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Ocular Manifestations in Inflammatory Bowel Disease Are Associated with Other Extra-intestinal Manifestations, Gender, and Genes Implicated in Other Immune-related Traits

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Abstract

Background: There has been considerable progress in identifying inflammatory bowel disease [IBD] susceptibility genes but little progress in examining the role of genetic variation in the development of the extra-intestinal manifestations [EIMs] of IBD. This study identified clinical, serological, and genetic factors associated with ocular EIMs [O-EIMs] in IBD.
Methods: We performed a retrospective case-control study of IBD patients, comparing those with and without O-EIMs using the Cedars-Sinai IBD Research Repository and the NIDDK IBD Genetics Consortium Repository. Genotyping was performed using Illumina whole genome platforms.
Results: In all, 124 cases and 3328 controls with available clinical data were identified; 103 cases and 2808 controls had genetic data available. Erythema nodosum and peripheral arthritis particularly were common in patients with O-EIMs [$p = 2.77 \times 10^{-13}$ and $p = 2.58 \times 10^{-13}$, respectively] with increasing odds ratios for O-EIMs with each additional non-ocular-EIM [for ≥ 2 EIMs, odds ratio 14.72]. Nominal association with O-EIMs was observed at several known IBD susceptibility single nuclear polymorphisms. One locus, containing RBM19, achieved genome-wide level of significance for association with O-EIMs.

Conclusions: In IBD, O-EIMs co-occur with musculoskeletal and skin manifestations and, in this study, are nominally associated with known IBD loci. Additional cohorts are needed to verify these results and identify additional genes.

Key Words: Crohn's disease; ulcerative colitis; IBD; eye; uveitis; genetics

1. Introduction

It is widely recognised that the inflammatory bowel diseases [IBD], Crohn's disease [CD], and ulcerative colitis [UC] occur in genetically susceptible individuals following exposure to, as yet poorly understood, environmental factors. Recently there have been considerable advances in the identification of IBD susceptibility loci,[1,2,3] but an understanding of the molecular associations with clinical sub-phenotypes has lagged behind. In addition to chronic, relapsing gastrointestinal [GI] inflammation, up to 40% of patients with IBD have extraintestinal manifestations including ocular inflammation which can cause significant morbidity including blindness.[4,5]

Episcleritis, scleritis, and anterior uveitis are the most common ocular-extraintestinal manifestations [O-EIMs] in IBD. Other less common eye manifestations with reported associations to IBD include retinal vasculitis, papillitis, corneal infiltrates, myositis, scleromalacia perforans, and optic neuritis. After peripheral arthritis, O-EIMs are the second most common extraintestinal manifestation [EIM], occurring in 2–6% of adult IBD patients, with a higher prevalence reported in children.[4,6,7]

In IBD, most patients who develop ocular inflammation do so at first presentation or with an established diagnosis of IBD, but eye disease can pre-date intestinal disease.[8,9] Uveitis is reported as occurring four times more commonly in women and has been strongly associated with sacroiliac joint abnormalities and arthritis.[10] Genetic susceptibility in part appears to explain the development of O-EIMs in IBD. Pheobe *et al.* demonstrated that a family history of IBD significantly increases the risk of ocular inflammation in subjects without IBD, suggesting shared immunological mechanisms for eye and intestinal disease.[10] Further evidence supporting shared aetiology comes from the observation that individuals with IBD and ocular disease are more likely to 'express' anti-outer membrane porin C [OmpC] antibodies and that patients with a family history of IBD and ocular inflammation are more likely to be anti-nuclear cytoplasmic antibody [ANCA] positive.[11,12]

Few studies have evaluated the association between O-EIMs and genetic variation and the studies to date have been limited by power and the depth of available genotypes. Orchard *et al.* examined major histocompatibility complex [MHC] associations with O-EIMs and identified that eye manifestations are strongly associated with human leucocyte antigen [HLA]-B*27, B*58 and HLA-DRB1*0103.[13] Studies examining known, non-HLA, IBD susceptibility genes including *ATG16L1, IL-23R*, and *NOD2/CARD15*, have found no association with O-EIMs and IBD.[14,15] We present the largest study to date investigating demographic, clinical, serological and genetic associations with O-EIMs in IBD.

