

Diagnostic performance and clinical associations of antibodies to the chromatin antigenic system in juvenile systemic lupus erythematosus

Silene Peres Keusseyan¹, Neusa Pereira da Silva², Maria Odete Esteves Hilário³, Eunice Mitiko Okuda⁴, Maria Teresa S. L. R. Ascenção Terreri⁵, Luis Eduardo Coelho Andrade⁶

ABSTRACT

Objectives: To determine the frequency of antibodies to chromatin components in juvenile systemic lupus erythematosus (JSLE), and to correlate the presence of these autoantibodies with clinical manifestations and disease activity. **Methods:** Anti-chromatin (anti-CHR), anti-nucleosome core particle (anti-NCS) and anti-dsDNA antibodies were measured in 175 individuals, including 37 patients with active JSLE and 41 with inactive disease, 47 non-lupus autoimmune disease patients (non-lupus AD), and 50 healthy children. An in-house ELISA was developed with purified nucleosome core particles from calf thymus to determine IgG and IgG3 anti-NCS antibodies. Anti-CHR and anti-dsDNA antibodies were detected by commercial ELISA kits (INOVA). **Results:** Anti-NCS and anti-CHR antibodies exhibited high specificity for JSLE and similar frequency in active and inactive JSLE. Anti-CHR and IgG/IgG3 anti-NCS serum levels did not differ between active and inactive JSLE. SLEDAI correlated with anti-dsDNA antibodies but not with antibodies to other chromatin components. There was association of anti-dsDNA, anti-CHR and IgG/IgG3 anti-NCS antibodies with proteinuria and low C4 serum levels. Anti-NCS antibodies in the absence of anti-dsDNA were observed in 14% of the JSLE patients. **Conclusions:** Our data indicate that anti-NCS and anti-CHR antibodies are relevant diagnostic markers for JSLE and appear to be correlated with JSLE lupus nephritis activity. IgG3 isotype anti-NCS antibodies do not seem to be more relevant than IgG anti-NCS antibodies as markers of disease activity or active nephritis in JSLE.

Keywords: nucleosomes, antinuclear antibodies, systemic lupus erythematosus, nephritis, chromatin.

© 2012 Elsevier Editora Ltda. All rights reserved.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by a wide range of clinical manifestations. SLE nephritis bears considerable morbidity and represents a major threat to long-term quality of life and survival. Renal involvement has been reported in 40%–80% of juvenile systemic lupus erythematosus patients (disease onset before 16 years old) and this high frequency contributes to the severity of juvenile systemic lupus erythematosus (JSLE).¹ SLE

is characterized by the presence of circulating autoantibodies against nuclear components (ANA).² More than 100 different autoantibodies have been identified in the serum of SLE patients³ and some of these are clinically useful as diagnostic markers and as ancillary parameters for disease activity monitoring.⁴ Among the clinically useful SLE autoantibodies, anti-native DNA, anti-Sm and anti-ribosomal P protein are considered to be specific diagnostic markers but their modest sensitivity restricts their diagnostic performance. This scenario has endorsed the search for novel and more efficient biomarkers for SLE.⁵

Received on 09/28/2011. Approved on 06/27/2012. The authors declare no conflict of interest. Financial Support: Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP. Ethics Committee: 1149/04. Universidade Federal de São Paulo – Unifesp.

1. Master in Health Science, Universidade Federal de São Paulo – Unifesp

2. PhD in Pediatrics and Science Applied to Pediatrics, Unifesp; Associate-Professor of Rheumatology, Unifesp

3. Associate-Professor of Pediatric Rheumatology, Discipline of Allergy, Immunology and Rheumatology, Unifesp

4. PhD in Medicine, Faculdade de Ciências Médicas da Santa Casa de Misericórdia de São Paulo – SCMSP

5. PhD in Pediatrics and Science Applied to Pediatrics, Unifesp; Adjunct Professor, Discipline of Allergy, Immunology and Rheumatology, Unifesp

6. Adjunct Professor, Rheumatology, Unifesp

Correspondence to: Luis Eduardo Coelho Andrade. Rua Botucatu, 740. CEP: 04023-900. São Paulo, SP, Brazil. E-mail: luis.andrade@unifesp.br

The nucleosome core particle is the fundamental chromatin unit and is composed of ~146 base pairs of DNA wrapped around a protein core, an octamer comprising two molecules of each of the histones H2A, H2B, H3 and H4. The nucleosome core particles are joined together by a linker DNA, which is associated with histone H1 located outside the nucleosome core particles.⁶ During cell apoptosis, nucleosomes are released in the intracellular milieu by endonuclease chromatin cleavage. In physiologic conditions, phagocytes engulf apoptotic cells and apoptotic bodies to prevent the release of cell constituents in the extracellular space.⁷ In the last 15 years, several pieces of evidence have suggested that the nucleosome is a major antigenic domain in SLE pathophysiology, and that antibodies to nucleosome core particles (anti-NCS/CHR) are associated with organ damage.^{8–10} Additionally, there is isolated evidence that IgG3 isotype anti-NCS antibodies might constitute a selective biologic marker of active SLE, and in particular, of lupus nephritis.¹¹ Although several studies evaluated the diagnostic performance of anti-NCS or anti-CHR antibodies in adult SLE patients, there are only a few studies about these autoantibodies in JSLE.^{12–14}

The aim of the present study was to investigate the diagnostic performance of antibodies to the chromatin antigenic system in JSLE as well as their associations with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and with individual clinical manifestations of the disease, with emphasis on lupus nephritis.

