Autoantibodies targeting ficolin-2 in systemic lupus erythematosus patients with active nephritis

Sophie Colliard, Noemie Jourde-Chiche, Giovanna Clavarino, Françoise
Sarrot-Reynauld, Evelyne Gout, Alban Deroux, Mélanie Fougere, Nathalie
Bardin, Laurence Bouillet, Jean-Yves Cesbron, et al.

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Authors: Sophie Colliard, MSc [1], Noémie Jourde-Chiche, MD, PhD [2,3], Giovanna Clavarino, PhD [1,4], Françoise Sarrot-Reynauld, MD, PhD [5], Evelyne Gout, BSc [6], Alban Deroux, MD [5], Mélanie Fougere, BSc [1], Nathalie Bardin, PharmD, PhD [3,7], Laurence Bouillet, MD, PhD [5], Jean-Yves Cesbron, MD, PhD [1,4], Nicole M. Thielens, PhD [6], Chantal Dumestre-Pérard, PharmD, PhD [1,4].

Postal addresses of all the authors:

[1] Laboratoire d'Immunologie, pôle de Biologie, CHU Grenoble Alpes, CS 10217, 38043 Grenoble Cedex 9, France.

[2] Aix-Marseille Univ, Assistance-Publique Hôpitaux de Marseille, Centre de Néphrologie et Transplantation Rénale, CHU de la Conception, 147 Bd Baille, 13005 Marseille, France.

[3] Aix-Marseille Univ, UMR_S 1076, Vascular Research Center of Marseille, Faculté de Pharmacie, 13005 Marseille, France.

[4] BNI TIMC-IMAG, UMR5525, CNRS-Université Grenoble Alpes, BP170, 38042 Grenoble, Cedex 9 France.

[5] Clinique universitaire de médecine interne, pôle pluridisciplinaire de médecine et de gérontologie clinique, CHU GrenobleAlpes, CS 10217, 38043 Grenoble Cedex 9, France.

[6] Univ. Grenoble Alpes, CNRS, CEA, IBS, F-38000 Grenoble, France.

[7] Laboratoire d'Immunologie, Hôpital de la Conception, 147 Bd Baille, 13005 Marseille, Aix-Marseille Université, France.

Corresponding author:

Dr C. Dumestre-Pérard

Laboratoire d'Immunologie, Pôle de Biologie

CHU de Grenoble

CS 10217

38043 Grenoble Cedex 9, France

Telephone : + 33 476 76 54 16

Fax number : + 33 476 76 52 66

E-mail address: cdumestre-perard@chu-grenoble.fr

Abstract

Objective. Systemic lupus erythematosus (SLE) is a multi-system inflammatory disease characterized by production of various autoantibodies. The aim of this study was to investigate the presence of anti-ficolin-2 antibodies in SLE patients and to evaluate the association between the levels of these autoantibodies, clinical manifestations, and disease activity.

Methods. This is a comparative study using a cohort of 165 SLE patients and 48 healthy subjects. SLE patients were further divided into two groups, with "low disease activity" (SLEDAI score ≤ 4, n = 88) and with "high disease activity" (SLEDAI score > 4, n = 77). Clinical manifestations were defined according to the physician in charge. Active lupus nephritis (LN) was documented by kidney biopsy. Detection of anti-ficolin-2 antibodies was performed by ELISA.

Results. Levels of the anti-ficolin-2 autoantibodies were significantly higher in SLE patients as compared to healthy subjects and associated with the SLEDAI score. They were found positive in 61/165 (37%) SLE patients. Presence of anti-ficolin-2 antibodies was significantly related only to renal involvement, with a very high prevalence (86%) of anti-ficolin-2 antibodies in SLE patients with active LN. Patients with active proliferative LN had significantly more positive anti-ficolin-2 antibodies than those with non-proliferative LN.

The combination of anti-ficolin-2, anti-ficolin-3 and anti-C1q demonstrated a very high specificity (98%) for the diagnosis of active LN.

