-studied epigenetic modification that occurs through the covalent addition of a methyl group to the 5' carbon of the cytosine ring in the context of CpG dinucleotides, resulting in 5-methylcytosine. DNA methylation is a key regulatory mechanism of gene transcription (in such a manner that when a gene is methylated, its expression is diminished) (8–10). These processes have been functionally implicated in the regulation of gene expression in patients with IBD, providing new insights into the pathogenesis of the disease (11). However, the contribution of the scientific community is especially limited in patients with CD. One study (12) had been performed on mucosa (normal and inflamed) of patients with CD and UC and another on the peripheral blood of patients with CD. Both studies identified some genes regulated by methylation (13). These findings await validation and/or further research, given that they have not yet been replicated by independent groups. One study had shown a strong correlation between methylation levels of selected genes in colon biopsies and in the peripheral blood in IBD, which supports the idea that peripheral blood reflects the methylation status and can be used for samples from patients with IBD (14).

Our group has characterized various proteins and metabolic pathways involved in the pathogenesis of CD. Initially, we found that antioxidant enzymes such as catalase (CAT) and its regulators (PKC ζ , PPA2) were altered in debut patients with CD, which are known to present reversible oxidative damage (15). This fact also had an impact on regulating other metabolic pathways, such as apoptosis (FasL, FasR) and PPAR γ (16–18). Other studies by our group have contributed to the characterization of probable biomarkers, such as α -defensin 5 (DEFA5) in ileal disease (19) or cytokine prediction for anti-tumor necrosis factor (TNF) treatment (TNF, TNFRSF1A, TNFRSF1B) (20). To better characterize these findings, we have analyzed the expression of their genes or pathway-related genes (*ABCB1*), observing that some gene expression correlates with the activity of the disease, but others are permanently over-/ underexpressed (21). This genetic expression could be regulated by epigenetic mechanisms that would indicate that the environment is playing a specific target role in the pathogenesis.

Thus, the aim of this study was to assess and validate DNA methylation changes on the referred genes by analyzing locus-specific DNA methylation patterns in the peripheral blood obtained from patients with onset CD and from patients with CD in morphologic remission. The identification of DNA methylation signatures could provide novel insights into the pathophysiology events regulating CD-implicated genes. Furthermore, these signatures could help identify new biomarkers, therefore improving the diagnostic tools for CD management possibilities.

MATERIAL AND METHODS

Participants: Patients and control subjects

This study comprised 2 cohorts of patients and healthy control subjects: a retrospective first cohort with 31 participants (active [onset] CD [aCD], $n = 11$; inactive CD [iCD], $n = 12$; and healthy control subjects, $n = 8$) and a prospective cross-sectional validation cohort including 72 participants (aCD [onset], $n = 24$; iCD, $n = 24$; and healthy control subjects, $n = 24$). In the first cohort, 11 consecutive patients with CD at the onset of disease (diagnosed according to the endoscopic, radiologic, histologic, and clinical

criteria by the European Crohn's and Colitis Organisation) (2) and yet to begin any specific medication were included. Patients with unclear diagnoses and those with additional diseases were excluded. Patients were classified according to Montreal Criteria (22). Harvey-Bradshaw Index values were also collected (23). Data on age, sex, smoking habits, clinical signs and symptoms, biochemical analyses, disease indexes, and localization were collected. Additionally, 12 patients with CD under specific treatment were included in the study as the inactive group when they achieved clinical, analytic, and morphologic remission. Clinical remission was established (2) with a cutoff of Harvey-Bradshaw Index ≤ 4 ; analytic remission was defined as when the inflammation parameters fell to within normal values (C-reactive protein, fibrinogen and erythrocyte sedimentation rate, calprotectin); and morphologic remission was defined as when there was mucosal healing, as evaluated by ileocolonoscopy or MRI.

The control group consisted of 8 healthy volunteers who were not taking any medication and had completely normal blood test results.

Independent validation of the methylation results was performed on a further cohort of 72 new patients (validation cohort) to assess the reproducibility of the associations found in the first cohort. The same inclusion and exclusion criteria mentioned earlier were applied. For the validation experiments, 24 new patients were included in each experimental group (onset CD, iCD, and healthy control subjects).

The information regarding the demographic and clinical characteristics of all participants in the study is shown in Table 1.

The study was conducted in a tertiary university teaching hospital. All participants gave their written informed consent. The study was approved by the Ethics Committee of the University Hospital La Fe (no. PI14/01702) and complied with the Declaration of Helsinki.

Genes and CpGs selection

We developed the methylation analysis for selected genes based on the importance of their established role in the pathogenesis of CD, as previously reported by our group and others (15–21,23). The list of the susceptibility genes, their main function, and their reported status in CD is briefly summarized in Table 2. As shown, the selected genes are mainly involved in the following: the cellular antioxidative defense mechanism (CAT) and its modulators (PKC ζ and PPA2); a transporter involved in the metabolism of corticosteroids (*ABCB1*); regulating the sensing for bacterial muramyl dipeptide (*NOD2*); or producing enteric antimicrobial peptides (α -defensin gene). We also selected genes involved in the PPAR γ regulation of local intestinal inflammation (PPAR γ gene) or the disruption of the integrity of intestinal inflammation (*FasL*, *FasR*, *TNF*, *TNFRSF1A*, *TNFRSF1B*), which also happen to regulate the proinflammatory function of T cells by controlling their proliferative, differentiation, and apoptotic capacities.

Blood sampling and DNA purification

Fasting blood samples were collected in K2-ethylenediaminetetraacetic acid vacutainer tubes. Within 1 hour after extraction, the blood was layered onto Histopaque 1077 solution (Sigma-Aldrich, UK) and centrifuged at 213g for 30 minutes (without breaks), all at room temperature. The upper-layer phases, containing the white cell-rich plasma, were collected and subsequently separated by centrifugation at 2,375 g for 10 minutes. The plasma supernatants were aliquoted and stored frozen. The pelleted mononuclear blood cells were then submitted to steps of erythrocyte lysis, washed to

