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Expression of ICAM1 and VCAM1 serum levels in rheumatoid arthritis clinical activity. Association with genetic polymorphisms

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Abstract. To investigate the association of sICAM-1 and sVCAM-1 with *ICAM1* 721G>A and *VCAM1* 1238G>C polymorphisms and rheumatoid arthritis (RA) clinical activity, sixty RA patients and 60 healthy non-related subjects (HS) matched for age and sex were recruited. Soluble adhesion molecules were determined by ELISA technique. Rheumatoid factor (RF), C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) were measured by routine methods. Disability and clinical activity was measured with Spanish-HAQ-DI and DAS28 scores, respectively. The *ICAM1* and *VCAM1* polymorphism were identified using the PCR-RFLP procedure. Inter-group comparison showed increased levels of sICAM-1 and sVCAM-1 in RA patients (284 and 481 ng/mL) *versus* HS (132 and 280 ng/mL); in the RA group, significant correlations between sVCAM-1 and RF ($r = 0.402$), ESR ($r = 0.426$), Spanish-HAQ-DI ($r = 0.276$), and DAS28 ($r = 0.342$) were found, whereas sICAM-1 only correlated with RF ($r = 0.445$). In RA patients, a significant association with the 721A allele of *ICAM1* polymorphism ($p = 0.04$), was found. In addition, the allele impact ($G/A + A/A$) of this polymorphism was confirmed, ($p = 0.038$, OR = 2.3, C.I. 1.1–5.0). sVCAM-1 and sICAM-1 serum levels reflected the clinical *status* in RA, independently of the *ICAM1* and *VCAM1* polymorphism. However, the *ICAM1* 721A allele could be a genetic marker to RA susceptibility.

Keywords: Rheumatoid arthritis, polymorphism, soluble adhesion molecules, ICAM-1, VCAM-1

1. Introduction

The rheumatoid arthritis (RA) natural history involves clinical manifestations characterized by remission and recurrent activity stages with variable severity, secondary mainly to chronic inflammation of the synovial membrane [1], this tissue is an exclusive microenvironment where the perpetuation of the abnormal immune response occurs [2–4]. The most important pathological mechanism at an early stage of the inflammation process occurs when leukocytes firmly attach to the activated synovial endothelium, infiltrate the vessel wall, activate and release interleukin-1 (IL-1) and tumor necrosis factor-α, (TNF-α) which in turn stimulates the endothelial cells (EC) within the joint, increasing the expression of cell adhesion molecules (CAMs). Finally, CAMs perform and mediate continuously in the leukocyte-endothelium interaction [3–6].

Along CAMs, intercellular (ICAM-1) and vascular cell (VCAM-1) adhesion molecules belong to the cytokine inducible immunoglobulin-like (Ig-like) superfamily, and are receptor-like membrane bound proteins that bind leukocyte integrins. Macrophage-1 antigen (Mac-1) and lymphocyte function associated antigen-1 (LFA-1) are the ligands of ICAM-1, while very late

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Table 1
ICAM1 and *VCAM1* SNPs data

Genes	*Locus*	SNP	Exon	Codon substitution	Protein domain	Sequence primers pairs
*ICAM1**	19p13.3–13.2	721G>A	4	241 Gly>Arg	Ig-like 3	F: 5'-CCGTGGTCTGTTCCCTGTAC-3' R: 5'-GAAGGAGTCGTTGCCATAGG-3'
*VCAM1**	1p32-31	1238G>C	6	413 Gly>Ala	Ig-like 5	F: 5'-GCTTTTTGCTTGCGATTTG-3' R: 5'-CCAGTATCTTCAATGGTAGGGATG-3'

*Information from references 18, 33, 15 and 24. *ICAM1*: intercellular adhesion molecule 1; *VCAM1:* vascular cell adhesion molecule 1; G: guanine; A: adenine; C: citosine; T: timine; Gly: glycine ; Arg: Arginine ; Ala: alanine; Ig: Immunoglobulin domain; F: forward ; R: reverse.

antigen activation-4 is the VCAM-1 ligand [7–9]. Circulating soluble CAMs (sCAMs) result either from alternating splicing of mRNA or proteolysis of the membrane-bound protein form. Increased sCAM levels are found in patients with infection, cancer, inflammatory and autoimmune diseases, as a consequence of endothelial activation. Thus, sCAM concentration reflects the endothelial expression [10,11]. Although RA has an unknown aetiology, it is considered multifactorial in origin with a polygenic component. Genetic contribution to RA, however, is still controversial [12, 13].

