Immunity against post-translationally modified proteins in autoimmune diseases

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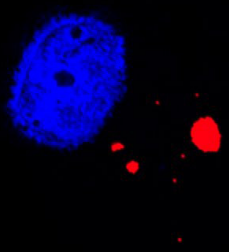

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Autoantibodies against specific post-translationally modified proteins are present in patients with lupus and associate with major neuropsychiatric manifestations

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Key messages

What is already known?

- Post-translationally modified (PTM) proteins and anti-PTM antibodies are described in many diseases, such as rheumatoid arthritis, in which anti-PTM antibodies are associated with disease progression;

What does this study add?

- We demonstrate the presence of several anti-PTM antibodies (anti-MAA, anti-AGE and anti-CarP) in patients with systemic lupus erythematosus (SLE) and their association with different aspects of disease activity in SLE and neuropsychiatric SLE (NPSLE);

How might this impact on clinical practice or future developments?

- As virtually no biomarker exists for NPSLE, anti-PTM Abs are a potential candidate. Future studies should further establish the potential role of anti-PTM antibodies in NPSLE.

Abstract

Background

Although autoantibodies are an important hallmark of systemic lupus erythematosus (SLE), most are not specific for SLE or any of its clinical manifestations. Autoantibodies against post-translationally modified (PTM) proteins have been studied extensively in rheumatoid arthritis and associate with disease progression. While PTMs have also been detected in SLE patients, studies on anti-PTM antibodies remain scarce. We studied the presence of anti-PTM antibodies in SLE and neuropsychiatric SLE (NPSLE), a manifestation that lacks serological markers.

Methods

IgG antibody responses against six PTMs (malondialdehyde-acetaldehyde adducts (MAA), advanced glycation end-products (AGE), carbamylation (CarP), citrullination (Cit), acetylation (AL) and nitration (NT)) were tested using ELISA in sera of 349 SLE patients (mean age 44 ± 13 years; 87% female) and compared to 108 healthy controls. Levels and positivity were correlated with clinical features and SLE manifestations.

Results

Anti-MAA, -AGE and -CarP antibodies were more prevalent in SLE compared to controls (MAA: 29 vs 3%, AGE: 18 vs 4%, CarP: 14 vs 5%, all $p \leq 0.0001$). Anti-MAA and anti-AGE antibodies correlated with clinical manifestations and serological inflammatory markers. Patients with major NPSLE showed higher positivity of anti-MAA (39 vs 24%, $p=0.01$) and anti-CarP antibodies (20 vs 11%, $p=0.04$) than patients without major NPSLE. In addition, anti-PTM Abs levels correlated with brain volumes, an objective measure of nervous system involvement.

Conclusions

In our NPSLE cohort, a subset of SLE patients have anti-PTM antibodies against MAA, AGE and CarP modified proteins. Interestingly, anti-MAA and anti-CarP were more prevalent in NPSLE, a manifestation for which no biomarkers exist.

Introduction

Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disease characterized by a global loss of self-tolerance. Although autoantibodies are an important hallmark of SLE, many autoantibodies are not specific for SLE or specific SLE manifestations, such as neuropsychiatric involvement (NPSLE) (1).

Many different types of biomarkers exist and may be used in different contexts for diagnostic, prognostic and predictive purposes. In rheumatoid arthritis (RA), the identification of anti-citrullinated protein antibodies (ACPA), antibodies directed against a post-translational modification (PTM), has facilitated the diagnostic process and created new insights in its pathophysiology (2,3). Noteworthy, the presence of specific anti-PTM antibodies (Abs) also facilitate discrimination between phenotypes within RA, as they associate with more severe RA (4-7). It is possible that antibodies against PTMs may also contribute to the identification of specific phenotypes in patients with SLE.

