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Polymorphisms in *CLEC5A* and *CLEC7A* genes modify risk for inflammatory bowel disease

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Abstract

Background Inflammatory bowel disease (IBD) seems to arise from an interplay between genetic and environmental factors. *CLEC5A* and *CLEC7A* genes code for 2 members of the C-type lectin receptor superfamily, which participate in the immune response against various pathogens, mediating inflammatory signaling. *CLEC5A* polymorphisms have been linked to the risk of Crohn's disease (CD), whereas *CLEC7A* has been implicated in fungal dysbiosis, chemically induced colitis in mice and undertreated ulcerative colitis (UC) in humans. This study aimed to explore how specific *CLEC5A* and *CLEC7A* polymorphisms contribute to the development of CD and UC.

Methods One hundred twelve CD patients, 94 UC patients and 164 sex- and age- matched healthy individuals were genotyped for the single nucleotide polymorphisms rs2078178 and rs16910631 of the *CLEC7A* gene, and rs1285933 of the *CLEC5A* gene.

Results The *CLEC7A* rs2078178 AA genotype was more frequent in UC patients compared to healthy individuals, The *CLEC7A* rs16910631 CT genotype was significantly associated with UC risk compared to healthy individuals, while there was no statistical correlation with CD. The *CLEC5A* rs1285933 GA genotype was found to be protective against UC and CD, and the AA genotype against CD. Carriers of the rs1285933 A allele appeared to have reduced susceptibility to CD, implying that the presence of the A allele could be protective against CD development.

Conclusions This is the first study to correlate the *CLEC5A* rs1285933 polymorphism with the risk for UC. The rs2078178 AA genotype and the *CLEC7A* rs16910631 CT could be promising biomarkers for UC susceptibility.

Keywords Inflammatory bowel disease, *CLEC5A*, *CLEC7A*, single-nucleotide polymorphism, biomarkers

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Introduction

Inflammatory bowel disease (IBD) is a collective term that comprises 2 clinically discrete nosological entities of the intestines, namely Crohn's disease (CD) and ulcerative colitis (UC). IBD is a global health concern that accounted for 4.9 million cases worldwide in 2019, marking an increase of over 47% compared to 1990 [1]. Because of its physical history, IBD is associated with high rates of hospitalization, an increased risk for abdominal surgery and colorectal cancer, and a large financial burden due to medical expenditures and a significant loss of productive potential [2,3]. Although the etiology of IBD has not been fully elucidated, the contemporary view of CD and UC highlights a multifactorial dynamic interplay between genetic variants and environmental factors that reciprocally incite and perpetuate a relapsing–remitting inflammatory cycle. In genetically susceptible subjects, a dysregulated immune

response functions against a dysbiotic commensal microbiome that has been shaped by environmental determinants. The influence of extrinsic triggers may account for the nonuniform geographic distribution of disease incidence and prevalence, since developed, newly industrialized and developing countries are in different epidemiological stages of IBD evolution [4]. Among the several factors that may contribute to the development of disease, major environmental drivers include dietary composition, smoking (increased risk for CD, yet protective for UC), low vitamin D levels, oral contraceptives, and pollution of air and water [5].

Regarding the genetic background of IBD, the use of genome-wide association studies has pinpointed more than 230 loci as contributory to IBD pathogenesis [6]. Although a fraction of them are implicated in both CD and UC, most loci exhibit disease specificity. *NOD2/CARD15* comprises the first and most studied locus, correlated with both adult [7] and pediatric-onset CD [8]. Polymorphisms in the autophagy genes *ATG16L1* and *IRGM* and the intelectins (*ITLN1*) also confer susceptibility, primarily to CD [9]. The panel of risk loci and gene mutations associated with IBD is being consistently enriched with new candidates [10-12].

