Association of haplotypes of the TLR8 locus with susceptibility to Crohn's and Behçet's diseases

L. Ortiz-Fernández[1], J.-R. García-Lozano[1], M.-A. Montes-Cano[1], M. Conde-Jaldón[1],
E. Leo[2], N. Ortego-Centeno[3], M. Gómez-García[4], F.-J. García-Hernández[5], J.-L. Márquez[2],
G. Espinosa[6], G. Graña-Gil[7], J. Sánchez-Bursón[8], M.-R. Juliá[9], R. Blanco[10],
A.-C. Barnosi-Marín[11], R. Solans[12], P. Fanlo[13], M. Rodríguez-Carballeira[14], T. Camps[15],
S. Castañeda[16], J. Martín[17], M.-F. González-Escribano[1]

Lourdes Ortiz-Fernández, PhD*
José-Raúl García-Lozano, MD, PhD
Marco-Antonio Montes-Cano, PhD
Marta Conde-Jaldón, PhD
Eduardo Leo, MD
Norberto Ortego-Centeno, MD
María Gómez-García, MD
Francisco-José García-Hernández, MD
José-Luis Márquez, MD
Gerard Espinosa, MD
Genaro Graña-Gil, MD
Juan Sánchez-Bursón, MD
María-Rosa Juliá, PhD
Ricardo Blanco, MD
Ana-Celia Barnosi-Marín, MD
Roser Solans, MD
Patricia Fanlo, MD
Mónica Rodríguez-Carballeira, MD
Teresa Camps, MD
Santos Castañeda, MD
Javier Martín, MD
María-Francisca González-Escribano, PhD

*Authors' affiliations on page S-122.

Please address correspondence to:
Dr J.R. García-Lozano,
Servicio de Inmunología,
Hospital Universitario Virgen del Rocío,
Avda Manuel Siurot s/n,
41013 Sevilla, Spain.
E-mail:
jraul.garcia.sspa@juntadeandalucia.es

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ABSTRACT

Objetive. *The aim of this study was to investigate the role of the TLR8, a mediator of innate inflammatory response, in susceptibility to two immune-mediated disorders characterised by dysregulation of the immune response, Crohn's and Behçet's diseases (CD and BD).*

Methods. *A total of 844 CD, 371 BD patients and 1385 controls were genotyped in 8 tag single nucleotide polymorphisms (tSNPs) in the locus TLR8 (chromosome X). All these tSNPs have a minor allele frequency greater than 0.05 in the Caucasian population.*

Results. *The rs2407992 and the rs5744067 were associated with susceptibility to BD and CD, respectively (OR=1.34, 95%CI=1.10–1.62, p=0.0025 and OR=0.82, 95%CI=0.68–0.99, p=0.045, respectively). Although after stratification by gender, statistically significant differences in the distribution of the aforementioned SNPs were only observed in the females groups (BD OR=1.31, 95%CI=1.06–1.64, p=0.012 and CD OR=0.84, 95%CI=0.72–0.98, p=0.044) the trend was similar among males. Since the rs5744067 and rs2407992 are located in the same linkage disequilibrium block, we performed a haplotypic analysis by combination of the tSNPs. One haplotype (H1) was identified as a protective factor in BD (OR=0.75, 95%CI=0.62–0.90, p=0.0027) and another (H2) as a protective factor in CD (OR=0.78, 95%CI=0.64–094, p=0.0102). No statistically significant differences in the mean of the levels of expression attributable to the haplotype variants were found in the* in silico *analysis performed.*

Conclusion. *Our results suggest a relationship between the TLR8 and the susceptibility to CD and BD. Nevertheless, these differences could not be imputed to the levels of expression.*

Introduction

Disorders with clinical manifestations as different as an inflammatory bowel disease such as Crohn's disease (CD) and a systemic vasculitis such as Behçet's disease (BD) are immune-mediated diseases characterised by dysregulation of the immune response (1). The exact aetiology of these diseases remains unclear, although there are many studies suggesting that both disorders are complex diseases that are triggered in genetically predisposed individuals by the concurrence of certain environmental factors. The genetic association studies have provided evidence of the key role that an impaired response, innate or inflammatory, plays in the pathogenesis of several diseases affecting different organs and systems. Thus, currently it is known that IL23R and IL10 affect susceptibility to CD and BD, a finding that supports a relationship between the innate immunity and both diseases (2-5).

