RESEARCH ARTICLE

Endocrine disruption in Crohn's disease: Bisphenol A enhances systemic inflammatory response in patients with gut barrier translocation of dysbiotic microbiota products

Raquel Linares[1] | Mariana F. Fernández[2,3,4] | Ana Gutiérrez[5,6] | Rocío García-Villalba[7] | Beatriz Suárez[2,3,4] | Pedro Zapater[5,6,8] | José Alberto Martínez-Blázquez[7] | Esther Caparrós[1,5] | Francisco A. Tomás-Barberán[7] | Rubén Francés[1,5,6] iD

[1]Departamento de Medicina Clínica, Universidad Miguel Hernández, San Juan de Alicante, Spain

[2]Centro de Investigación Biomédica (CIBM), Universidad de Granada, Granada, Spain

[3]Instituto de Investigación Biosanitaria (ibs. GRANADA), Granada, Spain

[4]CIBERresp, Instituto de Salud Carlos III, Madrid, Spain

[5]IIS ISABIAL, Hospital General Universitario de Alicante, Alicante, Spain

[6]CIBERehd, Instituto de Salud Carlos III, Madrid, Spain

[7]CEBAS-CSIC, Universidad de Murcia, Murcia, Spain

[8]Departamento de Farmacología, Universidad Miguel Hernández, San Juan de Alicante, Spain

Correspondence

Rubén Francés, Hepatic and Intestinal Immunobiology Group, Departamento de Medicina Clínica, Universidad Miguel Hernández de Elche, Carretera Alicante-Valencia, Km 8,7 03550 San Juan de Alicante, Spain.
Email: rfrances@umh.es

Abstract

The relevance of environmental triggers in Crohn's disease remains poorly explored, despite the well-known association between industrialization and disease onset/progression. We have aimed at evaluating the influence of endocrine disrupting chemicals in CD patients. We performed a prospective observational study on consecutive patients diagnosed of CD. Serum levels of endocrine disruptors, short-chain fatty acids, tryptophan and cytokines were measured. Bacterial-DNA and serum endotoxin levels were also evaluated. Gene expression of ER-α, ER-β and GPER was measured in PBMCs. All patients were genotyped for NOD2 and ATG16L1 polymorphisms. A series of 200 CD patients (140 in remission, 60 with active disease) was included in the study. Bisphenol A was significantly higher in patients with active disease versus remission and in colonic versus ileal disease. GPER was significantly increased in active patients and correlated with BPA levels. BPA was significantly increased in patients with bacterial-DNA and correlated with serum endotoxin levels, (r = 0.417; P = .003). Serum butyrate and tryptophan levels were significantly lower in patients with bacterial-DNA and an inverse relationship was present between them and BPA levels (r = −0.491; P = .001) (r = −0.611; P = .001). Serum BPA levels correlated with IL-23 (r = 0.807; P = .001) and IL-17A (r = 0.743; P = .001). The multivariate analysis revealed an independent significant contribution of BPA and bacterial-DNA to serum levels of IL-23 and IL-17A. In conclusion, bisphenol A significantly affects systemic inflammatory response in CD patients with gut barrier disruption and dysbiotic microbiota secretory products in blood. These results provide evidence of an endocrine disruptor playing an actual pathogenic role on CD.

Abbreviations: ATG16L1, Autophagy Related 16 Like 1 gene; BPA, bisphenol A; BT, bacterial translocation; BuPB, butylparaben; BzP, benzophenones; CD, Crohn's disease; CDAI, Crohn's disease activity index; EDCs, endocrine disrupting chemicals; ER, estrogen receptor; EtPB, ethylparaben; GPER, G Protein-Coupled Estrogen Receptor 1; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; MePB, methylparaben; NOD2, nucleotide binding oligomerization domain containing 2 gene; PBMCs, peripheral blood mononuclear cells; PrPB, propylparaben; SCFAs, short-chain fatty acids.

1 | BACKGROUND

Crohn's disease (CD) is a major form of inflammatory bowel disease with a relevant incidence worldwide.[1] The genetic background, several environmental factors and a deregulated immune interaction with the gut microbiota content are involved in the development and progression of this chronic multifactorial disease.[2]

The inflammatory response in CD patients shows important local and systemic exacerbations that characterize disease flares. Even during remission periods, a pathological Th17 response is expanded in gut tissue of CD patients, and the functional activity of epithelial cells is significantly decreased.[3] Gut bacterial antigen translocation into blood contributes to systemic dissemination of pro-inflammatory molecules such as TNF-alpha or IL-6, and it constitutes an independent risk factor of flare in CD patients.[4,5] Bacterial translocation events are facilitated by the microbiota dysbiotic state acquired during disease and the increased permeability induced by the barrier integrity loss and the inflammatory infiltration developed in response to microbiota composition changes.[6] The effect of bacterial antigen translocation on systemic inflammation is particularly important in patients bearing variant NOD2 genotypes.[7]

In the recent years, the role of environmental chemical pollutants in the pathogenesis of metabolic diseases has been investigated. Endocrine-disrupting chemicals (EDCs) are a group of pollutants able to interfere with different hormone actions.[8] The relationship between obesity and specific EDCs exposure has been proven in different experimental models.[9-11] This interaction has also been described to predispose to chronic diseases such as type 2 diabetes,[10,12,13] and non-alcoholic fatty liver disease (NAFLD).[14,15] Moreover, the relationship established between EDCs and increased prevalence of obesity and type 2 diabetes has been supported by epidemiological studies.[16,17]

Certain EDCs, such as bisphenol A (BPA) may affect intestinal microbial amino acid metabolism.[18] Dietary BPA intake is considered to influence the composition of gut microbiota.[19] In fact, the microbiota of mice fed with BPA significantly reduces its diversity of species.[20] In these animals, BPA favors increments in Proteobacteria while Clostridia populations are reduced, clearly pointing towards a state of microbiota dysbiosis. Alterations in the abundance of Proteobacteria have been associated with the instability of gut microbiota composition which, in turn, is considered as a driver of intestinal inflammation.[21] As gut dysbiosis in patients with Crohn's disease (CD) favors bacterial antigen translocation and an altered immune interaction with the host,

facilitating disease flares, EDCs may constitute a trigger for dysbiosis-related inflammatory exacerbation in CD patients.

While both genetic predisposition and microbiota-derived factors have been more intensively studied in CD, the relevance of metabolic associated triggers remain less explored, despite the known association between factors such as diet or hormones[22] with disease onset and progression. This proof-of-concept study has aimed at evaluating the influence of serum EDCs in disease activity and phenotype, their interaction with dysbiosis-derived bacterial products and the systemic inflammatory response in CD patients.

2 | PATIENTS AND METHODS

2.1 | Patients and study design

Prospective observational study on consecutive patients diagnosed of CD and managed at the IBD Unit of Hospital General Universitario de Alicante. Standard clinical, endoscopic, histological and radiographical criteria were used to establish diagnosis.[23] Clinical and analytical characteristics of patients at inclusion were recorded. Disease activity was determined by Crohn's disease activity index (CDAI) > 150 and presence of clinical symptoms of relapse. The use of antibiotics in the previous 4 weeks, signs of active infection and refusal to sign informed consent to participate in the study were considered as exclusion criteria. All patients were categorized by the Montreal classification. All included patients were provided with diaries in order to record their symptoms a week prior to inclusion in the study.

Peripheral blood samples were collected aseptically Vacutainer SST II (BD Diagnostics, Erembodegem, Belgium). Serum and peripheral blood mononuclear cells (PBMCs) were collected, aliquoted and immediately stored at −80°C. When available, ileal biopsies of patients undergoing colonoscopy as part of their routine clinical management were collected. All patients signed an informed consent to participate in the study and the Ethics Committee of Hospital General Universitario de Alicante approved the study protocol.

