

Association of the MCP-1 –2518 A/G Polymorphism and No Association of Its Receptor CCR2 –64 V/I Polymorphism with Lupus Nephritis

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ABSTRACT. Objective. To evaluate whether the A/G polymorphism at position –2518 in the regulatory region of the monocyte chemoattractant protein-1 (MCP-1) or the V/I polymorphism at position –64 of the receptor, CCR2, are associated with lupus nephritis (LN) or any clinical characteristics of the disease or with renal survival in a patient population.

Methods. We selected 197 patients with lupus nephritis and 220 matched healthy controls for study. MCP-1 and CCR2 genotyping was performed by polymerase chain reaction. Clinical and laboratory data were compiled from patients' charts over followup that ranged from 6 months to 10 years.

Results. The G/G genotype of MCP-1 was more common in LN patients ($p = 0.019$), while the A allele was associated with healthy controls ($p = 0.007$) as was the V allele of CCR2 ($p = 0.046$) compared to LN patients. Clinical index measures [SLE Disease Activity Index (SLEDAI)], immunological markers, renal histology, renal function at enrollment, and renal survival were not influenced by these polymorphisms. A less aggressive renal disease, measured by renal SLEDAI index, was associated with the V allele of the CCR2 gene polymorphism.

Conclusion. These findings support that MCP-1 –2518 G/G is associated with LN but there was no association of this genotype with renal function or renal survival. When studying CCR2 –64 V/I polymorphism we showed a positive association of the V allele with healthy controls but no association of the genotype with LN patients. (First Release March 15 2010; J Rheumatol 2010;37:776–82; doi:10.3899/jrheum.090681)

Key Indexing Terms:
LUPUS NEPHRITIS

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Lupus nephritis (LN), the kidney disease of systemic lupus erythematosus (SLE), contributes substantially to patient morbidity and mortality¹. While there is emerging knowledge about the regulatory elements of the inflammatory response in LN, less is known about genetic factors that may predispose to its development or progression². The effects of functional genetic polymorphisms of inflammatory mediators in LN, such as interleukin 10, RANTES, tumor necrosis factor- α , Fc γ receptors, and monocyte chemoattractant protein-1 (MCP-1), have been examined, but controversy about their role remains^{3–5}.

Two genetic MCP-1 polymorphisms have been reported but only the one at position –2518 influences transcription activity⁶. There are some data that MCP-1 expression is upregulated in glomerular and tubular cells and urinary concentrations of MCP-1 are elevated in patients with active LN⁷. This upregulation of MCP-1 expression could be genetically determined by its functional polymorphism, therefore some populations should be more “reactive” than others toward the production of this chemokine, and ethnicity of patients could play an important role in this response^{2,8}.

Tucci, *et al* showed a strong association of the MCP-1 –2518 G/G with SLE and LN in an American population composed of 51% whites, 35% blacks 12% mixed, and 2% Asians⁹. Sanchez, *et al* studied a Spanish population of Caucasian origin comparing MCP-1 genotype frequencies, and obtained no significant difference between SLE patients and healthy controls¹⁰. Another negative study was published by Kim, *et al*¹¹ from a Korean population. They showed similar frequencies of –2518 MCP-1 polymorphism in LN patients and controls in spite of increased urinary excretion of MCP-1, correlated to proteinuria, in their patients¹¹. Lima, *et al* studied a Mexican Mestizo population

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and showed that -2518 *MCP-1* polymorphism was moderately associated to SLE¹². Brown, *et al* studied an American population of 36.7% Caucasian and 53.7% African American, and showed that CCL-2 A (-2518) G genotype was a significant risk factor for SLE among Caucasians but not African Americans¹³. In summary, it is likely that the racial composition of the study population would be an important factor in the association of LN with *MCP-1* polymorphism.

CCR2 is a high affinity receptor of MCP-1 that is expressed mainly on T cells and monocytes¹⁴. However, CCR2 expression has recently been described in epithelial, endothelial, and vascular smooth-muscle cells¹⁵⁻¹⁷, suggesting a role of the MCP-1/CCR2 system in conferring a proliferative and migratory phenotype in cells other than monocytes, as reported by Burt, *et al* in podocytes *in vitro*¹⁸. In CCR2, a G to A nucleotide substitution has been detected at position 190 (CCR2 -V64I). It has not been proven whether the 64I mutation is a functional or just a neutral polymorphism in autoimmune diseases. There are few reports on CCR2 gene polymorphism in SLE or LN. Aguilar, *et al* studied a Spanish population and observed no association between the -64 V/I CCR2 polymorphism and SLE¹⁹; as similarly observed by Ye, *et al* among Chinese with SLE compared to controls²⁰.