Since the identification of the nucleotide-binding oligomerisation domain containing 2 (NOD2) gene as the major factor in susceptibility to CD (6), other genes encoding molecules involved in innate inflammatory response, such as nucleic acid sensor, have been related to susceptibility to this disease. In this regard, several studies have reported association of CD with several Toll-like receptor (TLR) genes, such as TLR4 (7), TLR9 (8,9) and TLR10 (10,11). Regarding BD, the contribution of the class I region to susceptibility to this disease has been repeatedly

and consistently demonstrated (12). As it stated above, other genes encoding molecules related with the innate immunity contribute to the genetic basis of this disease. In addition to those mentioned above, genes encoding of sensors of bacterial nucleic acid, such as NOD2 and TLR4, as well as molecules of related pathways, such as TIRAP (an important molecule of the signaling cascade for TLR4) have also been associated with BD (13, 14). In a recently published study, we have identified the cluster IFI16-AIM2 as a new locus of genetic susceptibility to BD in the Spanish population (15). This cluster was identified in a screening in which a total of nine candidate genes encoding nucleic acid sensors were studied. Among these candidate genes, several members of the TLR family, specifically TLR3, TLR7, TLR8 and TLR9, were included. The TLR7 and TLR8 genes are located on chromosome X and, in general, there is a lack of statistical power in the genetic studies for the genes located in the heterochromosomes (16). In our previous study, one SNP located in the TLR8 locus was found to have a suggestive association with BD (ie. association was lost after the correction of the *p*-value). TLR8, which is located in intracellular compartments, such as the endosomes, recognises single stranded RNA and induces type I IFN production (17), suggesting that TLR8 signalling is important in the pathogenesis of the immune-mediated diseases. In fact, the association between polymorphisms in this gene and allergic and autoimmunity diseases have been previously investigated (18-21).

We decided to further investigate whether TLR8 could play a gender dependent role in the BD susceptibility and whether this gene influences the susceptibility to CD, a pathology that is also associated with an impaired innate inflammatory response and widely related with TLR genes.

Materials and methods
Patients and controls
A total of 371 BD and 844 CD patients and 1385 ethnically matched bone marrow donors, as healthy controls were included in this study. All those,

patients and controls, were unrelated Spanish Caucasian individuals.

BD patients fulfilled the 1990 International Study Group classification criteria for BD (22) and were recruited from different Spanish hospitals: A Coruña (CHU A Coruña), Almería (H. Torrecárdenas), Barcelona (H. Clinic, Vall d'Hebron and Mútua Terrassa), Granada (H. Clínico San Cecilio), Madrid (H. de la Princesa), Málaga (H. Carlos Haya), Palma de Mallorca (H, Universitari Son Espases), Pamplona (H. Virgen del Camino), Santander (H. Marqués de Valdecilla) and Sevilla (H. Virgen del Rocío and H. Virgen de Valme). Clinical features of BD patients are presented in Table I.

CD patients, 448 males and 396 females, diagnosed according to established clinical, endoscopic, radiological and histopathological criteria were recruited from two hospitals: H. Virgen de las Nieves (Granada) and H. Virgen del Rocío (Sevilla). The CD patients were classified according to the Montreal classification (23) (Table I).

The study was approved by the local ethics committees of the corresponding hospitals and all the participants gave written informed consent to be included. Peripheral blood was used as starting material in the case of healthy controls while in patient groups, both blood and saliva were used for obtaining DNA. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturer´s recommendations and stored at -20°C.