Table 1. Characteristics of the participants

Characteristic	First cohort			Second validation cohort		
	Onset CD (n = 11)	iCD (n = 12)	Control (n = 8)	Onset CD (n = 24)	iCD (n = 24)	Control (n = 24)
Age (yr)						
Median (1st–3rd Q)	22 (19–38)	33 (27–40)	27 (26–30)	25 (20–30)	38 (34–46)	38 (27–41)
Mean (SD)	29 (14)	35 (11)	28 (3)	28 (13)	39 (11)	38 (12)
Sex, n (%)						
Female	9 (81.8)	8 (66.7)	6 (75)	11 (45.8)	11 (45.8)	18 (75)
Male	2 (18.2)	4 (33.3)	2 (25)	13 (54.2)	13 (54.2)	6 (25)
WBC (10 ³ /μL)						
Median (1st–3rd Q)	8.7 (6.9–9.6)	6.2 (4.7–7.2)	6.0 (5.5–6.9)	7.7 (6.3–11.4)	5.6 (4.4–6.3)	5.9 (5.4–6.7)
Mean (SD)	8.6 (4.5)	6.2 (1.4)	6.1 (0.5)	8.9 (3.5)	5.9 (2.2)	6.2 (0.8)
CRP (mg/L)						
Median (1st–3rd Q)	65.7 (2–94)	0.5 (0.3–1)	0.6 (0.3–1.1)	16.5 (11.1–88.6)	1.0 (0.5–3.0)	0.6 (0.4–1.0)
Mean (SD)	49.8 (48.4)	0.8 (0.6)	0.8 (0.5)	48.7 (55.6)	1.8 (1.8)	0.7 (0.8)
Calprotectin (μg/g)						
Median (1 st –3 rd Q)	1140 (480–1946)	40 (22–46)	n.a.	1360 (558–2084)	44.5 (28.3–98.8)	n.a.
Mean (SD)	1103 (988)	44.9 (39.6)	n.a.	1525 (1243)	82.6 (90.3)	n.a.
Smoking, n (%)						
Active	5 (45.5)	2 (16.7)	1 (12.5)	6 (25)	2 (8)	6 (25)
Ex	3 (27.2)	3 (25)	1 (12.5)	3 (12.5)	5 (20.8)	1 (4.2)
No	3 (27.2)	7 (58.3)	6 (75)	15 (62.5)	17 (70.8)	17 (70.8)
Disease duration (mo)						
Median (1st–3rd Q)		48 (29–69)			96 (51–180)	
Mean (SD)	n.a.	51.2 (26.8)	n.a.	n.a.	112.0 (71.7)	n.a.
Disease location, n (%)						
L1 ileal	6 (54.5)	7 (58.3)		18 (75)	13 (54.2)	
L2 colonic	0	0	n.a.	0	0	n.a.
L3 ileocolonic	5 (45.5)	5 (41.7)		5 (20.8)	10 (41.7)	
L1 + L4 (upper GI)	0	0		1 (4.2)	1 (4.2)	
Disease behavior, n (%)						
B1 nonstricturing, nonpenetrating	9 (81.8)	5 (41.7)		16 (66.7)	20 (83.3)	
B2 stricturing	1 (9.1)	5 (41.7)	n.a.	0	4 (16.7)	n.a.
B3 penetrating	1 (9.1)	2 (16.7)		8 (33.3)	0	
P perianal	4 (36.4)	3 (25)		3 (12.5)	6 (25)	
Maintenance therapy, n (%)						
Thiopurines		3 (25)			9 (37.5)	
Anti-TNFα mono	n.a.	3 (25)	n.a.	n.a.	7 (29.2)	n.a.
Combotherapy		1 (8.3)			3 (12.5)	
Mesalamine		5 (41.7)			5 (20.8)	
Surgery, n (%)	n.a.	5 (41.7)	n.a.	n.a.	11 (45.8)	n.a.

CD, Crohn's disease; CRP, C-reactive protein; GI, gastrointestinal; iCD = inactive CD; TNF, tumour necrosis factor; WBC, white blood cell.

remove erythrocyte contamination, and stored at -80°C until further analysis.

Genomic DNA was isolated from stored leukocytes and purified using the PureLink Genomic DNA Mini kit (Cat no. K1820-01)

from Invitrogen (Thermo Fisher Scientific, CA) following the manufacturer's instructions. The quantity and quality of the isolated DNA were determined with a spectrophotometer (NanoDrop 2000 Spectrophotometer Thermo Scientific). In all samples, DNA

Table 2. Description of the selected genes for the methylation studies

Gene	Function	Status in CD
<i>ABCB1</i>	Multidrug transporter (P-glycoprotein [P-gp]) controlling the accumulation of xenobiotic compounds and exogenous pharmacologic molecules (41).	Significantly lower mRNA levels at onset (21).
<i>CAT</i>	Antioxidant enzyme to defend against oxidative stress (16,17).	Decreased activity, protein, and mRNA levels (15,38).
<i>DEFA5</i>	Antimicrobial peptide of the innate immune system produced by ileal Paneth cells (32).	Decreased protein concentration (19,33,34).
<i>NOD2</i>	Intracellular sensor for small peptides derived from the bacterial peptidoglycan (cell wall). It mediates in defensin synthesis (35).	Failure of adequate bacterial recognition and control of microbial infections (innate immune system). Impaired autophagy pathway (36).
<i>FasR</i>	Induction of apoptosis with the selection of lymphocytes and downregulation of immunologic processes (42,43).	Higher mRNA levels in inactivity with a similar trend at onset (21).
<i>FasL</i>	Induction of apoptosis with the selection of lymphocytes and downregulation of immunologic processes (42).	Decreased mRNA levels at onset (21).
<i>TNF</i>	Proinflammatory cytokine involved in apoptotic pathways, metabolism, and inflammation (43).	Identified as one of the key cytokines in the pathogenesis of CD (44). Anti-TNF α therapy is able to reduce disease activity with mucosal healing (45,46).
<i>TNFRSF1A</i>	TNF receptor (1A) involved in the regulation of expression of other cytokines and immunoregulatory molecules (47).	Polymorphisms are related to infliximab (anti-TNF α) in patients with CD (48).
<i>TNFRSF1B</i>	TNF receptor (1B) involved in the regulation of expression of other cytokines and immune regulatory molecules (47).	Polymorphisms are related to infliximab (anti-TNF α) in patients with CD (47–49).
<i>PPA2</i>	Inhibition of inflammatory cytokines (39) and CAT (50).	Significantly higher protein levels at onset (38).
<i>PPARγ</i>	Essential for intestinal homeostasis (51) and maintains defensin expression (35,52), an important functional receptor mediating adipocyte differentiation and aminosalicylate activity in IBD (51,53).	Decreased intestinal mucosa expression in both aCD and iCD (52,53).
<i>PKCζ</i>	Positive regulator of CAT (50) and proinflammatory cytokines (TNF) (54).	Significantly higher protein levels at onset (38).

CD, Crohn's disease; DEFA, defensin 5; IBD, inflammatory bowel disease; iCD, inactive CD; mRNA, messenger RNA; TNF, tumour necrosis factor.

concentrations were between 50 and 100 ng/ μ L, with an optical density 260/280 ratio between 1.8 and 2.0, and an optical density 260/230 ratio above 1.5.

To prevent clustering of samples and batch differences (technical effects) (24), all samples were processed using the same method (protocols and commercial kits), and for each of the 2 cohorts (first and validation), DNA extractions were performed the same day. All purified DNA samples were randomly distributed into 96-well plates for the bisulphite conversion.

DNA methylation analysis

The DNA methylation analyses were performed using the MassARRAY EpiTYPER (Agena, San Diego, CA) platform (Faculty of Medicine, Valencia, Spain) with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and RNA base-specific

cleavage (3' to either rUTP or rCTP by RNase A, MassCLEAVE). Polymerase chain reaction primers were designed using Agena's EpiDesigner software (www.epidesigner.com; San Diego, CA). Sequences are shown in Table 1 (Supplementary Digital Content 1, <http://links.lww.com/CTG/A104>).