We examined the influence of functional *MCP-1* polymorphisms on risk of developing LN (compared to healthy controls), and whether any genotype pointed to a more inflamed phenotype and more aggressive renal disease. We also studied if receptor CCR2 -64 V/I polymorphism showed any particular role in this scenario.

MATERIALS AND METHODS

Study population. We selected 197 patients with lupus nephritis and 220 healthy controls for study. Patients with SLE fulfilled the American College of Rheumatology 1982 revised criteria²¹ and kidney biopsy was performed in all. Class of nephritis was determined according to the WHO classification²². All adult patients (age > 18 yrs) undergoing followup in the Nephrology Division between July 2005 and July 2007 were enrolled. Patients with diabetes, hepatitis B, hepatitis C, and HIV were excluded. Patient followup ranged from 6 months to 10 years and clinical and laboratory examinations were performed, with a regular outpatient recall system scheduled by the attending physicians. Conventional immune-suppression drugs were administered according to clinical and laboratory criteria. The most common therapeutic regimens for patients with class IV disease included oral and intravenous corticosteroids and intravenous cyclophosphamide, both administered according to the protocol devised by the US National Institutes of Health²³. Some patients responsive after 6 months of intensive treatment were converted to azathioprine or mycophenolate mofetil, according to the attending physician's opinion. For patients with class V disease we used the protocol devised by Austin, *et al*²⁴ with oral corticosteroids. Over the course of the study, laboratory testing was periodically performed to determine serum creatinine, complement, antinuclear antibody (ANA), anti-double-stranded DNA (dsDNA), anticardiolipin antibody (aCL), blood cell counts, proteinuria, and urinalysis. ANA was determined by immunofluorescence in Hep-2 cells, anti-dsDNA by immunofluorescence using *Crithidia luciliae* as substrate, complement by radial immunodiffusion, aCL by ELISA, and lupus anticoagulant by the coagulo-

metric method with simplified dilute Russell's viper venom test (DRVVT). Clinical and laboratory data were compiled from patient charts. Laboratory data were collected at baseline (around 1 week before renal biopsy) and at the last followup examination prior to July 2007. Disease activity was assessed using the SLE Disease Activity Index (SLEDAI) and renal disease activity by the renal SLEDAI score²⁵. Renal insufficiency was defined²⁶ as serum creatinine ≥ 1.2 mg/dL, and progression of renal disease by duplication of serum creatinine or endstage renal disease (dialysis or transplant) on followup. The control group for the genotyping study consisted of 220 healthy individuals matched by race and sex to patients with LN, obtained from a larger DNA bank in the Laboratory of Genetics and Molecular Cardiology, INCOR, Sao Paulo²⁷. Blood samples were collected for DNA genotype assay on enrolment. All subjects, controls and patients with lupus nephritis, were also classified by ethnicity according to a validated questionnaire for the Brazilian population²⁸. Subjects were classified as Brazilian Caucasians or Afro-South Americans by phenotypic characteristics (skin color, hair texture, shape of the nose, aspect of the lips, and jaw position). The Research Ethics Committee of the University of Sao Paulo approved the study protocol and all patients gave written informed consent.