2.2 | Quantification of endocrine disruptors in serum samples of CD patients

EDCs bisphenol-A (BPA), ethylparaben (EtPB), methylparaben (MePB), propylparaben (PrPB), butylparaben (BuPB), and

benzophenones (BzP-1 and -3) levels were quantified in serum samples using optimized techniques,[24,25] and analyzed by dispersive liquid–liquid micro-extraction (DLLME) and ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). Collecting, storing, and processing of serum biospecimens were performed under controlled conditions, avoiding potential external contamination from collection containers, equipment or labware.

2.3 | Estrogen receptors measurements in PBMCs from CD patients

RNA isolation and gene expression analysis for estrogen receptor (ER)-α, ER-β and G Protein coupled Estrogen Receptor 1 (GPER) were done using previously reported methodology.[26] Primer pairs are described in Supplementary Table 1.

2.4 | Microbial products in serum samples of CD patients

Bacterial DNA was detected by 16SrRNA gene amplification by polymerase chain reaction as previously described.[27] Patients above the 10pg detection limit were considered as bacterial DNA positive. Serum endotoxin was measured by handling a chromogenic limulus amoebocyte lysate test (BioWhittaker, Nottingham, UK) following manufacturer's instructions and as described elsewhere.[28] Short-chain fatty acids (SCFAs) were measured by gas chromatography–mass spectrometry (GC-MS) on plasma samples (200 μL). Samples processing and analysis were performed following methodology previously reported and optimized by García-Villalba et al.[29]

2.5 | Tryptophan and GPER quantification in serum of CD patients

Serum levels of tryptophan and GPER were measured by handling a fluorometric Tryptophan assay kit (Abcam, Cambridge, UK) and the human GPER ELISA Kit (Cloud-Clone Corp., Houston, TX), respectively, following manufacturer's instructions. Samples were run in triplicate and read at 440 nm for tryptophan and at 450 nm for GPER for quantification.

2.6 | Tight-junctions gene expression levels

RNA from available ileal samples conserved in RNAlater was isolated and used to analyze gene expression of zona occludens (ZO)-1, claudin-1 and occludin. Primer pairs are described in Supplementary Table 1.

2.7 | CD associated polymorphisms genotyping

Single-nucleotide polymorphisms for NOD2/CARD15 (SNP-8, R702W, rs2066844; SNP-12, G908R, rs2066845; SNP-13, L1007finsC, rs2066847) and the ATG16L1 variation rs2241880 were genotyped as previously described.[7] A variant genotype was considered either present in homozygosis or heterozygosis.

2.8 | Statistical analysis

The statistical analysis of results was run on SPSS 19.0 software (Chicago, IL). Qualitative variables are described as frequencies or percentages. Quantitative variables following normal distribution were described as mean and standard deviation. The differences between groups in the experimental qualitative and quantitative variables were studied by means of the chi-square test and either the Student's t test or the Mann-Whitney U test for quantitative variables. Bonferroni correction was used for multiple comparisons. The association between continuous variables was explored through correlation analyses, and multivariate linear regression analyses were carried out to explain the variability and possible associations of variables. Significance was considered for $P < .05$ values.

3 | RESULTS

3.1 | Clinical and analytical characteristics of CD patients

A series of 200 CD patients (140 patients in remission, 60 patients with active disease) were included in the study. Mean CDAI scores were 94 ± 27 versus 181 ± 38, $P = .001$; Fecal calprotectin levels were 95.8 ± 56.4 versus 840.3 ± 580.6, $P = .001$; and PCR values were 0.37 ± 0.60 versus 0.84 ± 0.90, $P = .09$, respectively. No differences in the Montreal classifications were present between patients in remission and active patients. Table 1 resumes clinical and analytical characteristics of both groups of CD patients.

3.2 | Serum endocrine disruptors in CD patients

Serum levels of selected endocrine disruptors are shown in Table 2. BPA levels showed the highest concentrations in blood among studied EDCs and, despite variability, its concentration was significantly elevated in active patients compared to patients in remission (Figure 1A). No correlations were found between serum BPA levels, calprotectin and CRP in patients in remission. However, a significant correlation

	Patients in remission (n = 140)	Patients with active disease (n = 60)
Gender, male (%)	67 (47.8%)	30 (50%)
Age (years)	40 ± 14	40 ± 15
Smoking habit, yes (%)	47 (33.5%)	25 (41.6%)
CDAI	72.7 ± 38.5	230 ± 83.7*
Disease duration (months)	110 ± 100	85 ± 100
Previous surgery, yes (%)	42 (30%)	16 (26.6%)
Montreal classification, n (%)		
Age of onset		
A1	10 (7.1%)	4 (6.7%)
A2	103 (73.6%)	43 (71.6%)
A3	27 (19.3%)	13 (21.7%)
Location		
Ileal	69 (49.3%)	34 (56.7%)
Ileocolonic	29 (20.7%)	9 (15%)
Colonic	42 (30%)	17 (28.3%)
Behavior		
1	73 (52.1%)	29 (48.4%)
1p	16 (11.4%)	8 (13.3%)
2	19 (13.6%)	11 (18.3%)
2p	5 (3.6%)	–
3	21 (15%)	8 (13.3%)
3p	6 (4.3%)	4 (6.7%)
Perianal activity, yes (%)	27 (19.3%)	11 (18.3%)
Extraintestinal manifestations, yes (%)	27 (19.3%)	7 (11.7%)
Therapy, n (%)		
No treatment	38 (27.2%)	15 (25%)
IS	57 (40.7%)	29 (48.3%)
Biologics	29 (20.7%)	11 (18.4%)
IS + Biologics	16 (11.4%)	5 (8.3%)
Use of steroids, yes (%)	9 (6.4%)	26 (43.3%)*
Total leukocytes (mm^3)	6946 ± 2520	7736 ± 2670
Hemoglobin (g/dL)	13.8 ± 1.4	13.1 ± 1.5
Albumin (g/dL)	4.1 ± 0.4	3.6 ± 0.6
CRP (mg/dL)	0.4 ± 0.6	1.7 ± 3.0*
Fecal calprotectin (µg/g)	95.6 ± 56.2	795.8 ± 545.5*

Abbreviation: CRP, C-reactive protein.

*$P < .01$ compared to patients in remission.

was found between fecal calprotectin and serum BPA levels among active CD patients (Figure 1B). Gene expression levels of ER-α (Figure 1C) and ER-β (Figure 1D) in PBMCs were not significantly different between patients in remission and active patients. GPER mRNA expression levels were significantly increased in patients with active disease (Figure 1E). GPER protein levels correlated positively with BPA levels ($r = 0.708$; $P = .001$) (Figure 1F), Phenotypically, serum BPA levels were significantly increased in patients with colonic versus ileal disease (Figure 1G). No differences in serum BPA levels were observed according to the presence of perianal disease or the use of biologics, either in regular or intensified regime. No correlations were found for the rest of EDCs investigated on CD disease activity or phenotype. No significant differences by gender were present in our cohort for EDCs and estrogen receptor levels (Supplementary

TABLE 2 Serum levels of evaluated endocrine disrupting chemicals.

Concentration (µM)	Patients in remission (n = 140)	Patients with active disease (n = 60)
BPA	5.57 ± 8.29	11.98 ± 20.25[*]
MePB	3.67 ± 5.72	3.26 ± 5.50
EtPB	0.90 ± 2.79	0.31 ± 0.55
PrPB	0.32 ± 0.82	0.15 ± 0.25
BuPB	0.07 ± 0.40	0.04 ± 0.13
BzP1	0.10 ± 0.52	0.05 ± 0.14
BzP3	0.21 ± 1.46	0.03 ± 0.09

Abbreviations: BPA, bisphenol A; BuPB, butylparaben; BzP-1, benzophenone-1; BzP-3, benzophenone-3; EtPB, ethylparaben; MePB, methylparaben; PrPB, propylparaben.