Since genetic variants that affect functional domains of the molecules, the *ICAM1* and *VCAM1* genes are possible factors for diseases with an inflammatory component, as well as RA.

Human *ICAM1* and *VCAM1* genes single-base polymorphisms with amino-acid substitution at the Ig-like domain are known [14,15]. These domains are related with leukocyte integrin binding. In fact, other *ICAM1* genetic polymorphisms have already been associated with RA [16–18]. Therefore, the purpose of this study was to investigate the association of genetic variants of adhesion molecules *ICAM1* 721G>A and *VCAM1* 1238G>C and their soluble-protein concentration with RA clinical activity.

2. Subjects, materials and methods

In a case-control study, 60 RA patients classified according to the American College of Rheumatology (ACR) criteria [19] and 60 healthy subjects (HS), matched for age and sex ethnicity were studied. RA patients were recruited at the outpatient Rheumatology Department in the Hospital Civil "Fray Antonio Alcalde" from Guadalajara, Jalisco, Mexico. The HS group was composed of healthy adult volunteers. In both study groups were excluded individuals with infection diseases, malignancy, renal and metabolic diseases such as diabetes mellitus. All individuals from both groups were non-related Mexican mestizos, according to the National Institute of Anthropology [20], i.e., an individual that was born in Mexico, with a Spanish last name, and a family history of Mexican ancestors for at least three generations. A written consent form was obtained from all participants before enrolment, fulfilling Helsinki Declaration guidelines.

Patients were evaluated and classified by two independent rheumatologists. Demographic and clinical variables included age, sex, disease evolution, history of drug use, and current therapy. Disability and disease activity was measured using the Spanish HAQ-DI (Spanish version of the Health Assessment Questionnaire Disability Index) and DAS28 (Disease Activity Score, 28 joints) scores [21,22]. Blood samples were obtained from antecubital venipuncture after an overnight fast. Rheumatoid factor (RF), C-reactive protein (CRP, IMMAGE® Immunochemistry Systems Beckman Coulter, Inc. Fullerton, CA), erythrocyte sedimentation rate (ESR, Wintrobe method), white blood cell count (WBC) and platelet count (PLT, Cell-dyn 3700 Abbott Diagnostics™. Abbott Park, Illinois, USA) were determined.

Serum concentrations of sICAM-1 and sVCAM-1 were determined using a commercial enzyme-linked immunosorbent assays (ELISA, R&D Systems Inc., Minneapolis, MN, USA). Sensitivity was 0.35 ng/mL for sICAM-1 and 0.6 ng/mL for sVCAM-1.

The genotypes were characterized using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Genomic DNA was extracted according to the Miller method [23]. Primer sequences for *ICAM1* and *VCAM1* amplification are shown in Table 1. In a 25 μL final volume, PCR was carried out as described previously [15,24–26]. In brief, amplified fragments (15 μL) were subjected to restriction enzyme digestion, 1 U of *Bsr* GI or *Cac* 8I, (New England Biolabs Inc., Ipswich, MA, USA), during 2 and 16 h at 37°C, for *ICAM1* or *VCAM1* genes, respectively. Electrophoresis was done at a constant voltage

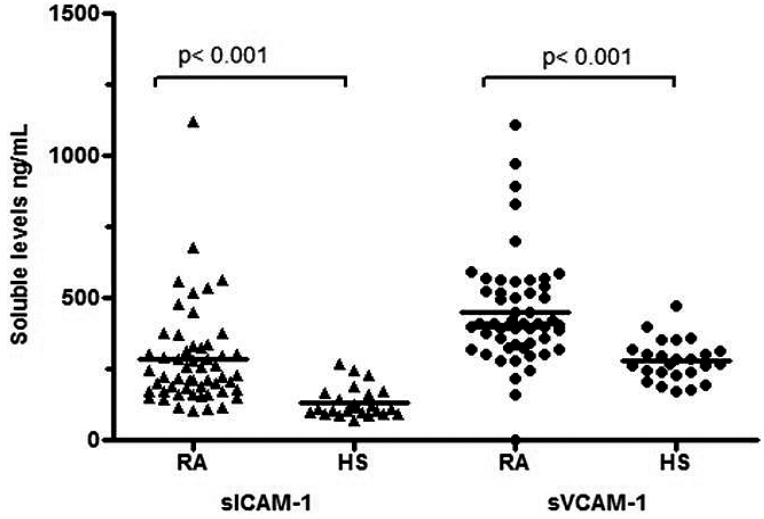

Fig. 1. Serum concentrations of sICAM-1 and sVCAM-1 in RA (rheumatoid arthritis) and HS (healthy subjects). CAM serum levels are expressed as mean ± SD.

of 80 V on 3% agarose gels stained with 0.1 μg of ethidium bromide.

