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# No evidence of association between genetic variants of the PDCD1 ligands and SLE

AK Abelson[1], CM Johansson[1], SV Kozyrev[1], H Kristjansdottir[1,2], I Gunnarsson[3], E Svenungsson[3], A Jönsen[4], G Lima[5], HR Scherbarth[6], S Gamron[7], A Allievi[8], SA Palatnik[9], A Alvarellos[10], S Paira[11], C Graf[12], C Guillerón[13], LJ Catoggio[14], C Prigione[15], CG Battagliotti[16], GA Berbotto[17], MA García[18], CE Perandones[19], L Truedsson[20], K Steinsson[2], G Sturfelt[4], B Pons-Estel[21,22], The Argentinean Collaborative Group and ME Alarcón-Riquelme[1]

[1]*Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden;* [2]*Center for Rheumatology Research, Landspitali, Reykjavik, Iceland;* [3]*Department of Medicine, Rheumatology Unit, Karolinska Institutet/Karolinska University Hospital, Solna, Sweden;* [4]*Department of Rheumatology, Lund University Hospital, Lund, Sweden;* [5]*Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición 'Salvador Zubirán', Mexico City, Mexico;* [6]*Servicio de Reumatología, Hospital Interzonal General de Agudos 'Dr Oscar Alende', Mar del Plata, Argentina;* [7]*Servicio de Reumatología de la UHMI 1, Hospital Nacional de Clínicas, Universidad Nacional de Córdoba, Córdoba, Argentina;* [8]*Hospital General de Agudos Dr Juán A Fernandez, Buenos Aires, Argentina;* [9]*Facultad de Ciencias Medicas, Universidad Nacional de Rosario y Hospital Provincial del Centenario, Rosario, Argentina;* [10]*Servicio de Reumatología, Hospital Privado, Centro Medico de Córdoba, Córdoba, Argentina;* [11]*Sección de Reumatología, Hospital José M Cullen, Santa Fe, Argentina;* [12]*Hospital San Martín, Paraná, Entre Ríos, Argentina;* [13]*Departamento de Inmunología, Instituto de Investigaciones Médicas 'Alfredo Lanari', Buenos Aires, Argentina;* [14]*Sección Reumatología, Servicio de Clínica Médica, Hospital Italiano de Buenos Aires y Fundación Dr Pedro M Catoggio para el Progreso de la Reumatología, Buenos Aires, Argentina;* [15]*Servicio de Reumatología, Hospital Provincial de Rosario, Rosario, Argentina;* [16]*Hospital de Niños Dr Orlando Alassia, Santa Fe, Argentina;* [17]*Servicio de Reumatología Hospital Escuela Eva Perón, Granadero Baigorria, Rosario, Argentina;* [18]*Servicio de Reumatología, Hospital Interzonal General de Agudos General San Martín, La Plata, Argentina;* [19]*Centro de Educación Médica e Investigaciones Clínicas (CEMIC), Buenos Aires, Argentina;* [20]*Institute of Laboratory Medicine, Lund University, Lund, Sweden and* [21]*Sanatorio Parque, Rosario, Argentina*

*PDCD1, an immunoreceptor involved in peripheral tolerance has previously been shown to be genetically associated with systemic lupus erythematosus (SLE). PDCD1 has two ligands whose genes are located in close proximity on chromosome 9p24. Our attention was drawn to these ligands after finding suggestive linkage to a marker (gata62f03, Z = 2.27) located close to their genes in a genome scan of Icelandic families multiplex for SLE. Here, we analyse Swedish trios (N = 149) for 23 single nucleotide polymorphisms (SNPs) within the genes of the PDCD1 ligands. Initially, indication of association to eight SNPs was observed, and these SNPs were therefore also analysed in Mexican trios (N = 90), as well as independent sets of patients and controls from Sweden (152 patients, 448 controls) and Argentina (288 patients, 288 controls). We do not find support for genetic association to SLE. This is the first genetic study of SLE and the PDCD1 ligands and the lack of association in several cohorts implies that these genes are not major risk factors for SLE.*
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## Introduction