*$P < .01$ compared to patients in remission.

Table 3). No significant differences among women distributed by the use of hormonal contraceptives or by being on menopause were present in our cohort of patients for estrogen receptor gene expression levels (Supplementary Table 4).

3.3 | Serum BPA levels and microbiota dysbiotic products in CD patients

We evaluated the interaction between BPA levels with several surrogated markers of microbiota dysbiosis in CD patients. First, thirty-eight patients among those in remission (27.1%) and 32 active patients (53.3%) showed detectable bacterial DNA in blood. Serum BPA levels were significantly higher in patients with bacterial DNA translocation, both in remission and active groups (Figure 2A), and a positive correlation between fecal calprotectin and BPA levels in the overall series of patients with bacterial DNA, irrespective of disease activity (Figure 2B). ZO-1 and claudin 1 mRNA levels in available ileal samples from patients with versus without bacterial DNA translocation were significantly decreased regardless disease activity (Figure 2C-E). No significant differences were found in ER-α and ER-β gene expression levels between remission and disease activity in available ileal samples. However, GPER transcriptional levels were significantly increased in ileal samples from active patients compared to those in remission (Supplementary Table 5).

To determine whether an active disease-independent association between bacterial DNA translocation and serum BPA levels was present, a linear regression analysis was run including both variables. Disease activity (OR 4.473 [1.472-7.474], $P = .004$) and bacterial DNA translocation (OR 6.346 [3.664-9.028], $P = .001$) remained as independent variables associated with serum BPA levels.

Second, serum endotoxin levels were significantly higher in the overall series of active patients versus patients in remission ($2.6 ± 1.1$ vs $1.1 ± 0.8$ UE/mL, $P = .001$). Serum endotoxin levels were significantly higher in patients with bacterial DNA in blood compared to those without translocation of bacterial genomic fragments, even among patients in remission (Figure 3A). Serum BPA levels significantly correlated with endotoxin levels, irrespective of disease activity ($r = 0.417$; $P = .003$) (Figure 3B).

Third, serum levels of SCFAs were measured in all included patients (Table 3). As shown, although serum levels of all detected SCFAs were higher among patients in remission compared to active patients, no statistically significant difference was reached for any SCFA. Valeric acid was not detected in the serum of our series of CD patients. However, butyric, isobutyric, and isovaleric acids were significantly decreased among patients with bacterial DNA translocation, irrespective of disease activity (Table 3), Serum butyric acid levels were significantly lower in patients with bacterial DNA in blood compared to those without bacterial DNA translocation, even among patients in remission (Figure 3C). An inverse relationship was present between serum butyric acid and BPA levels ($r = −0.491$; $P = .001$) (Figure 3D).

Finally, tryptophan levels were measured in the serum of CD patients. Although patients with active disease showed lower levels than patients in remission, only patients without evidence of bacterial antigen translocation in blood presented significantly lower levels of tryptophan compared to patients without bacterial DNA in blood (Figure 3E). Tryptophan levels showed an inverse significant correlation with BPA levels ($r = −0.611$; $P = .001$) (Figure 3F) in the overall series of patients.

3.4 | Serum BPA levels are not associated with variant NOD2 and ATG16L1 genotypes in CD patients