Recently, Elleisy *et al* [13] and Iliev *et al* [14] studied the associations of single nucleotide polymorphisms (SNPs) rs1285933 in the *CLEC5A* gene with CD, and rs2078178 and rs16910631 in the *CLEC7A* gene with CD and UC. C-type lectin domain family 5 member A (*CLEC5A*) and C-type lectin domain family 7 member A (*CLEC7A*) are type II transmembrane proteins that are members of group V of the C-type lectin receptor (CLR) superfamily [15]. *CLEC5A* is a spleen tyrosine kinase (SYK)-coupled CLR, primarily expressed on immune cells of the myeloid lineage. Upon binding microbial and nonmicrobial antigens, *CLEC5A* forms multivalent agglomerates with CLRs and toll-like receptors, leading to downstream signaling that promotes secretion of proinflammatory cytokines and chemokines and induces acquisition of the proinflammatory M1 phenotype in macrophages [15]. *CLEC5A* induction plays a critical role in the pathogenesis of severe Dengue virus [16] and influenza type A infection [17], *Pseudomonas* and SARS-CoV-2-induced neutrophil extracellular traps and thromboinflammation [18], and myocardial dysfunction following myocardial infarction [19]. On the other hand, *CLEC5A* deletion prevents macrophage polarization, NLRP3 inflammasome activation and pyroptosis [19]. Furthermore, in a pan-cancer analysis, *CLEC5A* was found to be consistently upregulated in analyses of single cancer cells, with higher levels of expression being correlated with macrophage infiltration and worse clinical outcomes [20]. In the context of IBD, significantly higher *CLEC5A* expression was reported in peripheral blood mononuclear cells of patients with CD compared to healthy individuals [21]. A high *CLEC5A* to *CDH2* ratio (high risk >3) in whole-blood transcriptomic analysis was indicative of the need for treatment escalation in UC [22].

CLEC7A (Dectin-1) is expressed predominantly on myeloid cells and to a lesser degree on subsets of B and T cells [23]. β-glucans comprise the primary Dectin-1 ligand, although recent studies have reported a set of endogenous molecules engaged by Dectin-1, including galectin-9, annexins, vimentin, tropomyosin and N-glycan [24]. Upon stimulation by fungal β-1,3 glycans, Dectin-1 signals via a SYK-dependent or independent route to regulate an antifungal defense response in the form of cytokine production, phagocytosis and respiratory burst. Consequently, *CLEC7A* -/- mice are highly vulnerable to invasive fungal disease, because of their impaired phagocytic response [25]. Importantly, apart from its protective role in the context of fungal infections, intestinal *CLEC7A* expression modifies the composition of gut microbiota, as it restricts colonization by opportunistic fungi, including *Candida* spp. and *Trichosporon* spp., preserves *Saccharomyces cerevisiae* proportion, and thus prevents fungal gut dysbiosis [14]. The studies of Iliev *et al*, Wang *et al* and Tang *et al* have addressed the functional implications of *CLEC7A* deletion in mouse models of colitis, yielding conflicting results [14,26,27].

The scarcity and relative discrepancy between currently published data implies a need for further studies in order to conclusively address the role of *CLEC7A* and *CLEC5A* in IBD pathogenesis. The present study aimed to investigate whether polymorphisms in the *CLEC5A* and *CLEC7A* genes contribute to the risk for IBD in a well-characterized cohort of Greek patients with IBD, and healthy individuals. Our work provides further insights into the association between *CLEC5A* and *CLEC7A* polymorphisms and the risk for CD and UC development. Furthermore, this is first study on the correlation between *CLEC5A* gene polymorphisms and the risk for UC.

Patients and methods

Patients

Two hundred six patients with an endoscopic and histological diagnosis of IBD, according to the ECCO-ESGAR guidelines [28], were included in the present study. The entire cohort was followed on an outpatient basis by the Department of Gastroenterology of Aretaieion University Hospital in Athens, Greece. One hundred twelve patients had CD (66 male, mean age 38.98±14.42 years) and 94 patients had UC (48 male, mean age 34.4±15.2 years). A total of 164 sex- and age-matched healthy individuals (94 male, mean age 37.43±13.33 years), evaluated in the same institution, served as the control group. Detailed demographic characteristics and clinical features of the enrolled patients and healthy individuals are presented in Table 1.