SNP selection and genotyping
Tag single nucleotide polymorphisms (tSNPs) were selected across each loci from the designated set of common SNPs genotyped in the CEU population (HapMap Project, Release 28, Phase II+III, NCBI build 36 assembly, dbSNP b126; http://www.hapmap.org). The tSNPs selection was done with pairwise $r^2 \geq 0.80$ and minor allele frequency (MAF)≥ 0.05 using the Haploview v. 4.0 software (http://www.broad.mit.edu/mpg/haploview/download.php) (24). According to the above rules, 8 tSNPs which permit the capture of 23 SNPs were selected (Fig. 1). BD pa-

tients and 854 controls were genotyped using the SEQUENOM iPlex MassARRAY platform (Sequenom, San Diego, CA, USA) (15) and CD patients and 531 controls by TaqMan SNP Genotyping Assays (Applied Biosystems, Barcelona, Spain) in a LightCycler 480 (Roche, Barcelona, Spain). To verify the inter-platforms reproducibility, 96 samples of controls were genotyped with both platforms.

Bioinformatic analysis of TLR8 expression
A gene-expression data set of lymphoblastoid cell lines derived from 270 unrelated individuals genotyped in the HapMap Project was obtained from the Gene Expression Omnibus (GEO) database (25, 26) to check the effect of the variants in the expression of the two most common TLR8 isoforms (transcripts 1 and 2). These two isoforms are similar from a functional point of view, although they differ in their N-terminus in both sequence and length.

Statistical analysis
Allele frequency distributions were compared using the χ^2 test and a corrected *p*-value (p_c) was calculated from 10,000 permutations (Haploview programme). The male haplotypes were directly assigned and female haplotypes were inferred using Famhap v. 19 (available at the website http://iweb.meb.uni-bonn.de/famhap/). In female group, genotypes of each SNP were assessed according to dominant [AA *vs.* AB+BB (A, major allele; B, minor allele)] or recessive (AA+AB *vs.* BB) models. The odds ratios (ORs) with their corresponding 95% confidence intervals (95% CI) were calculated using OpenEpi v. 2.3 software online (http://www.openepi.com). Statistical analysis of mRNA TLR8 expression was performed by the Joncheere-Terepstra method using the SPSS v. 18 software. *P*-values <0.05 were considered statistically significant.

Results
Table II shows the frequencies of the tSNPs of the TLR8 gene in BD and CD patients and healthy controls. The successful rate of genotyping was

Table I. Clinical features of the BD and CD patients.

Behçet's disease patients		Crohn's disease patients	
Male/female	160/211	Male/female	448/396
Age at diagnosis	38.7±13.8 years	Age at diagnosis	
Oral ulcers	100%	A1 < 16 years	10%
Genital ulcers	64%	A2 17-40 years	70%
Uveitis	59%	A3 > 40 years	20%
Arthritis	48%		
Vascular involvement	20%	Disease location	
Neurological involvement	22%	L1 ileal	32%
Gastrointestinal involvement	19%	L2 colonic	22%
		L3 ileocolonic	35%
		L4 isolated upper disease	11%
		Disease behaviour	
		B1 non-stricturing, non-penetrating	42%
		B2 stricturing	21%
		B3 penetrating	37%

>98% for all the SNPs included, and the study population was found to be in Hardy-Weinberg equilibrium for all the polymorphisms analysed ($p>0.05$). On the inter-platforms analysis, the concordance of the assigned genotypes in those samples studied using the two platforms was 100%.