Conclusion. Our results support the usefulness of anti-ficolin-2 as a complementary serological biomarker for the diagnosis of active lupus with renal manifestation.

Significance and Innovations

- We present here for the first time the presence of anti-ficolin-2 antibodies in the serum of patients affected by lupus.

- The titer of anti-ficolin-2 antibodies is correlated with the disease activity (SLEDAI score).

- Regarding associations with clinical manifestations, only the presence of active lupus nephritis is significantly associated with the presence of anti-ficolin-2 antibodies.

- The combination of anti-ficolin-2, anti-ficolin-3 and anti-C1q antibodies demonstrates a very high specificity for the diagnosis of renal manifestations in lupus patients.

Introduction

Systemic lupus erythematosus (SLE) is a multi-system inflammatory disease presenting a broad spectrum of clinical manifestations affecting numerous organs (heart, kidney, brain, blood vessels). The course of the disease is unpredictable, with flares alternating with remissions, thus making the monitoring of patients particularly difficult, especially regarding possible life threatening organ involvement. SLE is a prototypical autoimmune disease characterized by T and B lymphocyte dysfunction and production of various autoantibodies. Although the mechanisms of induction of autoantibodies are complex and not fully deciphered, a contribution of defects in apoptotic cell clearance leading to secondary cell necrosis and subsequent release of intracellular autoantigens has been proposed (1). In accordance with this hypothesis, molecules involved in the uptake of dying cells, such as complement recognition proteins, could play a role in the prevention of autoimmune pathologies such as SLE. These proteins include ficolin-2, a member of the complement

defense collagens family, which has recently been reported to bind late apoptotic material and to play a role in its clearance (2,3).

Ficolin-2 is an oligomeric protein composed of 35 kDa subunits assembled in triple helix, consisting of both collagen-like and fibrinogen-like domains. This protein is produced by hepatocytes with a median plasma concentration level of 5.4 μg/mL in healthy humans (4). Ficolin-2 plays an important role in innate immunity and the maintenance of tissue homeostasis, thanks to its capacity to bind specific pathogen- and apoptotic cells-associated molecular patterns. Target recognition triggers the innate immune response by either activating the lectin complement pathway or binding to collectin cellular receptors. Interestingly the serum levels of ficolin-2 in Japanese SLE patients were found lower than those in healthy individuals, but were not associated with disease activity (5). Two recent studies reported the association of low ficolin-2 plasma levels, more precisely with lupus nephritis (LN) (6,7). However ficolin-2 measurement has been shown to be strongly dependent on experimental variations and blood sample handling procedures, rendering the correlation with clinical features questionable in the absence of standardized measurement (8). In addition, a genetic study reported the association of ficolin-2 gene polymorphisms with nephritis in SLE patients (9).

Antibodies against several complement recognition proteins, including C1q, mannose-binding lectin (MBL) and ficolin-3, have been reported to contribute to the development of SLE, supporting the hypothesis that the complement system is deeply involved in the pathogenesis of this disease in multiple ways (10–12). To date, no study reported the presence of antibodies targeting ficolin-2 in SLE sera. This study aimed to investigate the presence of anti-ficolin-2 antibodies in SLE patients and to evaluate the association between the presence of these autoantibodies and clinical manifestations, and disease activity.

Material & Methods

Samples. All venous blood samples were obtained from patients referred for routine detection of autoantibodies and sera conserved in declared biobanks (DC 2012-1704 for Marseille and DC 2014-2268 for Grenoble), with respect of ethical directives. Samples from SLE patients, satisfying the revised 1997 American College of Rheumatology (ACR) classification criteria for SLE (n = 165), were selected to be further analyzed. Samples from healthy blood donors (n = 48) obtained from "Etablissement Français du Sang", matched for sex and age, served as controls. This study has been approved by the Institutional Review Board according to standards currently applied in France (Commission Nationale de