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease characterized by autoantibodies directed against molecules found in the nucleus, cytoplasm and cell surfaces, accompanied by a wide range of clinical manifestations, including chronic inflammation of various tissues and organs. The genetic component of SLE is strong, with a concordance rate estimated to 24–69% in monozygotic and 2–3% in dizygotic twins[1–3] and an overall increased risk for relatives of SLE patients.[4]

A regulatory polymorphism in the gene encoding the immunoreceptor PDCD1 has previously been reported to be involved in susceptibility for human SLE.[5] We reasoned that genes encoding molecules that interact with PDCD1 could also be susceptibility factors. In Icelandic families, suggestive linkage ($Z = 2.27$) was

Correspondence: Dr ME Alarcón-Riquelme, Department of Genetics and Pathology, Rudbeck Laboratory, Dag Hammarskjölds väg 20, 751 85 Uppsala, Sweden.
E-mail: marta.alarcon@genpat.uu.se
[22]Dr Pons-Estel is the coordinator of the Argentinean Collaborative group. All members are found in the appendix at the end.
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reported to marker gata62f03 on chromosome 9p24.[6] This region harbours the genes for the two ligands of PDCD1, *PD-L1* (also CD274) and *PD-L2* (also CD273), which rendered them our main candidates for further analysis.

PD-L1 is constitutively expressed on B, T, myeloid and dendritic cells and is upregulated upon activation. It is also expressed in a wide range of non-haematopoietic tissues including the heart, lung and pancreas. PD-L2 is induced on macrophages and dendritic cells.[7–10] Many studies have demonstrated that the PDCD1 pathway has an inhibitory effect on T cells,[11–13] whereas others have indicated a stimulating effect.[14–16] PDCD1$^{-/-}$ mice have been shown to develop arthritis and lupus-like glomerulonephritis.[17] Furthermore, in murine models of diabetes, colitis and multiple sclerosis, *in vivo* blockade of PDCD1 and/or one of its ligands has been shown to either trigger or augment the disease or to have a therapeutic effect.[18–20] Thus, the connection with autoimmunity and the role of the PDCD1 pathway in peripheral tolerance has been well established.

PD-L1 and PD-L2 are located together within a segment of 120 kilobasepairs (kb) on chromosome 9p24. PD-L1 is 18 kb in size with seven exons, whereas PD-L2 is located 42 kb downstream of PD-L1 and has equally many exons, but stretches up to 60 kb.

Owing to the role of the ligands of PDCD1 in autoimmunity and our previous linkage evidence, we reasoned these genes were interesting candidates for genetic association with SLE. However, the data from this study do not support such an association.

## Results

In a continued analysis of the Icelandic multicase families displaying linkage ($Z = 2.27$) to marker gata62f03, just 267 kb upstream from the location of PD-L1 and PD-L2, we sequenced promoter regions and all exons in eight index cases from these families and eight Icelandic unrelated controls and identified 11 SNPs within or adjacent to the exons. We investigated the inheritance pattern of these SNPs, but found no significant evidence of association. The genetic material was, however, relatively small and rather suited for linkage than association studies. We therefore proceeded with genotyping of a set of trios from another Scandinavian population, namely Swedish.

In total, 23 SNPs within the PD-L1 and PD-L2 genes were selected for analysis (see Materials and methods section). Eight were found to be associated to SLE in the Swedish trios ($N = 149$) when analysed by haplotype-based haplotype relative risk[21] (HHRR). The family-based association test[22] (FBAT) did not, however, detect any association (Table 1).