DNA isolation and genotype polymorphism of MCP-1 and CCR2. Genomic DNA was extracted from peripheral leukocytes obtained from EDTA-treated whole blood using the salting-out technique²⁹ and *MCP-1* and *CCR2* genomic variants were detected by polymerase chain reaction (PCR) followed by restriction enzyme fragment analysis with specific primers as described^{30,31}. *MCP-1* and *CCR2* gene regulatory regions were amplified by adding 1 μ L of genomic DNA to a solution of 10 μ M Tris-HCl (pH = 9), 50 mM KCl, 2.5 mM MgCl₂, 100 mM of each dNTP, 0.3 U of Easy Taq DNA Polymerase (Invitrogen[®]), and 5 pmol of each primer. The following primers were used for *MCP-1*: forward, 5'-CCG AGA TGT TCC CAG CAC AG-3' and reverse: 5'-CTG CTT TGC TTG TGC CTC TT-3'; and for *CCR2*: forward 5'-TTG TGG GCA ACA TGA TGG-3' and reverse: 5'-CTG TGA ATA ATT TGC ACA TTG C-3'. PCR for *MCP-1* was carried out in a thermocycling apparatus (PTC-DNA, Engine Tetrad 2; Bio-Rad/MJ Research, Hercules, CA, USA) by cycling at 94°C for 1 min, and then at 92°C for 30 s, 59.6°C for 3 min, 72°C for 1 min for 35 cycles, followed by 10 min at 72°C. The procedure was repeated for *CCR2* amplification with an adjusted 53.4°C temperature in the third sequence of cycling. The amplified *MCP-1* product with 930 bp was digested with PVU II restriction enzyme (Invitrogen) at 37°C overnight in a humid chamber while *CCR2* product amplified, 348 bp was digested by a Bsa BI restriction enzyme (New England Biolabs, Beverly, MA, USA) at 60°C overnight in a humid chamber. The products of digestion were analyzed by phototyping in 1.5% agarose gels containing ethidium bromide. *MCP-1* samples with a single 930 bp band were identified as A/A. Samples with 3 bands of 930, 708, and 222 bp were typed as A/G; those with 2 bands of 708 and 222 bp were typed as G/G¹³. *CCR2* samples with a single 348 bp band were identified as V/V, samples with 3 bands of 348, 183 and 165 bp were typed as V/I, and those with 2 bands of 183 and 165 bp were typed as I/I³².

Statistical methods. Continuous and semicontinuous variables were tested if parametric or not and expressed as mean \pm standard deviation. For independent variables, Student's unpaired t test (with Welch's correction if necessary) was used. In comparing 2 or more groups, ANOVA for categorical variables was used together with Tukey's modified post-test and these are also expressed as mean \pm standard deviation of the sample. Nonparametric data were expressed by median values and percentiles and compared by Mann-Whitney test or by Kruskal-Wallis test after Muller-Dunn post-test if more than 2 groups were compared. Categorical data were expressed as absolute (n) and relative (%) frequencies. For matrices in which the results were genotypic, the chi-square test for trend was used. For square matrices (2 \times 2) the chi-square test with Yates's correction was used. Hardy-Weinberg equilibrium for the genotypes was tested by chi-square goodness-of-fit test. Progression of renal disease and kidney survival were analyzed by Kaplan-Meier curves with log-rank test. The α risk adopted for the study was $\leq 5\%$ ($p \leq 0.05$) and β risk $\leq 20\%$.

RESULTS

Polymorphisms of *MCP-1* –2518 A/G and its receptor *CCR2* –64 V/I were studied in 197 women with lupus nephritis and 220 matched healthy controls. Both groups had similar ages, patients 28.6 ± 9.8 years, controls 30.1 ± 10.7 years, and similar race distribution: 65.5% and 72.3%, respectively, were Brazilian Caucasians and 34.5% and 27.7% were Afro-South Americans. Other clinical and laboratory characteristics of LN patients are shown in Table 1.

MCP-1 –2518 A/G and *CCR2* –64 V/I genotypes in patients and controls. There was a significant association of the GG genotype of *MCP-1* with LN patients compared to controls (p = 0.019, OR 2.27). Otherwise, for the allele frequency distribution of *MCP-1* there was a significant association of the A allele with controls (p = 0.007, OR 0.8; Table 2).

For *CCR2* –64 V/I polymorphism distribution, there was no association of the genotype with LN, while there was an association of the V allele with the control group (p = 0.046, OR 0.86; Table 3).

Analysis of MCP1 –2518 A/G and CCR2 –64 V/I genotypes in patients and SLEDAI scores. There was no association of the *MCP-1* and *CCR2* genotypes with SLEDAI index scores, but there was a significant association of the V allele frequency of the *CCR2* (p = 0.046) with a less aggressive renal disease, revealed by SLEDAI renal score < 8 (the median value in our population; Table 4).

Analysis of MCP1 –2518 A/G and CCR2 –64 V/I genotypes

Table 1. Characteristics of patients with lupus nephritis (LN) (n = 197) at enrollment. SLEDAI scores and proteinuria expressed as median (25%–75%); followup data are mean ± SD.

