

Autoantibodies against galectins are associated with antiphospholipid syndrome in patients with systemic lupus erythematosus

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Received on March 2, 2012; revised on August 1, 2012; accepted on August 2, 2012

The presence of autoantibodies against immunoregulatory effectors can be relevant for onset and/or the progression of autoimmune disease. Emerging insights into an immunological activity profile including a role as opsonins give reason to systematically monitor sera of patients for immunoglobulin G (IgG) autoantibodies, preferably for several galectins at the same time. Here, we report on a study of chronic inflammatory rheumatic diseases, i.e. systemic lupus erythematosus (SLE; pilot cohort p, *n* = 40; confirmation cohort c, *n* = 109), rheumatoid arthritis (RA; p, *n* = 32; c, *n* = 25) and primary antiphospholipid syndrome (APS; c, *n* = 64). Enzyme-linked immunosorbent assay-based series using galectin-1, -2, -3, -4, -7, -8 and -9 and natural processing products, i.e. the truncated version of galectin-3 and the N-terminal domains of galectin-4, -8 and -9, were performed. Normal healthy donors (p, *n* = 20; c, *n* = 21) and patients with paraproteins (c, *n* = 19) served as controls. Highly significant optical density-value readings for IgG autoantibodies were consistently detected for the proto-type galectin-7 (SLE) and the tandem repeat-type galectin-8 and -9 (SLE and RA). Their presence was independent from the autoantibody status

against double-stranded DNA (for patients with SLE) or a rheumatoid factor (for patients with RA), respectively. Importantly, anti-galectin-2 autoantibodies highly significantly correlated with the appearance of a secondary APS in patients with SLE so that this parameter may serve as an additional biomarker for APS. Equally of note, the presence of IgG autoantibodies against galectins capable to act as an opsonin may contribute to a sustained immune dysregulation in patients with chronic inflammatory rheumatic diseases.

Keywords: antiphospholipid syndrome / autoantibodies / galectins / systemic lupus erythematosus

Introduction

Autoimmune diseases are characterized by the occurrence of a wide panel of autoantibodies (Andreoli et al. 2008; Meroni and Shoenfeld 2008), which may contribute to impairment of clearance processes and hereby be linked to disease activity (Gaipl et al. 2007; Janko et al. 2008; Munoz et al. 2010). Therefore, antibodies can serve as a marker for onset and/or progression in autoimmune diseases such as systemic lupus erythematosus (SLE) or antiphospholipid syndrome (APS). Indeed, certain autoantibodies are known to be involved in specific organ manifestations in SLE (Rahman and Isenberg 2008), in fetal loss (Borchers et al. 2009), and can occasionally be found during infection (Doria et al. 2008; Praprotnik et al. 2008). Our study focuses on a class of endogenous lectins with immunoregulatory, cargo-routing and phagocytosis-promoting functions (Gabius 2006; Schwartz-Albiez 2009; Kaltner and Gabius 2012; Ledeen et al. 2012; Liu et al. 2012). In detail, we systematically screened for the presence of anti-galectin immunoglobulin G (IgG) in sera of patients with SLE, APS and rheumatoid arthritis (RA). Galectins exert diverse immune activities by binding distinct glycans on the cell surface, with notable target specificity regulated on six levels (Gabius et al. 2011). Glycoproteins, such as CD7, CD45 and the fibronectin receptor ($\alpha_5\beta_1$ -integrin) or ganglioside GM1, are involved in signaling and can induce immunosuppression in T effector/regulatory cells or cell death of activated T or tumor cells (Andre, Kaltner, et al. 2005; Fischer et al. 2005; Liu 2005; Gabius 2006; Wang et al. 2009; Sanchez-Ruderisch et al. 2011; Liu et al. 2012). Galectin-1, -2, -3, -7, -8 and -9

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induce the apoptosis of activated T cells with a differential profile of caspase activation, whereas galectin-4 appears to be a stimulator for T cells (Kashio et al. 2003; Sturm et al. 2004; Liu et al. 2012). The potential for galectins to act in immune dysregulation is, for example, supported by the expression of galectin-1, -3, -8 and -9 in synovial tissue promoting pro-inflammatory activity by cytokine secretion, and the regulation of apoptosis by galectin-3, -8 and -9 (Ohshima et al. 2003; Eshkar Sebban et al. 2007; Seki et al. 2007, 2008; Filer et al. 2009). Considering clearance, selective galectin binding was detected on dying and dead leukocytes (Beer et al. 2008), and capacity to serve as an opsonin for apoptotic cells and cellular debris has been proven for galectin-3, e.g. in connection with the Mer receptor tyrosine kinase (Karlsson et al. 2009; Caberoy et al. 2012).

To study the occurrence of anti-galectin IgG in autoimmune disease is hence of interest. Following the discovery of auto-antibodies for galectin-1 in the serum of healthy individuals (Lutomski et al. 1995), anti-galectin-1 was detected in patients with Chagas disease, multiple sclerosis, SLE and uveitis (Lutomski et al. 1997; Giordanengo et al. 2001; Romero et al. 2006; Montiel et al. 2010), anti-galectin-3 in normal sera and specimens from patients with tumors, SLE and inflammatory bowel disease, albeit with conflicting evidence (Jensen-Jarolim et al. 2001; Suarez-Alvarez et al. 2001; Hartmann et al. 2004); anti-galectin-4 in sera of colon cancer (Scanlan et al. 1998), anti-galectin-8 in SLE and RA (Massardo et al. 2009) and anti-galectin-9 in the normal serum (Suk et al. 1999). In overview, this survey of studies consistently focusing on a distinct lectin called for a systematic study testing the immunoreactivity in sera against a panel of galectins in parallel.

In our study, we observed increased optical density (OD) values for specific anti-galectin IgG in patients with primary APS (pAPS), RA and SLE, especially in those with secondary APS (sAPS). Anti-galectin IgG occurred independently from anti-double-stranded DNA (dsDNA) and rheumatoid factor (RF) for SLE and RA, respectively. Importantly, anti-galectin-2 IgG significantly correlated with sAPS. Since there are several patients with APS that do not show the classical serum markers lupus anticoagulant (LAC), anti- β 2GP1 IgG and anti-cardiolipin (anti-CL) IgG, we hypothesize that anti-galectin-2 IgG may be useful for the diagnosis of APS.