The frequency of the C allele of the rs2407992 SNP was significantly higher among BD patients (45.8 *vs.* 38.6% in the control group, $p_c=0.014$, OR=1.34, 95%CI=1.10-1.62). With respect to the analysis after stratification by gender, statistically significant differences were observed between patients and controls in the female group in both models, the allelic and the recessive (Table III). The frequency of rs2407992C allele was significantly higher among female patients than among female controls (44.5 *vs.* 38.0%, $p=0.012$, OR=1.31, 95%CI=1.06–1.64) and females with two copies of rs2407992C allele had a statistically significant risk of susceptibility to BD (OR=1.64, 95%CI=1.36–2.44, $p=0.009$). Regarding male patients, no significant differences in the distribution of the rs2407992 alleles were found on comparing with the male control group although the trend of the distribution was similar as those observed in the female group (OR=1.33, 95%CI=0.93–1.92, $p=0.11$) (Table III).

With regards to CD patients, the frequency of the C allele of the rs5744067 was significantly lower among patients (16.4 *vs.* 19.3% in the control group, $p=0.045$, OR=0.82, 95%CI=0.68–0.99), although it did not reach statistical significance after permutation testing (Table II). However, after stratification by gender, statistically significant
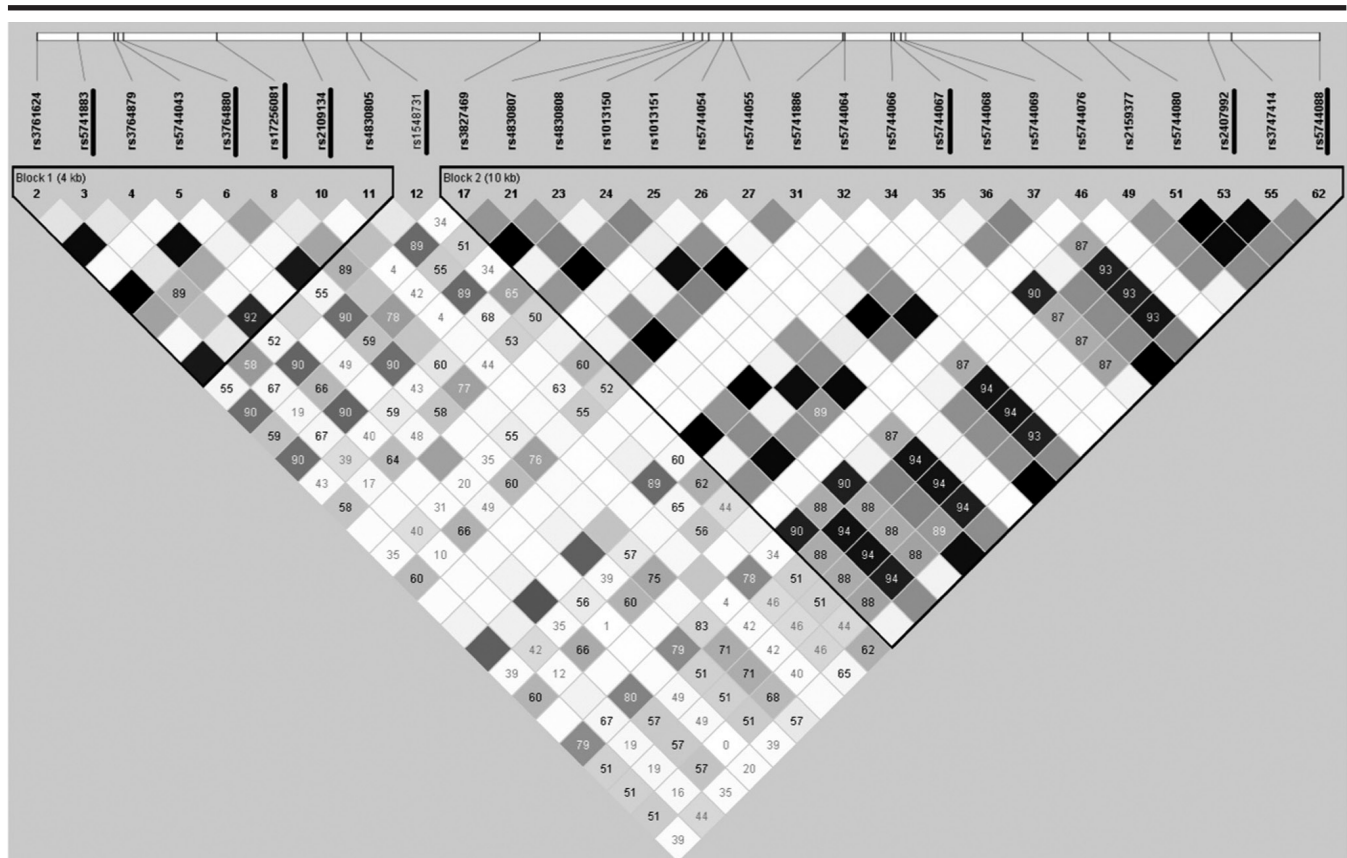