SLEDAI score	20 (16–24)
SLEDAI renal score	8 (8–12)
Protein excretion, g/day	4.4 (2.7–7.1)
Patients with PCr > 1.2 mg/dl, %	56.3
Patients with positive anti-dsDNA, %	74.6
Patients with positive anti-cardiolipin, %	37.6
Patients with low C3, %	81.2
Patients with low C4, %	49.7
WHO class of LN (no. subjects) III + IV/V/VI	131/61/5
Followup, mo	69 ± 37

PCr: plasma creatinine.

and ethnic distribution, immunological markers, and histological classes of LN. There was no association of genotype frequency of the 2 genes with LN patients compared to controls concerning ethnic distribution, immunological markers, or histological classes of LN.

Analysis of MCP1 –2518 A/G and CCR –64 V/I genotype and renal function. Renal function was studied by serum creatinine on a followup basis, from the time of the histological diagnosis of LN until the closure of this protocol. Creatinine value > 1.2 mg/dl was established to define renal failure. Using this criterion, 56.3% of our population had renal failure at the diagnosis of LN, while 39% (77 patients) showed it at the end of the followup (69 ± 37 mo). Analysis of patients showed there was no association of the studied genotypes with renal failure at diagnosis of nephritis, with AA 50.5%, AG 38.7%, and GG 10.8% for the group with creatinine > 1.2 mg/dl; and AA 46.5%, AG 38.4%, and GG 15.1% for the group with creatinine ≤ 1.2 mg/dl.

Progression and regression of renal failure of patients with LN and genotypes. Fifty-one (45.9%) of 111 patients that had creatinine > 1.2 mg/dl at onset of LN showed improved renal function (PCr ≤ 1.2 mg/dl) on followup; whereas 17 (19.8%) out of 86 with normal renal function (PCr < 1.2 mg/dl) showed diminished renal function on followup (PCr ≥ 1.2). There were no differences of genotype *MCP-1* polymorphism distribution between the group with disease progression (n = 17; AA 50%, AG 33%, GG 17%) and the group with improvement of renal function (n = 51; AA 55%, AG 38%, GG 7%). Time-course analyses of renal survival, defined by doubling of serum creatinine or endstage renal disease with respect to the *MCP-1* –2518 A/G and *CCR2* –64 V/I polymorphism genotypes, are shown in Figures 1 and 2. Kaplan-Meier analysis showed that there was no influence of the genotype studied on the progression of renal disease.

DISCUSSION

We examined the role of the polymorphism of the *MCP-1* distal regulatory region (–2518 A/G) and its receptor *CCR2* (–64 V/I) polymorphism in lupus nephritis. Our results showed that patients with the –2518 G/G *MCP-1* genotype had 2- to 3-fold higher risk of developing LN, whereas con-

Table 2. Genotype and allele distribution of *MCP1* –2518 A/G polymorphism in patients with lupus nephritis (LN) and controls. Values are number (%) of subjects.

	LN Patients n = 197	Controls, n = 220	p	OR	95% CI
Genotype					
A/A	96 (48.7)	125 (56.8)	0.059	0.77	0.53–1.13
A/G	76 (38.6)	84 (38.2)	0.527	0.90	0.58–1.40
G/G	25 (12.7)	11 (5.0)	0.019	2.27	1.74–3.53
Allele frequency					
A	268 (68)	334 (75.9)	0.007	0.80	0.81–0.84
G	126 (32)	106 (24.1)	0.189	1.19	0.82–1.71

Table 3. Genotype and allele distribution of *CCR2* -64 V/I polymorphism in patients with lupus nephritis (LN) and controls. Values are number (%) of subjects.

	LN Patients, n = 197	Controls, n = 220	p	OR	95% CI
Genotype					
VV	148 (75.1)	177 (80.5)	0.120	0.84	0.62–1.14
VI	44 (22.3)	41 (18.6)	0.664	1.01	0.60–2.0
II	5 (2.5)	2 (0.9)	0.445	3.33	0.36–30.72
Allele frequency					
V	340 (86.3)	395 (89.8)	0.046	0.86	0.84–0.86
I	54 (13.7)	45 (10.2)	0.421	1.22	0.70–2.14

Table 4. Genotype and allele distribution of *CCR2* -64 V/I polymorphism and renal SLEDAI in patients with lupus nephritis. Values are number (%) of subjects.