MATERIALS AND METHODS

Patients and controls

Over a two-year period, 125 patients aged 5–18 years old were sequentially recruited from the outpatient clinic of the Department of Pediatrics at Universidade Federal de São Paulo and Santa Casa de Misericórdia Medical School Hospital, in São Paulo, Brazil. Disease distribution among patients was: 37 children had active JSLE; 41 had inactive JSLE; 47 had non-lupus autoimmune diseases (non-lupus AD) comprising systemic sclerosis (SSc; n = 4), juvenile idiopathic arthritis (JIA; n = 28), dermatomyositis (DM; n = 7), and chronic autoimmune hepatitis (n = 8). SLE, JIA, SSc and DM were diagnosed according to the American College of Rheumatology criteria and International Autoimmune Hepatitis Group respectively.^{15–19} Clinical and laboratory information (renal, hematological and skin involvement, arthritis, fever, alopecia, mucous ulcers, chronic headache, neurological manifestations, vasculitis, and serositis) were obtained for each JSLE patient in order to determine the disease activity score using SLEDAI.²⁰

Inactive JSLE and active JSLE was arbitrarily defined as SLEDAI ≤ 2 and ≥ 6 , respectively.²¹ Patients with SLEDAI scores 3, 4, and 5 were excluded from the study. Sera from 50 gender and age-matched healthy children and adolescents were used as controls. These individuals were relatives from laboratory staff and otherwise healthy children referred for small surgical procedures. The study was approved by the Institutional Ethics Committee (# 1149/04).

Each participant provided 10 mL of blood for laboratory tests after having the informed consent signed by parents or legal guardians. The samples were stored at -20°C until used. At the time of serum harvest, most JSLE patients were under treatment with immunosuppressant drugs. Intravenous cyclophosphamide was used in 2.5%, pulse therapy with methylprednisolone in 7.7%, oral prednisone above 0.5 mg/kg/day in 65.4%, azathioprine in 33.3%, methotrexate in 6.4%, and hydroxychloroquine in 57.7% of the JSLE patients.

Serologic analysis

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence (IIF) with in-house HEp-2 cells slides according to the standard procedure;²² anti-dsDNA antibodies were determined by enzyme immunoassay (INOVA Diagnostics, San Diego, CA), according to the manufacturer's directions, and by IIF with in-house *C. luciliae* slides (CLIF assay) according to standard procedure.²³ Antibodies against extractable nuclear antigens (ENA) were detected by double immunodiffusion against calf spleen extract according to Ouchterlony's technique.²⁴ Anticardiolipin antibodies were determined by in-house enzyme immunoassay as previously described²⁵ and calibrated with international APL standards (Louisville APL Diagnostics Inc, Doraville, GA, USA). C3 and C4 serum levels were determined by radial immunodiffusion (The Binding Site Ltd., Birmingham, UK) according to the manufacturer's directions. Two assays were used to measure antibodies anti-chromatin/nucleosome. Since anti-chromatin and anti-nucleosome antibodies are normally synonymous we decided in this study to name the anti-chromatin commercial assay anti-CHR, and the in-house anti-nucleosome assay, anti-NCS.

Nucleosome core particle preparation and detection of antibodies to nucleosome core particles (NCS) and to chromatin (CHR)

The commercial kit QUANTA Lite™ Chromatin using highly purified calf thymus chromatin without histone H1 and non-histone proteins (INOVA Diagnostics, San Diego, CA) was processed according to the manufacturer's instructions. For

qualitative assessment, when using the cut-off point of 20 U/mL suggested by the manufacturer, results were expressed as anti-CHR-20. Alternatively, when using the cut-off point of 60 U/mL (moderate to strong positive sera, according to manufacturer), results were expressed as anti-CHR-60.

The in-house anti-NCS immunoassay was set up to detect IgG and IgG3 antibodies against the nucleosome core particles isolated from calf thymus as previously described,^{26,27} with slight modifications. Briefly, 10 g of calf thymus were ground and homogenized in 140 mL buffer A [0.3 M sucrose, 6 mM MgCl₂, 1.2 mM CaCl₂, 10 mM NaHSO₃, 10 mM Tris-HCl, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5] and passed through cheesecloth and miracloth. Nuclei were isolated by centrifugation at 3,300 g for 8 minutes at 4°C, washed twice in the same buffer, pelleted and resuspended in 8 mL 15 mM NaCl, 15 mM 2-mercaptoethanol, 60 mM KCl, 0.5 mM spermine, 0.15 mM spermidine, pH 7.4. The volume was adjusted to yield a final DNA concentration of ~2,500 µg/mL as judged by optical density at 260 nm. The nuclei suspension was digested for 2 minutes at 37°C with 40 IU/mg micrococcus nuclease (Worthington Biochemical Corp., Likewood, NJ) in the presence of 1 mM CaCl₂ and the reaction was terminated by the addition of 2 mM EDTA. The nuclei were pelleted at 8,000 g for 2 minutes at 4°C, resuspended in the original volume with 0.2 mM EDTA pH 7.0, and then homogenized in a tight-fitting Dounce homogenizer. The homogenate was centrifuged again for 2 minutes at 8,000 g at 4°C and the supernatant containing the soluble long chromatin was recovered. H1 and non-histone proteins were stripped by dropwise addition of ice cold 4 M NaCl to a final concentration 0.5 M NaCl. The non-histone proteins and H1 were separated from the stripped chromatin solution by gel filtration in a Sepharose 4B column (Sigma Chemical, St. Louis, USA) previously equilibrated with 0.45 M NaCl, 0.2 mM EDTA and 5mM Tris-HCl, pH 7.5. The fractions containing the nucleosome core particles were selected according to agarose gel electrophoresis pattern and pooled together.

The in-house anti-NCS ELISA was based on the assay developed by Burlingame and Rubin.²⁷ ELISA plates (Nunc-MediSorpT Surface, Denmark) were coated with 200 µL/well purified nucleosome core particles 5 µg DNA/mL in cold phosphate buffered saline (PBS) for 48 hours at 4°C, and blocked with 200 µL/well 0.1% gelatin in PBS for 2 hours at room temperature (RT). Patient samples (200 µL) diluted 1:100 in 0.1% gelatin in PBS were applied to each well and then allowed to react for 2 hours at RT. Plates were washed three times with 0.05% Tween 20 in PBS (250 µL/well) and then incubated for 2 hours at RT with 200 µL/well horseradish peroxidase (HRP) labeled mouse anti-human IgG (Sigma,

St. Louis, USA) 1:20,000 in 0.05% Tween 20, 0.1% gelatin, 0.1% BSA, and 0.5% fetal calf serum (FCS) in PBS or HRP-labeled mouse anti-human IgG3 (ZYMED Laboratories, San Francisco, CA) 1:2,000 in the same buffer. After washing as before, plates received 200 µL/well peroxidase chromogenic substrate (10 mg o-phenylenediamine, 10 µL H₂O₂ in 25 mL 0.1 M citrate buffer, pH 5.0). After incubation under shaking for 1 hour at RT in a dark chamber the OD was read at 492 nm after the addition of 50 µL/well of stop solution (1 N H₂SO₄). Serum background reactivity was checked in uncoated wells processed in parallel with the test wells. The cut-off value was determined as the mean plus three standard deviations of the OD values obtained with serum samples from 80 healthy blood donors (0.573 for IgG anti-NCS and 0.400 for IgG3 anti-NCS). The cut-off values obtained were compatible with the cut-off values derived from ROC curve analysis (see results). Three positive and three negative control samples, obtained from a private laboratory certified on-site by the US College of American Pathologists (CAP), were included in each plate.