Results

Pilot study: Detection of anti-galectin autoantibodies

The maintenance of immunoreactivity of all proteins when adsorbed to the surface of microtiter wells was ascertained by galectin-type-specific polyclonal antibodies from rabbits. Since these enzyme-linked immunosorbent assay (ELISA) tests with purified proteins consistently gave dose-dependent results, we defined conditions for autoantibody detection in each case so that a panel of galectins could be tested in parallel from serum aliquots of the same patient. In the sera of patients with SLE, we found antibodies against galectin-2, -4, -7, -8 and -9, the proteolytically truncated version of galectin-3 and the N-terminal domain of the tandem repeat-type galectin-9 (Figure 1, Table I). Beside the fact that these

forms are naturally produced by proteolysis this process may help expose otherwise hidden epitopes. In contrast, sera of RA patients showed less reactivity to galectins. Antibodies in these samples were directed against galectin-4, -7, -8, -9 and -9N (Figure 1, Table I). Importantly, anti-galectin IgG in SLE patients were not related to anti-dsDNA IgG. OD values of anti-galectin IgG in SLE mutually correlated significantly after correction with Bonferroni post-testing (Table II, Supplementary data, Figure S1).

Pilot study: Statistical correlations with anti-dsDNA/RF reactivity

The calculations revealed that certain galectin-dependent factors could be related to the standard markers. Significant correlations were initially found in SLE for dsDNA vs anti-galectin-3, -4 and -8 (Figure 2) and in RA for RF vs truncated galectin-3, -4 and -8 and the N-terminal domain of galectin-9 (not shown). However, after Bonferroni post-testing, no statistically significant correlations between anti-galectin and anti-dsDNA in SLE (Figure 2) and RF in RA (not shown) were observed. Although it had been reported that galectin-1 lost lectin activity when adsorbed to the surface of microtiter plate wells (Caron et al. 1994), what would exclude antibody binding via IgG glycans, we added controls with a haptenic inhibitor.

Pilot study: Profiling of sera in the presence of a lectin inhibitor

All experimental series were also carried out in the presence of 100 mM lactose to exclude that lectin-antibody interaction is not due to carbohydrate binding involving antibody glycans. About 100 mM lactose was ascertained to effectively interfere with galectin binding to glycoproteins, i.e. asialofetuin, asialo- α_1 -acid glycoprotein and asialoglycophorin in solid-phase assays. Running assays with sera in the absence and the presence of the haptenic inhibitor revealed no effect after correction for multiple testing (Figure 3). This result also excluded any modification of galectin reactivity to autoantibodies by loading with ligand; this process is known to reduce the gyration radius of galectin-1 from 19.1 to 18.2 Å (He et al. 2003).

Pilot study: Clinical implications of the anti-galectin autoantibodies

Next, we analyzed the correlation of anti-galectin autoantibodies in sera from patients with established disease markers, with medication and with clinical manifestations. In patients with RA, we observed significant correlations of OD values/readings for anti-galectin-7 with the age of patients, of OD values of anti-galectin-3t, -4, -8 and -9N autoantibodies with RF and OD values of anti-galectin-9 with anti-nuclear antibodies (ANAs; Table III). In patients with SLE, initially significant correlations were observed between anti-CL and anti-galectin-2 and -7 and between LAC and anti-galectin-2 and -8N (Table IV). However, after correction for multiple testing, anti-galectin IgG did not correlate significantly with age and the common disease markers C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), disease activity

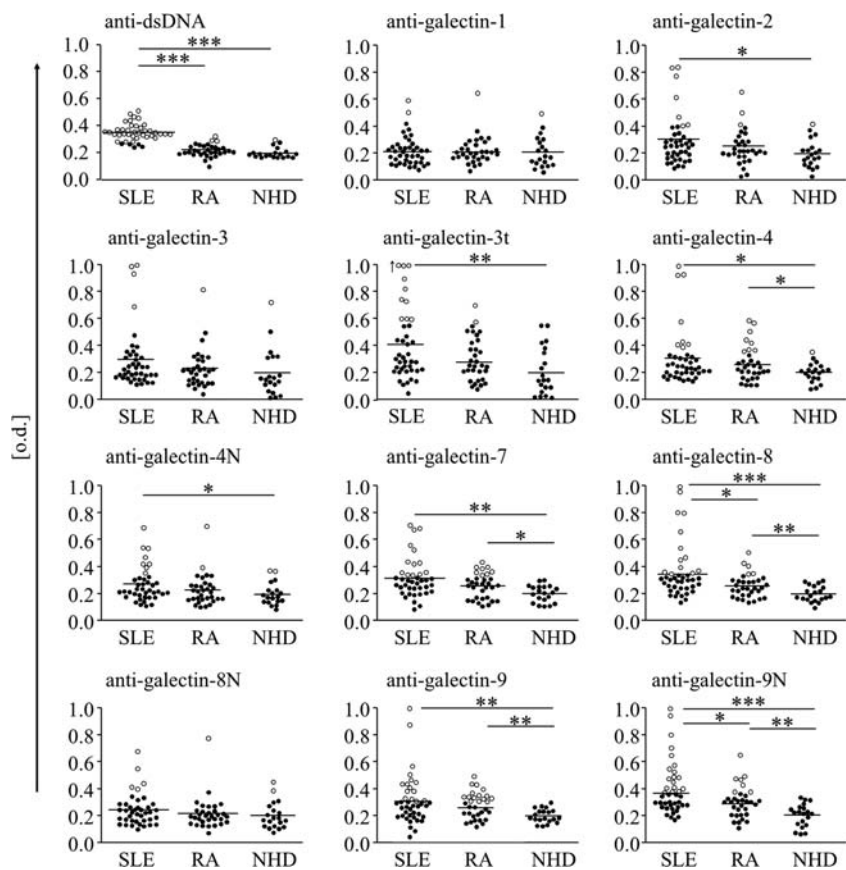


Fig. 1. Pilot study: Analysis of anti-dsDNA IgG and anti-galectin-1, -2, -3, -4, -7, -8 and -9 IgG in sera of SLE, RA and NHD. NHD, normal healthy donor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; horizontal line, the mean value; open circles, >mean of NHD + 2 SD; closed circles, <mean of NHD + 2 SD; o.d., optical density. *P*-values, two-tailed Mann–Whitney *U*-test with the Bonferroni post-test; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 1. Pilot study: Anti-galectin in sera of patients with SLE and RA

Galectin	Anti-galectin autoantibodies in SLE (%)	Anti-galectin autoantibodies in RA (%)	Clinical associations
1	5	3	sAPS
2	17.5*	9	
3	10	3	
4	20*	25*	
7	32.5**	25*	
8	40***	16**	
9	35**	47**	

P-values two tailed Mann–Whitney *U*-Test with Bonferroni post-test, **P* < 0.05; ***P* < 0.01, *P* < 0.001. Positivity for anti-galectin autoantibodies was considered for SLE sera with OD > mean of NHD sera + 2 SD of NHD. SLE, patients with systemic lupus erythematosus; RA, rheumatoid arthritis; NHD, normal healthy donors; sAPS, secondary antiphospholipid syndrome.