**Table 1** Allele frequencies of SNPs in PD-L1 and PD-L2 for transmitted and non-transmitted alleles using HHRR and for patients and population controls

| *SNP* | *Location*[a] | *Sequence feature* | *Transmitted (HHRR)* | *Patients* N/*total* N *of alleles* | *Non-transmitted (HHRR)* | *Controls* N/*total* N *of alleles* | P-*value (HHRR)* | P-*value (FBAT)* |
|---|---|---|---|---|---|---|---|---|
| *PD-L1* | | | | | | | | |
| L1 × 11 | −85 | Promoter | 1 (0.3%) | NT | 2 (1.9%) | NT | NS | NS |
| rs10815225 | −62 | Promoter | 27 (9.3%) | 36/276 (13.0%) | 22 (19.8%) | 98/979 (9.8%) | 0.0037 | NS |
| rs7866740 | −29 | Promoter | 21 (7.3%) | 17/274 (6.2%) | 18 (15.3%) | 74/978 (7.6%) | 0.0011 | NS |
| rs17742278 | 5965 | Intronic | 74 (30.1%) | NT | 34 (27.6%) | NT | NS | NS |
| rs1411262 | 8862 | Intronic | 77 (30.6%) | NT | 34 (27.6%) | NT | NS | NS |
| rs10815226 | 9148 | Intronic | 57 (23.0%) | NT | 25 (22.9%) | NT | NS | NS |
| rs7041009 | 12686 | Intronic | 77 (30.3%) | NT | 32 (25.8%) | NT | NS | NS |
| C39 | 15529 | CNS | 48 (17.5%) | 42/278 (15.1%) | 35 (31.8%) | 140/845 (16.6%) | 0.0020 | NS |
| L1 × 73 | 17244 | Intronic | 18 (6.3%) | 16/272 (5.9%) | 17 (14.2%) | 64/995 (7.0%) | 0.0083 | NS |
| rs2297136 | 17399 | 3′ UTR | 157 (55.3%) | 135/294 (45.9%) | 58 (40.3%) | 465/996 (46.7%) | 0.0044 | NS |
| rs4143815 | 17701 | 3′ UTR | 74 (27.4%) | NT | 38 (32.2%) | NT | NS | NS |
| rs10118693 | 33683 | CNS | 70 (29.7%) | NT | 36 (36.7%) | NT | NS | NS |
| C57:2 | 33799 | CNS | 2 (0.8%) | NT | 0 (0.0%) | NT | NS | NS |
| *PD-L2* | | | | | | | | |
| rs16923189 | 74 | 5′ UTR | 62 (26.1%) | NT | 32 (29.1%) | NT | NS | NS |
| C91:1 | 7641 | CNS | 2 (0.7%) | NT | 1 (0.9%) | NT | NS | NS |
| C91:2 | 7667 | CNS | 2 (0.7%) | NT | 0 (0.0%) | NT | NS | NS |
| rs12001295 | 7690 | CNS | 10 (3.4%) | 16/285 (5.6%) | 12 (10.1%) | 50/840 (5.6%) | 0.0053 | NS |
| rs7870226 | 19447 | CNS | 83 (33.5%) | NT | 47 (38.2%) | NT | NS | NS |
| C103:2 | 19500 | CNS | 1 (0.4%) | NT | 0 (0.0%) | NT | NS | NS |
| C103:3 | 19512 | CNS | 4 (1.5%) | NT | 3 (2.9%) | NT | NS | NS |
| rs6476989 | 45002 | CNS | 1 (0.4%) | NT | 0 (0.0%) | NT | NS | NS |
| rs7854413 | 47138 | Exonic | 15 (5.1%) | 27/294 (9.2%) | 12 (10.7%) | 94/1016 (9.3%) | 0.029 | NS |
| rs7852996 | 59290 | Intronic | 10 (3.6%) | 23/243 (8.6%) | 12 (11.1%) | 68/830 (8.2%) | 0.0038 | NS |

Abbreviations: CNS, conserved non-coding sequence; FBAT, family-based association test; HHRR, haplotype-based haplotype relative risk; SNPs, single nucleotide polymorphisms; NS, non-significant; NT, not tested.
Results of the association analysis using HHRR and FBAT are shown as well as the location of the SNPs. SNPs with rs-numbers can be found in databases, other names indicate previously unknown SNPs identified by us. Minor allele frequencies are shown.
[a]Basepairs from transcription start.