Fig. 1. Haplotype blocks described in the region ChrX:12832700..12851600 of HapMap Project in the CEU population. The linkage disequilibrium (LD) solid spine approach was used to define the haplotype blocks. Standard color-coding was used for LD plots: white ($r^2=0$), shaded gray ($0<r^2<1$), black ($r^2=1$). Squares without a number indicate D'=1. The SNPs included in this study are underlined.

Table II. Minor allele frequencies of the SNPs studied in the TLR8 gene in Behçet's and Crohn's disease patients and healthy controls.

SNP	Location (variant 1 / 2)	MA	Controls	BD patients	p	p_c	OR (95% CI)	CD patients	p	p_c	OR (95% CI)
rs5741883	Near 5' UTR	T	0.198	0.233	ns			0.187	ns		
rs3764880	5' UTR / Exon 1[1]	G	0.337	0.324	ns			0.326	ns		
rs17256081	Intron 2 / Intron 1	C	0.424	0.352	ns			0.459	ns		
rs2109134	Intron 2 / Intron 1	T	0.025	0.022	ns			0.024	ns		
rs1548731	Intron 2 / Intron 1	T	0.262	0.323	ns			0.288	ns		
rs5744067	Intron 2 / Intron 1	C	0.193	0.224	ns			0.164	0.045	ns	0.82 (0.68-0.99)
rs2407992	Exon 3[2] / Exon 2[2]	C	0.386	0.458	0.0025	0.0141	1.34 (1.10-1.62)	0.356	ns		
rs5744088	3' UTR	C	0.127	0.149	ns			0.140	ns		

[1]nonsynonymous polymorphism. [2]synonymous polymorphism. SNP: single-nucleotide polymorphism; MA: minor allele; OR (95%CI): odds ratio with 95% confidence interval; ns: not significant $p>0.05$. p_c: 10,000-fold permutation testing.

Table III. Distribution of allelic frequencies of the SNP rs2407992 (associated with BD) and the SNP rs5744067 (associated with CD) after stratification by gender.

rs2407992	MA			Allelic model		Dominant model		Recessive model	
				p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)
		Controls n=824	BD Patients n=211						
Female	C	0.380	0.445	0.012	1.31 (1.06-1.64)	0.11	1.31 (0.95-1.81)	0.009	1.64 (1.36-2.44)
		Controls n=561	BD Patients n=160						
Male	C	0.403	0.473	0.11	1.33 (0.93-1.92)				

rs5744067	MA			Allelic model		Dominant model		Recessive model	
				p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)
		Controls n=824	CD Patients n=396						
Female	C	0.191	0.167	0.044	0.84 (0.72-0.98)	0.0023	0.73 (0.59-0.89)	0.43	0.89 (0.67-1.18)
		Controls n=561	CD Patients n=448						
Male	C	0.190	0.157	0.17	0.79 (0.56-1.11)				

MA: minor allele; OR (95%CI): odds ratio with 95% confidence interval.

differences were observed in the allelic as well as the dominant model in the female group (OR=0.84, 95%CI=0.72–0.98, p=0.044; and OR=0.73, 95%CI=0.59–0.89, p=0.0023, respectively). No significant differences in the frequency distribution were found in males although, as in BD, the trend was similar to that observed in the female group (OR=0.79, 95%CI=0.56–1.11, p=0.17) (Table III).