PTMs can occur naturally, as part of physiological functions, or may be the result of enzymatic or chemical processes (8,9). SLE has been associated with a dysregulated metabolic state and elevated levels of reactive oxygen species (10), which enhances the formation of PTMs. In some situations, immune responses against PTMs can develop, leading to anti-PTM Abs (11). To date, anti-PTM Ab studies in SLE have mainly focused on ACPA and anti-carbamylated protein Abs (anti-CarP), which associated with increased joint damage (12-15). In addition, several anti-PTM Abs have been associated with general disease activity (SLE disease activity index (SLEDAI)) in lupus (16-18). Phospholipid B2-glycoproteine-1 is reported to be modified by PTMs making it more antigenic (19). Around 35% of all SLE patients are positive for antibodies against these phospholipids (aPL), which are associated with antiphospholipid antibody syndrome (APS) (20). Overall, studies on anti-PTM Abs in SLE patients remain limited and techniques to measure anti-PTM Abs vary greatly (17,18)

Based on previous studies, we hypothesized that SLE activity can lead to the generation of PTMs on relevant antigens and that there is specificity in breaking tolerance towards these neoantigens. In this study, we focused on IgG antibodies against six different PTMs, selected based on their association with activity in other diseases and variation in location in the protein, configuration and reversibility. We aimed to firstly study the presence of these six anti-PTM Abs in patients with SLE using a standardized method to assess specific anti-PTM Ab reactivities. Secondly, we aimed to assess the association between anti-PTM Abs and clinical phenotypes of SLE, in particular NPSLE, for which virtually no biomarkers exist. Additionally, both the subjective clinical diagnosis of NPSLE and objective evidence of nervous system involvement, namely radiological measurements, are assessed.

Methods

Study design and population

Patients visiting the NPSLE clinic of the LUMC between 2007-2019 with the clinical diagnosis of SLE and signed informed consent were included in this study. The NPSLE clinic is a tertiary referral center in which patients with neuropsychiatric (NP) symptoms, potentially caused by SLE, are assessed multidisciplinary. This evaluation process has been described in detail previously (21,22). In short, NP symptoms attributed to SLE by multidisciplinary consensus requiring immunosuppressive or anticoagulant treatment are classified as 'major NPSLE'. NP symptoms not attributed to SLE, mild NP symptoms that do not require additional treatment other than symptomatic treatment or NP symptoms due to other causes, are classified as minor/non-NPSLE. Patients with major NPSLE are further classified as having an ischemic, inflammatory or combined (both ischemic and inflammatory) phenotype, based on the suspected pathogenetic mechanism (21). Major NPSLE diagnoses are classified according to the 1999 ACR case definitions for NPSLE syndromes (23). Approval for this study was obtained (Leiden-the Hague-Delft ethical committee, *B18.040). Supplementary Figure 1* depicts the inclusion procedure.

Patient and Public Involvement statement

No patients were involved in the concept or design of this study.

Patient characteristics

Demographic and clinical patient characteristics were collected from electronical medical files of the visit to the NPSLE clinic: age, sex, smoking status, BMI, 1997 SLE classification criteria (24), SLE duration, SLEDAI-2K (25), SLICC/ACR Damage Index (SDI) (24), presence and phenotype of NPSLE and medication use. Current renal involvement was defined as hematuria, proteinuria, urinary casts or pyuria as according to the SLEDAI-2K (25). The presence of active arthritis was established during physical assessment. Serum samples from each participant were collected at time of visit to the NPSLE clinic. In some patients with inflammatory NPSLE, immunosuppressive treatment was already initiated at this timepoint (median treatment duration: 1 month). Details of routine laboratory assessment are provided in *Supplementary files Part II.*

Assessment of anti-PTMs

Generation of antigens

Modified proteins and their corresponding control non-modified protein were produced by either enzymatic or chemical reactions as previously described with some adaptations (6, 26-29). For more details, see *Supplementary files Part II.*