DNA isolation and genotyping

Genomic DNA was extracted from peripheral whole blood samples of recruited IBD patients and healthy individuals using the Nucleospin Blood kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany), according to the manufacturer's instructions. Three SNPs of 2 genes were evaluated in the present study, namely rs2078178 and

rs16910631 of *CLEC7A*, and rs1285933 of *CLEC5A*. The rs2078178 variant (G/A) and the rs16910631(C/T) in the *CLEC7A* gene were genotyped by allele-specific polymerase chain reaction (PCR) (Table 2). For the rs2078178 an amplification of a 188 bp fragment using 2 allele-specific forward primers F (G): 5'-AAACTGCCTAGGGGGACTGC-3' and F (A): 5'-AAACTGCCTAGGGGGACTGT-3' was used, in combination with a common reverse primer R: 5'-ACCTGACATCAACCTAGAGAGAAG-3', and for the rs16910631 an amplification of a 185 bp fragment using the allele-specific forward primers F(C): TCTCAAAGGATTATTGCGGGAATTAAAC and F(T): TCTCAAAGGATTATTGCGGGAATTAAAT, with the common reverse primer R: GGCAACCTATTGAGGAAGCG. The rs1285933 polymorphism of the *CLEC5A* gene was genotyped by RFLP-PCR using the primers F: GGGATCACTGGGTCAAATGGTAT and R: CCTTTCGTGTATTGTTCATCCAGC. The 191 bp product was digested overnight AluI (Enzyquest, Crete, Greece).

Statistical analysis

Genotype frequencies were analyzed by the χ^2 test with Yate's correction, using S-Plus (version 6.2 Insightful, Seattle, WA, USA) software. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with GraphPad (version 300, GraphPad Software, San Diego, CA, USA). All P-values are 2-sided. P-values <0.05 were considered significant.

Results

Genotype and allele distributions for SNPs rs2078178, rs16910631 in the *CLEC7A* gene and SNP rs1285933 in *CLEC5A* are detailed in Tables 3-5. The *CLEC7A* rs2078178 AA genotype presented a higher frequency in IBD patients compared to healthy individuals, but the AA genotype was significantly associated only with UC (P=0.0035; OR 14.000,

Table 1 Demographic characteristics of Crohn's disease patients, ulcerative colitis patients and healthy individuals

Characteristics	Crohn's disease (112) n (%)	Ulcerative colitis (94) n (%)	Healthy individuals (164) n (%)
Sex			
Male	66 (58.9)	48 (51.1)	94 (57.3)
Female	46 (41.1)	46 (48.9)	70 (42.7)
Age (years, mean±SD)	38.98±14.42	34.4±15.2	37.43±13.33
Disease location			
Ileal disease	25 (22.3)	N/A	N/A
Colonic disease	10 (8.9)		
Ileal and colonic disease	77 (68.8)		
Disease extent			
Pancolitis	N/A	51 (54.3)	N/A
Left colon		27 (28.7)	
Proctitis		16 (17.0)	
Disease behavior			
Non stenosing/non penetrating	96 (85.7)	N/A	N/A
Fibro-stenosing	2 (1.8)		
Penetrating	14 (12.5)		

N/A, non-applicable; SD, standard deviation; N/A, not applicable

Table 2 Polymerase chain reaction primers and reaction conditions for the studied SNPs

SNP	Primers (5'-3')	Primers Tm	Restriction enzyme and products (bp)
rs2078178	F (G): AAACTGCCTAGGGGGACTGC F (A): AAACTGCCTAGGGGGACTGT R: ACCTGACATCAACCTAGAGAGAAG	59°C	188bp
rs16910631	F (C): TCTCAAAGGATTATTGCGGGAATTAAAC F (T): TCTCAAAGGATTATTGCGGGAATTAAAT R: GGCAACCTATTGAGGAAGCG	59°C	185bp
rs1285933	F: GGGATCACTGGGTCAAATGGTAT R: CCTTTCGTGTATTGTTCATCCAGC	60oC	AluI (Enzyquest, Crete, Greece) G/G: 191bp A/A: 115bp+76bp

SNP, single nucleotide polymorphism; bp, base pair

Table 3 *CLEC7A* rs2078178 genotype and allele distributions among CD/UC cases and healthy individuals