The rs5744067 and rs2407992 are located in the same linkage disequilibrium (LD) block (Fig. 1), thus, we performed an analysis with the haplotypes constructed by combination of the SNPs included in this LD block: rs5744067, rs2407992 and rs5744088 (block 2 of Fig. 1). Four haplotype combinations with frequency greater than 0.05 and named (in order to their frequencies) from H1 to H4 were identified in our population (Table IV). The H1 haplotype tagged by the rs2407992G allele was identified as a protective haplotype in BD (OR=0.75, 95%CI=0.62–0.90, p=0.0027). While, the H2 haplotype, tagged by the rs5744067C allele, was identified as protective in CD (OR=0.78, 95%CI=0.64–0.94, p=0.0102).

Case-only phenotype analysis of CD patients revealed no association between H2 haplotype and mean age at diagnosis, disease location or disease behaviour (data not shown).

In order to explore whether differences in RNA expression among haplotypes could explain the association found, we analysed TLR8 expression levels of the variants of transcripts 1 and 2 using the expression profiles of GEO database. No statistically significant differences in the mean of the levels of expression attributable to the haplotype variants were found.

Discussion

In this study, we identified the TLR8 gene as a novel genetic susceptibility locus for BD and CD in the Spanish population. Analysing the possible relationship between nucleic acid sensors and BD, we identified association between the TLR8 gene and susceptibility to BD. In addition, this gene was involved in susceptibility to CD, a pathology which is also associated with impaired innate inflammatory response. Although the differences were only significant in female groups, we can not rule out a similar role for this gene in both genders, because the trend in male groups was the same as that observed in the corresponding groups of females. We have identified protec-

Table IV. Allelic frequencies of the major haplotypes constructed by combination of the 3 SNP located in the LD block 2 (Fig. 1) in Behçet's and Crohn's disease patients and healthy controls.

	Haplotypes[1]			Controls	BD Patients	p	OR (95% CI)	CD patients	p	OR (95% CI)
	rs5744067	rs2407992	rs5744088							
H1	T	G	G	0.607	0.536	0.0027	0.75 (0.62-0.90)	0.637	ns	
H2	C	C	G	0.192	0.220	ns		0.159	0.0102	0.78 (0.64-0.94)
H3	T	C	C	0.125	0.147	ns		0.140	ns	
H4	T	C	G	0.068	0.089	ns		0.058	ns	

[1]Haplotypes with frequency >0.05. OR (95%CI): odds ratio with 95% confidence interval; ns: not significant *p*>0.05.

tive haplotypes in BD (H1, tagged by the major allele of the rs2407992, G) and CD (H2, tagged by the minor allele of the rs5744067, C), in both cases, to have one or two copies of the corresponding associated haplotype has the same protective effect in the female groups. These result suggest that the dominant was the best fit model for the protective effect in the female group.

Genome-wide association (GWA) studies have identified common genetic variations associated with both CD and BD, a finding that supports the existence of shared pathways between these immune-mediated diseases (2-5). Most of the data from the chromosome X have not been analysed in these studies and, consequently, only a few SNPs located in this chromosome have been associated with the susceptibility to diseases (16). Regarding BD, only one previous study that investigated association between a single SNP in TLR8 and the disease has been published (27). This study was performed in the Chinese Han population and, in agreement with our data, it reported no association between the disease and the rs3764880. With regard to CD, one study has addressed the possible relationship between polymorphisms in TLR8 and CD (28). In this study, performed in an American cohort of European origin, the authors identified haplotypes that provide protection and risk to CD. Similarly to our study, the results did not reach statistical significance in the males and the association fits with a dominant model in females. Therefore both studies suggest a relationship between TLR8 gene and CD, although in contrast to our study, the haplotypes associated in the American cohort are located in the LD block 1