Sodium bisulphite conversion was performed using an EZ-96 DNA Lightning methylation kit according to the manufacturer's protocol (Zymo Research, Freiburg, Germany) on 1 μ g of genomic DNA. PCRs were performed in a 5 μ L format with 10 ng/mL bisulphite-treated DNA, 0.2 units of TaqDNA polymerase (Agena), 1 \times supplied Taq buffer, and 200 mM PCR primers. Amplification for the PCR was as follows: preactivation at 95 $^{\circ}$ C for 15 minutes, 45 cycles of 95 $^{\circ}$ C denaturation for 20 seconds, 56 $^{\circ}$ C annealing for 30 seconds, and 72 $^{\circ}$ C extension for 30 seconds, finishing with a 72 $^{\circ}$ C incubation for 4 minutes. Dephosphorylation

of unincorporated dNTPs was performed by adding 1.7 μ L of H₂O and 0.3 μ L of shrimp alkaline phosphatase (Agena), incubating at 37 °C for 40 minutes, and then for 10 minutes at 85 °C to deactivate the enzyme. For each reverse primer, an additional T7 promoter tag for *in vivo* transcription was added, and the transcription/cleavage reaction contained 27 units of T7 R&DNA polymerase (Agena), 0.64 \times of T7 R&DNA polymerase buffer, 0.22 μ L of T Cleavage Mix, 3.14 mM of dithiothreitol, 3.21 μ L of H₂O, and 0.09 mg/mL of RNaseA (Agena). The MassCLEAVE biochemistry was performed as follows: *in vivo* transcription and RNA cleavage was achieved by adding 2 μ L of PCR product to 5 μ L of transcription/cleavage reaction and incubating at 37 °C for 3 hours. The reactions were additionally diluted with 20 mL of H₂O and conditioned with 6 mg of CLEAN Resin (Agena) for optimal mass-spectra analysis.

The resulting spectra were analyzed using proprietary peak picking and signal-to-noise calculations, after which the spectra's methylation ratios were generated using EpiTYPER software v1.2 (Agena). We observed that not all exploratory CpG methylation levels exceeded the technical uncertainty threshold established (0.1) (25), and the data with an estimated error larger than this value (meaning imprecise data) were excluded for the subsequent analyses.

Statistical analysis

The mean value of the 2 replicate amplicons was analyzed using R software (version 3.5.1) (<http://www.R-project.org/>) to identify differentially methylated CpG sites across the samples to establish an association between DNA methylation level and the disease status.

We first performed an exploratory analysis using unsupervised techniques, such as clustering methods to divide the methylation results into groups with a high degree of similarities. These techniques do not require sample annotation and allow data exploration and visualization, suggesting directions for further study. We used hierarchical clustering with dendrograms (tree structures in which CpGs are located as leaves) and heatmaps (graphic representations of the data in which values are color coded) to visualize and interpret the results.

To assess the CpG sites able to discriminate among the 3 studied groups, we used an elastic net-penalized multinomial regression model. This regularization method, which is a combination of the ridge regression and LASSO (26), is suited for analyzing data with many variables and few observations, selecting those variables with higher influence on the disease and removing the others from the model.

The shape parameter of the elastic net was set at 0.5, and the penalization factor was selected using 500 repetitions of 10-fold cross-validation. Additionally, a penalized logistic regression model was fitted to assess the CpG sites discriminating between onset and iCD. To reinforce the results from the elastic net, a random forest analysis was performed to assess the importance of the variables in discriminating between onset and iCD. Beta regression was used when analyzing the validation data.

RESULTS

Demographic data and DNA methylation prefiltering

In the first cohort (clinical and demographic data on the participants are shown in Table 1), we analyzed a total of 419 CpGs in 20 different regions distributed throughout the selected genes previously presented in Table 2 (see Table 1, Supplementary Digital Content 1, <http://links.lww.com/CTG/A104>). Not all exploratory

CpG methylation levels exceeded the technical uncertainty threshold established (0.1); thus, these CpGs were excluded, and the remaining 240 CpGs were used in the subsequent exploratory and supervised analyses.

DNA methylation analysis and first cohort results

First, an unsupervised analysis of the results was performed using hierarchical clustering, which showed a similar methylation pattern in all analyzed samples, with no apparent homogenous subgroups among the observations (see Figure 1, Supplementary Digital Content 2, <http://links.lww.com/CTG/A105>). Next, a supervised analysis was performed, using a multinomial regression analysis penalized with the elastic net algorithm. This regularization method performs variable selection, providing a list of CpGs that are predictive of disease presence (and activity) based on their contribution to the model and consequently built on their methylation changes. Among all those CpGs, 16 appeared to discriminate between the groups (onset CD, iCD, and control subjects) based on their methylation levels. CpGs and their genes are shown in the heatmap in Figure 1: *TNFRS1B*_CpG_6 and 10.11.12; *FAS*_CpG_19.21; *PPA2*_CpG_12.13.14.15.16; *DEFA5*_CpG_11 and 13; *CAT*_CpG_6.11.12.13.14.17.18 and 31.32.33; *ABCBI*_CpG_21; and *TNF*_CpG_4.12. In this heatmap, samples were grouped using a hierarchical clustering algorithm according to their methylation status to visualize the differences between groups.

DNA methylation results in disease activity

Furthermore, we analyzed the methylation results to identify CpGs that showed differential methylation profiles between patients with aCD (onset) and iCD, which thus could help identify the disease status. In this case, the control group was not considered for the analysis. We first used an elastic net logistic regression analysis, but the estimation of the penalization parameter was highly unstable; therefore, as an alternative, we used a random forest analysis. Random forest is able to recognize unknown interactions among the variables, detecting potentially nonlinear relationships between our prediction variables (methylation profile) and the response (disease status). The random forest algorithm selected 7 CpGs able to discriminate between patients with aCD and iCD, with a good prediction rate of 74% for a cutoff of 0.2 (Figure 2): *CAT*_CpG_6.8.9 and 31.32; *FAS*_CpG_7.8.9; *TNF*_CpG_10; *ABCBI*_CpG_6.7.8; and *TNFRS1B*_CpG_10.11.12. However, these results were not validated in the new prospective cohort of patients, and we did not find differences in those selected CpGs between the 2 experimental groups (see Figure 2, Supplementary Digital Content 3, <http://links.lww.com/CTG/A106>). These results could be affected by the fact that the statistical analysis showed a bimodal distribution in the elastic net test in the inactive patient group (data not shown). That outcome could be related to an unexpected subclassification of patients: those with deep, iCD and those who are not in such a deep remission, even though all had a morphologic test showing inactivity.