Statistical analysis

Continuous variables were expressed as mean and standard deviation. Comparison between groups was performed with *t*-test (parametric variables) or Mann-Whitney test (non-parametric variables) for continuous variables and chi-square or Fisher's exact test for categorical variables, as needed. Correlations were determined by Spearman's correlation for non-parametric variables. All statistical analyses were performed with SPSS software (version 15.0 for Windows, Chicago, USA). *P* < 0.05 was considered significant.

RESULTS

Demographic data

Thirty-seven patients had active JSLE (SLEDAI score ≥ 6) and 41 had inactive JSLE (SLEDAI score ≤ 2). There was no significant difference in gender, age, or ethnicity among the groups of 37 active JSLE (29 girls, 13.2 ± 3.4 years old, 20 Caucasian-descendants), 41 inactive JSLE (35 girls, 12.0 ± 3.0 years old, 23 Caucasian-descendants), 47 non-lupus AD (35 girls, 11.0 ± 3.6 years old, 26 Caucasian-descendants), and 50 healthy controls (35 girls, 14.0 ± 4.3 years old, 22 Caucasian-descendants) (*P* = 0.153, 0.054, and 0.525, respectively). Patients with active JSLE had lower disease duration as compared to inactive JSLE (*P* = 0.013) (Table 1). The clinical and laboratory features of patients with active and inactive JSLE are depicted on Table 2. Active nephritis at the moment of

Table 1

Demographic data of patients with active JSLE, inactive JSLE, non-lupus AD, and healthy controls

	Active JSLE (n = 37)	Inactive JSLE (n = 41)	Non-lupus AD (n = 47)	Healthy controls (n = 50)	P
Gender (F/M)	31/6	36/5	35/12	35/15	0.153 ^a
Age (years)	13.2 ± 3.4	13.2 ± 3.0	11 ± 3.6	11.5 ± 4.3	0.054 ^a
Disease duration (months)	24 ± 23.4	41.8 ± 30.4	nd	nd	0.013 ^b
Ethnicity (C/NC)	20/17	24/17	26/21	22/28	0.525 ^a

Mean and standard deviation. Chi-square P value: ^acomparison of all groups; ^bComparison between active and inactive JSLE patients.
F: female; M: male; C: Caucasian; NC: non Caucasian; JSLE: juvenile systemic lupus erythematosus; AD: autoimmune disease.

Table 2

Clinical and laboratory features of patients with active JSLE, inactive JSLE, non-lupus AD, and healthy controls

	Active JSLE % (n = 37)	Inactive JSLE % (n = 41)	Non-lupus AD % (n = 47)	Healthy controls % (n = 50)
Renal involvement	84% (31)	None	nd	nd
Skin involvement	22% (8)	None	nd	nd
Arthritis	19% (7)	None	nd	nd
Hematological involvement	13% (5)	10% (4)	nd	nd
Fever	11% (4)	None	nd	nd
Alopecia	11% (4)	None	nd	nd
Mucous ulcers	8% (3)	None	nd	nd
Chronic headache	8% (3)	None	nd	nd
Neurologic manifestations	5% (2)	None	nd	nd
Vasculitis	5% (2)	None	nd	nd
Serositis	3% (1)	None	nd	nd

nd: not determined; JSLE: juvenile systemic lupus erythematosus; AD: autoimmune disease.

blood withdrawing, defined by proteinuria above 0.5 g/day, was identified in 15 of the 37 active JSLE patients.

Antibodies to nucleosome core particles (NCS), chromatin (CHR), and to native DNA (dsDNA)

Among all JSLE patients, anti-dsDNA antibodies were detected in 29% by ELISA and in 14% by CLIF. IgG anti-CHR-20 and anti-NCS antibodies were found in 40% and 23% of all JSLE patients, respectively. IgG3 anti-NCS antibodies were detected in 18% of all JSLE patients. Table 3 depicts the frequency of the several autoantibodies in each group as well as data about sensitivity, specificity, positive predictive value and negative predictive value for the diagnosis of JSLE calculated against non-lupus AD patients and healthy children altogether. Among the analyzed tests, CLIF assay for anti-dsDNA antibodies was the least sensitive and presented the highest specificity and positive predictive value. The ELISA assays for anti-dsDNA, anti-CHR-60, and IgG anti-NCS had equivalent performance

in all diagnostic parameters. The anti-CHR-20 assay presented higher sensitivity but lower specificity and positive predictive value as compared to the three former ELISA assays. There was considerable heterogeneity among JSLE sera with respect to ELISA OD values in the anti-dsDNA, anti-CHR and anti-NCS antibody assays (Figure 1). Patients with JSLE presented significantly higher levels of antibodies to chromatin components than those with non-lupus AD and healthy individuals ($P < 0.01$). ANA-HEp-2 and anti-ENA positive tests were observed, respectively, in 92% and 32% active JSLE, 90% and 27% inactive JSLE, 36% and 0% non-lupus AD patients, and 2% and 0% healthy children. Homogeneous and fine speckled were the most frequent ANA-HEp-2 patterns found in JSLE sera positive for any tested chromatin components.