2. Methods

2.1. Databases

The Cedars-Sinai IBD Research Repository [MIRIAD] and the National Institute of Diabetes and Digestive and Kidney Diseases IBD Genetics Consortium [NIDDK-IBDGC] database [Cedars-Sinai Medical Center, John Hopkins University, University of Chicago, University of Montreal, University of Pittsburgh, University of Toronto, and Yale University] were used to identify cases. We queried the two IBD databases for clinical, serological, and genetic factors associated with [+] O-EIMs and without [-] O-EIMs.

2.2. Subjects

We evaluated all IBD patients in MIRIAD and identified those with at least one documented episode of O-EIM. The paper and electronic charts for these patients were reviewed to verify the O-EIM. The diagnosis of O-EIMs was made based on a previous diagnosis of episcleritis, scleritis, or uveitis and/or after evaluation by an ophthalmologist. O-EIMs in MIRIAD were suspected in patients with ocular pain, double vision, sudden decreased visual acuity, redness of the eyes, and eye irritation or watering. Patients were referred to ophthalmology if they had any of these symptoms. We also included O-EIM positive cases from the NIDDK-IBDGC database that uses a validated phenotyping collection protocol.[16]

In addition to O-EIMs, we collected information on demographics [gender, age], disease location and behaviour [as per Montreal classification], smoking status, family history, and surgical history from both databases. Data regarding the presence of non-ocular extraintestinal manifestations [non-ocular EIMs] also were collected. Non-ocular EIMs included erythema nodosum [EN], pyoderma gangrenosum [PG], peripheral arthritis, ankylosing spondylitis [AS], and primary sclerosing cholangitis [PSC]. IBD serologies [anti-*Saccharomyces cerevisiae* antibodies [ASCA IgG and IgA], perinuclear antinuclear cytoplasmic antibody [pANCA], anti-flagellin [anti-CBir1], anti-OmpC, and anti-*Pseudomonas fluorescens* associated sequence I2 [anti-I2]] were measured by enzyme-linked immunosorbent assay [ELISA] and expressed and assessed as previously described.[17] Only patients from MIRIAD had serology data available for evaluation.

Genotypes on the MIRIAD samples were generated at Cedars-Sinai Medical Center [CSMC] using Illumina Human610-quad, HumanCNV370-quad, and the HumanOmniExpress whole genome platforms.[13,18] Five samples [on 610-quad], 3 samples [on 370-quad], and 14 samples [on OmniExpress] were genotyped in duplicate, yielding concordance rates of 100%, 99.999% and 99.997%, respectively. Heritability concordance for three genotyping control trios was 99.53%. Samples which passed genotyping quality control exhibited genotyping call rate > 97%, and the average genotyping call rate across three platforms was 99.85%. The NIDDK IBDGC samples were genotyped using the HumanHap300 and HumanHap550 platforms as previously described.[19,20] Quality control was maintained as previously described.[17]

To combine results for the two independent genome-wide association studies [GWAS] cohorts, we imputed untyped genotypes using Impute2 [http://hapmap.ncbi.nlm.nih.gov] and HapMap Phase III [www.hapmap.org] reference genotypes for the Cedars GWAS data and minimac software, and 1000 Genome Project data release 2010-08 genotypes for the NIDDK cohorts. Poorly imputed single nucleotide polymorphisms [SNPs], defined by an R-squared quality RSQR

< 0.30 with MACH1/minimac or an information measure Is < 0.30 with IMPUTE2, were excluded from the analyses.

The Institutional Review Board at CSMC approved the study. All patients in both databases provided informed consent before genetic analysis.

2.3. Statistics

Univariate analysis

Logistic regression was performed to detect association of each clinical parameter with O-EIM status. The source of the sample [MIRIAD or NIDDK] was included as covariate to control for potential confounding effect. A significance threshold of 2.96×10^{-3} was used to control for multiple testing.

Similarly, logistic regression was performed to detect the association of IBD serology as well as other EIMs with O-EIM. The significance threshold was set to be 8.33×10^{-3} for both analyses.