DNA methylation validation in a second cohort

From the results in Figure 1, we selected those with the greater differences between groups (Figure 3) to validate and confirm their importance in CD. Given the heatmaps represent Z-scores instead of methylation percentages, this selection is depicted as box plots to better visualize the differences between the

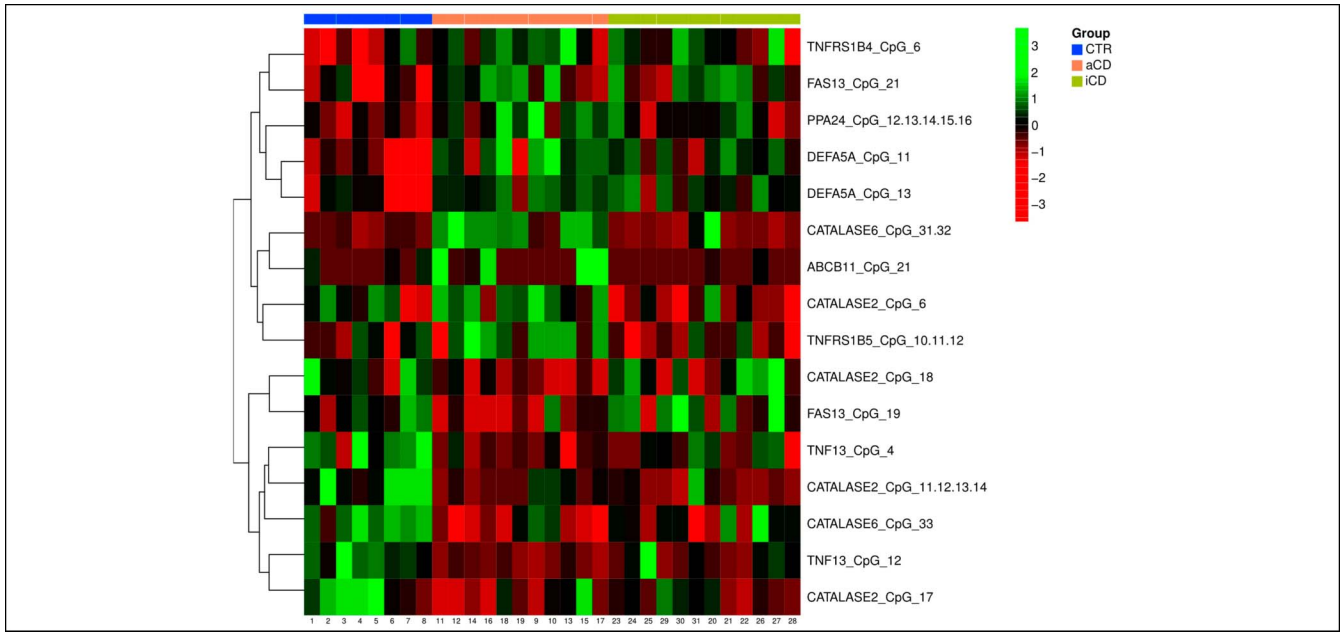


Figure 1. Heatmap including the CpGs for discriminating control, active CD and inactive CD groups, showing statistically significant differences in the elastic net analysis. Rows (CpGs) and columns (patients) are ordered according to the results of hierarchical clustering. Color scale ranges from red for hypomethylation to green for hypermethylation. aCD, active (onset) CD; CD, Crohn’s disease; CTR, healthy control subjects; iCD, inactive CD.

methylation values of each CpG: *DEFA5*_CpG_11 and CpG_13; *CAT*_CpG_31.32; *TNF*_CpG_4 and CpG_12; and *ABCB1*_CpG_6.7.8. A new cohort of patients and healthy control subjects was prospectively recruited (Table 1). To maintain consistency, these participants had demographic and clinical characteristics similar to the first cohort. In this new prospective group, various beta regression models were adjusted to assess differences in the methylation status between groups (shown in Figure 4). For *DEFA5* (CpG11, and CpG13) and *TNF* (CpG4 and

CpG12), the results strongly confirmed our previous outcomes, given differences were found in the methylation levels of the different groups ($P < 0.001$) (Table 3). In particular, *DEFA5* showed a higher methylation profile at the onset of disease and in inactivity. Contrarily, *TNF* showed a lower methylation profile in patients with both aCD and iCD. Nevertheless, the results for the CpGs in the genes of *ABCB1* (CpG_6.7.8) and *CAT* (CpG_31.32) were not validated because the percentage of methylation did not differ between the groups (Figure 4).

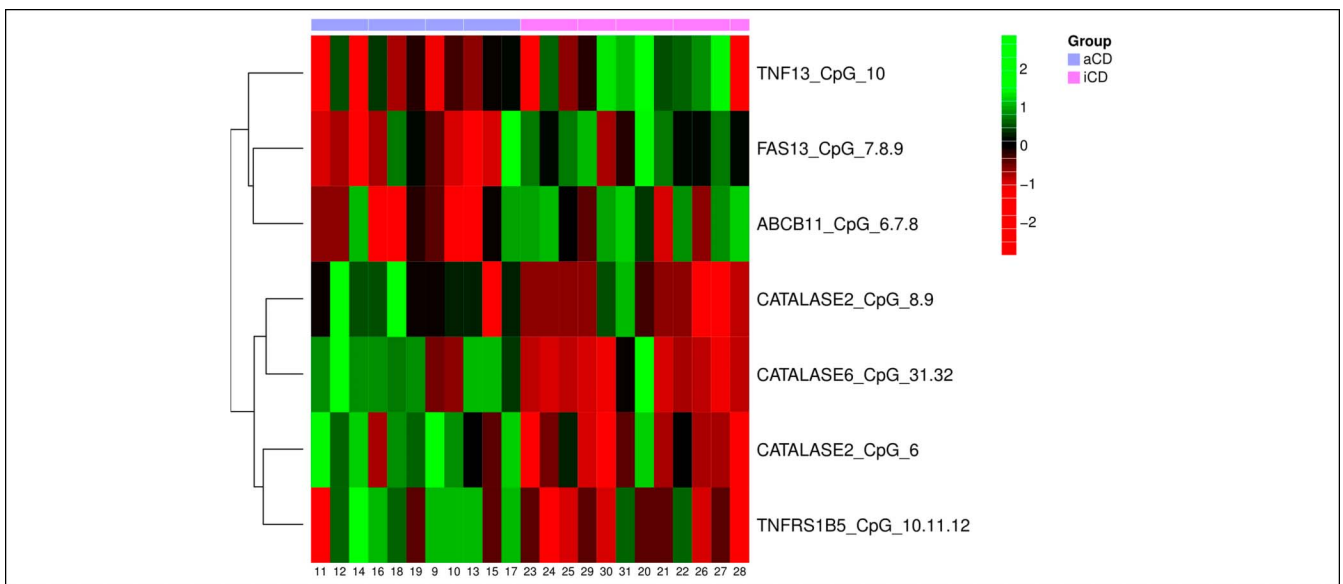


Figure 2. Heatmap including the CpGs for discriminating active CD and inactive CD groups, showing statistically significant differences in the random forest algorithm. Rows (CpGs) and columns (patients) are ordered according to the results of hierarchical clustering. Color scale ranges from red for hypomethylation to green for hypermethylation. aCD, active (onset) CD; CD, Crohn’s disease; iCD, inactive CD.