Several parameters of disease activity showed correlation with antibodies to chromatin components. Anti-dsDNA antibodies (CLIF assay) were more frequent in patients with active JSLE (SLEDAI ≥ 6) as compared to those with inactive disease (SLEDAI ≤ 2) (24% vs. 5%, $P < 0.001$). In addition, SLEDAI

Table 3

Positivity, sensitivity, specificity, positive predictive value and negative predictive value of different autoantibody assays for the diagnosis of JSLE in comparison with patients with non-lupus AD and healthy controls altogether

	Autoantibody assay positivity (%)					
	CHR-20	CHR-60	NCS IgG	NCS IgG3	dsDNA CLIF	dsDNA ELISA
Active JSLE (n = 37)	43%	27%	27%	22%	24%	38%
Inactive JSLE (n = 41)	36%	22%	19%	15%	5%	22%
Non-lupus AD (n = 47)	11%	4%	2%	4%	0%	6%
Healthy controls (n = 50)	0%	0%	2%	2%	0%	0%
Diagnostic parameters*						
Sensitivity	40%	24%	23%	18%	11%	27%
Specificity	96%	98%	98%	95%	100%	97%
PPV	88%	90%	90%	74%	100%	87%
NPV	66%	62%	61%	58%	58%	62%

*Diagnostic parameters were calculated for the diagnosis of JSLE against non-lupus AD patients and healthy children altogether. PPV: positive predictive value; NPV: negative predictive value; JSLE: juvenile systemic lupus erythematosus; AD: autoimmune disease; CHR-20: commercial anti-chromatin antibody assay with cut-off at 20 U/mL; CHR-60: commercial anti-chromatin antibody assay with cut-off at 60 U/mL; NCS-IgG: in-house anti-nucleosome antibody assay for total IgG antibodies; NCS-IgG3: in-house anti-nucleosome antibody assay for IgG3 antibodies; CLIF: anti-dsDNA antibody assay based on indirect immunofluorescence on *C. luciliae*; ELISA: Enzyme-Linked Immunoabsorbent Assay.

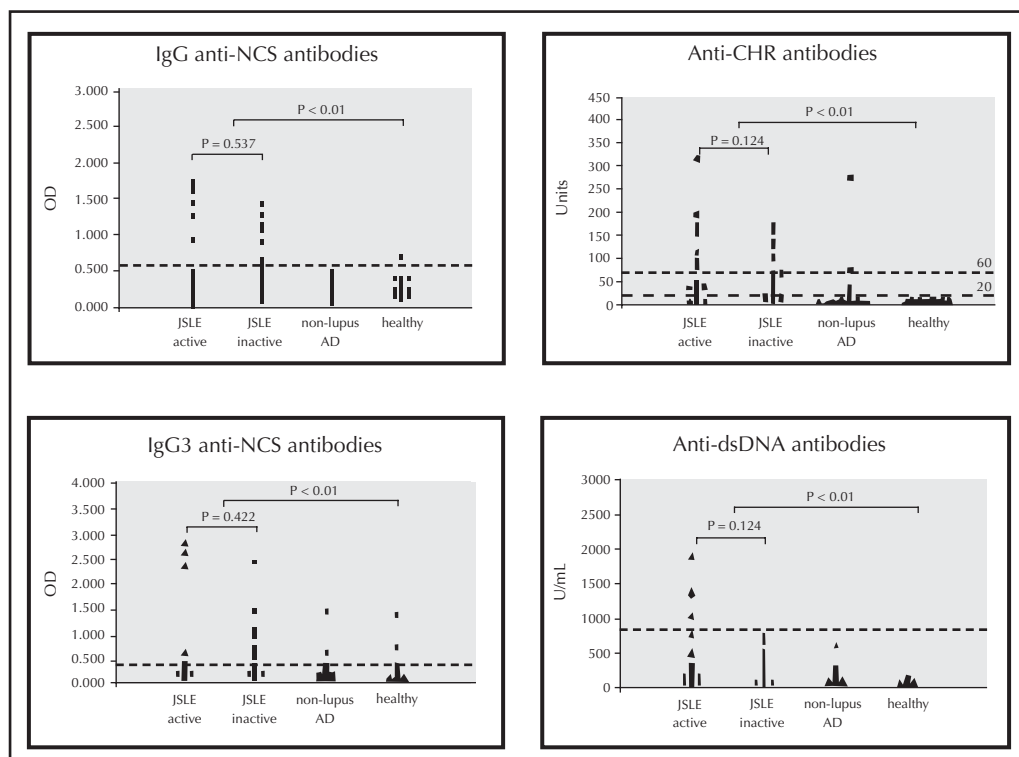


Figure 1
Distribution of JSLE patients, non-lupus AD patients and healthy controls according to ELISA serum levels of antibodies to NCS, CHR and dsDNA. Dashed lines correspond to the cut-off threshold for each assay (for the anti-CHR assay there were cut-off levels at 20 U and 60 U).

Anti-CHR: commercial anti-chromatin antibody assay (with cut-off at 20 U/mL); IgG anti-NCS: in-house anti-nucleosome antibody assay for total IgG antibodies; IgG3 anti-NCS: in-house anti-nucleosome antibody assay for IgG3 antibodies; anti-dsDNA: commercial anti-DNA double helix antibody assay; ELISA: enzyme-linked immunoabsorbent assay.

correlated with ELISA anti-dsDNA antibody levels ($r = 0.235$; $P = 0.038$) but not with antibody levels to anti-CHR and anti-NCS. Patients with active JSLE did not differ significantly from those with inactive JSLE with respect to the frequency and O.D. levels of anti-dsDNA (424.8 ± 540.9 UI/mL vs. 208.2 ± 202.9 UI/mL; $P = 0.124$),

anti-CHR-20 (56.3 ± 71.8 U/mL vs. 35.4 ± 43.7 U/mL, $P = 0.537$), IgG anti-NCS (0.589 ± 0.528 vs. 0.429 ± 0.336 ; $P = 0.432$), and IgG3 anti-NCS (0.536 ± 0.810 vs. 0.343 ± 0.442 ; $P = 0.422$). There was a significantly higher frequency of proteinuria in patients with antibodies to any of the tested chromatin components versus those

Table 4

Distribution of 78 JSLE patients according to the presence of autoantibodies to chromatin components and relevant laboratory parameters