Detection of anti-PTM IgG antibodies by ELISA

Modified Fetal Calf Serum (FCS) and non-modified FCS were coated at 10µg/mL in 0.1M carbonate-bicarbonate buffer pH 9.6 (CB) on Nunc Maxisorp plates (430341, Thermofisher) overnight at 4°C. All sequential incubation steps were done at 4°C, blocking for 6 hours, serum incubation overnight and detection antibody for 3.5 hours. In between each step plates were washed with PBS/0.05% Tween (P1379, Sigma). After washing, plates were blocked using PBS/1%BSA for 6 hours at 4°C. Following washing, wells were incubated with serum at a 1/50 dilution in PBS/0.05%Tween/1%BSA (PTB) for CarP, Cit, AL and NT and at a 1/100 or 1/1000 dilution in PTB for AGE and MAA respectively. For each PTM, a standard of a pool of anti-PTM positive sera was taken along in serial dilutions on each plate. Human IgG was detected using rabbit anti-human IgG-HRP (P0214, Dako) diluted in PTB and incubated at 4°C for 3.5 hours. After the final wash, HRP enzyme activity was visualized using ABTS (A1888, Merck) with 0.05% H202 (107209, Merck) and absorbance at 415 nm was read using a microplate reader (Bio-Rad). Serum samples of 108 healthy controls from the Leiden area were also tested.[30 31] Absorbance was transformed to arbitrary units per milliliter (aU/mL) using a corresponding standard line for each PTM. Background aU/mL of FCS was subtracted from the aU/mL signal on FCS-PTM to analyze specific anti-PTM reactivity. Negative outcomes were changed to 0. Positivity for specific anti-PTM Abs was defined as a value larger than the mean plus two times the standard deviation (SD) in the healthy controls (HC). HCs with a value ≥10x the mean were excluded in calculating the cut-off. Additionally, serum of 54 NPSLE patients at their second visit (<2 years after 1st visit) were analyzed for anti-PTM antibody reactivity.

Brain volume

Brain volume measurements were available for 182 patients visiting the NPSLE clinic between 2007-2015. An extensive description thereof has been published previously (32,33). In short, white matter volume, grey matter volume, white matter hyperintensity volume and total brain volume were assessed using the CAT12 toolbox from the statistical parametric mapping software and the Lesion Segmentation Toolbox version 2.0.15 (34).

Statistical analysis

Differences in levels of anti-PTM Abs between HC, SLE patients and specific SLE manifestations were assessed using the Mann-Whitney test and Chi-Square test. Median, median difference and 95% confidence intervals (CI) were calculated using quantile regression. Further analyses were only performed if anti-PTM Ab positivity between HC and SLE differed by at least a factor of two. Spearman rank analyses were used to assess correlation between anti-PTM Ab levels and all continuous clinical variables (including brain volumes). Point-biserial correlations were used to assess correlation between the level of anti-PTM Abs (continuous) and other antibodies (dichotomous). As anti-PTM Ab level was non-normally distributed, the levels were natural log transformed for the point-biserial

correlations. Kruskal-Wallis test was used to compare level of anti-PTM Abs in patients with different NPSLE phenotypes. Wilcoxon signed-rank test was used to compare anti-PTM Ab level at baseline vs follow-up. P-values ≤0.05 were considered significant.
All statistical analyses were performed using STATA statistical software version 16.

Results

Study cohort

349 SLE patients were included in this study: 87% female and mean age 43.7 ± 13.4 years. At time of enrollment, median disease duration was four years [interquartile range (IQR): 1 – 13] and median disease activity (SLEDAI-2K) was four [IQR: 2 – 8] (*Table 1A)*. The most common ACR 1997 criteria were ANA positivity ever (97%), immunologic disorder (76%) and non-erosive arthritis (59%). 104 patients were diagnosed with major NPSLE (30%), of which 51 patients had an inflammatory, 28 patients an ischemic and 25 patients a combined phenotype. NPSLE syndromes (1999 ACR case definitions) are presented in *Supplementary Table 1*. Active nephritis and arthritis were present in 85 and 17 patients respectively. Most patients were ANA positive at inclusion (89%) and complement consumption was present in 34% of patients (*Table 1B)*.

Anti-MAA, anti-AGE and anti-CarP levels and positivity differ between HC and lupus patients

IgG antibody levels against six PTMs (MAA, AGE, CarP, Cit, AL and NT) from serum of all SLE patients were compared to serum of 108 HCs (*Table 2 and Supplementary Figure 2A-F*). Median difference (95% CI) between antibody levels in SLE patients vs HCs were 12 (95% CI: 7; 18) for anti-MAA, 32 (3; 60) for anti-AGE, 91 (60; 123) for anti-CarP, 0 (-1; 1) for anti-Cit, 4 (-2; 9) for anti-AL and 33 (-1; 67) for anti-NT.