score (DAS-28), RF, anti-citrullinated protein/peptide antibody and ANA for RA (Table III) and with CRP, C3, C4, ESR, α-CL, α-β2GP-1, LAC and ANA for SLE (Table IV). Therefore, anti-galectin IgG may be considered as an independent marker for these diseases. The comparison of anti-galectin IgG with various medications (methotrexat, prednisone, chloroquin, azathioprine) revealed no statistically

significant correlations in patients with SLE and RA (not shown). In contrast, the analysis of the correlation of anti-galectin IgG with various disease parameters delineated a statistically significant correlation between anti-galectin-2, -3, -4 and -7 and sAPS in patients with SLE, which remained highly significant after correction for multiple testing with the Bonferroni post-test (Table V). The associations of anti-galectin-2 in SLE patients with sAPS, with seropositivity for anti-CL and LAC, are shown in Figure 4. The anti-galectin-2 status was more significantly associated with the clinical appearance of sAPS (arterial and venous thrombosis, recurrent miscarriages and thrombocytopenia) than with anti-CL and LAC, established serological markers of APS (Figure 4). When calculating the single diagnostic test comparison we observed that specificity, sensitivity and negative as well as positive predictive values for anti-galectin-2 were in the same range as for the established classical biomarkers of APS, anti-CL and LAC (Table VI).

Confirmation cohorts: Detection of anti-galectin autoantibodies

The pilot study revealed that anti-galectin-8 showed the strongest reactivity in sera of patients with SLE [40% > mean + 2 standard deviation (SD) of normal healthy donors (NHDs)]

Table II. Pilot study: OD values of anti-galectin mutually correlate

	Gal-1	Gal-2	Gal-3	Gal-4	Gal-7	Gal-8	Gal-9	PGA	dsDNA
Gal-1		2.07E-09	8.23E-08	2.38E-08	2.37E-06	3.25E-04	1.52E-04	n.s.	n.s.
Gal-2	2.07E-09		3.27E-10	1.88E-11	5.46E-11	6.01E-06	6.61E-06	n.s.	n.s.
Gal-3	8.23E-08	3.27E-10		2.45E-30	2.23E-08	2.62E-07	2.04E-06	n.s.	n.s.
Gal-4	2.38E-08	1.88E-11	2.45E-30		4.04E-09	1.92E-08	3.90E-07	n.s.	n.s.
Gal-7	2.37E-06	5.46E-11	2.23E-08	4.04E-09		2.04E-06	2.71E-07	n.s.	n.s.
Gal-8	3.25E-04	6.01E-06	2.62E-07	1.92E-08	2.04E-06		5.19E-04	n.s.	n.s.
Gal-9	1.52E-04	6.61E-06	2.04E-06	3.90E-07	2.71E-07	5.19E-04		n.s.	n.s.
PGA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.
dsDNA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Corrected *P*-values (Bonferroni) are displayed; n.s., not significant; PGA, poly-guanilic acid; dsDNA, double-stranded DNA; Gal, galectin.

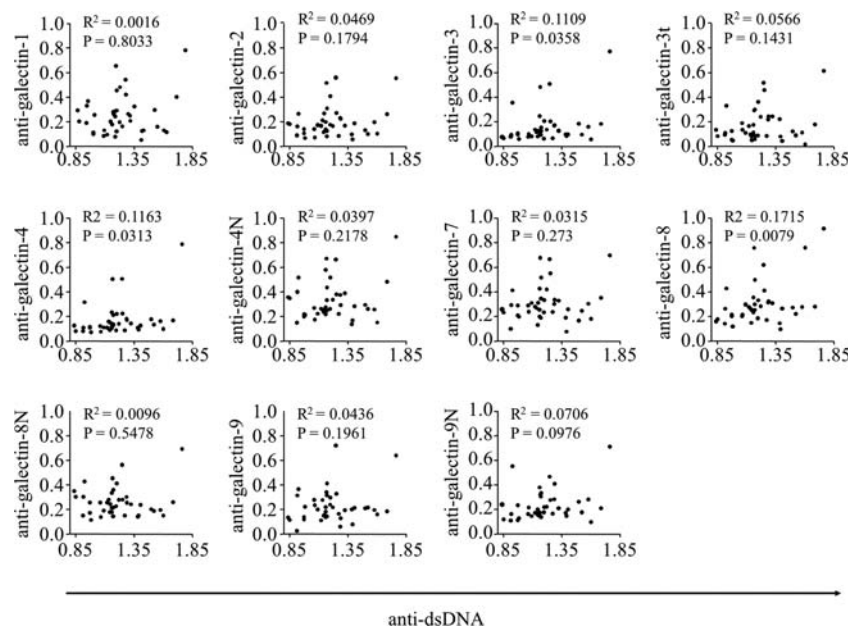


Fig. 2. Pilot study: ODs of IgG autoantibodies against galectin-3, -4 and -8 significantly correlate with ODs of anti-dsDNA IgG autoantibodies in SLE before correction for multiple testing (Bonferroni).

and that anti-galectin-2 IgG is associated with the appearance of sAPS (Table I). Anti-galectin IgG mutually correlated in the patients' sera (Table II, Supplementary data, Figure S1). Therefore, we selected anti-galectin-2 IgG and anti-galectin-8 IgG for further analysis in the confirmation cohorts.

The ELISAs to detect autoantibodies were extended to sera from patients with SLE, SLE with sAPS, pAPS and RA from further clinical centers to enlarge the database. Sera from NHDs and patients with paraproteins (PPs; incomplete IgG, e. g. in tumor patients) served as negative controls. Statistically significant differences compared with NHDs were detected in the case of anti-galectin-2 and -8 for patients with SLE only, SLE/sAPS and pAPS (Brescia), but not for anti-galectin-8 in the London cohort (Figure 5A and B). OD values of anti-dsDNA IgG were significantly elevated in patients with SLE only, SLE/sAPS and pAPSs from Brescia and London (Figure 5C).

Confirmation cohorts: Clinical implications of anti-galectin autoantibodies

Finally, we analyzed correlations of anti-galectin IgG with established disease markers and clinical manifestations. When we compared the OD values of anti-galectin-2 IgG from patients with SLE only and patients with SLE/sAPS (Heidelberg and London cohorts), we observed a statistically significant positive correlation. Again, the occurrence of anti-galectin-2 autoantibodies was more significantly associated with that of a clinical APS than with the occurrence of typical serum markers of APS such as LAC (Figure 6A and B). These results confirm the findings from the pilot cohort, where an association of anti-galectin-2 with sAPS in SLE was detected (Figure 4, Table V). We therefore consider anti-galectin-2 autoantibodies as an independent biomarker for APS.