Laboratory parameter	Autoantibodies				
	dsDNA CLIF n = 11	dsDNA ELISA n = 23	CHR-20 n = 33	IgG NCS n = 18	IgG3 NCS n = 14
Proteinuria > 0.5 g/24 h	6 (54.5%)*	9 (39.1%)*	11 (33.3%)*	7 (38.9%)*	6 (42.9%)*
Proteinuria < 0.5 g/24 h	5 (45.4%)	14 (60.9%)	22 (66.7%)	11 (61.1%)	8 (57.1%)
Decreased C3	3 (27.3%)	8 (34.8%)	13 (39.4%)*	7 (38.9%)	6 (42.9%)
Normal C3	8 (72.7%)	15 (65.2%)	20 (60.6%)	11 (61.1%)	8 (57.1%)
Decreased C4	4 (36.4%)	10 (43.5%)*	15 (45.5%)*	9 (50%)*	8 (57.1%)*
Normal C4	7 (63.6%)	13 (56.5%)	18 (54.5%)	9 (50%)	6 (42.9%)

* P < 0.05 (P values refer to Chi-square test comparing the presence/absence of each autoantibody with the presence/absence of the analyzed parameter).
 CHR-20: commercial anti-chromatin antibody assay with cut-off at 20 U/mL; IgG NCS: in-house anti-nucleosome antibody assay for total IgG antibodies; IgG3 NCS: in-house anti-nucleosome antibody assay for IgG3 antibodies; CLIF: anti-dsDNA antibody assay based on indirect immunofluorescence on *C. luciliae*; ELISA: Enzyme-Linked Immunoabsorbent Assay.

without them (Table 4). Correspondingly, the levels of autoantibodies were significantly higher in patients with proteinuria as compared to those without proteinuria for ELISA anti-dsDNA (691.1 ± 730.4 vs. 220.4 ± 217.6 , $P = 0.049$) and IgG anti-NCS antibodies (0.814 ± 0.598 vs. 0.431 ± 0.365 , $P = 0.019$). There was also a similar trend for anti-CHR antibodies (95.5 ± 93.1 vs. 33.3 ± 40.6 , $P = 0.093$) but not for IgG3 anti-NCS antibodies (0.811 ± 1.03 vs. 0.340 ± 0.485 , $P = 0.700$). There was association between decreased C3 levels and the frequency of anti-CHR-20 positive assay. Among JSLE patients with decreased C4 levels there was higher frequency of positive assays to dsDNA (ELISA), CHR-20, IgG NCS and IgG3 NCS (Table 4). Antibodies to isolated or combined extractable nuclear antigens were observed in 23 JSLE patients (13 anti-SS-A/Ro, four anti-SS-B/La, six anti-Sm, and 14 anti-U1-RNP) and there was no association with the presence of antibodies to chromatin components. Only seven of the 78 JSLE patients had moderate levels of anti-cardiolipin antibodies [two IgG (2.5%) and five IgM (6.4%)] and no association was observed between reactivity to cardiolipin and presence of antibodies to chromatin components. Other clinical manifestations were equally frequent in JSLE patients with and without any of the tested antibodies to chromatin components (data not shown).

As depicted on Figure 2 there was good agreement between the ELISA assays for anti-dsDNA and anti-CHR antibodies (85% and 87% for CHR-20 and CHR-60, respectively), between anti-dsDNA and IgG anti-NCS antibodies (86%), and between IgG anti-NCS and anti-CHR antibodies (78% and 91% for CHR-20 and CHR-60, respectively). In fact, we found a great similarity between the in-house anti-NCS assay and the commercial anti-CHR assay when it was analyzed using 60 U/mL as cut-off value (anti-CHR-60).

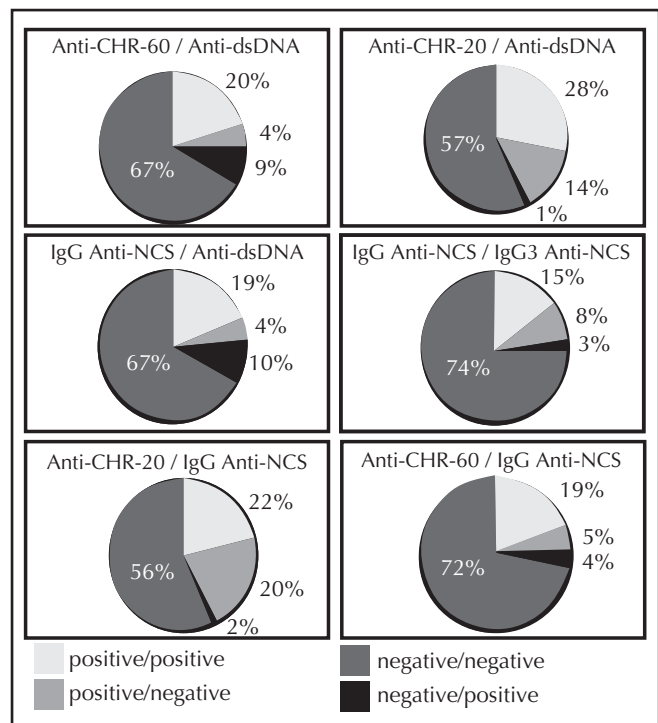


Figure 2

Concordance between ELISA assays for anti-NCS, anti-CHR-20, anti-CHR-60, and anti-dsDNA antibodies in JSLE patients. Anti-CHR-20 and anti-CHR-60 refer to the ELISA anti-CHR assay with cut-off points at 20 U/mL and 60 U/mL, respectively. CHR-20: commercial anti-chromatin antibody assay with cut-off at 20 U/mL; CHR-60: commercial anti-chromatin antibody assay with cut-off at 60 U/mL; IgG anti-NCS: in-house anti-nucleosome antibody assay for total IgG antibodies; IgG3 anti-NCS: in-house anti-nucleosome antibody assay for IgG3 antibodies; anti-dsDNA: commercial anti-DNA double helix antibody assay; ELISA: enzyme-linked immunoabsorbent assay.

Additionally, the agreement rate between IgG anti-NCS and IgG3 anti-NCS antibodies was 89%. The disagreement rate between the same pairs of tests ranged from 9%–21%. In particular, ELISA for anti-dsDNA and ELISAs for anti-NCS or anti-CHR showed disagreement rates around 15%. Regarding the 23 anti-dsDNA ELISA positive patients, 22 were also anti-CHR-20 positive.