NOD2 and ATG16L1 were genotyped in all patients. One hundred and nineteen patients (59.5%) presented any of the studied NOD2 variant genotypes, whereas 83 patients (41.5%) showed an ATG16L1 variant genotype. The combination of both variant genotypes was present in 55 CD
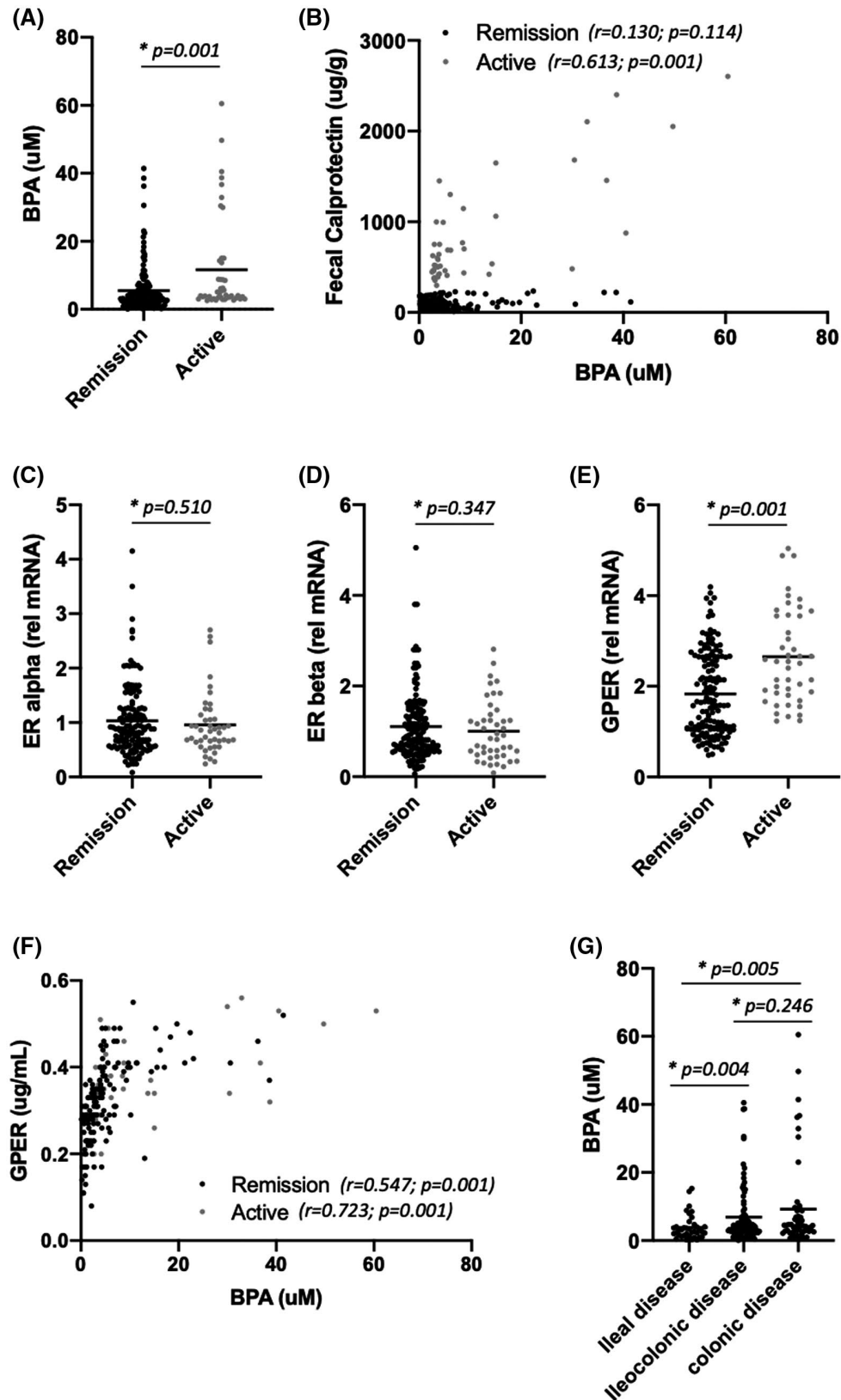
FIGURE 1 A, BPA levels in serum of CD patients distributed by disease activity. B, Correlation between serum BPA and fecal calprotectin in CD patients distributed by disease activity. C-E, Relative gene expression levels of ER-α (C), ER-β (D) and GPER (E) in PBMCs of CD patients distributed by disease activity. F, Correlation between serum BPA and GPER in CD patients distributed by disease activity. G, BPA levels in serum of CD patients distributed by disease location. BPA, bisphenol A; ER, estrogen receptor; GPER, G protein-coupled estrogen receptor
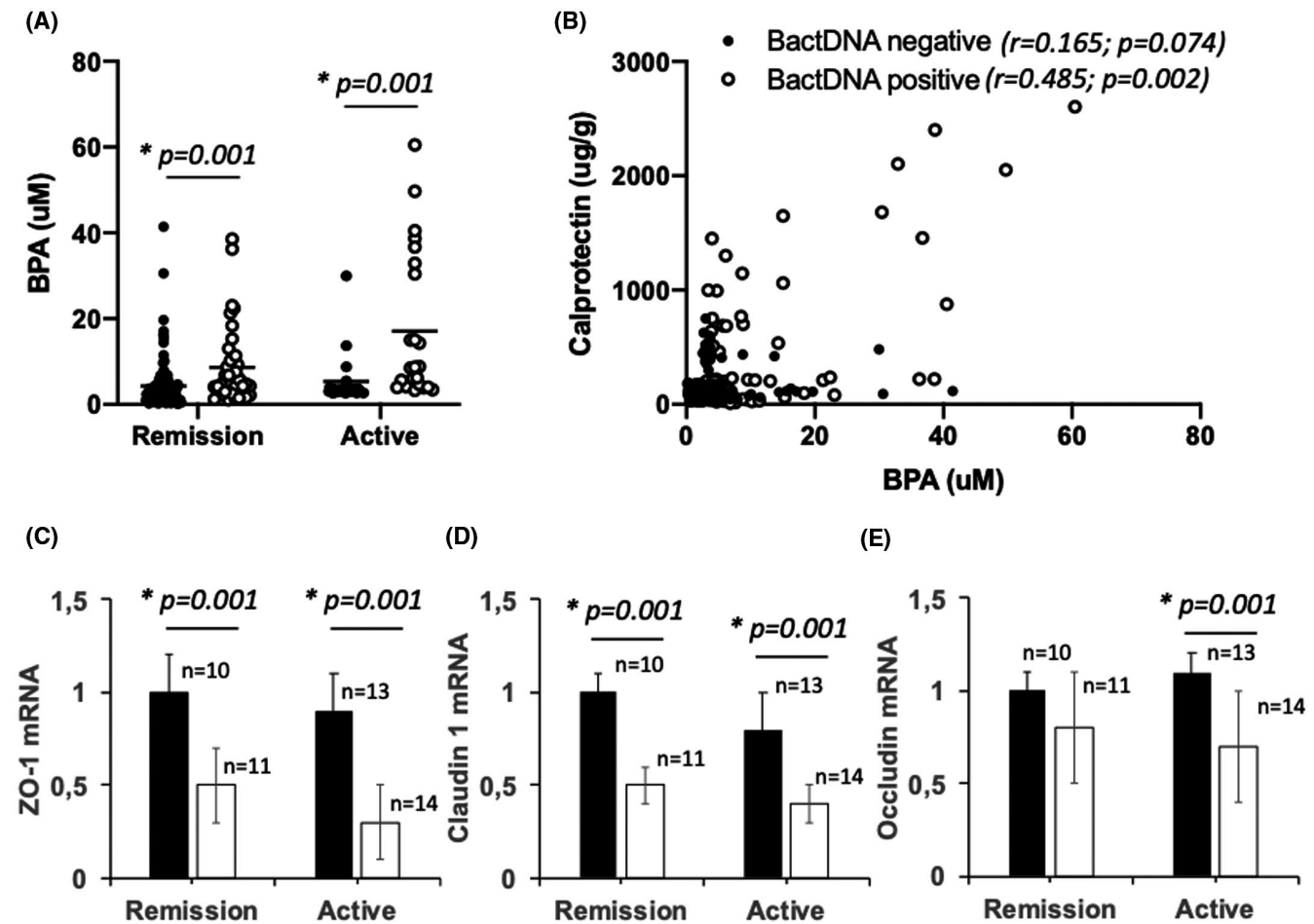
FIGURE 2 A, BPA levels in serum of CD patients sorted by the evidence of bacterial DNA translocation. B, Correlation between serum BPA and fecal calprotectin in CD patients distributed by the presence of bacterial DNA translocation. C-E, Relative gene expression levels of ZO-1 (C), claudin 1 (D) and occludin (E) in available ileal samples of CD patients distributed by the presence of bacterial DNA translocation. BPA, bisphenol A; ZO-1, zona occludens 1

patients (27.3%). No differences in serum BPA levels were present between CD patients according to this genotyping distribution (Supplementary Table 2).

3.5 | Serum BPA levels influence the systemic inflammatory response in CD patients

Table 4 resumes serum cytokine levels in our series of CD patients. Pro-inflammatory effector cytokines IFN-g and IL-17A, and their regulatory cytokines IL-12, IL-6 and IL-23 were increased in active patients, although differences didn't reach statistical significance. However, the presence of bacterial DNA in blood significantly increased IL-23 and IL-17A, irrespective of disease activity. Serum BPA levels showed a weak correlation with IL-12 ($r = 0.353$; $P = .005$) and IFNg ($r = 0.315$; $P = .007$). A stronger positive correlation was present between serum BPA and IL-23 ($r = 0.807$; $P = .001$) (Figure 4A) and IL-17A ($r = 0.743$; $P = .001$) (Figure 4B). Serum LPS, butyric acid and tryptophan levels were also

associated with IL-23 and IL-17A levels in our series of CD patients. Treatments were not associated with significant differences in any cytokine levels. Among all variables, the multivariate analysis revealed an independent significant contribution of BPA and bacterial DNA to serum levels of IL-23 and IL-17A (Table 5).

4 | DISCUSSION

The present proof-of-concept study shows the multifactorial interaction of BPA on Crohn's disease. Serum BPA is associated with activity and phenotype of disease, it correlates with BT markers and microbiota secretory products, and it independently influences patients' systemic inflammatory response. These results provide evidence of an endocrine-disrupting factor playing an actual pathogenic role on CD.