ICAM1 allele G lacks *Bsr*GI a restriction site and is defined by a 110 bp fragment, while allele A, that contains this restriction site, as two digested bands of 90-20 bp. *VCAM1* allele G (absent *Cac 8I* restriction site) is represented by a 251 bp fragment, and allele C (containing the restriction site) by two bands 201 and 50 bp in length. On both polymorphisms the homozygote showed the corresponding single-band pattern of each allele, and heterozygote exhibit a three-band pattern. For confirmation purposes, representative samples of each genotype were sequenced in an ABIPRISM 310 Sequencer (Applied Biosystems Foster, City, CA, USA).

All data were captured and analyzed using SPSS version 10.0 (SPSS Inc. Chicago, Illinois). Arithmetic mean, minimum, and maximum values for quantitative data are presented. Mean comparison of two independent samples between groups was performed (Student t test). Data from serum concentrations of CAMs, the laboratorial assessment and disease variables were subjected to Pearson and Spearman's correlation tests. Genotype inter-group comparisons by means of all variables were done with the Kruskal-Wallis and the Mann-Whitney U tests. An X^2 test, with Yates' correction when was applicable, was used to test genotype proportions against Hardy-Weinberg expectations. Inter-group allele comparisons were performed by the Fisher exact test. Odds ratios (ORs, with 95% confidence intervals, CI_{95}; Epi Info 6.04, CDC) were calculated for allele and RA status. A $p < 0.05$ value was considered as the statistically significant threshold.

3. Results

3.1. Clinical features

The HS mean age was 39 ± 12 years whereas in RA group was 46 ± 13 years and the ratio male/female was 9/51 in both groups. The mean body mass index was similar in HS and RA groups (26 ± 4.0 kg/m^2 and 27 ± 4 kg/m^2, respectively). The disease mean duration was 10.7 ± 9 years. The extraarticular manifestations and drug treatment of the RA patients are shown in Table 2. None of the patients were treated with any TNFα blockers.

3.2. Comparison of sICAM-1 and sVCAM-1 levels between RA and HS

The RA group showed higher levels of sICAM-1 and sVCAM-1 (284 and 481 ng/mL) than HS (132 and 280 ng/mL, respectively) (Fig. 1).

3.3. sCAM correlations

sICAM-1 and sVCAM-1 were correlated between them ($r = 0.40$, $p = 0.002$). Significance between sVCAM-1 and RF (53 seropositives and 7 seronegatives), ESR levels, Spanish HAQ-DI, and DAS28 was found, whereas sICAM-1 only correlated with RF. The correlations are shown in Table 3.

Table 2

Relationship of sICAM-1 and sVCAM-1 levels with extraarticular manifestations and drug treatment in RA

	Extraarticular manifestations						Drug treatment					
	Rheumatoid nodules		*Sicca* syndrome		Cutaneous vasculitis		Prednisone		DMARDs		NSAIDs	
$n = 60$	Present	Absent	Present	Absent	Present	Absent	With	Without	With	Without	With	Without
	13	47	18	42	2	58	13	47	36	24	53	7
sICAM-1	369 ± 275	261 ± 127	228 ± 72	258 ± 189	173 ± 36	289 ± 176	286 ± 146	285 ± 184	289 ± 196	279 ± 142	290 ± 186	253 ± 47
p	NS		NS		**0.038**		NS		NS		NS	
sVCAM-1	638 ± 410	429 ± 177	408 ± 96	490 ± 281	445 ± 82	476 ± 263	524 ± 266	459 ± 257	485 ± 277	461 ± 234	450 ± 200	646 ± 494
p	**0.012**		NS		NS		NS		NS		NS	

The sICAM-1 and sVCAM-1 levels are expressed in ng/mL (mean $\pm$ SD), sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1. DMARDs: Disease modifying anti-rheumatic drugs; NSAIDs: non steroidal anti-inflammatory drugs; NS: No significant.