In order to confirm the association or lack of association, we analysed the eight SNPs in a set of Mexican trios ($N = 90$). Neither HHRR nor FBAT tests were able to confirm the association (Table 2). The same eight SNPs were also examined in two sets of patients and unrelated controls from Sweden (152 patients and 448 controls) and Argentina (288 patients and 288 controls). No association was seen in either population (Tables 1 and 3), with the exception of allele T of L1 × 73, which in Argentina is found in 2.0% of patients and 4.5% in controls ($P = 0.0157$). However, for several reasons stated in Discussion section, we consider this a false–positive result.

**Table 2** Frequencies of PD-L1 and PD-L2 polymorphisms in Mexican trios

| *SNP* | *Transmitted* | *Non-transmitted* |
|---|---|---|
| | N/*total* N (%) | N/*total* N (%) |
| rs10815225 | 20/168 (11.9) | 15/118 (12.7) |
| rs7866740 | 8/174 (4.6) | 6/120 (5.0) |
| C39 | 39/174 (22.4) | 34/127 (26.8) |
| L1 × 73 | 8/174 (4.6) | 6/122 (4.9) |
| rs2297136 | 40/166 (24.1) | 31/113 (27.4) |
| rs12001295 | 9/176 (5.1) | 9/126 (7.1) |
| rs7854413 | 18/176 (10.2) | 13/125 (10.4) |
| rs7852996 | 16/174 (9.2) | 9/120 (7.5) |

**Table 3** Frequencies of PD-L1 and PD-L2 polymorphisms in Argentinean patients and controls

| *SNP* | *Patients* N/*total* N (%) | *Controls* N/*total* N (%) |
|---|---|---|
| rs10815225 | 69/566 (12.2) | 81/560 (14.5) |
| rs7866740 | 34/568 (6.0) | 32/562 (5.7) |
| C39 | 139/572 (24.3) | 147/544 (27.0) |
| L1 × 73 | 11/560 (2.0) | 25/552 (4.5) |
| rs2297136[a] | 208/552 (37.7) | 201/522 (38.5) |
| rs12001295 | 45/570 (7.9) | 37/540 (6.9) |
| rs7854413 | 60/566 (10.6) | 64/566 (11.3) |
| rs7852996 | 52/570 (9.1) | 57/570 (10.0) |

Abbreviation: SNP, single nucleotide polymorphisms.
Minor allele frequencies are shown.
[a]For rs2297136 the opposite allele is minor compared with Swedish and Mexican cohorts.

The Swedish and Argentinean populations are not similar enough for the two cohorts to be analysed as one, but a meta-analysis is applicable. We analysed all patients and controls from Sweden and Argentina using the Mantel–Haenszel method, and found no association. Haplotype frequencies estimated using PHASE[23,24] did not differ significantly between patients and controls (data not shown). A comparison of the degree of linkage disequilibrium (LD) in patients and controls in our cohorts found no indications of a conserved risk haplotype in the patients. The only noticeable difference was found in the Swedish cohort, where the patients actually appeared to have a slightly lower degree of LD than both healthy controls and healthy trio parents (data not shown).

Ten of the SNPs analysed here have now been included in the HapMap Project[25] (http://www.hapmap.org). In the European HapMap trios, there are three large blocks of LD in the region of the PDCD1 ligands. Roughly estimated, the first block corresponds to PD-L1, the second block to the intergenic region and the first exon of PD-L2, and the third block comprises the remaining six exons of PD-L2. The markers analysed in this study are localized in 5′, middle and 3′ regions of both genes, as well as in the intergenic region and all LD blocks are represented by at least one SNP (Figure 1).