Identifying the causes driving CD is a complex task. While a consensus exists regarding the implication of genetic,
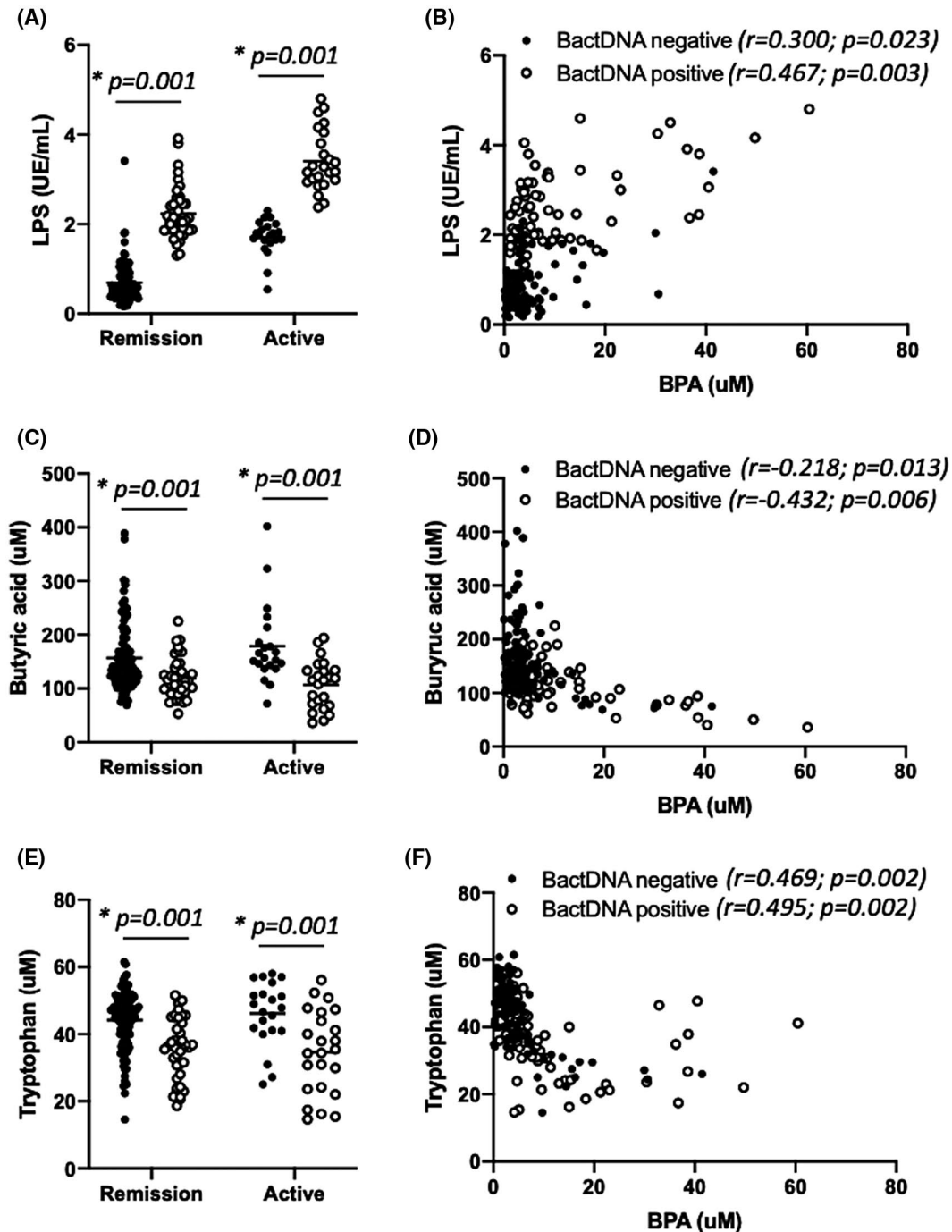
FIGURE 3 A, LPS levels in serum of CD patients sorted by disease activity and the evidence of bacterial DNA translocation. B, Correlation between serum BPA and LPS in CD patients distributed by the presence of bacterial DNA translocation. C, Butyric acid levels in serum of CD patients distributed by disease activity and the presence of bacterial DNA translocation. D, Correlation between serum BPA and butyric acid in CD patients sorted by the evidence of bacterial DNA translocation. E, Serum tryptophan levels in serum of CD patients distributed by disease activity and the presence of bacterial DNA translocation. F, Correlation between serum BPA and tryptophan in CD patients sorted by the presence of bacterial DNA translocation. BPA, bisphenol A; LPS, lipopolysaccharide

epithelial, immune, microbial and environmental factors, the relationships established between some of these factors remain partially unraveled. Although data analyzed from several GWAS identified 30 CD-specific loci, along with additional 110 loci shared with ulcerative colitis,[30] genetic background seems to only explain partially the susceptibility

to the disease.[31] The relevance of a fine-tuned gut immunity to achieve homeostasis reveals the central role of immune alterations in developing local inflammation that may lead to systemic dissemination.[32,33] In intimate relation with this, microbiota eubiosis is lost during CD and this fact centers microbial community and their products in the onset and

TABLE 3 Serum levels of SCFAs.

	Patients in remission	Patients with active disease	Patients without bactDNA	Patients with bactDNA
Acetic acid (µM)	592.8 ± 674.4	597.4 ± 382.4	588.9 ± 724.6	603.8 ± 320.6
Propionic acid (µM)	69.6 ± 66.7	66.3 ± 22.5	70.4 ± 71.8	65.9 ± 19.2
Butyric acid (µM)	146.3 ± 58.2	140.6 ± 69.5	160.4 ± 63.8	114.3 ± 39.7[*]
Isobutyric acid (µM)	104.6 ± 43.2	93.6 ± 23.1	106.5 ± 46.2	95.0 ± 24.9[*]
Isovaleric acid (µM)	251.3 ± 111.1	227.7 ± 71.1	257.3 ± 114.5	227.7 ± 80.3[*]

*$P = .001$ compared to patients without bactDNA.

TABLE 4 Serum levels of cytokines

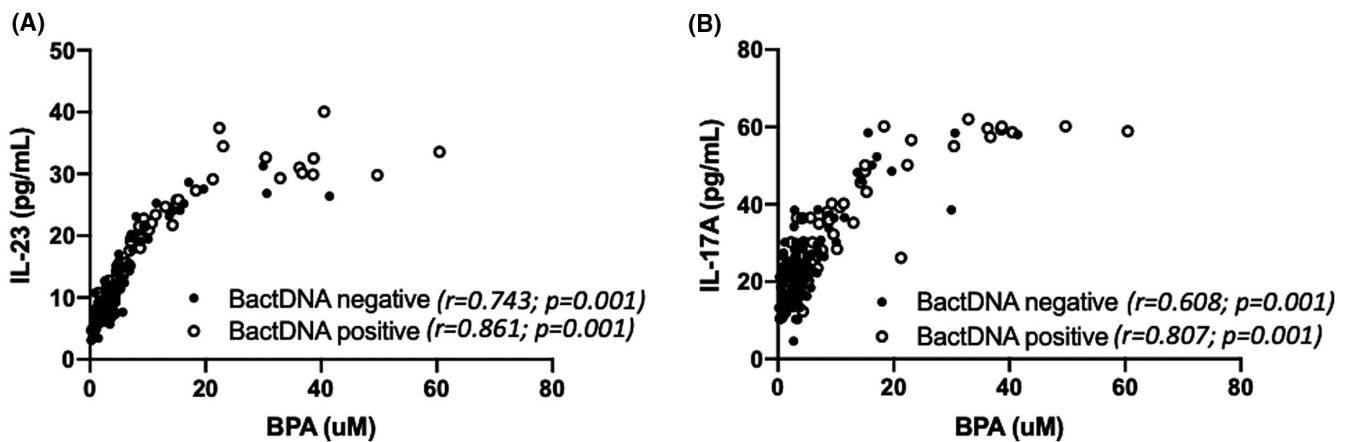|	Patients in remission	Patients with active disease	Patients without bactDNA	Patients with bactDNA
IL-12 (pg/mL)	38.6 ± 17.2	42.5 ± 16.9	37.7 ± 16.2	43.1 ± 18.5
IFNg (pg/mL)	21.1 ± 10.9	26.1.3 ± 11.5	22.1 ± 10.2	27.7 ± 12.4
IL-6 (pg/mL)	28.9 ± 16.3	27.7 ± 13.5	28.2 ± 16.3	29.5 ± 14.5
IL-23 (pg/mL)	12.6 ± 10.4	16.5 ± 8.9	11.3 ± 6.3	18.1 ± 9.1[*]
IL-17A (pg/mL)	26.6 ± 11.6	32.0 ± 16.6	23.9 ± 10.8	33.3 ± 15.2[*]

*$P = .01$ compared to patients without bactDNA.