Cut-off for anti-PTM positivity was defined as values larger than 2 times standard deviation above the mean of HCs. Anti-MAA, -AGE and -CarP showed significant higher positivity in SLE patients compared to HCs *(Table 2*).

Anti-MAA and anti-AGE correlate with measures of systemic inflammation

Next, we sought to investigate whether these increased anti-PTM Abs correlated with clinical and serological markers (*Figure 1A-C* and *Supplementary Table 2*). Anti-MAA and anti-AGE both negatively correlated with complement factors C3 and C4 ($p \leq 0.002$) and correlated positively with ESR ($p < 0.001$), ANA ($p = 0.02/0.03$), anti-dsDNA ($p \leq 0.005$) and anti-SM ($p = 0.02$). Anti-MAA negatively correlated with disease duration ($p = 0.03$) and showed positive correlations with disease activity ($p = 0.03$) and anticardiolipin ($p = 0.04$). Compared to anti-MAA, anti-AGE correlated slightly stronger with disease activity ($p = 0.001$). All correla-

tions found were modest (correlation coefficients ≤0.30). Anti-CarP only correlated significantly with age (p = 0.01). There was no significant difference in anti-PTM antibody levels between patients with and without immunosuppressive treatment.

Table 1A: Characteristics of study population with systemic lupus erythematosus at time of inclusion

Patient characteristics	**SLE (n = 349)**
Female	303 (87)
Age (years)	43.7 ± 13.4
Duration of SLE (years)	4 [1 – 13]
SLEDAI-2K	4 [2 – 8]
SDI	1 [0 – 2]
BMI	24.9 ± 5.1
Current Smoking	99 (28)
Comorbidities	
Hypertension	120 (35)
Diabetes	17 (4.9)
ACR 1997 criteria for SLE	
Malar rash	135 (39)
Discoid rash	65 (19)
Photosensitivity	179 (51)
Oral ulcers	149 (43)
Non-erosive arthritis	206 (59)
Pleuritis or pericarditis	90 (26)
Renal disorder (ever)	94 (27)
Neurologic disorder (psychosis/epilepsy)	43 (12)
Hematologic disorder	175 (50)
Immunologic disorder	265 (76)
Positive ANA	340 (97)
Current immunosuppressive medication	
Hydroxychloroquine	226 (65)
Prednisolone	187 (54)
Azathioprine	55 (16)
Methotrexate	22 (6)
Belimumab	2 (1)
Other*	11 (3)
Current organ involvement	
Major NPSLE	
Inflammatory	51 (15)
Ischemic	28 (8)
Combined	25 (7)
Renal involvement	67 (19)
Arthritis	17 (5)

Results are presented as n (%), mean ± sd or median [interquartile range; IQR]
*Other: includes cyclophosphamide (n = 9), tacrolimus (n = 2)
ANA, anti-nuclear antibodies; SDI, SLICC/ACR damage index; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index.

Table 1B: Routine laboratory assessment of study population with systemic lupus erythematosus at time of inclusion*

	SLE (n = 349)
Nuclear antibodies	
ANA	309 (89)
Anti-dsDNA	91 (26)
Anti-ENA	166 (48)
Anti-SSA	131 (38)
Anti-SSB	39 (11)
Anti-SM	24 (7)
Anti-RNP	46 (13)
Antiphospholipid antibodies	
Lupus anticoagulant	101 (29)
Anti-cardiolipin IgG	46 (13)
β2-glycoprotein IgG	40 (15)
Complement factors	
Low C1q	42 (12)
Low C3	119 (34)
Low C4	86 (25)
Inflammation	
CRP	0.8 [0.8 - 7]
ESR	17 [9 - 39]

Results are presented as n (%) or median [interquartile range; IQR]
* Missing data nuclear antibodies: ANA + anti-dsDNA: n = 1, other: n = 2; antiphospholipid antibodies: β2-glycoprotein n = 79, LAC n = 5, aCl = 1. Complement factors: n = 2, Inflammation: n = 2. Percentages are given for the number of positive patients divided by the number of patients tested
ANA, anti-nuclear antibodies; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ENA, extractable nuclear antigen antibodies; SLE, systemic lupus erythematosus.