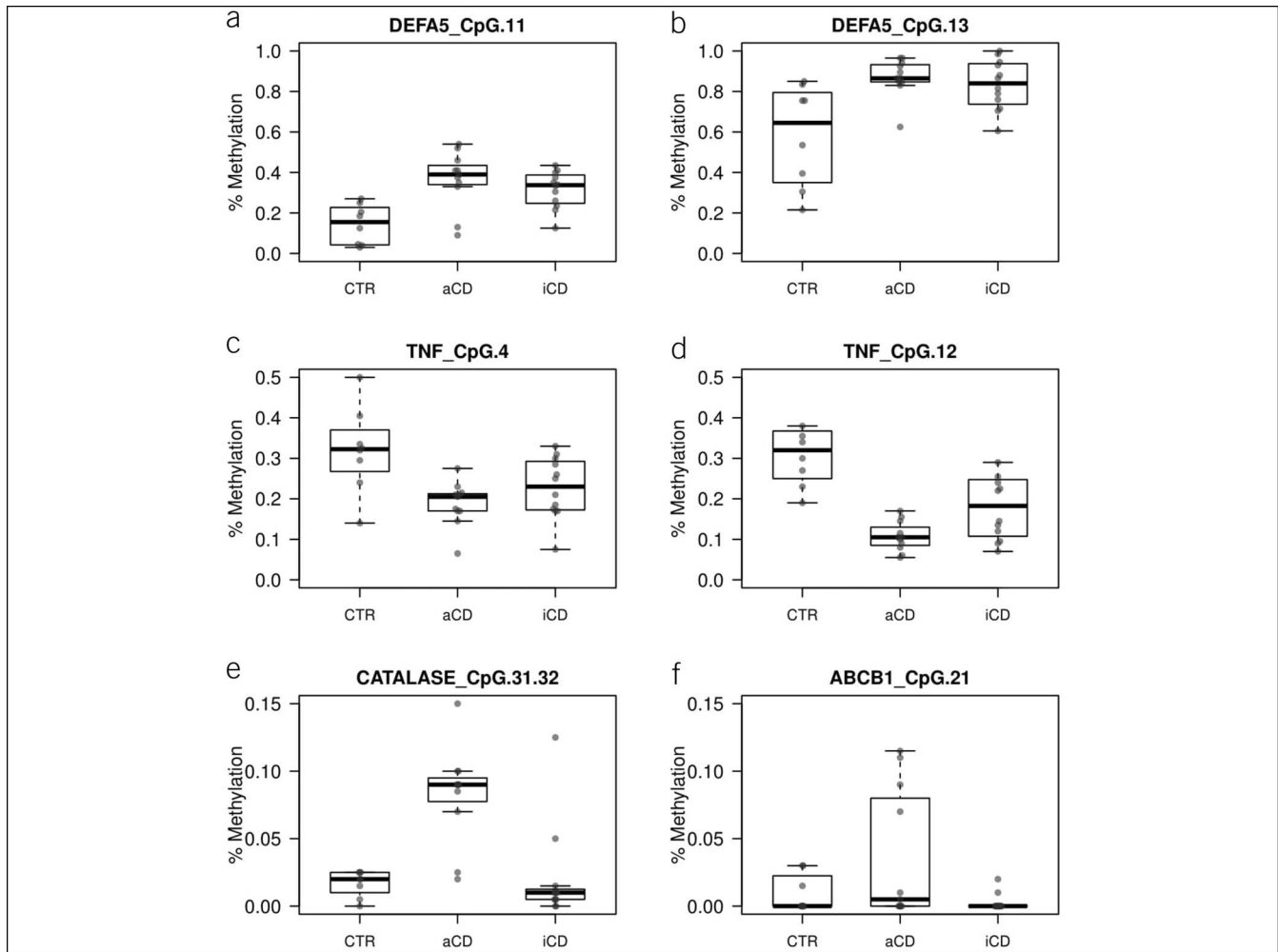


Figure 3. Box plots displaying the distribution of β -values of the selected CpG for validation. aCD, active (onset) CD; CD, Crohn's disease; CTR, healthy control subjects; DEFA, defensin 5, iCD, inactive CD; TNF, tumour necrosis factor.

Analysis for DNA methylation regions

The CpG methylation profiles of the selected genes for validation were further analyzed to identify specific patterns in the analyzed genetic sequences (see Figure 3, Supplementary Digital Content 4, <http://links.lww.com/CTG/A107>). There was no pattern of methylation level distribution throughout the sequences between the experimental groups.

DISCUSSION

We report a state-of-the-art and specific methylation panel of CpGs from a set of selected genes related to CD pathogenesis: 2 CpGs in the *TNF* gene (CpG4 and CpG12) and 2 CpGs in the *DEFA5* gene (CpG11, and CpG13) can differentiate between patients with CD and healthy control subjects, providing a predictive model that could be used as a clinical tool to discriminate between healthy control subjects and patients with CD. This predictive model was validated in a prospective cohort, which gives strength to the findings and supports their clinical use for identifying these patients. Therefore, by using a novel statistical analysis with a strict selection of patients with CD, we have found a methylation signature that could be translated into clinical practice to help in CD diagnosis.

Although CD is known to be an inflammatory disease with a chronic evolution, its etiology and the reasons for its chronicity and flare-ups are not yet understood. Within the genetic approaches, epigenetic studies have been focused mainly on epigenome-wide association study DNA methylation profiling (8–12,27). Although epigenome-wide association studies in CD are scarce, the epigenetic changes identified by this massive analysis are frequently difficult to interpret, given changes are detected at some CpGs in novel sequences/genes with an unknown implication in the pathology. Furthermore, massive data analyses with numerous CpGs to analyze entail the possibility that real effects get lost in such a complex statistical analysis. For these reasons, the study of DNA methylation in CD-related genes that have previously been implicated in the disease appears more rational. We have found a characteristic signature for CD which could indicate a therapeutic target, given that methylation can be reversed.

The implication of TNF in CD is widely reported, given it was the first cytokine identified as a target element for developing biologic treatments in IBD (15,28,29). However, there is still an important lack of knowledge about TNF, including the genetic mechanisms that cause its altered expression in CD. Likewise, its receptors, TNFRSF1A and B, and other related members from