## Discussion

We initially found indications of association between SLE and eight SNPs within PD-L1 and PD-L2 in Swedish trios analysed by the HHRR test. However, other statistical methods applied to the same data set failed to confirm this association.

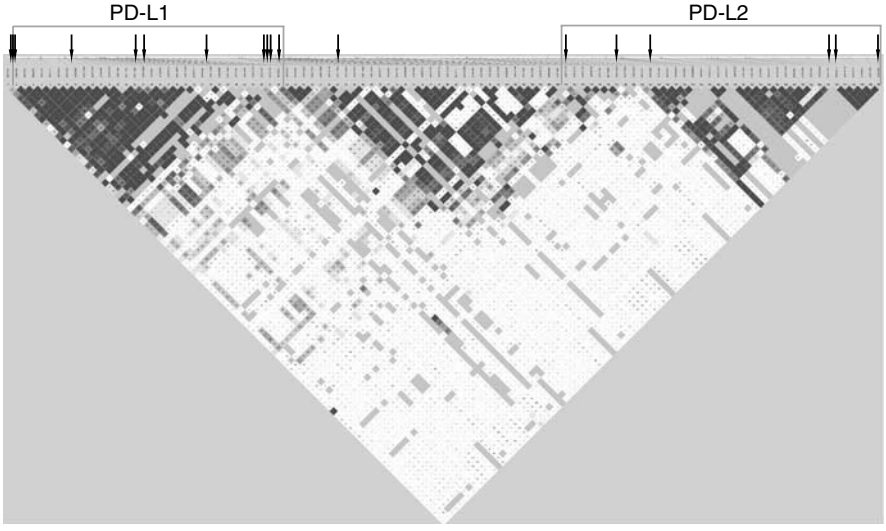


**Figure 1** Pattern of LD according to HapMap data (www.hapmap.org). Dark grey indicates strong LD, light grey to white indicates weak or no LD. Position of SNPs analysed in this study are indicated by arrows.

The SNPs that were associated in the Swedish trios were also analysed in Mexican trios, and in two independent sets of patients and controls from Sweden and Argentina. No association was seen in these three cohorts, except the highly questionable protective association of L1 × 73 in Argentina. For several reasons we believe this association to be, most likely, false positive. First, the risk of type I error is greater for a rare allele than for a common.[26] Second, this Argentinean cohort has shown to be somewhat heterogeneous with about 20% Amerindian admixture,[27] which further increases the risk of false-positive results owing to population substructure. Third, a large number of markers have now been analysed in several cohorts, increasing the probability of spurious association. If corrected for multiple testing, the association would become nonsignificant.

There are many different statistical methods for analysing genetic association. Family-based studies have the advantage of lower sensitivity to bias owing to population substructure. On the other hand, bias can be introduced by inclusion of incomplete families or by reconstruction of parental genotypes from their offspring, causing inflated frequencies of rare alleles in the family-based controls.[28–31] Why we observe association with the HHRR test and not FBAT, could perhaps be explained by the incomplete trios included in our material. FBAT should be less sensitive to this type of bias, as it is designed to apply to any type of pedigree, including incomplete trios.[22] Comparison of non-transmitted allele frequencies from our HHRR analysis with frequencies in the healthy controls further supports this theory: the frequency of rare alleles is often considerably higher among nontransmitted than among healthy controls. Estimated haplotype frequencies did not differ significantly between patients and controls, and the degree of LD is not higher in patients than controls, which provides further evidence for lack of association in the region.