	CCR2				
	Genotype	Allele			
	VV	VI	II	V	I
Renal SLEDAI ≥ 8 , n = 157	113 (72)	39 (24.8)	5 (3.2)	265 (84.4)	49 (15.6)
Renal SLEDAI < 8, n = 40	35 (87.5)	5 (12.5)	0 (0)	75 (93.8)*	5 (6.2)

SLEDAI: SLE Disease Activity Index. * p = 0.046.

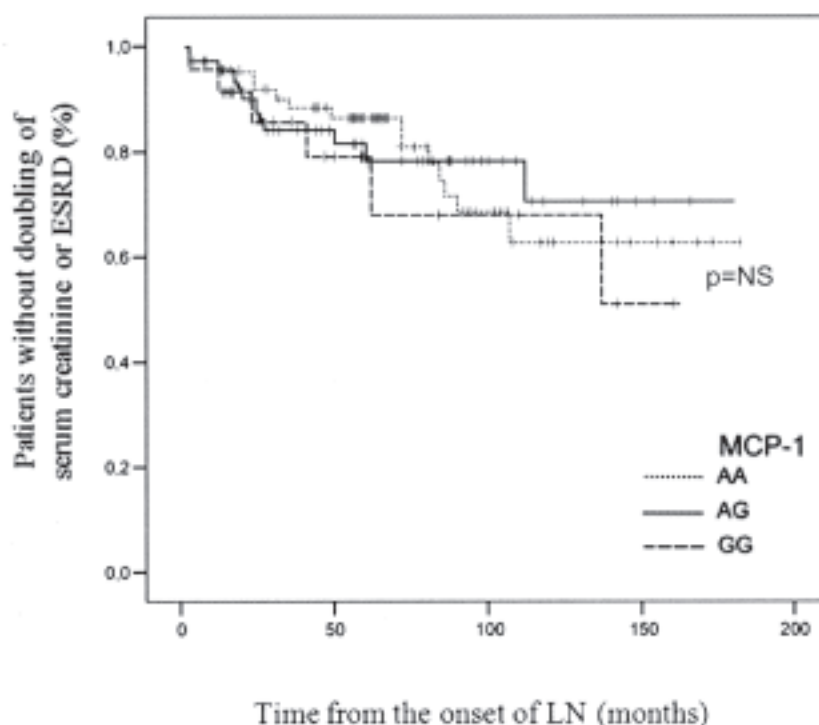


Figure 1. Kaplan-Meier analysis of renal survival in patients with lupus nephritis (LN) with respect to doubling of serum creatinine or endstage renal disease (ESRD) and *MCP-1* -2518 genotype.

trols showed association with the A allele (Table 2). Similar results were obtained by Tucci, *et al* studying an American sample⁹, while reports from Spain¹⁰ and Korea¹¹ showed no association, pointing to ethnicity as an impor-

tant factor in this gene distribution and association with LN disease.

We examined ethnicity and stratified our patients into Caucasians and Afro-South Americans. There were no dif-

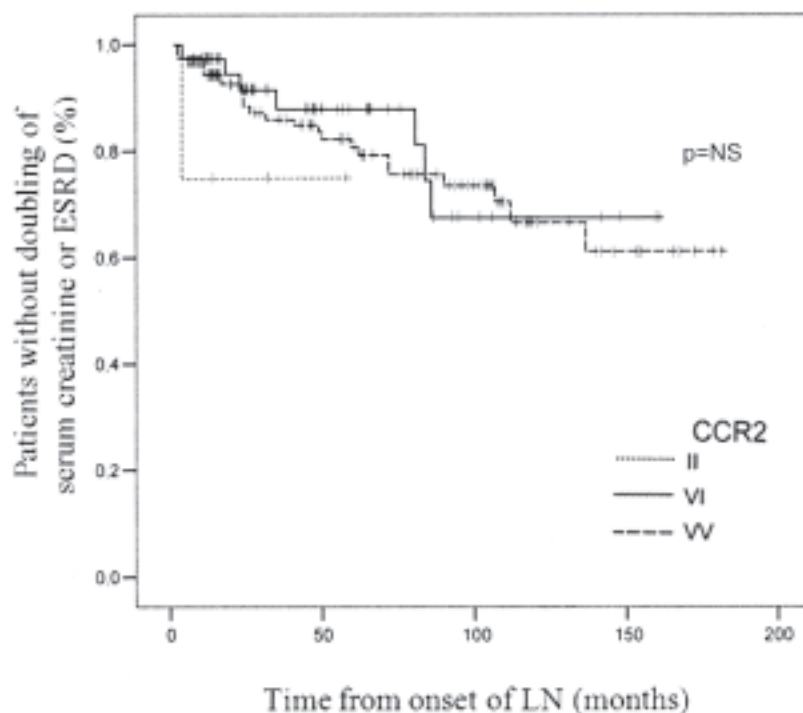


Figure 2. Kaplan-Meier analysis of renal survival in patients with lupus nephritis (LN) with respect to doubling of serum creatinine or endstage renal disease (ESRD) and *CCR2* -64 genotype.