l'Informatique et des Libertés", CNIL N°2030950vO). The study has also been registered to clinicaltrial.gov (N° NCT03063281). The study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. For SLE patients, clinical and biological manifestations and disease activity, evaluated using Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) at the time of sampling, were recorded. The SLE patients were divided into two groups for further analyses: a group with "low disease activity" (SLEDAI score $\leq$ 4, n = 88) and a group with "high disease activity" (SLEDAI score > 4, n = 77), according to the physician in charge. All patients with renal involvement had an active LN, defined by the presence of a significant proteinuria ($\geq$ 0.5 g/day) and/or the presence of hematuria, aseptic leukocyturia or urinary casts, which was documented by renal biopsy and classified according to the ISN/RPS classification. Samples were collected just before kidney biopsy. Active proliferative LN was defined as a class III or IV LN with activity, +/- class V, as opposed to non-proliferative LN (classes I, II or isolated V).

Anti-ficolin-2 antibodies assessment. Recombinant ficolin-2 was produced in Chinese hamster ovary cells and purified using a one-step affinity chromatography on *N*-acetylcysteine-Sepharose, as previously described (13). Detection of anti-ficolin-2 antibodies was adapted from an ELISA previously described for measurement of anti-ficolin-3 antibodies (12). Microtiter plates (96 wells) were coated overnight at 4°C with 4 μg/mL of recombinant ficolin-2 in 15 mM Na_2CO_3, 35 mM $NaHCO_3$ (pH 9.6). The plates were washed three times with Phosphate Buffer Saline (PBS) containing 0.1% Tween-20 (w/v) (PBS-T) and blocked for 1 h at room temperature (RT) with PBS-T containing 1% BSA (w/v). After washing as above, serum samples were added to the wells and incubated overnight at 4°C. After four washings as above, horseradish peroxidase (HRP)-conjugated goat polyclonal anti-human IgGs (The Binding Site, dilution 1:15000) were added for 1 h at RT. Subsequently tetramethylbenzidine substrate was added to each well, the reaction was stopped by addition of H_2SO_4 and optical densities (ODs) were measured at 450 nm. The threshold value of 95 AU was calculated using 98th percentile on OD reading for the control group. A value was considered significantly elevated when the concentration was above 95 AU (arbitrary units). The concentration was defined as (OD sample/OD control) x 100 AU.

Anti-dsDNA antibodies, complement fractions, anti-C1q and anti-ficolin-3 antibodies assessment. Anti-double stranded (ds)DNA were detected by ELISA (BIORAD) with normal value defined as < 50 IU/mL. Complement levels were measured by nephelometry (BN II,

Siemens), according to manufacturer's instructions, with normal range for C4 being > 100 mg/l and for C3 > 880 mg/l. Anti-C1q and anti-ficolin-3 antibodies were detected by ELISA, as previously described (12). A value was considered significantly elevated when the concentration was above 100 AU and 70 AU, respectively.

Statistics. Data analyses were carried out using Statview software version 5.0. Comparisons of continuous variables between subgroups were done using Mann-Whitney non parametric U-test. Correlations between continuous variables were assessed calculating Spearman's rank correlation coefficients. Comparison of categorical variables, expressed as counts (%), were done using Chi^2 or Fisher's exact-tests. P-values lower than 0.05 were considered statistically significant.

Results

Characteristics of SLE patients. Characteristics of the 165 SLE patients, including 36 biopsy-proven active LN, and matched healthy controls are provided in Table 1. At inclusion, the median SLEDAI score was 4 (range 0-24). 88 patients were classified in the "low disease activity" group and 77 in the "high disease activity" group, as previously defined (see Material & Methods). Biopsy-proven active LN patients belong to this latter group. The median age of the SLE patients was 42 years (range 16-84 years) with 88% women.

Detection of anti-ficolin-2 antibodies in SLE patients. Anti-ficolin-2 antibodies were quantified in serum samples from the 165 SLE patients and 48 healthy controls. Titers of anti-ficolin-2 antibodies were significantly higher in SLE patients than in healthy controls (median 74 vs 61 AU, p = 0.0403, Mann-Whitney U-test) (Figure 1A). Using a cutoff value of 95 AU (see Material & Methods) anti-ficolin-2 antibodies were detected as positive in 61 (37%) of 165 SLE patients. No anti-ficolin-2 antibodies were found in patients with rheumatoid arthritis (n = 14) and Sjögren's syndrome (n = 15) (Supplementary Figure 1).