Power estimations using Quanto[32–34] (http://hydra.usc.edu/GxE/) shows that our case-control cohorts from Sweden and Argentina each have high power to detect risk alleles with odds ratio (OR) above 1.5 (Table 4). Our trios have somewhat lower power than the case–control cohorts, and as many of our trios are incomplete, the actual power should be even lower. However, this study comprises several cohorts, which increases the overall power of the study substantially. We estimate that the risk of failing to detect true association in all of the investigated cohorts is low. Data for risk allele frequencies 0.1, 0.2 and 0.3 and ORs 1.5 and 2.0 are shown in Table 4. Although the SNPs analysed in this study were selected as possible functional polymorphisms rather than 'tag SNPs' for the total-genetic variation in these genes, they are spread across both genes and represent all LD blocks according to the HapMap data. The existence of association to a polymorphism of strong effect in the investigated populations does therefore not appear very likely. Nevertheless, we cannot exclude the possibility of a risk factor that is not in LD with any of the markers analysed in this study. It should be noted that although the cohorts in this study are mainly of European descent, the Argentinean and Mexican are admixed with Amerindians, who have not yet been included in the HapMap project. Consequently, HapMap LD patterns may not apply as well to these populations. We also want to clarify that the functional role that the ligands of PDCD1 may have in human SLE has not been the subject of this study.

PDCD1 has in many studies been shown to be associated not only to SLE, but also rheumatoid arthritis, type I diabetes and multiple sclerosis.[5,35–37] It has been implied that its ligands also are involved in autoimmunity.[18–20] The result of this study, however, does not find a role for genetic variants of the PDCD1 ligands in SLE susceptibility. To our knowledge, this is the first study of genetic association between the PDCD1 ligands and an autoimmune disease. The absence of association in the cohorts studied here suggests that these genes are not major risk factors for SLE.

## Materials and methods

*Subjects*

We studied 149 Swedish trios consisting of SLE patients and unaffected parents, as well as some unaffected siblings (58 patients in complete trios, 71 with one parent sample and 20 with only information from siblings). In total, 98 healthy siblings were included. We also studied 90 Mexican trios (56 patients in complete trios, 16 with one parent, and 18 patients with only siblings). In total, 27 siblings and five other close relatives are included. In addition, two cohorts of patient–control material including 152 independent patients and 448 controls from Sweden, and 288 patients and 288 controls from Argentina were studied. Ten Icelandic multicase families previously described[6] were also examined. All affected individuals fulfilled four or more of the American college of rheumatology's criteria for SLE.[38] This study was approved by local ethics committees in all countries and all subjects studied have given their informed consent.

*Identification/selection of SNPs*

Three approaches were used to identify SNPs: first, sequencing of all exons of *PD-L1* and *PD-L2* in SLE

**Table 4** Power of cohorts to detect risk factor with frequency of 0.1, 0.2 and 0.3 and odds ratio (OR) of 1.5 or 2.0

| *Cohort* | *Frequency = 0.1* | | *Frequency = 0.2* | | *Frequency = 0.3* | |
|---|---|---|---|---|---|---|
| | *OR = 1.5* | *OR = 2.0* | *OR = 1.5* | *OR = 2.0* | *OR = 1.5* | *OR = 2.0* |
| Swedish trios ($N = 149$) | 43.0% | 82.7% | 54.3% | 91.2% | 56.0% | 91.2% |
| Mexican trios ($N = 90$) | 30.8% | 64.3% | 38.9% | 75.3% | 40.2% | 75.4% |
| Swedish cases ($N = 301$) and controls ($N = 488$) | 74.7% | 99.3% | 85.8% | 99.9% | 86.3% | 99.8% |
| Argentinean cases ($N = 288$) and controls ($N = 288$) | 64.6% | 97.2% | 76.9% | 99.2% | 77.6% | 99.1% |

patients and healthy controls identified 11 SNPs within or adjacent to the exons. Second, sequences of potential regulatory importance were identified by species sequence comparison between human and murine *PD-L1* and *PD-L2*, including the 42 kb intergenic region and 20 kb upstream of *PD-L1* using Vista.[39,40] Thirteen regions were conserved non-coding sequences (CNS) with a threshold of 75% identity in at least 100 bp. Within these 13 CNS, we identified 10 SNPs. We also searched the introns of *PD-L1* for clusters of transcription factor binding sites (TFBS), with a focus on haematopoietic transcription factors. Five such clusters were located and sequenced in patients and controls, identifying one SNP. SNPs listed in databases were analysed for alteration of TFBS. One SNP was found to disrupt the binding of RUNX1, which was implicated in the association of SLE and PDCD1.[5] Thus, a total of 23 SNPs were selected for analysis.