DISCUSSION

Previous studies have addressed the analysis of autoantibodies against chromatin components in SLE and related diseases; however, few surveys have addressed anti-NCS antibodies in JSLE. In the present study we have observed a considerable variation in the diagnostic performance of the tests for diverse antibodies against chromatin components in JSLE. The traditional CLIF anti-dsDNA was the most specific and least sensitive test. The ELISA anti-dsDNA and the anti-CHR-20 were the most sensitive tests, though slightly less specific. Although SLEDAI correlated only with anti-dsDNA antibodies, there was association between several of the tested autoantibodies to chromatin components and parameters indicative of disease activity, such as proteinuria and low complement levels.

Nucleosome core particles are the fundamental units of chromatin and a normal product of cell apoptosis. Apoptosis defects are well known to be associated with certain animal models of lupus, and have also been discussed in connection with human SLE.^{28–31} Recent evidence obtained in murine models of SLE suggests that nucleosome core particles are a preferential target for lupus autoantibodies and they are accepted as genuine autoantigens triggering the production of antibodies against the nucleosome core particles themselves, dsDNA and histones.^{32–34} According to recent literature data, the presence of glomerular extracellular nucleosomes derived, for instance, from apoptotic cells is a pre-requisite for the binding of anti-chromatin antibodies to the glomeruli and may be involved in nephritic processes.^{33,34} Since humoral autoimmune response is accepted to be antigen-driven,² a comprehensive analysis of autoantibodies against individual components of a supra-molecular complex is justified. In addition, technical details in the preparation of the antigenic substrate may be determinant in preserving fastidious non-linear epitopes. With respect to the chromatin system, this is crucial due to the delicate and complex interaction of native DNA and several histone and non-histone proteins. Therefore, each methodological platform favors the exposure of an unique set of epitopes and this may influence considerably the clinical significance of these tests.³⁵

One widely accepted method for nucleosome core particle purification consists in the solubilization of native chromatin by micrococcal nuclease digestion and removal of H1 histone and other proteins by 0.5 M NaCl extraction at neutral pH. Most commercial anti-NCS/chromatin kits use poly or mononucleosomes extracted from calf thymus chromatin as antigenic substrate. The present study utilized both in-house and commercial enzyme immunoassays. The anti-chromatin antibody commercial kit (anti-CHR) (INOVA Diagnostics, San Diego, CA) and the in-house anti-nucleosome core particle immunoassay (anti-NCS) used chromatin-derived antigenic substrates, both isolated and purified from calf thymus and devoid of H1 histone and non-histone proteins. In addition, antibodies to dsDNA were determined by an ELISA commercial kit and by indirect immunofluorescence on *Crithidia luciliae* (CLIF). The performance variability of the several assays in the present series of patients is probably due to the cumulative effect of the differences in the epitope panel offered by the different antigenic substrates and the heterogeneous biochemical assay conditions of the different tests. In fact, the present study did not aim to compare the various tests but rather to explore the variability in the reactivity of JSLE sera against different epitope panels in assays targeting apparently related autoantigens.

The present study confirmed the high specificity of anti-nucleosome/anti-chromatin antibodies in JSLE (anti-CHR-20, 96% and anti-NCS, 98%) when compared with children with non-lupus AD and healthy children. This observation is in agreement with the diagnostic specificity of anti-nucleosome/anti-chromatin antibodies for adult lupus (mean 95%; range 85%–98.8%). The sensitivity of the various tests for the different autoantibodies against chromatin components was relatively low (11%–40%) when compared to the literature data on adult SLE.^{11,36–45} This may be related to specific features of JSLE and to the ethnic makeup of the studied sample or to the fact that most JSLE patients were under immunosuppressive therapy at the moment of serum harvest. In the present study 22 of 23 (96%) anti-dsDNA ELISA positive samples were also anti-CHR-20 positive. This finding is consistent with the concept that most of the anti-dsDNA antibodies in SLE patients are a subset of antibodies directed against chromatin.⁴⁰ However, 14% JSLE patients were reactive in the anti-CHR-20 assay but not in the dsDNA ELISA assay. Conversely, 1% of the JSLE samples were anti-dsDNA ELISA positive but anti-CHR-20 negative. On the other hand, 15 of 23 (65%) anti-dsDNA ELISA positive samples were also positive in the IgG anti-NCS and anti-CHR-60 assays, and 4% of the JSLE patients were negative in these assays. Such dissociation has been reported

previously^{13,38,40,42,43,46-48} and indicates that these autoantibody systems have complementary roles in the diagnosis of SLE. This may have an impact in clinical practice inasmuch as the demonstration of anti-NCS antibodies in the absence of other autoantibodies may be particularly helpful in patients with few clinical manifestations (e.g. < 3 classification criteria). In these cases, early therapy may be helpful.

The present data confirm the importance of defining the cut-off value according to the studied population in order to determine the diagnostic accuracy of the various immunoenzyme assays in different ethnic and social frameworks. Using the cut-off recommended by the manufacturer in our sample, the commercial anti-chromatin kit (INOVA) display considerably less specificity.

Disease activity, as measured by SLEDAI, was associated with the presence of anti-dsDNA (CLIF assay) and correlated with the serum levels of ELISA anti-dsDNA, but was not associated with any of the other tested assays. However, the low correlation level ($r = 0.235$) suggests that this parameter should be used with caution in the clinical practice. Interestingly, however, the presence of antibodies against chromatin components was largely associated in the literature with markers of active lupus nephritis. Several studies have previously demonstrated an association of anti-nucleosome antibodies and disease activity or active nephritis in SLE^{11,13,14,35,38,40,43,48}. It has been also pointed that anti-nucleosome antibodies are highly correlated with renal failure and progression to kidney transplantation in SLE.⁴⁹ The present study confirmed such association also for JSLE and further extended the observation to several autoantibody specificities within the supra-molecular chromatin system. Despite the wide dispersion of the serum levels of these autoantibodies in patients with active and inactive disease, they were associated with markers of active nephritis. This suggests that there may be an intra-individual association of disease activity with anti-nucleosome and anti-dsDNA antibody serum levels. Therefore, prospective longitudinal studies are warranted in order to further explore this possibility.