2.4. Multivariate analysis

To account for the complicated correlation structure in the clinical characteristics, we also performed a multivariate logistic regression. Only variables with $p < 0.05$ in univariate analysis were included in the model. Serological factors were not included in this multivariate model as the serology data were only available in the MIRIAD cohort.

2.5. Genetic analysis

SNPtest and PLINK were used for genetic analyses in the Cedars and NIDDK cohorts, respectively. The DNA strand alignment was double-checked in the meta-analysis and confirmed to be on the same strand. Most of the SNPs had the same minor and major alleles, although a very low proportion of SNPs had switched alleles, with an MAF close to 0.5.

Logistic regression was performed to investigate the association between O-EIMs and genetic variations, with adjustment for the top four principal components from the population stratification analysis, to control for potential confounding. Meta-analysis was performed for SNPs that were common in both Cedars and NIDDK imputed GWAS dataset, using inverse variance weighting.

We assessed genetic associations with O-EIMs using both a genome-wide approach and also specifically at previously identified IBD susceptibility loci with an a priori nominal statistical significance defined as $p < 5 \times 10^{-5}$ for O-EIMs and for any -EIM analyses. A priori significance of $p < 3.1 \times 10^{-4}$ was determined for association at any known IBD loci. A Manhattan plot was completed to show chromosomal locations of associations with O-EIMs [Figure 1].

3. Results

3.1. Clinical factors

Among 3452 IBD patients, we identified 124 [3.6%] [+] O-EIMs [Table 1]. In the MIRIAD database, 95% of [+] O-EIMs cases had been diagnosed before presentation at CSMC. Data regarding family history were available for 96.0% of cases and 97.0% of controls. Details regarding distribution of disease were available in 97.6% of cases and 98.6% of controls. Data on gender, age at diagnosis, smoking status, and surgical history were present for all patients among both cases and controls. On univariate analysis, no demographic or clinical factor was associated with O-EIMs in a statistically significant manner, though O-EIMs were more prevalent in females and CD patients [Table 1]. On multivariable analysis, female association with O-EIMs approached statistical significance [$p = 8.49 \times 10^{-3}$] [Table 2].

Overall, 122 O-EIM [+] [98.4%] cases and 3322 O-EIM [-] [99.8%] controls had complete data on presence of the five non-ocular EIMs. Skin and joint manifestations had clear associations with ocular inflammation in both CD and UC/IBD-U [IBD unclassified]. Among patients with CD, univariate analysis revealed peripheral arthritis was significantly associated with O-EIMs ($p = 7.72 \times 10^{-10}$, odds ratio [OR] 4.29) [Table 3]. UC/IBD-U subjects with O-EIMs had a particularly strong association with pyoderma gangrenosum [$p = 9.28 \times 10^{-23}$] [Table 4]. On multivariate analysis, both skin and joint manifestations were significantly associated with O-EIMs [Table 2].

When IBD patients had at least one non-ocular-EIM, they were at increased risk for developing ocular inflammation [OR 4.79, $p < 1.18 \times 10^{-15}$] [Figure 2]. Additionally, the risk of O-EIMs increased with each additional non-ocular-EIM [for ≥ 2 EIMs, OR 14.72, $p < 1.62 \times 10^{-23}$] [Figure 2].

3.2. Serology

Serological data were available for 52 MIRIAD cases and 2068 controls. There was no significant association with serology.

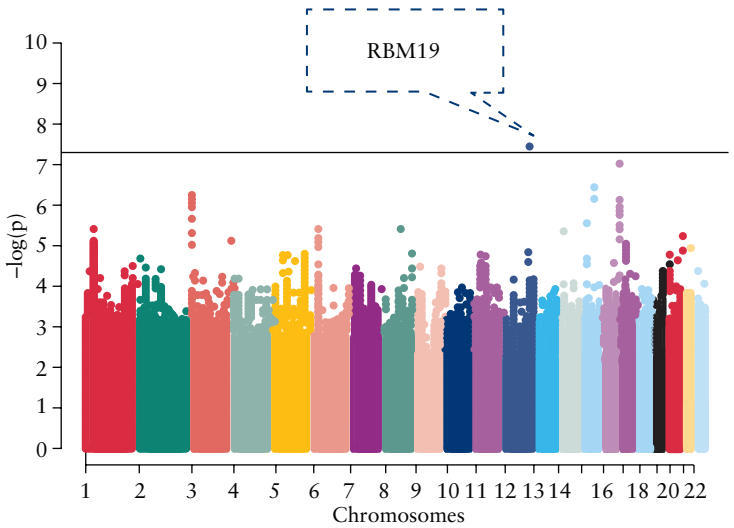

Figure 1. Manhattan plot highlighting loci associated with O-EIMs.