Association of anti-ficolin-2 antibodies with lupus activity. SLE patients were divided into two groups: a group with "low disease activity" (SLEDAI score ≤ 4) and a group with "high disease activity" (SLEDAI score > 4), according to the physician in charge. Anti-ficolin-2 antibodies were detected as positive in 34 (44%) of the 77 SLE patients with "high disease activity" and in 26 (30%) of the 88 SLE patients (26/88) with "low activity" Titers of anti-ficolin-2 antibodies were significantly higher in SLE patients with "high disease activity"

than in those with "low disease activity" (median 84 vs 68 AU, p = 0.0123, Mann-Whitney U-test) (Figure 1B). A low positive correlation between anti-ficolin-2 antibodies titers and lupus activity using SLEDAI score was observed (r = 0.25, p = 0.0011, Spearman test) (Supplementary Figure 2). In SLE patients, titers of anti-ficolin-2 antibodies were significantly positively correlated with the titers of anti-dsDNA antibodies (r = 0.43, p < 0.0001, Spearman test), anti-C1q antibodies (r = 0.28, p = 0.0003, Spearman test), anti-ficolin-3 antibodies (r = 0.28, p = 0.0003, Spearman test) and negatively correlated with ficolin-2 (r = -0.32, p < 0.0001, Spearman test) (Supplementary Figure 2). SLE patients with low complement levels had significantly more positive anti-ficolin-2 antibodies (p < 0.05, Chi2-test) (data not shown).

Association of anti-ficolin-2 antibodies with active LN. The relationship between the presence of anti-ficolin-2 antibodies in sera and the clinical characteristics of SLE was assessed. Anti-ficolin-2 antibodies were detected as positive in 31 (86%) of 36 SLE patients with active disease and LN. Titers of anti-ficolin-2 antibodies were significantly higher in SLE patients with active LN than in patients with active disease but without renal involvement (median 152 vs 71 AU, p < 0.0005, Mann-Whitney U-test) (Figure 1C).

Interestingly, among various clinical manifestations presented by our patients (Table 1), the presence of anti-ficolin-2 antibodies was significantly associated only with renal involvement (p ≤ 0.01, Chi2-test) (Figure 2A). We then further investigated the possible link between the presence of anti-ficolin-2 antibodies and immunohistological characteristics of the kidney biopsies of the 36 active LN patients. Patients with active proliferative LN (i.e. Classes III and IV) had significantly more positive anti-ficolin-2 antibodies than those with non-proliferative LN (p = 0.005, Fisher's exact test) (Figure 2B). We previously demonstrated in this cohort of SLE patients that low complement C3 and/or C4, anti-dsDNA, anti-C1q, and anti-ficolin-3 levels were associated with renal disease activity (12). As in the present study anti-ficolin-2 antibodies were associated with renal disease activity, we then described the diagnostic performances of this biomarker alone and in combination with the other ones. Sensitivity and specificity of anti-ficolin-2 antibodies for SLE renal involvement were respectively 64% and 68%. Predictive positive and negative values were respectively 64% and 68%. Interestingly, the combination of anti-ficolin-2, anti-ficolin-3 and anti-C1q showed a higher specificity of 95% for renal involvement than without anti-ficolin-2 (Supplementary Table 1).

Discussion

Our study is the first one assessing the presence of antibodies targeting ficolin-2 and measuring their titers using ELISA in our cohort of SLE patients. Levels of anti-ficolin-2 autoantibodies were significantly higher compared to healthy subjects. They were found positive in 61/165 (37%) SLE patients. These antibodies were found negative in other autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome. SLE is characterized by a systemic inflammation involving multiple organs including skin, kidney, joints, heart and brain. Interestingly, whereas a low positive correlation of anti-ficolin-2 antibodies with the SLEDAI score which reflects the disease activity was found, the presence of anti-ficolin-2 antibodies was significantly related to renal involvement. No significant association between the positivity of anti-ficolin-2 antibodies and other SLE clinical outcomes was observed. In line with these findings, we observed a very high prevalence (86%) of anti-ficolin-2 antibodies in the subset of SLE patients with active LN.