The behavior of anti-nucleosome antibodies in adult lupus has been analyzed according to the antibody isotype (IgG and IgM classes and IgG subclasses) by Amoura *et al.*¹¹ Interestingly, IgG3 anti-NCS antibodies were present in high levels only in active SLE patients, predominantly in active lupus nephritis. When testing for IgG3 anti-NCS antibodies in JSLE we did confirm that IgG3 anti-NCS antibodies were associated with markers of active lupus nephritis such as proteinuria and low C4 serum level. There was a diagnostic sensitivity of 18% and specificity of 95% for JSLE diagnosis. However, the frequency of IgG3 anti-NCS was similar in children with active and inactive JSLE and IgG3 anti-NCS serum levels did not correlate with SLEDAI. In summary, this is the first report on JSLE regarding a comprehensive survey of autoantibodies against several autoantigens of the chromatin complex, including IgG3 anti-NCS antibodies. We have found high specificity and moderate sensitivity of these antibodies for the diagnosis of JSLE, signaling them as helpful tools in the differential diagnosis of JSLE among systemic autoimmune diseases. There was a moderate disagreement rate between anti-NCS/chromatin and anti-dsDNA antibodies, indicating that these are complementary autoantibodies for lupus diagnosis. The association of anti-NCS/chromatin antibodies with proteinuria and low C4 levels suggests a possible role for these antibodies as markers of lupus nephritis activity. Further longitudinal studies are warranted to define the clinical utility of anti-NCS/chromatin antibodies in the monitoring of JSLE activity.

ACKNOWLEDGMENTS

The authors acknowledge Rufus Burlingame, Ph.D., for technical supervision on nucleosome purification and anti-nucleosome ELISA set up as well as for general advising on the project. This study was supported by São Paulo State Research Foundation (FAPESP) through grant #05/00658-0.

REFERENCES

1. Baqi N, Moazami S, Singh A, Ahmad H, Balachandra S, Tejani A. Lupus nephritis in children: a longitudinal study of prognostic factors and therapy. *J Am Soc Nephrol* 1996; 7(6):924–9.
2. Tan EM, Chan EKL. Molecular biology of autoantigens and new insights into autoimmunity. *Clin Investig* 1993; 71(4):327–30.
3. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum* 2004; 34(2):501–37.
4. Tan EM, Chan EKL, Sullivan KF, Rubin RL. Antinuclear antibodies (ANAs): diagnostically specific immune markers and clues toward the understanding of systemic autoimmunity. *Clin Immunol Immunopathol* 1988; 47(2):121–41.
5. Amoura Z, Piette JC, Bach JF, Koutouzov S. The key role of nucleosomes in lupus. *Arthritis Rheum* 1999; 42(5):833–43.
6. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997; 389(6648):251–60.
7. Flora PK, Gregory CD. Recognition of apoptotic cells by human macrophages: inhibition by a monocyte/macrophage-specific monoclonal antibody. *Eur J Immunol* 1994; 24(11):2625–32.
8. Decker P. Nucleosome autoantibodies. *Clin Chim Acta* 2006; 366(1–2):48–60.
9. Mohan C, Adams S, Stanik V, Datta SK. Nucleosome: a major immunogen for pathogenic autoantibody-inducing T cells of lupus. *J Exp Med* 1993; 177(5):1367–81.
10. Bruns A, Bläss S, Hausdorf G, Burmester GR, Hiepe F. Nucleosome are major T and B cell autoantigens in systemic lupus erythematosus. *Arthritis Rheum* 2000; 43:2307–15.
11. Amoura Z, Koutouzov S, Chabre H, Cacoub P, Amoura I, Musset L *et al.* Presence of antinucleosome autoantibodies in a restricted set of connective tissue diseases: antinucleosome antibodies of the IgG3 subclass are markers of renal pathogenicity in systemic lupus erythematosus. *Arthritis Rheum* 2000; 43(1):76–84.
12. Massa M, De Benedetti F, Pignatti P, Albani B, Di Fuccia G, Monestier M *et al.* Anti-double stranded DNA, anti-histone, and antinucleosome IgG reactivities in children with systemic lupus erythematosus. *Clin Exp Rheumatol* 1994; 12(2):219–25.
13. Campos LM, Kiss MH, Scheinberg MA, Manguiera CL, Silva CA. Antinucleosome antibodies in patients with juvenile systemic lupus erythematosus. *Lupus* 2006; 15(8):496–500.
14. Wu JF, Yang YH, Wang LC, Lee JH, Shen EY, Chiang BL. Antinucleosome antibodies correlate with the disease severity in children with systemic lupus erythematosus. *J Autoimmun* 2006; 27(2):119–24.
15. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25(11):1271–7.
16. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40(9):1725.
17. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J *et al.* International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol* 2004; 31(2):390–2.
18. Bohan A, Peter JB. Polymyositis and Dermatomyositis (first of two parts). *N Engl J Med* 1975; 292(7):344–7.
19. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL *et al.* International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; 31(5):929–38.
20. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* 1992; 35(6):630–40.
21. Petri M, Genovese M, Engle E, Hochberg M. Definition, incidence, and clinical description of flare in systemic lupus erythematosus. A prospective cohort study. *Arthritis Rheum* 1991; 34(8):937–44.
22. Miyachi K, Fritzler MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cell. *J Immunol* 1978; 121(6):2228–34.