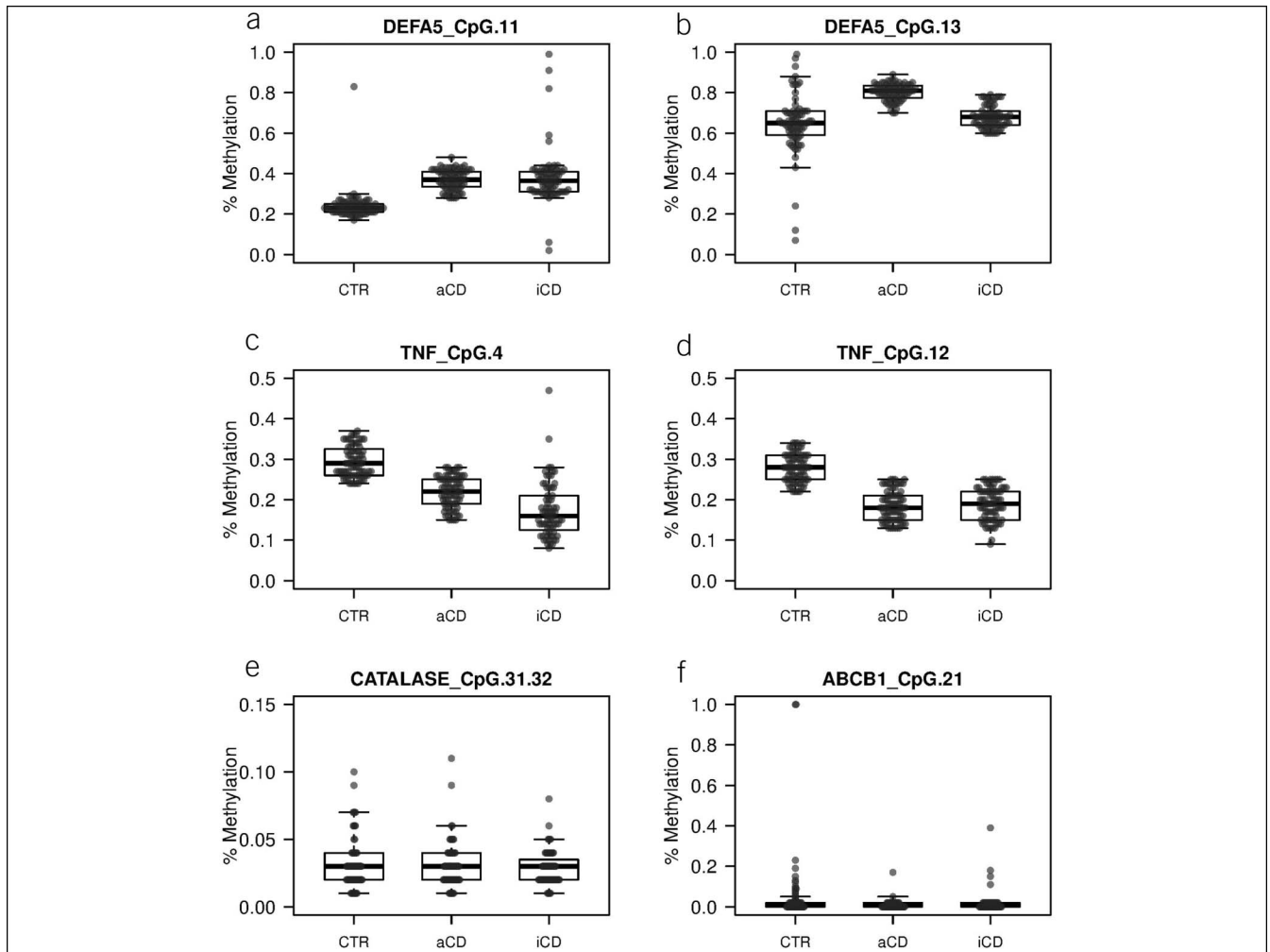


Figure 4. Box plots depicting the validation results for the selected CpG. aCD, active (onset) CD; CD, Crohn's disease; CTR, healthy control subjects; iCD, inactive CD; TNF, tumour necrosis factor.

this family, such as the death receptors, FasR and FasL, are all under analysis to identify the biologic origin of their impaired expression in CD. Our study initially showed that differential methylation profiles were present in genes related to the *TNF* family. However, only *TNF* gene methylation was confirmed by the validation analysis, showing a permanent low methylation profile for the *TNF* gene, both at the onset of disease and in inactivity. Thus, *TNF* genes maintain hypomethylation independent of the inflammatory activity of the disease, even when patients are receiving anti-TNF treatment (data not shown). Although some studies have indicated that medication can affect DNA methylation (29), our results are in agreement with previously published results suggesting no effect of specific medication, such as anti-TNF α , on the methylome (30). Our results explain the continuous higher production occurring in patients with CD and suggest that reversion of hypomethylation could be a new pharmacologic approach to modifying TNF production. However, functional experimental analyses are lacking to support this hypothesis.

Regarding the *TNF* family gene methylation status, few but consistent results have been published which align with our data. Studies performed on intestinal biopsies showed that genes coding for other members of the TNF family (*TNFSF4* and *TNFSF12*) are

differentially methylated in CD (27,31). Nimmo et al. (13) found, in a childhood cohort, differentially methylated loci for FasL, suggesting the importance of the TNF pathway in the disease. In a cohort of female patients with CD, some differentially methylated loci for *TNF* and *TNFSF4* were found, though the sample size and technical limitations restricted the study's conclusions (30). To the best of our knowledge, ours is the first study that not only characterizes but validates the methylation status of *TNF* genes in CD. Furthermore, our results help explain the chronic nature of CD, given the loss of methylation remains even when patients achieve remission, which confirms it is a pathogenic signature of CD and that patients in remission are not truly healed.

Defensins function as endogenous antibiotics released into the crypt lumen to defend against microbial colonization. Defects in the antimicrobial barrier and a loss of intestinal homeostasis are both processes commonly observed in CD (32–34). The messenger RNA and protein expression of *DEFA5* are impaired in inflamed intestinal biopsies of patients with CD, which affects the innate immunity pathways (33–36). Our previous study also demonstrated that *DEFA5a* alterations were characteristic of CD and were not occurring in other inflammatory conditions (19). Whether the methylation status of the *DEFA5* gene is regulating

Table 3. Differences in the validation cohort

Factor	Estimate	Standard error	Lower 95%	Upper 95%	P value
DEFA5 CpG11					
Intercept	-1.10	0.073	-1.244	-0.959	<0.001
Group aCD	0.587	0.099	0.393	0.781	<0.001
Group iCD	0.653	0.099	0.459	0.846	<0.001
Group aCD group iCD	0.066	0.14	-0.25	0.38	0.88
DEFA5 CpG13					
Intercept	0.649	0.074	0.504	0.795	<0.001
Group aCD	0.712	0.109	0.498	0.925	<0.001
Group iCD	0.094	0.106	-0.113	0.3	0.38
Group aCD group iCD	-0.61	0.15	-0.97	-0.27	<0.001
TNF CpG4					
Intercept	-0.866	0.035	-0.934	-0.797	<0.001
Group aCD	-0.4	0.051	-0.5	-0.299	<0.001
Group iCD	-0.719	0.053	-0.823	-0.615	<0.001
Group aCD group iCD	-0.319	0.077	-0.497	-0.14	<0.001
TNF CpG14					
Intercept	-0.95	0.025	-0.998	-0.901	<0.001
Group aCD	-0.527	0.038	-0.600	-0.453	<0.001
Group iCD	-0.508	0.037	-0.581	-0.435	<0.001
Group aCD group iCD	0.019	0.055	-0.108	0.146	0.93
ABCB1 CpG_6.7.8					
Intercept	-1.315	0.037	-1.387	-1.243	<0.001
Group aCD	0.081	0.052	-0.02	0.182	0.12
Group iCD	0.081	0.052	-0.02	0.182	0.12
Group aCD group iCD	0	0.073	-0.169	0.168	1
CAT CpG_31.32					
Intercept	-3.46	0.07	-3.61	-3.32	<0.001
Group aCD	-0.01	0.10	-0.22	0.19	0.93
Group iCD	-0.07	0.11	-0.28	0.13	0.52
Group aCD group iCD	-0.06	0.10	-0.12	0.002	0.58

aCD, active CD; DEFA, defensin 5; iCD, inactive CD; TNF, tumour necrosis factor.

its expression is not completely understood. A recent study (37) has reported the importance of DNA methylation patterns for UC pathogenesis, including the altered methylation status of *DEFA6* in mucosal biopsies. As we confirm here, the *DEFA5* gene presents a persistent altered hypermethylation in CD, which remains independent of disease status, as occurs with TNF. Therefore, lower *DEFA5* protein production could explain the consequent loss of intestinal homeostasis reported in CD.

CAT and *ABCB1* were other genes included in the validation cohort, but their methylation status could not be validated; thus, *CAT* and *ABCB1* expression could be independent of the methylation mechanism. In the experimental analysis, the *CAT* and *ABCB1* genes showed higher methylation in patients with aCD and returned to healthy control levels in iCD. This change could lead to low expression of *CAT* and *ABCB1* proteins, which would be consistent with our previous observations (15,21,38).