Lupus nephritis, one of the most frequent and serious complications in SLE patients, and a major predictor of poor outcome, is a real challenge in the management of SLE. In clinical practice, conventional parameters such as proteinuria, abnormal creatinine and an elevated anti-dsDNA antibody titer concomitant with reduced complement levels (C3, C4) are disease-activity biomarkers of SLE and LN. However, they still lack sensitivity and specificity for detecting ongoing disease activity in lupus kidneys and early relapse of nephritis (14). Our results indicate that anti-ficolin-2 antibodies might be an additional parameter to distinguish active LN from active non-renal SLE flare. A significant correlation between anti-ficolin-2 antibodies and other SLE biological biomarkers was found, including anti-dsDNA antibodies, low complement levels, anti-C1q antibodies and also anti-ficolin-3 antibodies, recently reported to be significantly associated with active LN (12). As the combined measurement of a variety of autoantibodies is expected to be more valuable, all these biomarkers were tested in combination. Interestingly, the combination of anti-ficolin-2, anti-ficolin-3 and anti-C1q outperformed anti-C1q, anti-dsDNA or low complement alone but also anti-ficolin-3 and anti-C1q in combination, demonstrating a higher specificity (98%) than any other serological biomarker (12). This combination of anti-C1q, anti-ficolin-3 and anti-ficolin-2 could open new perspectives for LN evaluation, with future directions in LN biomarker research focused on new multi-marker panels, including these antibodies. A high negative predictive value of anti-ficolin-2 antibodies for nephritis was found as reported for anti-C1q antibodies (10). In addition, even if the moderately sized cohort of LN limits the

statistical power of the present study, it is interesting that patients with active proliferative LN (i.e. Classes III and IV) showed significantly more positive anti-ficolin-2 antibodies than those with non-proliferative LN.

Although a causal relationship between the presence of anti-ficolin-2 antibodies and LN remains to be determined, the possible role of anti-ficolin-2 antibodies in the pathogenesis of LN and its activity is a key issue. We postulate that anti-ficolin-2 antibodies would affect the occurrence or the course of SLE disease by interfering with ficolin-2 functions, especially with apoptotic cells clearance, or by binding to ficolin-2 deposited on various tissues. It has been previously demonstrated that ficolin-2 is deposited in the glomerular tissue in patients with LN together with IgG, C3, C1q, terminal complement cascade components and MBL (15). From a physiopathological point of view, ficolin-2 could be a key component in tissue inflammation as a target for autoantibodies. Anti-ficolin-2 antibodies may contribute to the formation of circulating immune complexes that are deposited in the kidney or to the local formation of immune complexes at the level of glomerular basement membrane, leading to complement activation and subsequent tissue injury. This in agreement with our results showing a negative correlation between anti-ficolin-2 antibodies and ficolin-2. In the case of C1q, based on observations in patients and murine models, it has been demonstrated that C1q present in glomerular immune deposits serves as a target for anti-C1q autoantibodies, which triggers complement activation once sufficient solid-phase C1q is available (10). This mechanism could apply to ficolin-2 directly deposited on the glomerular membrane. Investigation of this hypothesis would require the identification of the ficolin-2 ligands at the cell surface as well as the specificity of the anti-ficolin-2 autoantibodies (collagen-like or fibrinogen-like regions).

In conclusion, this is the first report pointing out the presence of anti-ficolin-2 antibodies in the serum of SLE patients. Even if additional longitudinal studies are needed to validate the diagnostic and/or prognostic role of this new SLE parameter, these data support the usefulness of anti-ficolin-2 as an additional serological biomarker for the diagnosis of active lupus with renal manifestation.