Table 1. Clinical characteristics of subjects.

Clinical characteristic	% [+] O-EIM [*n* = 124]	% [-] O-EIM [*n* = 3328]	*p*-Value	OR [95% CI]
Male [%]	37.9	53.0	1.18×10^{-3}	0.54 [0.37–0.79]
CD	72.6	66.7	0.013	1.79 [1.13–2.85]
Mean age at diagnosis	26.7	26.7	0.931	-
FH of IBD	18.5	21.5	0.502	0.93 [0.61–1.41]
Smoking	28.6	31.6	0.349	0.82 [0.55–1.24]
Previous surgery	44.2	40.5	0.288	1.22 [0.84–1.77]
CD location:				
L1	31.0	28.6	0.945	1.02 [0.63–1.63]
L2	13.8	14.6	0.723	1.12 [0.59–2.16]
L3	55.2	56.8	0.771	0.93 [0.61–1.44]
L4	18.7	14.4	0.189	1.49 [0.82–2.72]
CD phenotype:				
B1	44.2	44.7	0.940	1.01 [0.66–1.57]
B2	29.1	29.9	0.958	0.98 [0.61–1.59]
B3	26.7	25.0	0.920	1.03 [0.63–4.97]
Perianal	39.0	31.9	0.135	1.41 [0.89–2.30]
UC/IBD-U Phenotype:				
E3	85.3	69.1	0.061	2.50 [0.96–6.52]

OR, odds ratio; CI, confidence interval; O-EIM, ocular extra-intestinal manifestation; CD, Crohn's disease; FH, family history; IBD-U, inflammatory bowel disease unclassified.

Table 2. Multivariable analysis of clinical factors associated with ocular-extraintestinal manifestations.

Variable	OR [95% CI]	*p*-Value
Male gender	0.59 [0.40–0.87]	8.49×10^{-3}
Crohn's disease	0.90 [0.58–1.40]	0.65
Peripheral arthritis	3.09 [2.01–4.75]	2.87×10^{-7}
Ankylosing spondylitis	2.73 [1.61–4.61]	1.78×10^{-4}
Erythema nodosum	5.73 [2.92–11.27]	4.13×10^{-7}
Pyoderma gangrenosum	5.14 [1.89–14.03]	1.38×10^{-3}

OR, odds ratio; CI, confidence interval.

3.3. Genetics

Genetic analyses were performed on 103 [+] O-EIM cases and 2808 [-] O-EIM controls. No known IBD loci were significantly associated with O-EIMs. Single nucleotide polymorphisms [SNPs] at two known IBD loci had nominal association with O-EIMs; the locus containing *C10ORF58* and *TSPAN14* were protective for O-EIMs [OR 0.65, p = 0.035]; the *TNFSF14* locus was associated with O-EIMs [OR 1.47, p = 0.010].

An unbiased approach examining all genotyped/imputed SNPs across the genome identified 14 loci with nominal significance associated with O-EIM, including one locus achieving genome-wide significance [rs4766697, p = 3.75E-8, OR = 3.31] [Table 6]. The imputation score was 0.879, indicating very good imputation quality. This locus contains a long intergenic non-protein coding RNA, *LINC01234*, and *RBM19*, a regulator of ribosome biogenesis expressed in crypt cells of the intestinal epithelium.