Table 2: Prevalence of antibodies against specific post-translational modifications in patients with systemic lupus erythematosus (n = 349) and healthy controls (n = 108)

	SLE (n = 349)		Healthy controls (n = 108)		
	aU/mL	***n, % positive***	**aU/mL**	***n, % positive***	**Median difference (95% CI)**
Anti-MAA	35 [23 – 52]*	101 (29)	23 [18-29]	3 (3)	12 (7;18)
Anti-AGE	112 [51 – 200]*	63 (18)	80 [41 – 122]	4 (4)	32 (3; 60)
Anti-CarP	126 [50– 206]*	49 (14)	35 [0 – 11]	5 (5)	91 (60; 123)
Anti-Cit	3 [2 – 6]	22 (6)	3 [2 – 6]	3 (3)	0 (-1; 1)
Anti-AL	8 [0 – 23]	29 (8)	4 [0 – 19]	8 (7)	4 (-2; 9)
Anti-NT	44 [0 - 177]	17 (5)	11 [0 – 132]	8 (7)	33 (-1; 67)

Results are presented as n (%) or median [IQR].
* Statistically significant difference between SLE patients and heathy controls ($p \leq 0.0001$)
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

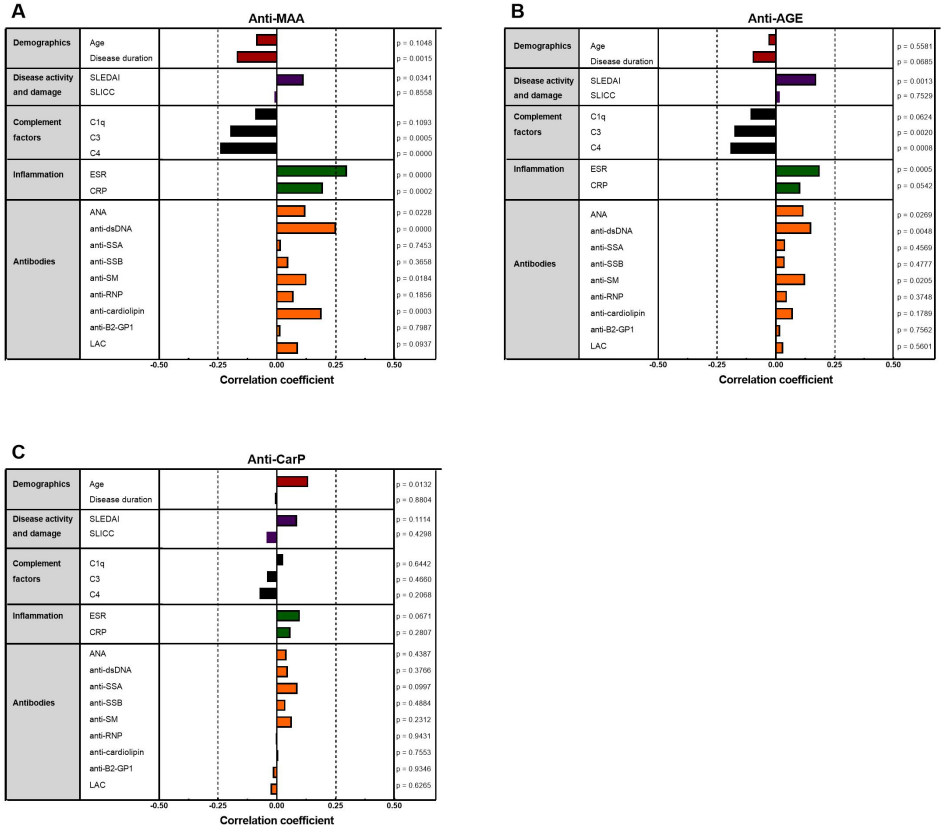

Figure 1: Correlation between (A) anti-MAA IgG (B) anti-AGE IgG and (C) anti-CarP IgG and clinical and laboratory markers in patients with systemic lupus erythematosus (n = 349). Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies). AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.