FIGURE 4 A, Correlation between serum BPA and IL-23 levels in CD patients sorted by the presence of bacterial DNA translocation. B, Correlation between serum BPA and IL-17A levels in CD patients sorted by the detection of bacterial DNA translocation. BPA, bisphenol A; IL, interleukin

progression of disease.[34-36] While the interaction between all these factors have been intensively studied, the role for certain environmental aspects in this multifactorial disease is yet to be placed.[22,37] Studies focused in causative environmental factors are challenging and have shown conflicting data.[38] However, the relevance of endocrine and metabolic factors is demonstrated by the rapid growth of IBD incidence in newly developing countries, probably as a consequence of an increasing westernized lifestyle.[39] Moreover, studies show that disease incidence in migrants is more similar to that in their adopted countries rather than in their country of origin, reflecting the importance of the environmental risk factors such as lifestyle, pollution or diet.[40-42]

EDCs are environmental pollutants that have shown to affect chronic metabolic diseases,[11,12] and to interact with gut microbiota favoring dysbiosis.[19] Among different non-persistent EDCs evaluated, our results show that BPA is significantly increased in serum of patients with active disease *versus* patients in remission. Regarding disease phenotype, serum BPA levels were higher in colonic *versus* ileal disease forms. Experimental studies have shown that BPA worsens disease activity during DSS-induced colitis, exacerbating intestinal inflammation,[18] and a study evaluating urinary phenolic compounds observed an association between increased 4-tert-octylphenol levels and ulcerative colitis in a small subpopulation of IBD patients.[43] Estrogen receptors (ERs) may

TABLE 5 Multivariate analysis on serum cytokine levels

	β	IC 95%	P
IL-23			
Disease activity (yes)	0.064	−2.072 to 2.201	.953
Bacterial DNA (yes)	**3.503**	**1.047 to 5.959**	**.005**
LPS	−0.959	−2.424 to 0.506	.198
BPA	**1.685**	**1.602 to 1.769**	**.001**
Butyric acid	−0.002	−0.014 to 0.010	.722
IL-17A			
Disease activity (yes)	−0.885	−4.540 to 2.770	.633
Bacterial DNA (yes)	**1.813**	**1.388 to 3.015**	**.006**
LPS	−0.348	−2.854 to 2.159	.785
BPA	**1.146**	**1.003 to 1.289**	**.001**
Butyric acid	0.001	−0.020 to 0.020	.969

Bold value is used to remark statistically significant variables.

Abbreviations: BPA, bisphenol A; IL, interleukin; LPS, lipopolysaccharide.

serve as signal transducers for xenoestrogens EDCs.[44] ERs have been implicated in CD activity. While lower levels of ER-β have been described in CD patients' peripheral T cells, a higher expression of ER-α has been reported in this same population.[45] In fact, the ratio ER-β/ER-α has been proposed as a tool for non-invasively monitoring CD activity.[46] While we observe no significant differences in either ER-β or ER-α, a significant increase in GPER gene and protein expression levels can be observed in PBMCs, as well as in available ileal samples, from active CD patients. In line with this, Jacenik et al found GPER overexpression in intestinal mucosa samples from male CD patients and, by handling a murine model of CD, they showed that lower GPER expression is related with reduced colonic inflammation.[47] Also, human mature adipocytes obtained from subcutaneous mammary adipose tissue and exposed to BPA increased GPER levels more than two-fold.[48] Although we don't find differences in ERs transcriptional levels among women according to the use of hormonal contraceptives or menopause, this effect cannot be completely discarded, as the number of patients in those subgroups is short in our cohort.

Different experimental studies have also presented evidence of microbiota modification in response to BPA exposure. We were interested in evaluating this association in CD, as a gut microbiota dysbiotic state is present in these patients,[34,36] contributing to inflammatory exacerbation.[2,49] Different surrogated markers of dysbiosis such as translocation of bacterial DNA, increased serum endotoxin levels or a reduced concentration of SCFAs in blood were studied in CD patients. Bacterial DNA translocation and endotoxin levels in blood were increased in active CD patients and a significant correlation could be established between both markers and BPA serum levels by linear regression analyses. These results support a disease activity-independent association between BPA and circulating bacterial products in the blood of CD patients. Accordingly, exposure of C57BL/6 mice to dietary BPA has been reported to reduce gut microbiota diversity and to increase *Proteobacteria*,[20] common signatures of microbiota changes present in chronic inflammatory disorders. Additionally, BPA has been implicated in inducing hepatic steatosis in CD-1 mice after promoting microbiota dysbiosis and gut-liver axis activation.[50] These exposed mice show increased serum LPS, as shown by us in CD patients, and a disrupted gut barrier integrity, a hallmark in the pathophysiology of CD.[2,49]

Further evidence on BPA interaction with bacterial-derived metabolites in CD patients is supported by the reduction observed in serum SCFAs, particularly butyric acid, and its significant inverse correlation with BPA. SCFAs are important modulators of gut homeostasis and their decrease is associated with a reduction in SCFA-producing bacterial clusters such as *Clostridia*, which are decreased in chronic inflammatory bowel and liver diseases.[34,36,51,52] It is plausible that BPA contribution to the dysbiotic state may be influencing SCFAs serum levels in CD patients. In fact, an inverse significant correlation is observed between BPA and butyric acid levels in these patients. Also, a significant reduction in Tryptophan levels, which have been related to disease activity in CD,[53] is observed among patients with evaluated markers of dysbiosis in our series of patients. Actually, the reduction in Tryptophan and metabolites derived from aromatic aminoacids (MDAs) has been reported in feces of BPA-treated mice.[18] Also, as microbiota content is more abundant in colon versus ileal segments, changes in microbiota composition, and their metabolite interaction with other inflammatory triggers becomes especially relevant in this tissue. This may partially explain the association observed between BPA and colonic disease in our cohort of CD patients.