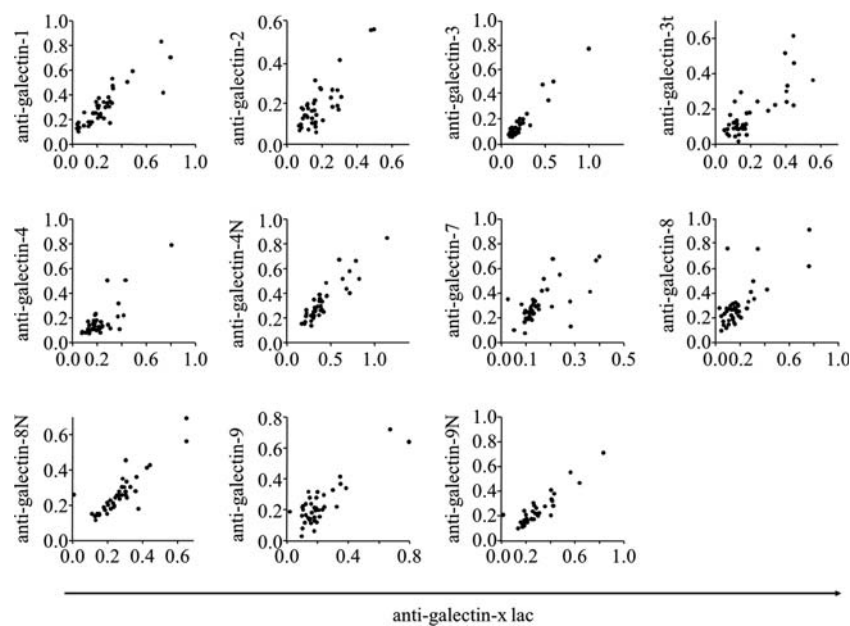


Fig. 3. Pilot study: Binding of anti-galactin autoantibodies is not altered by loading the lectin site of galectins with lactose. Correlation of anti-galactin IgG in the absence of lactose in sera with anti-galactin IgG in the presence of lactose in sera of SLE patients. lac, lactose.

Table III. Pilot study: Anti-galactin autoantibodies initially correlated with age, RF and ANA in patients with RA

RA	Age	CRP	ESR	DAS-28	RF	ACPA	ANA
Gal-1							
r^a							
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-2							
r^a							
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-3							
r^a							
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-3t							
r^a					0.403		
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.022, 0.528	n.s.	n.s.
Gal-4							
r^a					0.397		
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.024, 0.576	n.s.	n.s.
Gal-4N							
r^a							
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-7							
r^a	-0.369						
p^b , Bonferroni	0.037, 0.888	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-8							
r^a					0.383		
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.031, 0.744	n.s.	n.s.
Gal-8N							
r^a							
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-9							
r^a							-0.491
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.036, 0.864
Gal-9N							
r^a					0.445		
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.011, 0.264	n.s.	n.s.

However, after correction with the Bonferroni post-test for multiple testing, the statistical significance of correlation was lost. The correlation statistics of OD values of autoantibodies against galectins (Gal) in patients with RA vs established clinical markers of RA. ACPA, anti-citrullinated protein/peptide antibodies; ANA, anti-nuclear antibodies; CRP, C-reactive protein; DAS-28, disease activity score; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor.

^ar, correlation coefficient according to Pearson.

^bp, two-tailed *P*-value. No correction for multiple testing, Bonferroni, *P*-values of the two-tailed, Pearson test with the Bonferroni post-test.

Table IV. Pilot study: Anti-galectin autoantibodies initially correlated with anti-CL and LAC in patients with SLE

SLE	CRP	C3	C4	ESR	α -CL	α - β 2GP-1	LAC	ANA
Gal-1								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-2								
r^a					0.439		0.401	
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.008, 0.192	n.s.	0.025, 0.6	n.s.
Gal-3								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-3t								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-4								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-4N								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-7								
r^a					0.442			
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	0.008, 0.192	n.s.	n.s.	n.s.
Gal-8								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-8N								
r^a							0.364	
p^b , Bonferroni	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.044, 1.056	n.s.
Gal-9								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Gal-9N								
r^a								
p^b	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

However, after correction with the Bonferroni post-test for multiple testing, statistical significance of correlation was lost. Correlation statistics of OD values of autoantibodies against galectins (Gal) in patients with SLE vs established clinical markers of SLE. ANA, anti-nuclear antibodies; α -CL, anti-cardiolipin; CRP, C-reactive protein; C3, complement factor 3; C4, complement factor 4; ESR, erythrocyte sedimentation rate; LAC, lupus anticoagulant.

^a r , correlation coefficient according to Pearson.

^b p , two-tailed P -value. No correction for multiple testing. Bonferroni, P -values of the two-tailed, Pearson test with the Bonferroni post-test.

Table V. Pilot study: Anti-galectin autoantibodies in patients with SLE significantly correlate with clinical signs of APS after correction for multiple testing but not with other clinical manifestations

	Gal-1	Gal-2	Gal-3	Gal-4	Gal-7	Gal-8	Gal-9
Blood	1	1	1	1	1	1	1
CNS	1	1	1	1	1	1	1
sAPS	0.642	0.001	0.033	0.039	0.015	0.230	1
Joint	1	1	1	1	1	1	1
Kidney	1	1	1	1	1	1	1
Serositis	1	1	1	1	1	1	1
Skin	0.195	0.426	0.170	0.293	0.054	1	1

P -values of the two-tailed, Mann-Whitney U -test with the Bonferroni post-test. Values >1 after the Bonferroni post-test are listed as "1". sAPS, secondary antiphospholipid syndrome. SLE symptoms vary widely. SLE can harm blood vessels, CNS, joints, kidneys, skin or can cause serositis or clinical signs of APS. Anti-galectin autoantibodies are associated with clinical signs of APS as arterial and venous thrombosis, recurrent miscarriages and thrombocytopenia. All clinical manifestations occurring in SLE were taken from the patients' chart.

Discussion

Our data reveal the occurrence of antibody reaction against different galectins in sera of patients with SLE, APS and RA.