Finally, given the co-occurrence of EIMs, we performed an analysis looking at genetic associations with the development of any extra-intestinal manifestation. We identified 504 IBD cases with any EIM and 2407 cases with no history of EIMs. A genome wide analysis revealed nominal significant associations with variants tagging, a DNA mismatch repair gene [*MSH3*], a transmembrane protein [TMTC2], a post-transcriptional gene regulator [MIR548T], an integral membrane protein important in cell-cell recognition and adhesion [PCDH7], a tumour suppression gene [CSMD1], and a glutamate-regulated ion channel gene [GRIN3A] [Table 7]. One IBD-associated SNP tagging the *IL12B* locus achieved our a priori level of nominal significance but did not achieve genome-wide significance [p = 0.027, OR 1.16]. In contrast to previous studies, we found no association between HLA DRB*0103 and the presence of either ocular [p = 0.60] or non-ocular-EIMs [p = 0.81].

4. Discussion

We present the largest study, to date, describing characteristics associated with the development of ocular manifestations of IBD. We found that approximately 4% of IBD patients had a history of uveitis, scleritis, or episcleritis, consistent with previous findings.[4,21] Several previous studies have reported higher prevalence rates, mainly due to inclusion of dry eyes, conjunctivitis, and glaucoma, eye conditions not directly linked with IBD.[22,23,24] We identified higher prevalence of O-EIMs in women and Crohn's disease, consistent with previous studies.[4,13,21,24]

Little has been published on the relationship between O-EIMs in IBD and genetic variation. Although there was no association with known IBD loci and O-EIMs, after Bonferroni correction we observed a 'nominal' association between O-EIMs and the IBD loci containing *TSPAN14* and *TNFSF14*. Further studies, in larger cohorts, will be needed to assess whether these are valid observations or not. The unbiased approach implicated genes involved in eye diseases including: *COL8A2* [a major component of corneal endothelium], linked to Fuchs endothelial corneal dystrophy and posterior polymorphous corneal dystrophy type 2, and CHST6, associated with macular corneal dystrophy. Other processes potentially implicated in O-EIM development by these genetic findings include cell adhesion [*C2CD4A, CNTN4*], golgi and endoplasmic reticulum maintenance [*TPAPPC3, GABARAPL2, RAB2B*], response to viral infection [*AGO3, SLCO5A1*], metabolic syndromes [*ADAT1,*

Table 3. Extra-intestinal manifestations in Crohn's disease subjects

EIM	% [+] O-EIM	% [-] O-EIM	p-Value	OR [95% CI]
Peripheral arthritis	35.6	12.3	$7.72x10^{-10}$	4.29 [2.70–6.81]
Ankylosing spondylitis	18.9	8.8	$4.33x10^{-3}$	2.74 [1.56–4.80]
Erythema nodosum	14.9	2.3	$1.99x10^{-10}$	8.88 [4.53–17.39]
Pyoderma gangrenosum	8.9	1.3	$2.34x10^{-6}$	7.14 [3.16–16.16]
PSC	1.1	1.0	0.89	1.15 [0.15–8.63]
Any EIM	54.6	22.9	$7.11x10^{-12}$	4.81 [3.07–7.54]

OR, odds ratio; CI, confidence interval; O-EIM, ocular extra-intestinal manifestation; CD, Crohn's disease; PSC, primary sclerosing cholangitis.

Table 4. Extra-intestinal manifestations in UC/IBDU subjects.

EIM	% [+] O-EIM	% [-] O-EIM	*p*-Value	OR [95% CI]
Peripheral arthritis	23.5	6.4	$8.92x10^{-5}$	5.38 [2.32–6.52]
Ankylosing spondylitis	17.6	1.6	$1.14x10^{-7}$	16.92 [5.95–48.14]
Erythema nodosum	6.1	0.2	$5.00x10^{-4}$	93.0 [7.25–1194.74]
Pyoderma gangrenosum	8.8	0	$9.28x10^{-23}$	N/A
PSC	8.8	3.2	0.119	2.66 [0.78–9.13]
Any EIM	32.4	10.9	$1.04x10^{-4}$	4.42[2.08–9.35]

OR, odds ratio; CI, confidence interval; O-EIM, ocular extra-intestinal manifestation; UC, ulcerative colitis; IBD-U, inflammatory bowel disease unclassified; NA, not available.