Genotype	CD (112) n (%)	UC (94) n (%)	Healthy individuals (164) n (%)	CD P-value; OR (95%CI)	UC P-value; OR (95%CI)
G/G	66 (58.9)	52 (55.3)	104 (63.4)	1.00 (ref.)	1.00 (ref.)
G/A	42 (37.5)	35 (37.2)	59 (35.9)	0.7009; 1.122 (0.6791-1.853)	0.5841; 1.186 (0.6951-2.025)
A/A	4 (3.57)	7 (7.4)	1 (0.6)	0.0839; 6.303 (0.6891-57.656)	0.0035; 14.000 (1.677-116.88)
G	174 (77.7)	139 (73.9)	267 (81.4)	1.00 (ref.)	1.00 (ref.)
A	50 (22.3)	49 (26.1)	61 (18.6)	0.3305; 1.258 (0.89265-1.914)	0.0573; 1.543 (1.005-2.368)

CD, Crohn's disease; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval

Table 4 *CLEC7A* rs16910631 genotype and allele distributions among CD/UC cases and healthy individuals

Genotype	CD (112), n (%)	UC (94), n (%)	Healthy individuals (164), n (%)	CD P-value; OR (95%CI)	UC P-value; OR (95%CI)
C/C	80 (71.4)	63 (67.0)	127 (77.4)	1.00 (ref.)	1.00 (ref.)
C/T	30 (26.8)	29 (30.9)	30 (18.3)	0.1366; 1.588 (0.8903-2.831)	0.0309; 1.949 (1.077-3.527)
T/T	2 (1.8)	2 (2.1)	7 (4.3)	0.4880; 0.4536 (0.9189-2.239)	0.7207; 0.5760 (0.1162-2.855)
C	190 (84.8)	155 (82.4)	284 (86.6)	1.00 (ref.)	1.00 (ref.)
T	34 (15.2)	33 (17.6)	44 (13.4)	0.6190; 1.155 (0.7119-1.874)	0.2477; 1.374 (0.8401-2.248)

CD, Crohn's disease; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval

Table 5 *CLEC5A* rs1285933 genotype and allele distributions among CD/UC cases and healthy individuals

Genotype	CD (112), n (%)	UC (94), n (%)	Healthy individuals (164), n (%)	CD P-value; OR (95%CI)	UC P-value; OR (95%CI)
G/G	45 (40.2)	23 (24.4)	24 (14.6)	1.00 (ref.)	1.00 (ref.)
G/A	60 (53.6)	56 (59.6)	118 (72.0)	<0.0001; 0.2712 (0.1511-0.4868)	0.04; 0.4952 (0.2573-0.9529)
A/A	7 (6.2)	15 (16.0)	22 (13.4)	0.0003; 0.1697 (0.0634-0.4542)	0.5110; 0.7115 (0.2979-1.699)
G	150 (67.0)	102 (54.2)	166 (50.6)	1.00 (ref.)	1.00 (ref.)
A	74 (33.)	86 (45.8)	162 (49.4)	0.0002; 0.5055 (0.3552-0.7193)	0.4642; 0.8640 (0.6031-1.238)

CD, Crohn's disease; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval

95%CI 1.677-116.88). The heterozygous genotype was not significantly correlated with either CD or UC. A marginally non-significant association was observed between the rs2078178 A allele and UC (P=0.0573; OR 1.543, 95%CI 1.005-2.368). The *CLEC7A* rs16910631 CT genotype was significantly over-represented in UC patients compared to healthy individuals (P=0.0309; OR 1.949, 95%CI 1.077-3.527), while the correlation with CD did not reach statistical significance. The *CLEC5A* rs1285933 GA genotype appeared to confer a protective effect against UC (P=0.04; OR 0.4952, 95%CI 0.2573-0.9529) and CD (P<0.0001; OR 0.2721, 95%CI 0.1511-0.4868). Moreover, the presence of the rs1285933 AA genotype also conferred a significantly lower susceptibility to CD development (P=0.0003; OR 0.1697, 95%CI 0.0634-0.4542).