Table 3
Correlations of sICAM-1 and sVCAM-1 with the laboratorial assessments and RA activity indexes

Laboratorial assessment	Mean ± SD	sICAM-1		sVCAM-1	
		r	p	r	p
sICAM-1 (ng/mL)	285 ± 174	–	–	–	–
sVCAM-1 (ng/mL)	475 ± 258	**0.404**	0.002	–	–
RF (UI/mL)	607.9 ± 1142	**0.445**	0.005	**0.402**	0.005
#ESR (mm/h)	40.3 ± 11	0.270	NS	**0.426**	0.003
CRP (mg/L)	29.7 ± 38	0.005	NS	0.029	NS
Activity indexes					
#HAQ-DI (score 0–3)	1.20 ± 0.8	0.097	NS	**0.276**	0.046
#DAS 28 (score 0–10)	6.23 ± 1.2	0.120	NS	**0.342**	0.048

sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1. RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C reactive protein; HAQ-DI: Health Assessment Questionnaire Disability Index (Spanish version); DAS28: Disease Activity Score using 28 joint counts; r: correlation coefficient; *Pearson correlation; #Spearman correlation.

Table 4
Genotype and allele frequency of *ICAMI 721 G* > A polymorphism

Group	*Genotype, n (%)*			*Impact of allele A, n (%)*	*Allele, n (%)*	
	G/G	*G/A*	*A/A*	*G/A plus A/A*	*G*	*A*
RA	31 (53)	26 (42)	3 (5)	∗29 (47)	88 (73)	*32 (27)
HS	43 (63)	15 (33)	2 (4)	17 (37)	101 (83)	19 (17)

RA: rheumatoid arthritis HS: Healthy subjects $n = 60$ by group. Genotype inter-group comparison yielded a non-significant difference. *Allele frequency ($p = 0.040$) and impact of A allele (genotypes G/A plus A/A) [$p = 0.038$; OR = 2.3 (1.1 to 5.0)], in RA group *versus* HS group was different.

Table 5
Genotypes and allele frequency of *VCAM1* 1238 G>C polymorphism

Group	*Genotype, n (%)*			*Allele, n (%)*	
	G/G	*G/C*	*C/C*	*G*	*C*
RA	58 (97)	2 (3)	0 (0)	118 (98)	2 (2)
HS	59 (98)	1 (2)	0 (0)	119 (99)	1 (1)

Allele and genotype inter-group comparison (exact test) yielded non-significant differences.

3.4. Genetic polymorphisms

Genotype and allele frequencies in RA and HS are shown in Tables 4 and 5. For both polymorphisms, genotype proportions in HS group did not deviate from the ones predicted by the Hardy-Weinberg law ($p > 0.05$).

ICAM1 polymorphism analysis (Table 4) showed a higher frequency of A allele in RA than HS groups (27% vs 17%, $p = 0.04$). The genotype analysis did not show statistical significance ($p = 0.10$). When we analyzed the allele impact, including the genotypes that containing A allele (represented by genotypes G/A plus A/A) a significant association in RA group was found ($p = 0.038$, OR = 2.3, CI 1.1–5.0). No differences ($p > 0.10$) in other variables [HAQ-DI, DAS28, RF, CRP, ESR, WBC, and PLT] were observed. With respect to *VCAM1* polymorphism non significant association was found.

4. Discussion

In this case-control study, elevated levels of sICAM-1 and sVCAM-1 reflected the clinical activity in RA. This finding is supported because we identified a significant correlation between sVCAM-1 with sICAM-1, RF and ESR levels; Spanish-HAQ-DI and DAS28 indexes.

High levels of sCAMs have been observed in RA, juvenile RA, psoriatic arthritis, juvenile idiopathic arthritis, synovitis and osteoarthritis [27–32]. In our study, the correlations between sCAMs levels with the clinical activity suggest that they have a significant role in the pathogenesis of the disease. Klimiuk et al., observed high serum levels of sICAM-1 and sVCAM-1 in RA patients with synovitis, especially with follicular type of synovitis [31]. In the present study, the positive correlation between sVCAM-1 and clinical scores, RF and ESR, was observed, whereas, we only identified a positive correlation between sICAM-1 with RF. These

findings suggest that, sVCAM-1 could play a preferential role in RA. These results are supported by previous reports that described a significant positive correlation between sVCAM-1 with ESR and CRP, meanwhile sICAM-1 did not correlate either with disease markers or clinical activity scores [27,28,31,32]. The possible explanation is that ICAM-1 is a molecule of constitutive expression, whereas VCAM-1 is inducible by cytokine stimulation such as TNF-α and IL-1β, two abundant cytokines in inflamed RA synovium [24,28, 33].