For the validation of the results, CpGs along the promoter section of *CAT* and *ABCB1* were selected. It is possible that other CpGs not included in the analysis could play a role in the expression of *CAT* and *ABCB1*. Further studies are needed to clarify this point.

A good correlation between the methylation levels in peripheral blood and intestinal mucosa in IBD has been previously reported (14). Thus, experiments have been performed using peripheral blood, given this type of sample is easy to obtain, is minimally invasive, and will favor the future clinical translation of our results (39). We have included in the study newly diagnosed active patients who were treatment-naïve at the time of sampling to avoid pharmacologic bias of the methylation results. However, the results appear to indicate that the drugs typically prescribed for CD did not interfere with the methylation status. Other variables (such as smoking habit, disease location, and phenotype) did not show differences between our groups of patients, though

they could have had some effect on the methylation results, as well as other unknown variables.

To evaluate changes in the methylation profiles in patients with iCD, morphologic remission was confirmed. The methylation differences between patients with aCD and iCD were not validated in our study, although the first cohort had given positive results. The statistical analysis showed that the inactive patient group presented a bimodal distribution in the elastic net test. This bimodal distribution could be related to an unexpected subclassification of patients in the inactivity phase: those who had real iCD and others who did not, or not as deep a remission as the others (i.e., histologic remission) (40), even though all patients had a morphologic test that confirmed the inactive status of the disease. This result led us to use the random forest as a sensitivity analysis. Further studies are needed to clarify whether the depth of remission should be considered and whether a signature to distinguish active and inactive disease should be further explored.

Further experiments are needed to clarify whether methylation affects all immunologic subsets equally. Similarly, most of our patients had ileal involvement. Given that DEF-5 is principally implicated in ileal disease, the validation of this signature should be confirmed in patients with colonic CD only. Our results provide a basis for future experiments to manipulate the methylation status in search of therapeutic targets. Moreover, the fact that we have analyzed specific genes implicated in CD pathogenesis gives strength to our findings.

In conclusion, we have shown that methylation status can differentiate patients with CD from healthy individuals and thus could be used as a biomarker for diagnosis. Our experiments also indicate that its correlation with disease activity deserves further study, especially when defining the inactive cohorts, given different degrees of inactivity could reflect different methylation statuses. The higher methylation of *DEFA5* and the lower methylation of *TNF* genes in CD provide a signature biomarker that characterizes patients with CD and supports the implication of the environment and the innate immunity in the pathogenesis of CD.

CONFLICTS OF INTEREST

Guarantor of the article: Belén Beltrán, MD, PhD.

Specific author contributions: I.M.-T. and B.B.: study design, data collection, experimental analysis and interpretation, manuscript writing, and critical review. E.C. and E.S.-G.: data collection, manuscript writing, and critical review. J.S. and E.B.: experimental analysis and interpretation and critical review. D.H.: statistical analysis and interpretation. L.T.: data collection and critical review. M.I. and P.N.: study design and critical review. All authors reviewed and approved the final manuscript.

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Previous presentation: These results have been presented to the Annual Meetings of the American Gastroenterological Association (Digestive Disease Week 2019) in San Diego, California, USA (Gastroenterology, 2019 Vol. 156, Issue 6, S-1117) and to the European Crohn's and Colitis Organisation Congress 2019 (JCC, Volume 13, Issue Supplement_1, 25 January 2019, Pages S111–S112).

Study Highlights

WHAT IS KNOWN

- ✓ The estimated contribution of genetic variants to CD etiology accounts for only a small fraction, indicating a role for other factors, such as epigenetic alterations which link genotype with environment and disease.
- ✓ DNA methylation is a reversible and inheritable epigenetic mechanism that regulates gene expression.
- ✓ There are key biologic elements that are well-known to have an impaired gene expression in CD.

WHAT IS NEW HERE

- ✓ There are differential methylation profiles in selected genes among patients with CD and healthy controls: *DEFA5* CpG_11, CpG_13; *CAT* CpG_31.32; *TNF* CpG_4, CpG_12; and *ABCB1* CpG_21.
- ✓ Validation experiments in a prospective cohort confirm our results: *DEFA5* (methylation gain) and *TNF* (methylation loss) in CD.
- ✓ The methylation status of *DEFA5* and *TNF* genes is a signature biomarker that characterizes patients with CD.

TRANSLATIONAL IMPACT

- ✓ We provide a clinical tool to discriminate between CD and healthy control subjects.

REFERENCES

1. Bernstein CN. Review article: Changes in the epidemiology of inflammatory bowel disease—clues for aetiology. *Aliment Pharmacol Ther* 2017;46:911–9.
2. Gomollón F, Dignass A, Annesse V, et al. 3rd European evidence-based consensus on the diagnosis and management of Crohn's disease 2016: Part 1: Diagnosis and medical management. *J Crohns Colitis* 2017;11(1):3–25.
3. Jostins L, Ripke S, Weersma RK. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012;491:119–24.
4. Verstockt B, Smith KG, Lee JC. Genome-wide association studies in Crohn's disease: Past, present and future. *Clin Transl Immunol* 2018;7(1):e1001.
5. 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.
6. de Lange KM, Moutsianas L, Lee JC, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet* 2017;49:256–61.
7. Huang H, Fang M, Jostins L. Fine-mapping inflammatory bowel disease loci to single-variant resolution. *Nature* 2017;547(7662):173–8.
8. Bird A. Perceptions of epigenetics. *Nature* 2007;447:396–8.
9. Yung RL, Julius A. Epigenetics, aging, and autoimmunity. *Autoimmunity* 2008;41:329–35.
10. Holliday R. Epigenetics: A historical overview. *Epigenetics* 2006;1:76–80.
11. Marcin W, Michael S. Genetics and epigenetics of inflammatory bowel disease. *Swiss Med Wkly* 2018;148:w14671.
12. Lin Z, Hegarty JP, Cappel JA. Identification of disease-associated DNA methylation in intestinal tissues from patients with inflammatory bowel disease. *Clin Genet* 2011;80:59–67.
13. Nimmo ER, Prendergast JG, Aldhous MC. Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. *Inflamm Bowel Dis* 2012;18(5):889–99.
14. Karatzas PS, Mantzaris GJ, Safioleas M, et al. DNA methylation profile of genes involved in inflammation and autoimmunity in inflammatory bowel disease. *Medicine (Baltimore)* 2014;93(28):e309.
15. Beltrán B, Nos P, Dasí F, et al. Mitochondrial dysfunction, persistent oxidative damage, and catalase inhibition in immune cells of naïve and treated Crohn's disease. *Inflamm Bowel Dis* 2010;16(1):76–86.