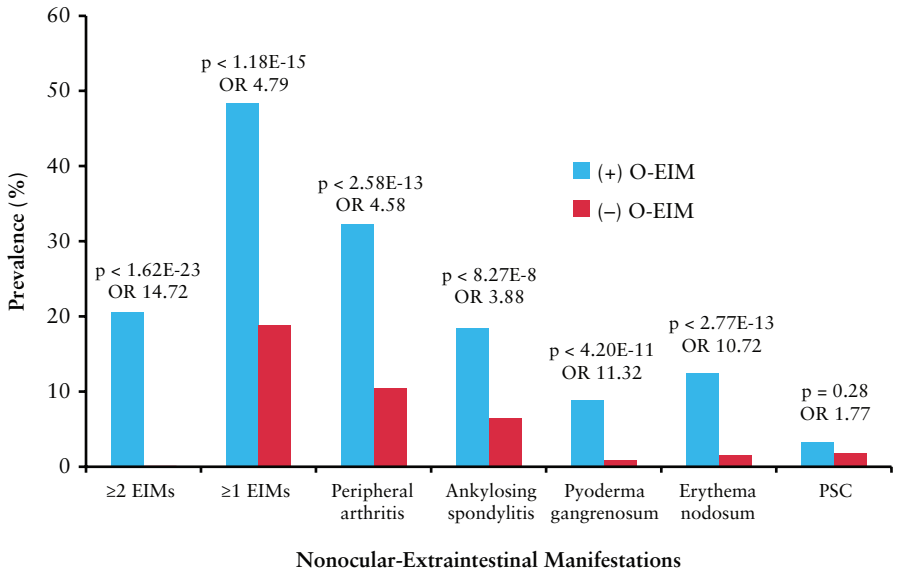

Figure 2. Prevalence of nonocular-extraintestinal manifestations in [+] O-EIMs and [-] O-EIMs subjects.

VPS13C], and T cell receptor alpha variable region genes. It is important to emphasise that these loci did not achieve genome-wide significance and therefore first require validation and then more detailed investigation before 'causal' genes and processes can be confirmed. rs4766697 tagging *RBM19* achieved genome-wide significance. Little is known about this gene, but it may be implicated in regulation of ribosome biogenesis and is expressed in crypt cells of the intestinal epithelium. Despite being the largest IBD O-EIMs cohort investigated to date, it is important to stress that our study is very significantly underpowered and that the associations observed do not meet criteria for association after correction for multiple testing for known IBD loci, and only one locus achieved a genome-wide level of significance from the unbiased approach. Nevertheless these findings may provide the basis for future studies in expanded IBD cohorts with O-EIMs characterised.

Overall, our clinical and genetic data suggest that ocular manifestations are commonly associated with other EIMs, particularly those involving the skin and joints [Figure 2]. Prior publications have suggested the possibility of a common antigen present in the eye, skin, joints, and gastrointestinal tract in these patients.[25] Our analysis examining genetic associations with the development of any EIMs, although not achieving genome-wide significance, are of interest given previous associations between *CSMD1* and psoriasis and between *MSH3* and rheumatoid arthritis. Additionally, *IL12B* variants have previously been associated with psoriasis, psoriatic arthritis, spondyloarthropathy, and multiple sclerosis as well as IBD. Furthermore, therapies targeted at this pathway are currently under evaluation in IBD.

Despite its size, this study has some limitations. Overall, O-EIMs occur so infrequently that our sample size may not be appropriately

powered to show more subtle clinical and serological differences between those IBD subjects with and without O-EIMs. However, where data do exist, our demographic and clinical results are consistent with previous published studies. Because the databases in this study included multiple referral medical centres, and due to the retrospective nature of the study, we were limited in our ability to verify the specific ocular diagnosis. However, phenotyping of subjects from the NIDDK database has been previously validated.[18] At CSMC, experienced IBD physicians questioned patients regarding eye disease at every visit, according to standardised encounter forms. When necessary, we verified the ocular disease diagnosis of CSMC patients through an ophthalmology encounter.

In the largest study to date, we have identified several putative novel clinical and genetic parameters of O-EIMs in IBD that may

Table 5. Extra-intestinal manifestations in inflammatory bowel disease subjects.