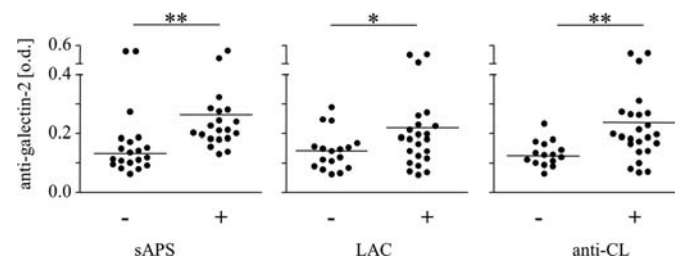


Fig. 4. Pilot study: Association of anti-galectin-2 IgG in SLE patients with sAPS, with seropositivity for anti-CL and LAC. The status of anti-galectin-2 autoantibodies was more significantly associated with the clinical appearance of APS than with the serological parameters. Anti-CL, anti-cardiolipin; LAC, lupus anticoagulant; sAPS, secondary antiphospholipid syndrome; o.d., optical density; horizontal line, the mean value. P -values, two-tailed Mann-Whitney U -test with the Bonferroni post-test; * $P < 0.05$; ** $P < 0.01$.

Galectins are effectors in apoptosis with emerging relevance for clearing dead cells and their remnants, compounds involved in processes initiating the immune response against nuclear proteins in SLE. First, the presented results confirm

Table VI. Pilot study: Single diagnostic test comparison of OD values of anti-galectin-2 IgG, anti-CL and LAC

	Mean of the 95% CI	95% CI	
		Lower limit	Upper limit
Specificity (%)			
Anti-Gal-2	78.95	57.98	99.91
Anti-CL	68.75	42.91	94.59
LAC	92.86	75.8	100
Sensitivity (%)			
Anti-Gal-2	80.95	61.78	100
Anti-CL	73.68	51.25	96.12
LAC	72.22	48.75	95.69
Negative predictive value (%)			
Anti-Gal-2	78.95	57.98	99.91
Anti-CL	68.75	42.91	94.59
LAC	72.22	48.75	95.69
Positive predictive value (%)			
Anti-Gal-2	80.95	61.78	100
Anti-CL	73.68	51.25	96.12
LAC	92.86	75.8	100

CI, confidence interval; anti-CL, anti-cardiolipin autoantibodies; LAC, lupus anticoagulant.

previous reports on galectin-1 autoantibodies in SLE (Montiel et al. 2010), galectin-3 (12 of 63 specimen positive; Lim et al. 2002) and galectin-8 autoantibodies in SLE and RA (Massardo et al. 2009). More important, when extending these analyses to a broad panel of galectins, we could present the complexity of the immune response to galectins in a more detailed way. Parallel assays of several galectins made inter-galectin comparisons possible, revealing so far not described positivities and a non-uniform response to galectins. The response profile characterizes the type of the disease. The significant immune reaction in relation to age- and sex-matched controls was found to be directed against the proto-type galectin-7 and the tandem repeat-type proteins, here particularly galectin-8 and -9, with indications for the positivity against galectin-7 (in SLE) and -9 to be independent from anti-dsDNA or RF. These results suggest that the autoantibodies present in the serum may contribute to immunologic dysregulation. A further function identified for distinct galectins that may be related to the induction and maintenance of autoimmunity is their reactivity to dead cells and their phagocytosis-promoting activity (Beer et al. 2008; Karlsson et al. 2009; Caberoy et al. 2012). Antibodies binding to opsonins of dead cells such as C1q (Kallenberg 2008), β 2GPI (Bassi et al. 2007), annexin-V (Szodoray et al. 2009) and also the collectin mannan-binding lectin (Tsutsumi et al. 2005), as well as antiphospholipid autoantibodies (Tincani et al. 2009), are frequently described in patients with SLE. These autoantibodies may shift clearance to a pro-inflammatory process, since apoptotic and post-apoptotic remnants are phagocytosed in an Fc receptor-dependent manner (Munoz et al. 2010). Our results thus inspire to monitor presence of anti-galectin IgG in other types of autoimmune diseases and to examine effects of their presence in functional assays.

Anti-galectin-8 was found to appear in SLE independently from anti-dsDNA (Table IV). Investigating the organ involvement in SLE, the OD values of anti-galectin IgG did not correlate with hematological disease, CNS, joint, kidney, serositis

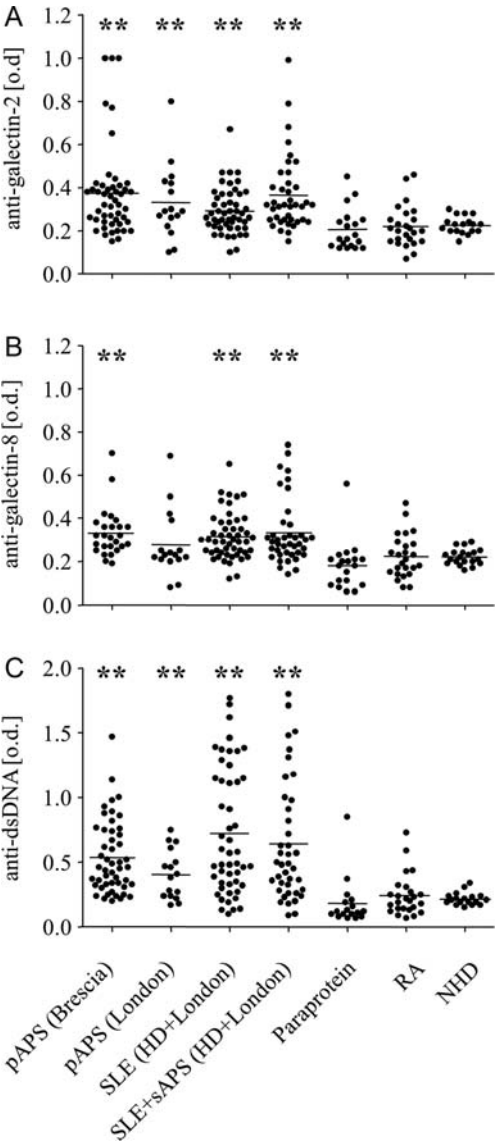


Fig. 5. Confirmation cohorts: Analysis of anti-galectin-2, anti-galectin-8 and anti-dsDNA in patients with pAPS, SLE, SLE + sAPS, RA and NHD. NHD, normal healthy donors; pAPS, primary antiphospholipid syndrome; RA, rheumatoid arthritis; sAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus; o.d., optical density; horizontal line, mean value. *P*-values, two-tailed Student's test with the Bonferroni post-test; ***P* < 0.01.