(Fig. 1). Bacterial nucleic sensors such as TLR4 and NOD2, previously described as involved in CD, have been recently implicated in susceptibility to BD (13). Altogether, these results suggest that the inflammatory pathways triggers by the recognition of bacterial and virus nucleic acid could contribute to the pathogenesis of both pathologies. There are some studies that establish a relationship between levels of expression of TLR8 and disease. Thus, an increased expression of TLR8 in different cell types (such as peripheral mononuclear blood cells, CD4+ T cells or monocyte) obtained from BD patients compared with controls (29), has been demonstrated. Likewise, expression of TLR8 mRNA and protein are highly up-regulated in the colonic epithelium from patients with active CD (30). The rs2407992 is a synonymous polymorphism and, therefore it does not introduce functional alterations in the TLR8 protein. Nevertheless, association between this polymorphism and susceptibility to allergic diseases has been reported, suggesting the authors a functional impact of this polymorphism on TLR8 splicing (19). However, this influence on splicing is not well established and, in any case, its consequences at the protein level are unknown. For these reasons, we asses a possible relationship between the variants of the genes and level of expression of the two most common isoforms. According to the results of the *in silico* analysis of the data set of lymphoblastoid cell lines of GEO, no differences in the transcription levels of TLR8 attributable to the alleles of rs2407992 were found. Regarding the rs5744067, located in an intron, no differences in the transcription levels

of the TLR8 were found in the *silico* analysis for this SNP. Influence of these variants in the mRNA levels of the other lesser common isoforms cannot be excluded because they were not included in the study. Nevertheless, the most likely situation is that the variants that we found as associated in the present study are just markers of the causal variants. The influence of the hypothetical causal variants could be related with differences in the 3' UTR region, the post-transcriptional control or alternative forms of the protein affecting its function.

In conclusion, our results suggest a relationship between the TLR8 locus and the susceptibility to immune-mediated diseases as CD and BD.

Authors' affiliations

[1]*Servicio de Inmunología, Unidad de Gestión Clínica "Intercentros de Laboratorios Clinicos", Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/ Universidad de Sevilla, Sevilla;*
[2]*Unidad de Gestión Clínica "Enfermedades Digestivas", Hospital Universitario Virgen del Rocío, Sevilla;*
[3]*Servicio de Medicina Interna, Hospital Clínico San Cecilio, Granada;*
[4]*Unidad de Enfermedad Inflamatoria Intestinal, Hospital Universitario Virgen de las Nieves, Granada;*
[5]*Servicio de Medicina Interna, Hospital Universitario Virgen del Rocío, Sevilla;*
[6]*Servicio de Enfermedades Autoinmunes, Hospital Clinic, Barcelona;*
[7]*Servicio de Reumatología, Complejo Hospitalario Universitario, A Coruña;*
[8]*Servicio de Reumatología, Hospital Universitario de Valme, Sevilla;*
[9]*Servei d´Inmunología. Hospital Universitari Son Espases. Palma de Mallorca;*
[10]*Servicio de Reumatología, Hospital Marqués de Valdecilla, Santander;*
[11]*Servicio de Medicina Interna, Hospital de Torrecárdenas, Almería;*
[12]*Servicio de Medicina Interna, Hospital Vall d´Hebron, Barcelona;*
[13]*Servicio de Medicina Interna, Hospital Virgen del Camino, Pamplona;*
[14]*Servicio de Medicina Interna, Hospital Universitari Mútua, Terrassa;*
[15]*Servicio de Medicina Interna, Hospital Universitario Carlos Haya, Málaga;*
[16]*Servicio de Reumatología, Hospital de la Princesa, Madrid;*
[17]*Instituto de Parasitología y Biomedicina López Neyra, Consejo Superior de Investigaciones Científicas, Granada.*

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