Discussion

The genetic background of IBD remains the focus of extensive research aiming at elucidating susceptibility genes and causal variants. Although current studies have identified more than 230 high-risk loci [6], only limited data have been published about candidate genes and their causal genetic variants. In the present study, we present the genotype and allele frequencies for the rs2078178 and rs16910631 polymorphisms of the *CLEC7A* gene and the rs1285933 polymorphism of the *CLEC5A* gene in a Greek cohort of 206 IBD patients, compared to their frequencies in healthy individuals. The correlation between *CLEC7A* SNPs rs2078178 and rs16910631 and UC has been previously discussed by Iliev *et al* [14], while an association analysis of the abovementioned SNPs and *CLEC5A* SNP rs1285933 with CD has been published by Elleisy *et al* [13]. The latter study reported a significant positive association between the SNP rs1285933 in the *CLEC5A* gene and the risk for CD (P=0.0523) [13]. Interestingly, *CLEC7A* gene SNPs rs2078178 and rs16910631, previously significantly associated with medically refractory UC by Iliev *et al* (P=0.007) [14], were not significantly correlated with the risk for CD. In view of the scanty data, our study aimed to provide further evidence regarding a potential association

between *CLEC5A* and *CLEC7A* gene polymorphisms and the risk for IBD development. Importantly, this is the first genetic analysis to study the possible association of *CLEC5A* rs1285933 with the risk for UC development. Furthermore, this is the first study of the *CLEC5A* and *CLEC7A* genes in a Greek cohort: we report allele frequencies for the rs2078178 and rs16910631 SNPs of the *CLEC7A* gene, and for rs1285933 of *CLEC5A*, in both IBD patients and healthy individuals.

The *CLEC5A* rs1285933 GA genotype was significantly under-represented in UC and CD patients compared to healthy individuals, implying a protective effect. This negative association between the GA genotype and the risk for CD development was also described by Elleisy *et al*, although with marginal non-significance (P=0.054; OR 0.65, 95%CI 0.42-1.01) [13]. The AA genotype was protective against CD, but not against UC. Similarly, A-allele carrier status was linked to a significantly lower susceptibility to CD, whereas this correlation did not reach statistical significance for UC. These findings are not in accordance with previously published results by Elleisy *et al*, who reported a positive association between genotype AA of rs1285933 and risk for CD development (P=0.009; OR 1.90, 95%CI 1.18-3.05) [13]. The disparity observed between our findings and the published literature warrants further studies with larger cohorts to test the data provided in our study. Investigating these correlations in an ethnically diverse study population would also be appropriate, as interethnic variations could potentially account for these conflicting results. Supporting this rationale, Walker *et al* reported that polymorphisms of genes *ATG16L1*, *IRGM* and *IL23R* that were significantly associated with IBD development in western populations, were not associated with a heightened risk for IBD in Indian Asians. In the same study, the SNP frequencies also differed considerably between European and South Asian IBD patients [29].

CLEC5A shows a high expression in leukocytes (including monocytes, neutrophils and dendritic cells) and has been reported to interact directly with virions. This interaction is mediated by terminal fucose and mannose moieties of viral-derived glycans [15]. There is a great deal of evidence to suggest a link between inflammatory disorders, such as IBD, and *CLEC5A*. Activation of *CLEC5A* leads to enhanced recruitment of inflammatory macrophages and neutrophils in autoimmune inflammatory conditions [30]. *CLEC5A* has been reported to participate in innate immunity through the production of different proinflammatory cytokines and chemokines after stimulation with pathogens [16,31], while it is known that impaired bacterial clearance is an aggravating factor in CD pathogenesis [32]. *CLEC5A* expression has been found to be greater in patients with IBD compared with age and sex-matched controls [33], and more specifically to be highest in CD patients who are heterozygous for *NOD2* disease-causing mutations, thus supporting the hypothesis that both proteins may interact within a regulatory network that is involved in the pathophysiology of CD [21,34]. Targeting *CLEC5A* either directly, or via its receptor or signaling pathways in which it is involved, such as TREM-1/*CLEC5A*, reduced the release of proinflammatory cytokines and improved the clinical signs of different pathological states such as chronic obstructive pulmonary disease [35], autoimmune arthritis [30], and intestinal inflammation during colitis [36,37]. Macrophages highly expressing *CLEC5A* that present proinflammatory characteristics were found to be abundant in the intestinal *lamina propria* of IBD patients [38].