and skin involvement. Instead, the presence of autoantibodies against galectin-2, -3, -4 and -7 (Table V) was highly significantly associated with sAPS (clinically characterized by arterial and venous thrombosis, recurrent miscarriages and thrombocytopenia), even after Bonferroni correction for multiple testing. We found positive correlations for anti-galectin-2 with LAC and anti-CL (Figures 4 and 6), whereas specificity, sensitivity and negative and positive predictive values for anti-galectin-2 were in the same range as the established classical biomarkers of APS (anti-CL, anti- β 2GPI and LAC; Table VI). On this experimental basis, we postulate anti-galectin-8 and anti-galectin-2 antibodies as new

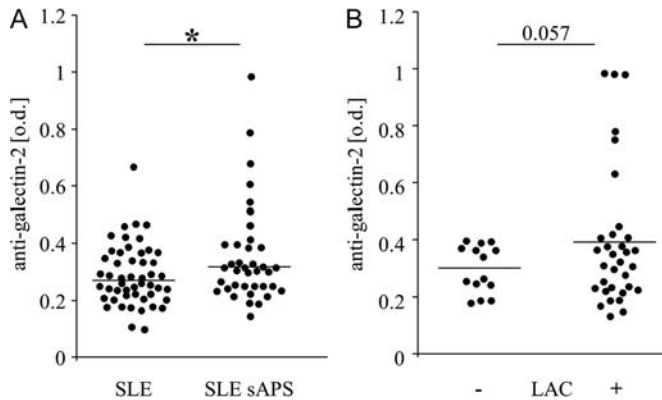


Fig. 6. Confirmation cohorts: Association of anti-galectin-2 in SLE patients with sAPS and with seropositivity for LAC. (A) Sera from SLE patients from Heidelberg and London. (B) Sera from pAPS patients from Brescia. LAC, lupus anticoagulant; SLE, systemic lupus erythematosus; sAPS, secondary antiphospholipid syndrome; o.d., optical density; horizontal line, the mean value. *P*-values, two-tailed Student's *t*-test with the Bonferroni post-test; **P* < 0.05.

independent biomarkers for SLE and sAPS in SLE, respectively. The detection of galectin-2-specific IgG autoantibodies in SLE patients could help to identify patients with seronegative sAPS prior to the occurrence of clinical APS with its obstetric and thrombotic complications. Furthermore, the analysis of anti-galectin-2 may help to elucidate the etiology of APS in patients lacking the classical autoantibodies.

Materials and methods

Pilot study group

The pilot study group included 40 SLE patients and 32 RA patients from our outpatient clinic at the Department of Internal Medicine 3 of the Erlangen University Medical Centre. All patients met the classification criteria of the American College of Rheumatology for SLE (Hochberg 1997) and RA (Arnett et al. 1988), respectively. Twenty age- and sex-matched NHDs served as controls. An informed consent was obtained from all blood donors and the study received the final approval from the ethics committee of the University Hospital Erlangen. The clinical manifestations and serological parameters of the patients were obtained from the clinical chart and the laboratory register of each patient (Supplementary data, Tables S1 and S2). Heparinized venous whole blood was collected by venipuncture and processed within 2 h. Serum samples were obtained by centrifugation at $1000 \times g$ for 15 min of clotted blood, frozen, stored at -20°C and thawed once for further analysis. All samples were measured on a single ELISA plate in monoplexates. This pilot study group was only used to identify the best galectin candidates to be investigated in the confirmation cohort.

Further clinical cohorts (confirmation cohorts)

In order to broaden the experimental basis, we obtained sera from other European autoimmune disease centers (Brescia,

London, Milano and Heidelberg). The cohort from Brescia comprised 48 patients with pAPS, from London 16 patients with pAPS, 20 patients with SLE and 20 patients with sAPS, from Milano 12 patients with SLE and 7 patients with sAPS and from Heidelberg 30 patients with SLE and 20 patients with sAPS. For further control purposes, additional 21 NHDs, 25 patients with RA and 19 patients with PPs were recruited. The sera were analyzed in triplicates. In order to improve the stability of the measurement, the outer rim of the ELISA plates was excluded. For the standardization of each up to 10 ELISA plates, nine sera of NHDs were employed on every single plate in monoplexates. For the ELISAs of the confirmation cohorts, fresh galectin preparations were used.

Galectins

The human galectins were obtained by recombinant production and purified by affinity chromatography on lactosylated Sepharose 4B, additionally freed of lipopolysaccharide contamination as described (Lensch et al. 2006; Andre, Maljaars, et al. 2007; Andre, Sanchez-Ruderisch, et al. 2007; Sarter et al. 2009). Quality controls included one- and two-dimensional gel electrophoresis as well as mass spectrometry of the proteins and tryptic peptides (Purkrabkova et al. 2003; Andre, Sanchez-Ruderisch, et al. 2007). Collagenase digestion of human galectin-3 yielded the truncated derivative (tGal-3) representing the carbohydrate recognition domain (Kubler et al. 2008). The immunoreactivity of adsorbed galectins was ascertained in ELISAs using polyclonal rabbit anti-galectin antibodies prepared and refined by removing intergalectin cross-reactivity as described (Saal et al. 2005; Lensch et al. 2006; Cada et al. 2009; Saussez et al. 2010). Solid-phase assays in microtiter plates were performed to verify the sensitivity of galectin binding to asialofetuin as model glycoprotein to the presence of 100 mM lactose as a haptenic inhibitor and to the probe reactivity of lactose-loaded galectins to their polyclonal antibodies as described (Andre, Specker, et al. 2009).

Anti-galectin IgG ELISA

To quantify anti-galectin IgG autoantibodies, home-made ELISA tests were performed. In detail, the surface of Maxisorb immunoplate wells (Nalgene Nunc, New York, NY) was coated with $0.5 \mu\text{g}$ of galectin in $100 \mu\text{L}$ coating buffer ($15 \text{ mM Na}_2\text{CO}_3$ and 35 mM NaHCO_3) overnight at 4°C . After coating, the plates were washed three times with phosphate-buffered saline (PBS)/0.05% Tween. Thereafter, the coated plates were blocked for 1 h at room temperature with PBS/1% bovine serum albumin (BSA). After coating, the plates were washed three times with PBS/0.05% Tween. Then, $100 \mu\text{L}$ of serum dilution (1/100 in PBS/1% BSA/0.05% Tween) per well was added and incubated for 1 h at room temperature. After washing three times with PBS/0.05% Tween, $100 \mu\text{L}$ /well of horseradish peroxidase-labeled anti-human IgG F_{ab}-fragment (Southern Biotech, Birmingham, AL), diluted 1:10,000 in PBS/1% BSA, was added and incubated for 1 h at room temperature. After thoroughly washing three times with PBS/0.05% Tween, $100 \mu\text{L}$ of development solution was added to each well and this setting incubated for

15 min at room temperature in the dark. The development solution was freshly prepared by mixing 1 mL of 3,3',5,5'-tetramethylbenzidine (1 mg/mL in DMSO, Sigma-Aldrich, Munich, Germany) with 9 mL of substrate buffer (0.1 M Na₂HPO₄, 0.05 M citric acid, pH 4.5–5.5) and 2 µL of H₂O₂ per plate. The enzymatic reaction was stopped with 100 µL of 25% H₂SO₄ per well. All sera were tested in duplicates. The OD was measured at 450 and 650 nm as a correction factor. On every plate, nine samples of sera of NHDs were routinely included as controls. The mean value obtained for these sera were calculated and normalized to an OD of 0.2. According to the normalized value of the control NHD sera, the OD sera of the patients' sera were calculated and compared.