16. Moret-Tatay I, Iborra M, Cerrillo E, et al. Possible biomarkers in blood for Crohn's disease: Oxidative stress and micrornas-current evidences and further aspects to unravel. *Oxid Med Cel Longev* 2016;2016:2325162.
17. Iborra M, Moret I, Rausell F. Role of oxidative stress and antioxidant enzymes in Crohn's disease. *Biochem Soc Trans* 2011;39(4):1102-6.
18. de Bruyn M, Vermeire S. NOD2 and bacterial recognition as therapeutic targets for Crohn's disease. *Expert Opin Ther Targets* 2017;21(12):1123-39.
19. Cerrillo E, Moret I, Iborra M, et al. Alpha-defensins (α -Defs) in Crohn's disease: Decrease of ileal α -Def 5 via permanent methylation and increase in plasma α -def 1-3 concentrations offering biomarker utility. *Clin Exp Immunol* 2018;192(1):120-8.
20. Bek S, Nielsen JV, Bojesen AB. Systematic review: Genetic biomarkers associated with anti-TNF treatment response in inflammatory bowel diseases. *Aliment Pharmacol Ther* 2016;44(6):554-67.
21. Iborra M, Moret I, Rausell F, et al. Different genetic expression profiles of oxidative stress and apoptosis-related genes in Crohn's disease. *Digestion* 2018;9:1-10.
22. Satsangi J, Silverberg MS, Vermeire S, et al. The montreal classification of inflammatory bowel disease: Controversies, consensus, and implications. *Gut* 2006;55(6):749-53.
23. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980;1(8167):514.
24. Jankipersadsing SA, van der Vlies P. Guidelines DNA quantity and quality for methylation projects. *UMCG Genet* 2013.
25. van den Boom D, Ehrlich M. Mass spectrometric analysis of cytosine methylation by base-specific cleavage and primer extension methods. *Methods Mol Biol* 2009;507:207-27.
26. Hui Z, Hastie T. Regularization and variable selection via the elastic net. *J R Statist Soc B* 2005;67(2):301-20.
27. Venthham NT, Kennedy NA, Nimmo ER, et al. Beyond gene discovery in inflammatory bowel disease: The emerging role of epigenetics. *Gastroenterology* 2013;145(2):293-308.
28. Pereira C, Coelho R, Grácio D. DNA damage and oxidative DNA damage in inflammatory bowel disease. *J Crohns Colitis* 2016;10(11):1316-23.
29. Chu CQ. Molecular probing of TNF: From identification of therapeutic target to guidance of therapy in inflammatory diseases. *Cytokine* 2018;101:64-9.
30. Li Yim AYY, Duijvis NW, Zhao J. Peripheral blood methylation profiling of female Crohn's disease patients. *Clin Epigenetics* 2016;8:65.
31. Cooke J, Zhang H, Greger L, et al. Mucosal genome-wide methylation changes in inflammatory bowel disease. *Inflamm Bowel Dis* 2012;18(11):2128-37.
32. Ehmann D, Wendler J, Koeninger L, et al. Paneth cell α -defensins HD-5 and HD-6 display differential degradation into active antimicrobial fragments. *Proc Natl Acad Sci U S A* 2019;116(9):3746-51.
33. Hu X, Deng J, Yu T, et al. ATF4 deficiency promotes intestinal inflammation in mice by reducing uptake of glutamine and expression of antimicrobial peptides. *Gastroenterology* 2019;156(4):1098-111.
34. Wehkamp J, Wang G, Kübler I. The Paneth cell alpha-defensin deficiency of ileal Crohn's disease is linked to Wnt/Tcf-4. *J Immunol* 2007;179(5):3109-1.
35. Gerseman M, Wehkamp J, Stange EF. Innate immune dysfunction in inflammatory bowel disease. *J Intern Med* 2012;271(5):421-8.
36. Cleynen I, Boucher G, Jostins L. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: A genetic association study. *Lancet* 2016;387(10014):156-67.
37. Taman H, Fenton CG, Hensel IV, et al. Genome-wide DNA methylation in treatment-naïve ulcerative colitis. *J Crohns Colitis* 2018;12(11):1338-47.
38. Iborra M, Nos P, Moret I, et al. Permanent catalase activity impairment due to minor expression of catalase protein in peripheral whitemononuclear cells of patients with naive and treated Crohn's disease. *J Crohn's Colitis* 2009;3(1):S128-P298.
39. Harris RA, Nagy-Szkal D, Pedersen N, et al. Genome-wide peripheral blood leukocyte DNA methylation microarrays identified a single association with inflammatory bowel diseases. *Inflamm Bowel Dis* 2012;18(12):2334-41.
40. Bryant RV, Winer S, Travis SP, et al. Systematic review: Histological remission in inflammatory bowel disease. Is "complete" remission the new treatment paradigm? An IOIBD initiative. *J Crohns Colitis* 2014;8(12):1582-97.
41. Ramesh R, Kozhaya L, McKeivitt K, et al. Pro-inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to glucocorticoids. *Jexp Med* 2014;211(1):89-104.
42. Eder P, Łykowska-Szuber L, Krela-Każmierczak I, et al. Disturbances in apoptosis of lamina propria lymphocytes in Crohn's disease. *Arch Med Sci* 2015;11(6):1279-85.
43. Ślebioda TJ, Kmiec Z. Tumour necrosis factor superfamily members in the pathogenesis of inflammatory bowel disease". *Mediators Inflamm* 2014;2014:15.
44. Clark IA. How TNF was recognized as a key mechanism of disease. *Cytokine Growth Factor Rev* 2007;18(3-4):335-43.
45. van Deventer SJ. Transmembrane TNF-alpha, induction of apoptosis, and the efficacy of TNF-targeting therapies in Crohn's disease. *Gastroenterology* 2001;121(5):1242-6.
46. Cholapranee A, Hazlewood GS, Kaplan GG, et al. Systematic review with meta-analysis: Comparative efficacy of biologics for induction and maintenance of mucosal healing in Crohn's disease and ulcerative colitis controlled trials. *Aliment Pharmacol Ther* 2017;45(10):1291-302.
47. Medrano LM, Taxonera C, Márquez A. Role of TNFRSF1B polymorphisms in the response of Crohn's disease patients to infliximab. *Hum Immunol* 2014;75(1):71-5.
48. Ananthakrishnan S, Andersen PS, Burisch J. Genetically determined high activity of IL-12 and IL-18 in ulcerative colitis and TLR5 in Crohns disease were associated with non-response to anti-TNF therapy. *Pharmacogenomics J* 2018;18(1):87-97.
49. Steenholdt C, Enevold C, Ainsworth MA, et al. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1b and fas ligand are associated with clinical efficacy and/or acute severe infusion reactions to infliximab in Crohn's disease. *Aliment Pharmacol Ther* 2012;36(7):650-9.
50. Yano S, Yano N. Regulation of catalase enzyme activity by cell signaling molecules. *Mol Cel Biochem* 2002;240(1-2):119-30.
51. Peyrin-Biroulet L, Beisner J, Wang G, et al. Peroxisome proliferator-activated receptor gamma activation is required for maintenance of innate antimicrobial immunity in the colon. *Proc Natl Acad Sci U S A* 2010;107(19):8772-7.
52. Dubuquoy L, Rousseaux C, Thuru X, et al. PPAR γ as a new therapeutic target in inflammatory bowel diseases. *Gut* 2006;55(9):1341-9.
53. Fernandes P, MacSharry J, Darby T, et al. Differential expression of key regulators of toll-like receptors in ulcerative colitis and Crohn's disease: A role for tollip and peroxisome proliferator-activated receptor gamma? *Clin Exp Immunol* 2016;183(3):358-68.
54. Diaz-Meco MT, Moscat J. The atypical PKCs in inflammation: NF- κ B and beyond. *Immunol Rev* 2012;246(1):154-67.

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