In addition to the close interaction observed between BPA serum levels and gut microbiota dysbiotic circulating products, this EDC pollutant seems to independently relate to systemic pro-inflammatory cytokine levels in CD patients (Figure 4). In line with these results, the ability of BPA to induce an inflammatory response has been reported previously in several studies. The amount of IL-12, among other cytokines, is increased in colonic samples from DSS-mice treated with BPA.[18] Serum TNF-α, IL-6 and IL-18 levels were significantly increased in BPA-fed CD-1 mice compared to controls.[50] Studies in humans have also found positive correlations between BPA exposure and increased serum proinflammatory cytokines such as TNF-α or IL-6.[54,55] Of interest, we here show a direct relationship with IL-23 and IL-17, the cytokines driving stabilization and expansion and the effector Th17 response,[56] mainly responsible for the inflammatory response in CD.[57,58] Since diet is one of the major sources of exposure to BPA,[59] and considering the lack of association found between BPA levels and distinct
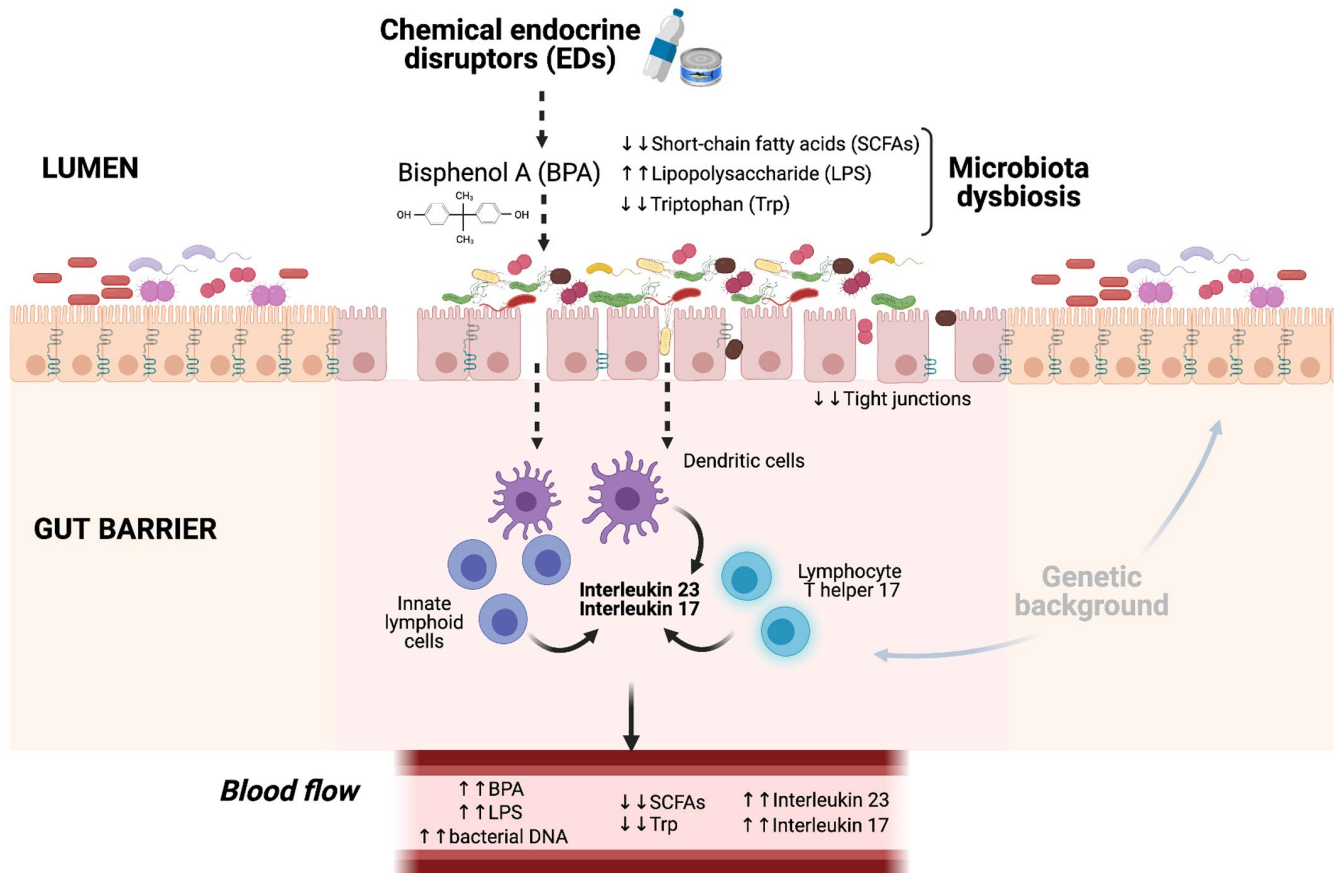
FIGURE 5 Proposed interaction of bisphenol A in the multifactorial etiologic scheme in Crohn's disease

CD-related genes variant genotypes, these results strengthen the benefits of a healthy lifestyle in helping maintain the immune homeostasis.

We acknowledge the limitations derived from this study. In first place, other EDCs such phthalates, perfluorinated compounds, and polycyclic aromatic hydrocarbons have not been evaluated and might play as well a role in disease activity and patients' inflammatory outlook. Also, BPA exposure was assessed in a single serum sample, which may lead to exposure misclassification due to its non-persistent nature and short-term viability.[60] However, this might result in attenuation bias, rather than an overestimation of effects. Nevertheless, we consider that presented data constitute a proof-of-concept showing BPA interactions with known CD inflammatory triggers, mainly derived from dysbiotic gut microbiota in a large real-life cohort of CD patients. A proposed multifactorial interacting scheme is presented in Figure 5.

In summary, results presented herein provide evidence on endocrine perturbation influencing disease activity through the BPA association with increased bacterial DNA translocation and serum endotoxin levels, and with decreased SCFAs and tryptophan levels, affecting the systemic inflammatory response that allows a sustained Th17 activation in CD patients. Further studies are needed to characterize the functional interaction of other EDCs with gut microbiota-derived metabolites in CD.

CONFLICT OF INTEREST

The authors state explicitly that there are no conflicts of interest in connection with this article.

AUTHORS' CONTRIBUTIONS

RL, EC: genetic, bacterial antigen translocation analysis, and inflammatory markers measurements. BS, MFF: measurement of endocrine-disrupting chemicals in serum samples. RGV, AMB, FATB: short-chain fatty acids evaluation in serum samples. AG: Inclusion of patients and scientific conclusions. PZ, RF: statistical analysis. RF: study design, data integration and manuscript writing.