Anti-dsDNA IgG ELISA

For measuring anti-poly-G and anti-dsDNA, 100 µL/well of 20-µg/mL poly-L-lysine solution (Sigma-Aldrich) in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM ethylenediamine-tetraacetic acid, pH 7.4) was precoated overnight at 4°C. After washing three times with TE buffer, 100 µL/well of 20-µg/mL calf thymus dsDNA (Sigma-Aldrich) in TE buffer was incubated with the poly-L-lysine matrix overnight at 4°C. After washing three times with PBS/0.05% Tween, the plates were blocked with 100 µL PBS/2% fetal calf serum (FCS) per well. After washing three times with PBS/0.05% Tween, 100 µL of serum diluted 1:500 in PBS/2% FCS was added per well and incubated for 1 h at room temperature. Subsequent steps were similar to the anti-galectin IgG ELISA.

Statistical analysis

Experimental values were expressed as a mean OD of the duplicates of each serum. The optimal cut-off levels for each group were determined relative to the SD of the NHD values. Differences among the study groups were assessed using the two-tailed *t*-test. Associations between data sets in the absence or the presence of lactose, between OD values of anti-galectin and of anti-dsDNA in SLE or of RF in RA, respectively, as well as associations among anti-galectin ODs and established disease markers were evaluated by the parametric Pearson's correlation test. Associations among anti-galectin ODs, organ involvement and medication were assessed by serial distributive Mann-Whitney *U*-tests for two independent samples. Unless stated otherwise, significance values were adjusted by the Bonferroni multiple testing correction procedure. The confidence level was set to 95%. SPSS 17.0 software was used for statistic calculations.

Supplementary Data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

Funding

This work was supported by the Masterswitch project of the European Union (EU), the Verein zur Förderung des biologisch-technologischen Fortschritts in der Medizin e. V., an

EC Marie Curie Research Training Network grant (MCRTN-2005-19561), grant agreement no. 260600 (GlycoHIT) from the EC Seventh Framework Programme, by the Emerging Fields Initiative (EFI) of the FAU Erlangen-Nuremberg, by the K & R Wucherpfennigstiftung, and by the Interdisciplinary Center of Clinical Research (IZKF) at the University Hospital Erlangen-Nuremberg. KS was supported by the training grant GK592 from the German Research Community (DFG) and an intramural grant from the ELAN-fonds of the Medical Faculty at the Friedrich-Alexander-University, CJ was supported by an intramural grant of the Friedrich-Alexander-University Erlangen-Nürnberg (Frauenförderung), and by SFB 643 TP B5.

Acknowledgements

We gratefully acknowledge the inspiring discussions with Drs B. Friday and S. Namirha as well as expert technical assistance by B. Hofer and L. Mantel.

Conflict of interest

The authors declare that they have no competing interests.

Abbreviations

ANA, anti-nuclear antibody; APS, antiphospholipid syndrome; BSA, bovine serum albumin; c, confirmation study; CL, cardiolipin; CRP, C-reactive protein; DAS-28, disease activity score; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; FCS, fetal calf serum; Ig, immunoglobulin; LAC, lupus anticoagulant; NHD, normal healthy donor; OD, optical density; p, pilot study; pAPS, primary APS; PBS, phosphate-buffered saline; PP, paraprotein; RA, rheumatoid arthritis; RF, rheumatoid factor; sAPS, secondary antiphospholipid syndrome; SD, standard deviation; SLE, systemic lupus erythematosus; TE, Tris-EDTA.

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Supplementary Table S1. Pilot study: Clinical and serological characteristics and medication of the patients with RA.

No	sex	Age	ESR 1h	CRP (mg/L)	RF (IU/ml)	ACPA (U/ml)	DAS-28	C3 (mg/dl)	C4 (mg/dl)	ANA-Titer (1/xxx)	treatment				
											Prednison (mg/week)	Methtrexat (mg/week)	Chloroquin (mg/week)	NSAID	Other
1	f	61	4	2	35.5	0	n.d.	n.d.	n.d.	40	0	12.5			
2	f	73	6	1	103	n.d.	n.d.	n.d.	n.d.	0	35	15		x	Rem
3	f	52	29	5	0	n.d.	n.d.	n.d.	n.d.	80		15		x	Rem
4	m	83	6	1	27	880	n.d.	91	17.9	0	42				
5	f	43	17	1	0	147	n.d.	n.d.	n.d.	0	0	20			
6	f	42	12	5	0	82.2	n.d.	n.d.	n.d.	0	35	15			
7	f	59	9	30	584	43	3.25	146	35	40	140	15			
8	f	79	n.d.	1	140	0	n.d.	100	25	0	35				
9	f	83	71	29	21	0	n.d.	148	29	0	52.5			x	
10	f	60	13	2	55	1336	2.33	107	28	100	21	20		x	
11	f	58	16	6	0	0	n.d.	162	27	0	17.5	15		x	
12	m	55	15	6	0	1442	n.d.	n.d.	n.d.	n.d.	0				
13	f	73	33	8.2	0	0	n.d.	130	23.5	0	35				
14	f	53	n.d.	1	35	918	n.d.	104	21	0	0			x	
15	f	73	32	23	0	0	n.d.	100	25	0	70				Cic
16	f	87	46	24	0	0	n.d.	n.d.	n.d.	0	35				
17	m	44	14	6	0	0	6.6	181	25	0	0				
18	f	52	31	24	255	0	4.95	113	18	0	35	20	1750	x	
19	f	65	n.d.	3	0	0	n.d.	157	28	100	35	15			Oxi
20	m	61	5	1	0	0	2.81	n.d.	n.d.	n.d.	70				Eta
21	f	66	40	13	39	560	n.d.	116	20	0	35				Lef
22	f	51	7	1	45	729	1.54	109	20	0	0				Lef/Eta
23	f	43	27	2	140	439	n.d.	89	16	0	7				
24	f	48	n.d.	12	177	1600	n.d.	n.d.	n.d.	100	0				Eta
25	f	79	14	1	0	1600	n.d.	100	25	n.d.	0				
26	f	82	33	5	52	175	n.d.	n.d.	n.d.	n.d.	28	10			Rem
27	f	59	n.d.	8	0	0	4.26	n.d.	n.d.	n.d.	35				
28	f	64	21	8	0	365	4.46	125	26	100	35	16		x	
29	f	63	n.d.	5.2	34	0	n.d.	133	24	0	0	25			
30	m	34	8	1	423	3200	4.76	n.d.	n.d.	n.d.	0				
31	f	54	n.d.	87	0	0	6.16	n.d.	n.d.	100	52.5	20		x	
32	m	64	n.d.	11	920	1607	3.7	n.d.	n.d.	n.d.	35				