ferences between the 2 groups in the distribution of the *MCP-1* genotypes, in contrast to data reported by Brown, *et al*¹³, studying an American population, which suggested that Caucasian carriers of the G allele are 4-fold more likely to have SLE than those with the AA genotype.

As a functional polymorphism⁶ it is known that monocytes/macrophages from SLE patients with the G/G genotype produced more MCP-1, with increased urinary levels⁷. Thus, we could expect that our patients with the G/G genotype would respond with amplified inflammation, mainly in the kidney. We therefore looked for any association of the *MCP-1* genotype polymorphism distribution with kidney function at the onset of LN and on followup, as well as any association with any immunological marker of systemic severity of the disease.

There was no association of the studied genotypes with plasma creatinine levels at the time of diagnosis of nephritis or at the last followup with the patients. Time-course analysis of renal survival showed no influence of the genotypes in the progression of renal disease (Figures 1 and 2).

Literature data on genotype distribution and renal function are scarce. Kim, *et al* found no differences in blood urea nitrogen levels or creatinine clearance among different genotype groups¹¹. We found no publications on time-course analysis of renal survival with which to compare our data.

Considering SLEDAI scores and immunological markers and genotype distributions, we found no association except

for patients with renal SLEDAI index < 8 and the V allele frequency of the *CCR2* ($p = 0.046$). We could find no published data on this issue, but our interpretation is that V allele is protective against an aggressive form of LN. Our protocol design could not determine how this occurs. There are 2 reports about *CCR2* polymorphisms in LN^{19,20}, but the authors do not mention any association of the polymorphisms with severity of renal disease. It is well known that chemokine receptors are involved in the cascade of inflammation, and genetic variability might predispose individuals to an altered response.

Some literature data show that there is a strong association between *CCR2* and *CCR5* activity, and that *CCR2* V64I polymorphism decreased reexpression of *CCR5* after ligand-induced internalization in CD4+ T cells, modulating T cell function³³. Further, the *CCR2* -64I variant is thought to provide protection against progression of HIV infection, but it does not influence the acquisition of HIV infection³⁴. Ortlepp, *et al* showed that the presence of the rare allele of the *CCR2* gene was significantly associated with a higher prevalence of myocardial infarction³⁵. Abdi, *et al* showed in data from a kidney transplant population that the percentage of recipients with a rejection episode was more than 2-fold lower in individuals possessing a *CCR2* -64I allele compared to those who lacked this allele; but they were unable to demonstrate association of those genes with renal function 3 years post-transplant³⁶.

Thus the literature is not clear about *CCR2* polymorphism and expression of inflammation, but our data suggest a protective role of allele V against aggressiveness of LN measured by renal SLEDAI index scores.

Considering the immunological markers (ANA, anti-dsDNA, aCL, and lupus anticoagulant and complement levels), there was no association of markers with genotype distributions of the 2 genes in our study. Lima, *et al*, however, showed an association of the *MCP-1* 2518 G/G genotype with anti-dsDNA and antiphospholipid autoantibodies¹². On examining the histological classes of LN and genotypes, we found no associations in accord with the data published by Tucci, *et al*⁹.

Our data show that the G/G *MCP-1* genotype is associated with lupus nephritis. According to literature reports, patients' leukocytes with this genotype would produce more MCP-1 when stimulated. Nevertheless, we could observe no association of the *MCP-1* genotype distribution with renal function, renal survival, histological WHO class, SLEDAI index scores, or immunological markers. Of note, we observed an association of the V allele with a less aggressive expression of renal disease, but with no other disease characteristics.

Screening for *MCP-1* and *CCR2* polymorphisms may be applied to determine the relative risk of developing lupus nephritis, but more studies are needed to establish the association of genotype distribution with the clinical and immunological aspects of the disease and its progression.

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