In addition, when classified the RA patients according to extraarticular manifestations, we identified a significant association between the presence of rheumatoid nodules with high levels of sVCAM-1. This finding is significant because previously Corona-Sánchez et al., reported high TNF-α levels in RA patients with extraarticular manifestations [47]. Alternately, Elewaut et al., 1998 and Edwards et al., 1993, confirms the low or absent expression of VCAM-1 *versus* ICAM-1 expression that was more pronounced in the RA-nodules [48, 49]. The probable justification is that TNF-α induce *de novo* expression of VCAM-1 and upregulate ICAM-1 on vascular endothelium [27]. Rheumatoid nodules are the most frequent extraarticular sign in RA, classic rheumatoid nodules commonly occur in geneticall-ly predisposed patients and correlated with severe and seropositive arthritis [50].

In contrast we did not find association between the presence of rheumatoid-vasculitis and high levels of sICAM-1. However, this finding is important because systemic rheumatoid-vasculitis frequently affects small and medium-size blood vessels, is one of the most harmful complications of RA and more often than not occurs in patients who have longstanding disease, generally of more than 10 year duration.

Other studies support the existence of histological patterns of CAMs in cutaneous necrotizing vasculitis and endothelial cells, which expressed increased levels of ICAM-1 and VCAM-1. In RA patient's formerly low frequency of clinical features of RA-associated vasculitis has been reported, on the other hand a typical predictors of vasculitis in patients with RA consist of clinical and genetic factors these to broadcast especially influence on the occurrence of the disease in the susceptible host [51–53]. Nonetheless, although rheumatoid-vasculitis is an unusual but well described complication of RA, this result cannot be completely explained because, first the RA-vasculitis pathophysiology continues to be imperfectly understood and, second we only indentified two RA patients with vasculitis.

Given their central role in the inflammatory response, suggested by other authors [15,34–36] the *ICAM1* and *VCAM1* genes are potential candidate genes for inflammatory diseases.

Here, we did not find an association between genetic variants of *ICAM1* 721G>A and *VCAM1* 1238G>C polymorphism with the sICAM-1 and sVCAM-1 expression respectively (data not shown). However, an associated study in healthy subjects, reported a significant effect of *ICAM1* (721G>A/241Gly>Arg) with serum sICAM-1 levels, but this was a very weak association [37].

The genetic contribution to RA susceptibility is well accepted [12,13]. The *ICAM1* 721G>A polymorphism has been associated in several diseases including: Behcet's disease [34], endometriosis [35,36], protection from transplant associated vasculopathy after cardiac transplantation [38], Graves disease [39], polymyalgia rheumatica/giant cell arteritis [40], chronic renal allograft failure [41], whereas other studies failed to find a significant contribution of the 721A allelic variant in inflammatory diseases [14,25,42–46]. In our RA group, a significant association with the 721A allele variants of *ICAM1* polymorphism ($p < 0.04$), was found. However, when we analyzed the allele impact (G/A+AA) of this polymorphism a significant association for the 721A allele was confirmed ($p < 0.038$, OR $= 2.3$, C.I. 1.1–5.0). Our results are in agreement with the study of Macchioni et al., whom reported association with the 721A (R241) allele in Italian RA patients. Moreover, this study showed a frequency of 12.8% 721A/R241 allele in RA patients [16], whereas in the present study a 27% frequency was found. Another study of Korean RA patients this polymorphism was not identified [18]. These differences between populations can be explained by the genetic background that influences the inter-population variability of the Mexican population.

VCAM1 polymorphism, was not previously studied in RA patients, and we not find an association in these patients. However, in healthy African Americans was reported a high frequency of the *VCAM1*G/C genotype (27%) whereas in German Caucasians, they reported a 23% G/C frequency [14,15]. In this study, the frequency of *VCAM1* G>C polymorphism was very low [3% (RA) versus 2% (HS)].

In summary, our results suggest that the sVCAM-1 and sICAM-1 serum levels reflect the clinical activity status in RA because they are associated with RF, ESR, HAQ-DI and DAS28 indexes independently of *ICAM1* and *VCAM1* polymorphism, but the *ICAM1* 721A allele could be a genetic marker to RA susceptibility in Western of Mexico.

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