23. Aarden LA, de Groot ER, Feltkamp TE. Immunology of DNA III. Crithidia luciliae, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique. *Ann NY Acad Sci* 1975; 254:505–15.
24. Hardin JA, Craft JE. Patterns of autoimmunity to nucleoproteins in patients with systemic lupus erythematosus. *Rheum Dis Clin North Am* 1987; 13(1):37–46.
25. Wong RC; Australasian aCL Working Party. Consensus guidelines for anticardiolipin antibody testing. *Thromb Res* 2004; 114(5–6):559–71.
26. Lutter LC. Kinetic analysis of deoxyribonuclease I cleavages in the nucleosome core: evidence for a DNA superhelix. *J Mol Biol* 1978; 124(2):391–420.
27. Burlingame RW, Rubin RL. Subnucleosome structures as substrates in enzyme-linked immunosorbent assays. *J Immunol Methods* 1990; 134(2):187–99.
28. Amoura Z, Chabre H, Koutouzov S, Lotton C, Cabrespines A, Bach JF *et al.* Nucleosome-restricted antibodies are defected before anti-ds-DNA and/or antihistone in serum of MRL-Mp lpr/lpr and +/- mice, and are present in kidney eluates of lupus mice with proteinuria. *Arthritis Rheum* 1994, 37(11):1684–8.
29. Tax WJM, Kramers C, van Bruggen MCJ, Berden JH. Apoptosis, nucleosomes, and nephritis in systemic lupus erythematosus. *Kidney Int* 1995; 48(3):666–73.
30. Koutouzov S, Jeronimo AL, Campos H, Amoura Z. Nucleosomes in the pathogenesis of systemic lupus erythematosus. *Rheum Dis Clin North Am* 2004; 30(3):529–58.
31. Berden JHM, Groostscholten C, Jürgen WC, van der Vlag J. Lupus nephritis: a nucleosome waste disposal defect? *J Nephrol* 2002; 15(Suppl. 6): S1–10.
32. Kalaaji M, Mortensen E, Jørgensen L, Olsen R, Rekvig OP. Nephritogenic lupus antibodies recognize glomerular basement membrane-associated chromatin fragments released from apoptotic intraglomerular cells. *Am J Pathol* 2006; 168(6):1779–92.
33. Kalaaji M, Fenton KA, Mortensen ES, Olsen R, Sturfelt G, Alm P *et al.* Glomerular apoptotic nucleosomes are central target structures for nephritogenic antibodies in human SLE nephritis. *Kidney Int* 2007; 71(7):664–72.
34. Mjelle JE, Rekvig OP, Fenton KA. Nucleosomes possess a high affinity for glomerular laminin and collagen IV and bind nephritogenic antibodies in murine lupus-like nephritis. *Ann Rheum Dis* 2007; 66(12):1661–68.
35. Simón JA, Cabiedes J, Ortiz E, Alcocer-Varela J, Sánchez-Guerrero J. Anti-nucleosome antibodies in patients with systemic lupus erythematosus of recent onset. Potential utility as a diagnostic tool and disease activity marker. *Rheumatology (Oxford)* 2004; 43(2):220–4.
36. Burlingame RW, Boey ML, Starkebaum G, Rubin RL. The central role of chromatin in autoimmune responses to histones and DNA in systemic lupus erythematosus. *J Clin Invest* 1994; 94(1):184–92.
37. Wallace DJ, Lin HC, Shen GQ, Peter JB. Antibodies to histone (H2A-H2B)-DNA complexes in the absence of antibodies to double-stranded DNA or to (H2A-H2B) complexes are more sensitive and specific for scleroderma-related disorders than for lupus. *Arthritis Rheum* 1994; 37(12):1795–7.
38. Chabre H, Amoura Z, Piette JC, Godeau P, Bach JF, Koutouzov S. Presence of nucleosome-restricted antibodies in patients with systemic lupus erythematosus. *Arthritis Rheum* 1995; 38(10):1485–91.
39. Mohan C, Liu F, Xie C, Williams Jr RC. Anti-subnucleosome reactivities in systemic lupus erythematosus (SLE) patients and their first-degree relatives. *Clin Exp Immunol* 2001; 123(1):119–26.
40. Min DJ, Kim SJ, Park SH, Seo YI, Kang HJ, Kim WU *et al.* Anti-nucleosome antibody: significance in lupus patients lacking anti-double-stranded DNA antibody. *Clin Exp Rheumatol* 2002; 20(1):13–8.
41. Ghillani-Dalbin P, Amoura Z, Cacoub P, Charuel JL, Diemert MC, Piette JC *et al.* Testing for anti-nucleosome antibodies in daily practice: a monocentric evaluation in 1696 patients. *Lupus* 2003; 12(11):833–7.
42. Cairns AP, McMillan SA, Crockard AD, Meenagh GK, Duffy EM, Armstrong DJ *et al.* Antinucleosome antibodies in the diagnosis of systemic lupus erythematosus. *Ann Rheum Dis* 2003; 62(3):272–3.
43. Cervera R, Viñas O, Ramos-Casals M, Font J, García-Carrasco M, Sisó A *et al.* Anti-chromatin antibodies in systemic lupus erythematosus: a useful marker for lupus nephropathy. *Ann Rheum Dis* 2003; 62(5):431–4.
44. González C, García-Berrocal B, Herráez O, Navajo JA, González-Buitrago JM. Anti-nucleosome, anti-chromatin, anti-dsDNA and anti-histone antibody reactivity in systemic lupus erythematosus. *Clin Chem Lab Med* 2004; 42(3):266–72.
45. Ghirardello A, Doria A, Zampieri S, Tarricone E, Tozzoli R, Villalta D *et al.* Antinucleosome antibodies in SLE: a two-year follow-up of 101 patients. *J Autoimmun* 2004; 22(3):235–40.
46. Gómez-Puerta JA, Molina JF, Anaya JM, Molina J. Clinical significance of anti-chromatin antibodies in systemic lupus erythematosus. *Lupus* 2001; 10(suppl 1):S73.
47. Braun A, Sis J, Max R, Mueller K, Fiehn C, Zeier M *et al.* Anti-chromatin and anti-C1q antibodies in systemic lupus erythematosus compared to other systemic autoimmune diseases. *Scand J Rheumatol* 2007; 36(4):291–8.
48. Sardeto GA, Simas LM, Skare TS, Nisihara RN, Utiyama SR. Antinucleosome in systemic lupus erythematosus. A study in a Brazilian population. *Clin Rheumatol* 2011; 31(3):553–6.
49. Stinton LM, Barr SG, Tibbles LA, Yilmaz S, Sar A, Benediktsson H *et al.* Autoantibodies in lupus nephritis patients requiring renal transplantation. *Lupus* 2007; 16(6):394–400.