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Figure legends

Figure 1: Serum anti-ficolin-2 antibodies titers in patients with SLE

A, Anti-ficolin-2 titers in healthy controls (n = 48) and SLE patients (n = 165)

B, Anti-ficolin-2 titers in SLE patients with high disease activity disease (SLEDAI > 4) (n = 77) and SLE patients with low disease activity (SLEDAI ≤ 4) (n = 88)

C, Anti-ficolin-2 titers in SLE patients with high disease activity (SLEDAI > 4) (n = 77) with renal involvement (n = 36) or without renal involvement (n = 41)

Horizontal lines in each group indicate the median values. Statistical analysis were performed by Mann-Whitney tests.

Figure 2: Presence of anti-ficolin-2 antibodies in active lupus nephritis

A, Detection of anti-ficolin-2 antibodies in SLE patients with active disease (SLEDAI > 4, n = 77) according to SLE clinical damage. Statistical analysis were performed by Chi^2 test.

B, Detection of anti-ficolin-2 antibodies in LN with active (n = 27) or non active (n = 9) proliferation. Statistical analysis was performed by Fisher's exact-test

Table 1: Demographic and clinical variables for SLE patients

Demographic variables	Healthy controls (n = 48)	SLE (n = 165)	Active LN (n = 36)
Age, mean ± SD (median)	41 ± 13.3 (38)	44 ± 15.5 (42)	36 ± 14.6
Gender, female, n (%)	42 (88)	145 (88)	24 (67)
Patients with active disease, n (%)	NA	77 (47)	36 (100)
Type of flare, n (%)			
Kidney		36 (22)	36 (100)
Joint		44 (27)	12 (33)
Haematological		9 (6)	0 (-)
Cutaneous		19 (12)	4 (11)
Cardiac		8 (5)	3 (8)
Neurological		3 (2)	0 (-)
Renal abnormalities, n (%)	NA	NA	
Urinary casts (heme-granular or RBC casts)			24 (69)
High blood pressure			6 (17)
Proteinuria			34 (94)
Abnormal creatinine			13 (36)
ISN/RPS 2003 class, n (%)	NA	NA	
I			2 (6)
II			1 (3)
III +/- V			4 (11)
IV-S or IV-G +/- V			23 (64)
V			6 (17)
Patients with active disease were defined according to the physician in charge and with a SLE Disease Activity Index score > 4. It should be noted that some patients may have lupus flare involving several organs. NA = Not Applicable RBC = Red Blood Cell ISN/RPS = International Society of Nephrology/Renal Pathology Society			

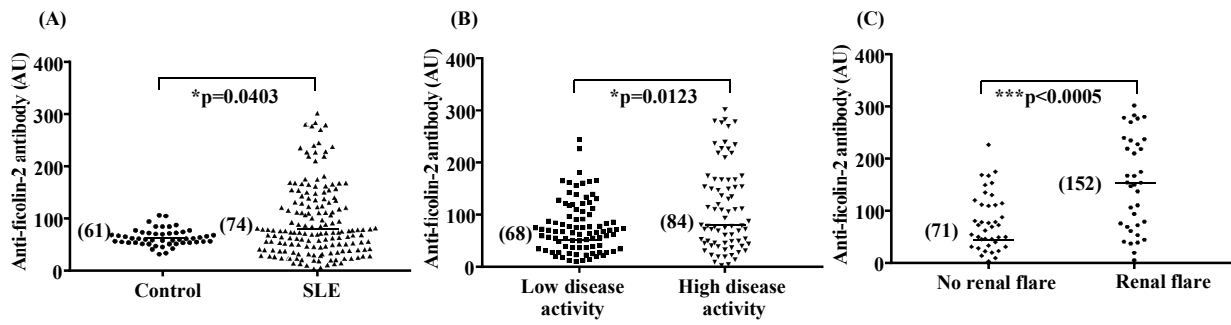SLE: Systemic Lupus Erythematosus; LN: Lupus Nephritis

Figure 1

Figure 2