EIM	% [+] O-EIM	% [-] O-EIM	*p*-Value	OR [95% CI]
Peripheral arthritis	32.2	10.4	$2.58x10^{-13}$	4.58 [3.05–6.88]
Ankylosing spondylitis	18.5	6.4	$8.27x10^{-8}$	3.88 [2.36–6.38]
Erythema nodosum	12.5	1.6	$2.77x10^{-13}$	10.72 [5.67–20.25]
Pyoderma gangrenosum	8.87	0.9	$4.20x10^{-11}$	11.32 [5.50–23.28]
PSC	3.2	1.8	0.276	1.77 [0.63–4.97]
Any EIM	48.4	18.9	$1.18x10^{-15}$	4.79 [3.26–7.03]
≥ 2 any EIM	20.5	0.02	$1.62x10^{-23}$	14.72 [8.69–24.94]

OR, odds ratio; CI, confidence interval; O-EIM, ocular extra-intestinal manifestation; PSC, primary sclerosing cholangitis.

Table 6. Genetic associations with O-EIMs using a genome-wide approach.

SNP	Chr	*p*-Value	OR [95% CI]	Gene
rs4766697	12	3.75E-8	3.31 [1.51–5.07]	*RBM19*
rs11640406	16	9.50E-8	3.55 [0.95–5.66]	*TERF2IP, ADAT1, CHST5/6*
rs12050616	15	3.50E-7	2.21 [1.67–3.00]	*C2CD4A, VPS13C*
rs11710167	3	5.65E-7	2.23 [1.72–3.05]	*CNTN4*
rs8031119	15	2.71E-6	2.69 [1.39–4.07]	*LINC00929*
rs645123	1	3.88E-6	2.49 [1.29–3.68]	*AGO1/3/4, TEKT2, COL8A2, TPAPPC3, MAP7D1, THRAP3*
rs16936449	8	3.91E-6	2.98 [1.78–4.73]	*SULF1, PRDM14, SLCO5A1*
rs6910165	6	3.97E-6	2.21 [1.79–3.10]	*LOC101928519*
rs35355940	14	4.57E-6	2.62 [1.51–3.96]	*CHD8, RAB2B, TOX4, METTL3,OR10G3, OR10G2, OR4E2,* *TCRα* variable region gene cluster
rs12624442	20	5.84E-6	2.65 [1.47–4.03]	*SLCO4A1, NTSR1, MRGBP, OGFR, TCFL5, DIDO1*
rs4401383	3	7.27E-6	2.15 [1.67–3.01]	*MCF2L2, KLHL6/24, YEATS2*

SNP, single nucleotide polymorphism; chr, chromosome; OR, odds ratio; CI, confidence interval.

Table 7. Genetic associations with all extra-intestinal manifestations, using a genome-wide approach.

SNP	Chromosome	p-Value	OR [95% CI]	Gene	Reference allele	Variant allele
rs28678153	4p15	1.40E-07	0.58 [0.47–0.71]	-	A	G
rs2055340	8p23	6.70E-07	0.71 [0.61–0.81]	*CSMD1*	A	T
rs16878216	5q14	1.54E-06	1.67 [1.35–2.06]	*MSH3, DHFR, RASGRF2*	C	G
rs12370878	12q21	7.72E-06	1.58 [1.29–1.92]	*TMTC2*	T	A
rs107608	9q31	8.59E-06	0.74 [0.65–0.85]	-	C	G

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

help better identify those at risk in the future as well as potentially provide targets for future pharmacological intervention. Additional cohorts are needed to verify and extend our findings in order to achieve these aims.

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Conflict of Interest

There is no conflict of interest.

Author Contributions

ST conceived the study, participated in its design, participated in data analysis, and drafted the manuscript. DL participated in data analysis and helped draft parts of the manuscript. SRT, AP, SB, JC, RD, JR, MS, EV, JR, DS, MD, and GM participated in collection of data and revised it critically for important intellectual content. TH participated in sample analysis and helped draft parts of the manuscript. DM conceived and supervised the study, participated in its design and coordination, participated in collection of data and data analysis, and helped draft the manuscript. All authors read and approved the final manuscript.

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