*Genotyping*
Swedish trios and Icelandic families were genotyped by sequencing using BigDye Terminator (Applied Biosystems, Foster City, CA, USA) for all SNPs except rs16923189, which was genotyped by restriction fragment length polymorphism (RFLP) using the enzyme *PvuII*. Mexican trios as well as Argentinean and Swedish patients and controls were genotyped by TaqMan SNP genotyping assays (Applied Biosystems). Genotypes determined by RFLP or TaqMan SNP genotyping were verified by sequencing of randomly selected samples. Rs7852996 was genotyped by sequencing in all materials, as other methods failed.

*Statistical analysis*
All SNPs were in Hardy–Weinberg equilibrium in all investigated populations. Swedish and Mexican trios were analysed by HHRR included in the Analyze software[21] and by FBAT.[22] For the case–control cohorts, $\chi^2$ analysis of $2 \times 2$ contingency tables was applied. Haplotypes were estimated using the PHASE v2.1 software.[23,24]

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## Appendix

The Argentine Collaborative group is composed of the following members: Pilar C Marino, MD, Estela L Motta, MD Servicio de Reumatología, Hospital Interzonal General de Agudos 'Dr Oscar Alende', Mar del Plata, Argentina; Cristina Drenkard, MD, Emilia Menso, MD Servicio de Reumatología de la UHMI 1, Hospital Nacional de Clínicas, Universidad Nacional de Córdoba, Córdoba, Argentina; Guillermo A Tate, MD Organización Médica de Investigación, Buenos Aires, Argentina; Jose L Presas, MD Hospital General de Agudos Dr Juán A Fernandez, Buenos Aires, Argentina; Marcelo Abdala, MD, Mariela Bearzotti, PhD Facultad de Ciencias Medicas, Universidad Nacional de Rosario y Hospital Provincial del Centenario, Rosario, Argentina; Francisco Caeiro, MD, Ana Bertoli, MD Servicio de Reumatología, Hospital Privado, Centro Medico de Córdoba, Córdoba, Argentina; Susana Roverano, MD, Hospital José M Cullen, Santa Fe, Argentina; Cesar E Graf, MD (*) Griselda Buchanan, PhD (**), Estela Bertero, PhD (*), (*) Hospital San Martín, Paraná, (**) Hospital Felipe Heras, Concordia, Entre Ríos, Argentina; Sebastian Grimaudo, PhD, Jorge Manni, MD Departamento de Inmunología, Instituto de Investigaciones Médicas 'Alfredo Lanari', Buenos Aires, Argentina; Enrique R Soriano, MD, Carlos D Santos, MD Sección Reumatología, Servicio de Clínica Medica, Hospital Italiano de Buenos Aires y Fundación Dr Pedro M Catoggio para el Progreso de la Reumatología, Buenos Aires, Argentina; Fernando A Ramos, MD, Sandra M Navarro, MD Servicio de Reumatología, Hospital Provincial de Rosario, Rosario, Argentina; Marisa Jorfen, MD, Elisa J Romero, PhD Servicio de Reumatología Hospital Escuela Eva Perón, Granadero Baigorria, Rosario, Argentina; Juan C Marcos, MD, Ana I Marcos, MD Servicio de Reumatología, Hospital Interzonal General de Agudos General San Martín, La Plata, Argentina; Alicia Eimon, MD Centro de Educación Médica e Investigaciones Clínicas (CEMIC), Buenos Aires, Argentina.