DATA AVAILABILITY STATEMENT

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

ORCID

Rubén Francés https://orcid.org/0000-0001-5105-1201

REFERENCES

1. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's disease. *Lancet*. 2017;389:1741-1755.
2. Chang JT. Pathophysiology of inflammatory bowel diseases. *N Engl J Med*. 2020;383:2652-2664.
3. de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol*. 2016;13:13-27.
4. Gutierrez A, Frances R, Amoros A, et al. Cytokine association with bacterial DNA in serum of patients with inflammatory bowel disease. *Inflamm Bowel Dis*. 2009;15:508-514.
5. Gutierrez A, Zapater P, Juanola O, et al. Gut bacterial DNA translocation is an independent risk factor of flare at short term in patients with Crohn's disease. *Am J Gastroenterol*. 2016;111:529-540.
6. Lavelle A, Sokol H. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol*. 2020;17:223-237.
7. Gutierrez A, Scharl M, Sempere L, et al. Genetic susceptibility to increased bacterial translocation influences the response to biological therapy in patients with Crohn's disease. *Gut*. 2014;63:272-280.
8. Zoeller RT, Brown TR, Doan LL, et al. Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society. *Endocrinology*. 2012;153:4097-4110.
9. Grun F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology*. 2006;147:S50-S55.
10. Gore AC, Chappell VA, Fenton SE, et al. EDC-2: The Endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocr Rev*. 2015;36:E1-E150.
11. Heindel JJ, Newbold R, Schug TT. Endocrine disruptors and obesity. *Nat Rev Endocrinol*. 2015;11:653-661.
12. Alonso-Magdalena P, Quesada I, Nadal A. Endocrine disruptors in the etiology of type 2 diabetes mellitus. *Nat Rev Endocrinol*. 2011;7:346-353.
13. Neel BA, Sargis RM. The paradox of progress: environmental disruption of metabolism and the diabetes epidemic. *Diabetes*. 2011;60:1838-1848.
14. Cave M, Falkner KC, Ray M, et al. Toxicant-associated steatohepatitis in vinyl chloride workers. *Hepatology*. 2010;51:474-481.
15. Heindel JJ, Blumberg B, Cave M, et al. Metabolism disrupting chemicals and metabolic disorders. *Reprod Toxicol*. 2017;68:3-33.
16. Kuo CC, Moon K, Thayer KA, Navas-Acien A. Environmental chemicals and type 2 diabetes: an updated systematic review of the epidemiologic evidence. *Curr Diab Rep*. 2013;13:831-849.
17. Braun JM. Early-life exposure to EDCs: role in childhood obesity and neurodevelopment. *Nat Rev Endocrinol*. 2017;13:161-173.
18. DeLuca JA, Allred KF, Menon R, et al. Bisphenol-A alters microbiota metabolites derived from aromatic amino acids and worsens disease activity during colitis. *Exp Biol Med (Maywood)*. 2018;243:864-875.
19. Galvez-Ontiveros Y, Paez S, Monteagudo C, Rivas A. Endocrine disruptors in food: impact on gut microbiota and metabolic diseases. *Nutrients*. 2020;12:1158.
20. Lai KP, Chung YT, Li R, Wan HT, Wong CK. Bisphenol A alters gut microbiome: comparative metagenomics analysis. *Environ Pollut*. 2016;218:923-930.
21. Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol*. 2012;13:R79.
22. Ananthakrishnan AN, Bernstein CN, Iliopoulos D, et al. Environmental triggers in IBD: a review of progress and evidence. *Nat Rev Gastroenterol Hepatol*. 2018;15:39-49.
23. Sands BE. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology*. 2004;126:1518-1532.
24. Salamanca-Fernandez E, Rodriguez-Barranco M, Arrebola JP, et al. Bisphenol-A in the European prospective investigation into cancer and nutrition cohort in Spain: levels at recruitment and associated dietary factors. *Environ Res*. 2020;182:109012.
25. Vela-Soria F, Ballesteros O, Zafra-Gomez A, Ballesteros L, Navalon A. A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid-liquid microextraction. *Talanta*. 2014;129:209-218.
26. Juanola O, Pinero P, Gomez-Hurtado I, et al. Regulatory T cells restrict permeability to bacterial antigen translocation and preserve short-chain fatty acids in experimental cirrhosis. *Hepatol Commun*. 2018;2:1610-1623.
27. Frances R, Benlloch S, Zapater P, et al. A sequential study of serum bacterial DNA in patients with advanced cirrhosis and ascites. *Hepatology*. 2004;39:484-491.
28. Gomez-Hurtado I, Gallego-Duran R, Zapater P, et al. Bacterial antigen translocation and age as BMI-independent contributing factors on systemic inflammation in NAFLD patients. *Liver Int*. 2020;40:2182-2193.
29. Garcia-Villalba R, Gimenez-Bastida JA, Garcia-Conesa MT, Tomas-Barberan FA, Carlos Espin J, Larrosa M. Alternative method for gas chromatography-mass spectrometry analysis of short-chain fatty acids in faecal samples. *J Sep Sci*. 2012;35:1906-1913.
30. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491:119-124.
31. Graham DB, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature*. 2020;578:527-539.
32. Choy MC, Visvanathan K, De Cruz P. An overview of the innate and adaptive immune system in inflammatory bowel disease. *Inflamm Bowel Dis*. 2017;23:2-13.
33. Park JH, Peyrin-Biroulet L, Eisenhut M, Shin JI. IBD immunopathogenesis: a comprehensive review of inflammatory molecules. *Autoimmun Rev*. 2017;16:416-426.
34. Halfvarson J, Brislawn CJ, Lamendella R, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol*. 2017;2:17004.
35. Mondot S, Lepage P, Seksik P, et al. Structural robustness of the gut mucosal microbiota is associated with Crohn's disease remission after surgery. *Gut*. 2016;65:954-962.
36. Yilmaz B, Juillerat P, Oyas O, et al. Microbial network disturbances in relapsing refractory Crohn's disease. *Nat Med*. 2019;25:323-336.
37. Tenailleau QM, Lanier C, Gower-Rousseau C, Cuny D, Deram A, Occelli F. Crohn's disease and environmental contamination:

current challenges and perspectives in exposure evaluation. *Environ Pollut*. 2020;263:114599.

38. Ho S-M, Lewis JD, Mayer EA, et al. Challenges in IBD research: environmental triggers. *Inflamm Bowel Dis*. 2019;25: S13-S23.

39. Kedia S, Ahuja V. Epidemiology of inflammatory bowel disease in India: the great shift East. *Inflamm Intest Dis*. 2017;2: 102-115.

40. Benchimol EI, Mack DR, Guttmann A, et al. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol*. 2015;110:553-563.

41. Li X, Sundquist J, Hemminki K, Sundquist K. Risk of inflammatory bowel disease in first- and second-generation immigrants in Sweden: a nationwide follow-up study. *Inflamm Bowel Dis*. 2011;17:1784-1791.

42. Hammer T, Lophaven SN, Nielsen KR, et al. Inflammatory bowel diseases in Faroese-born Danish residents and their offspring: further evidence of the dominant role of environmental factors in IBD development. *Aliment Pharmacol Ther*. 2017;45:1107-1114.

43. de Silva PS, Yang X, Korzenik JR, Goldman RH, Arheart KL, Caban-Martinez AJ. Association of urinary phenolic compounds, inflammatory bowel disease and chronic diarrheal symptoms: evidence from the National Health and Nutrition Examination Survey. *Environ Pollut*. 2017;229:621-626.

44. Acconcia F, Pallottini V, Marino M. Molecular mechanisms of action of BPA. *Dose Response*. 2015;13:1559325815610582.

45. Pierdominici M, Maselli A, Varano B, et al. Linking estrogen receptor beta expression with inflammatory bowel disease activity. *Oncotarget*. 2015;6:40443-40451.

46. Linares PM, Algaba A, Urzainqui A, et al. Ratio of circulating estrogen receptors beta and alpha (ERbeta/ERalpha) indicates endoscopic activity in patients with Crohn's disease. *Dig Dis Sci*. 2017;62:2744-2754.

47. Jacenik D, Zielinska M, Mokrowiecka A, et al. G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease. *Sci Rep*. 2019;9:6749.

48. Cimmino I, Oriente F, D'Esposito V, et al. Low-dose Bisphenol-A regulates inflammatory cytokines through GPR30 in mammary adipose cells. *J Mol Endocrinol*. 2019;63:273-283.

49. Roda G, Chien Ng S, Kotze PG, et al. Crohn's disease. *Nat Rev Dis Primers*. 2020;6:22.

50. Feng D, Zhang H, Jiang X, et al. Bisphenol A exposure induces gut microbiota dysbiosis and consequent activation of gut-liver axis leading to hepatic steatosis in CD-1 mice. *Environ Pollut*. 2020;265:114880.

51. Chen Y, Ji F, Guo J, Shi D, Fang D, Li L. Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci Rep*. 2016;6:34055.

52. Leung C, Rivera L, Furness JB, Angus PW. The role of the gut microbiota in NAFLD. *Nat Rev Gastroenterol Hepatol*. 2016;13:412-425.

53. Nikolaus S, Schulte B, Al-Massad N, et al. Increased tryptophan metabolism is associated with activity of inflammatory bowel diseases. *Gastroenterology*. 2017;153:1504-1516 e1502.

54. Yang YJ, Hong YC, Oh SY, et al. Bisphenol A exposure is associated with oxidative stress and inflammation in postmenopausal women. *Environ Res*. 2009;109:797-801.

55. Savastano S, Tarantino G, D'Esposito V, et al. Bisphenol-A plasma levels are related to inflammatory markers, visceral obesity and insulin-resistance: a cross-sectional study on adult male population. *J Transl Med*. 2015;13:169.

56. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol*. 2010;28:445-489.

57. Monteleone I, Pallone F, Monteleone G. Interleukin-23 and Th17 cells in the control of gut inflammation. *Mediators Inflamm*. 2009;2009:297645.

58. Sarra M, Pallone F, Macdonald TT, Monteleone G. IL-23/IL-17 axis in IBD. *Inflamm Bowel Dis*. 2010;16:1808-1813.

59. Michalowicz J. Bisphenol A–sources, toxicity and biotransformation. *Environ Toxicol Pharmacol*. 2014;37:738-758.

60. Vernet C, Philippat C, Agier L, et al. An empirical validation of the within-subject biospecimens pooling approach to minimize exposure misclassification in biomarker-based studies. *Epidemiology*. 2019;30:756-767.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.