Dectin-1, the gene product of *CLEC7A*, is involved in the regulation of antifungal defense mechanisms and the modification of fungal and bacterial gut microbiota. Fungal microbiota distortion is implicated in IBD pathogenesis, as elevated levels of *Candida albicans (C. albicans)* [39-41] and decreased levels of Ascomycota, especially *Saccharomyces cerevisiae (S. cerevisiae)* [40,41], have been consistently reported in patients with IBD. *C. albicans* has been identified as an inducer of anti-*S. cerevisiae* antibodies in humans, which serve as valuable immunological markers for CD [42]. Yu *et al* have reported that fungal dysbiosis enhances oxidative phosphorylation by enhancing glutaminolysis in CD4 cells through the Dectin-1-Syk-NF-κB pathway, an effect that is thought to support proinflammatory cytokine production [43]. An upregulation of Dectin-1 expression is observed in macrophages and other immune cell types participating in the inflammatory cascades associated with IBD; however, this finding is not limited to IBD, as it extends to inflammation associated with non-IBD conditions, such as diverticulitis [44].

Several studies [14,25-27] have associated *CLEC7A* deletion with modified risk and severity of UC, supporting the notion that *CLEC7A* SNPs could lead to the formation of a variant Dectin-1 with impaired function. Iliev *et al* [14] reported that *CLEC7A* -/- led to more severe colitis in mice, which was alleviated by the use of antifungals. Contrary to these findings, in the study of Wang *et al* Dectin-1 and Dectin-2 knockout altered bacterial but not fungal microbiota, and conferred a protective effect against dextran sodium sulfate (DSS)-induced colitis in mice [27]. Similarly, Tang *et al* showed that *CLEC7A* deletion or antagonism rendered mice refractory to DSS-induced colitis, an effect attributable to an increase in *Lactobacillus marinus* that triggers Treg expansion in the intestines [26]. This alteration was concomitant with a reduction in antimicrobial peptides induced by Dectin-1 signaling [26]. Furthermore, in human patients suffering from IBD a diminished proportion of closely related *Lactobacillus* species has been observed, suggesting a possible similar influence of diminished Dectin-1 expression on microbiota that can cause Treg expansion. In mice, upon experimental *Candida tropicalis* colonization, the protective effect of the *CLEC7A* -/- deletion was reversed and the mice became largely susceptible to colitis, implying a complex dual effect for *CLEC7A* on fungal and bacterial gut microbiota. These collective findings suggest that Dectin-1 may play a role in regulating intestinal immune homeostasis through its influence on Treg cell differentiation, achieved via both the modulation of the gut microbiota and the prevention of fungal invasion [26]. A better understanding of how fungi elicit gut inflammation may be useful in the development of better therapies for IBD, particularly for individuals with severe UC carrying Dectin-1 risk alleles.

To conclude, the present study explored the association between *CLEC5A* and *CLEC7A* SNPs and the risk for development of CD and UC. Our results highlight the protective effect of *CLEC5A* rs1285933 GA and AA genotypes against CD development, a finding that contrasts with previously published data. The correlation between *CLEC5A* and UC was first explored in the present study, yielding a protective effect of the rs1285933 GA genotype. *CLEC7A* gene SNPs were not associated with a heightened risk for CD, confirming the current literature. *CLEC7A* rs2078178 AA genotype conferred a 14-fold risk increase for UC development, and could be evaluated as a potential genotypic biomarker in subsequent larger cohorts. *CLEC7A* rs16910631 CT genotype frequency was also higher among UC patients. Larger and preferably ethnically diverse cohorts are required in order to consolidate the presented findings and shape a robust understanding of the role of *CLEC7A* and *CLEC5A* genes in the etiopathogenesis of IBD. Such research could determine whether polymorphisms in genes involved in the explored pathways can serve as prognostic biomarkers for IBD, and whether the associated gene products are candidate molecules for targeted therapeutic interventions.

Summary Box

What is already known:

- Genome-wide association studies have identified more than 230 loci as contributory to inflammatory bowel disease pathogenesis
- *CLEC5A* SNP rs1285933 has been associated with a high risk for Crohn's disease (CD)
- *CLEC7A* SNPs rs2078178 and rs16910631 have been correlated with a high risk for medically refractory ulcerative colitis (UC)

What the new findings are:

- The correlation between *CLEC5A* and UC was first explored in the present study, which identified a protective effect of the rs1285933 GA genotype
- *CLEC7A* gene SNPs were not associated with a heightened risk for CD
- *CLEC7A* rs2078178 AA genotype is associated with a 14-fold higher risk for UC development and could be considered as a potential biomarker

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