ACPA: Anti-citrullinated protein/peptide antibodies; ANA: anti-nuclear antibodies (normal < 1:100); C3: complement factor 3 (normal 79-152 mg/dl); C4: complement factor 4 (normal 16-47 mg/dl); Cic: ciclosporin; CRP: C-reactive protein (normal < 0.8mg/dl); DAS 28: disease activity score based on 28 previously defined joints; Eta: etacemept; ESR: erythrocyte sedimentation rate; Lef: leflunomid; n.d. = not determined; NSAID = non-steroidal anti-inflammatory drugs; Oxi: oxicodon; Rem: remicade; RF: rheumatoid factor.

Supplementary Table S2. Pilot study: Clinical and serological characteristics and medication of the patients with SLE.

No	sex	Age	ESR 1h	CRP (mg/L)	Anti-CL (U/ml)	anti-β2gp-1 (U/ml)	Lupus anticoagulant	C3 (mg/dl)	C4 (mg/dl)	ANA-Titer (1/xxx)	DNA-Ab (U/ml)	treatment					Other
												Prednison (mg/week)	Methotrexat (mg/week)	Chloroquin (mg/week)	Azathioprin (mg/week)	NSAID	
1	f	24	65	1	1,9	0.5	neg	37	10	10000	444	420				x	
2	m	64	n.d.	46	7,7	n.d.	n.d.	36	5	1000	966	105		1400	700	x	
3	f	30	n.d.	39	19.8	4.9	pos	46	2,9	1000	12,3	100	15				
4	m	77	5	1	14.7	0	neg	95	10	0	0	54			1050		
5	f	71	11	1	5	n.d.	neg	131	23.8	80	9.1	35					
6	f	52	14	1	14.6	n.d.	pos	79	6.3	0	20.6	35		1750	1050		
7	f	43	22	1	19	n.d.	pos	118	16	0	23	140					
8	f	51	30	1	17.3	1.3	pos	99	13	40	47.6	52.5	7.5				
9	f	52	17	1	100	n.d.	pos	59	12.7	0	9.9	70					
10	f	52	7	1	7.2	n.d.	n.d.	72	4.1	0	0	0	15	1750			
11	f	33	n.d.	8	58	n.d.	neg	82	11	0	0	63				x	
12	f	50	10	1	9	0	n.d.	98	12	0	3.6	35		1750			
13	f	48	40	1	9	4.8	neg	65	16.8	0	104	35					
14	f	50	23	1	20.1	n.d.	neg	86	11	160	3.2	0	10	1750			
15	f	39	22	1	n.d.	n.d.	n.d.	82	11	40	0	140		1750	1050		
16	m	69	6	1	100	1.2	pos	111	18	0	35	0					
17	f	70	2	1	34.2	1.3	neg	119	26.5	0	9.5	35					
18	f	77	37	1	14.2	1.3	pos	76	16.1	0	15.3	17.5		1750		x	
19	f	52	8	1	22.3	0.1	n.d.	105	16.9	0	0	21					
20	f	75	40	6	34.8	0.3	neg	n.d.	n.d.	0	0	35					
21	f	72	12	13	100	2.3	neg	110	31	80	4.3	0			350		
22	f	63	9	1	n.d.	n.d.	neg	87	17.7	0	0	210			1050		
23	f	56	70	21	12	10.3	neg	90	17.4	80	8.2	35			350		
24	f	40	10	1	56.4	0.9	pos	50	7.85	0	12.1	70			700		
25	f	46	2		23.2	n.d.	pos	n.d.	n.d.	160	n.d.	0					
26	f	41	6	5	13.1	0	neg	114	23.4	0	0	0					
27	m	65	40	14	11.4	1.9	neg	78	2	20	0.1	70	20	1400		x	
28	f	67	39	14	22	0.3	pos	49	10	160	336	210			1050		
29	f	56	18	9	42.8	1.9	pos	87	8.4	80	2.9	56			700		
30	m	56	10	5	39.4	11.6	pos	102	25.5	20	28.4	17.5					
31	f	52	52		13.9	n.d.	pos	75	7.4	40	69	70	7.5				
32	f	58	2	4	20.7	5	neg	60	12.2	0	3.7	0	15			x	
33	m	48	51	10	26.8	0.1	neg	95	24.2	40	487	56				x	Lef
34	f	58	23	85	7.9	0.1	n.d.	95	13.2	40	23	70		2100			
35	f	33	4	2	5.6	0.1	neg	95.2	15.2	40	8	49		1750		x	
36	f	39	53	10	28.6	5.7	pos	64	13	80	88	35			350		
37	f	33	4	2	5.6	0.1	neg	95.2	15.2	40	8	49		1750		x	
38	f	48	10	1	n.d.	n.d.	n.d.	47	10	80	29	52.5			875		
39	f	71	n.d.	4	n.d.	n.d.	n.d.	86	16	320	4.8	70		700			
40	f	50	8	1	n.d.	n.d.	n.d.	107	12.4	40	11.7	0	10	1750		x	

ANA: anti-nuclear antibodies (normal < 1:100); anti-CL: anti-cardiolipin autoantibodies; anti-dsDNA: anti-double-stranded DNA autoantibodies (normal < 7 U/ml); C3: complement factor 3 (normal 79-152 mg/dl); C4: complement factor 4 (normal 16-47 mg/dl); CRP: C reactive protein (normal < 0.8mg/dl); ESR: erythrocyte sedimentation rate; Lef: leflunomid n.d. = not determined; NSAID = non-steroidal anti-inflammatory drugs.

Supplementary Figure 1. Pilot study: In patients with SLE the occurrence of anti-galectins mutually correlates. Exemplarily, correlations for anti-galectin-8 and -9; anti-galectins-3 and -4 and anti-galectins-4 and-8 are shown. o.d.: optical density.