Anti-MAA and anti-CarP are more common in major NPSLE than in other SLE organ manifestations

Levels and positivity for different anti-PTM Abs were compared between patients with and without specific SLE manifestations (*Table 3* and *Figure 2A-C*). As our cohort comprises patients visiting an expertise center for NPSLE, we primarily focused on this manifestation. Patients with major NPSLE more frequently harbored anti-MAA Abs (39 vs 24%, p = 0.01) and anti-CarP Abs (20 vs 11%, p = 0.04) compared to patients without major NPSLE, whereas the prevalence of anti-AGE Abs did not differ as clearly (23 vs 16%, p =

0.13). In our cohort, for patients with active nephritis or active arthritis, differences in anti-PTM Ab positivity were less pronounced. Associations between the different anti-PTM Abs and ever having major organ manifestations is presented in *Supplementary Table 3*.

Table 3: The association between anti-PTMs (IgG) antibodies and specific organ manifestations of systemic lupus erythematosus (n = 349)

	NPSLE		**Active nephritis**		**Arthritis**	
	Yes *n* = 104	No *n* = 245	Yes n = 85	No *n* = 264	Yes n = *17*	No *n* =332
Anti-MAA						
aU/mL	41 [24 – 61]	34 [23 – 48]	36 [22 – 52]	35 [23 – 52]	30 [24 - 71]	35 [22 - 51]
Positive	**41 (39)***	60 (24)	24 (28)	77 (29)	6 (35)	95 (29)
Anti-AGE						
aU/mL	134 [48 - 217]	103 [52 –188]	124 [70 - 220]	107 [50 - 187]	115 [70 - 325]	112 [50 – 198]
Positive	24 (23)	39 (16)	20 (23)	43(16)	6 (35)	57 (17)
Anti-CarP						
aU/mL	133 [52– 245]	123 [50– 203]	139 [55 - 261]	121 [50 - 204]	157 [65 - 258]	126 [50 - 206]
Positive	**21 (20)****	28 (11)	16 (19)	33 (13)	3 (18)	46 (14)

Results are presented as n (%) or median [IQR].
*Chi-Square tests were used to assess the difference between the presence and absence of the specific manifestations. NPSLE yes vs no: *p = 0.01 ** p = 0.04, other values were not significant.
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

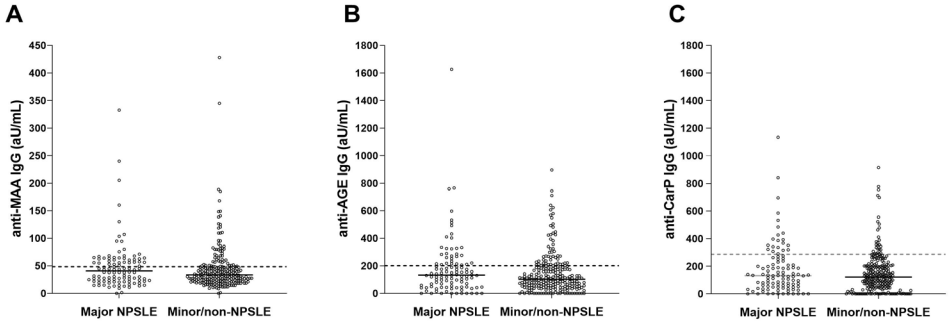

Figure 2: Levels of (A) anti-MAA, (B) anti-AGE and (C) anti-CarP IgG in patients with (n = 104) and without (n = 245) major NSPLE. Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls (dashed line), as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). AGE, advanced glycation end-product; CarP, carbamylated protein; NSPLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct.

Anti-PTM Abs are similarly present in different major NPSLE phenotypes

As NPSLE has different pathophysiological origins, levels and positivity for different anti-PTM Abs were compared within specific phenotypes of major NPSLE (*see Supplementary Table 4*). Patients with a combined NPSLE phenotype showed the most anti-PTM Ab positivity (anti-MAA = 40%, anti-AGE = 28%, anti-CarP = 32%), followed by patients with an inflammatory phenotype (41, 27 and 14% respectively) and an ischemic phenotype (36, 11 and 21% respectively). These differences were not statistically significant. In addition, no difference was observed in the presence of anti-PTM Abs between inflammatory NPSLE patients that did and did not initiate immunosuppressive treatment prior to the clinic visit.

Brain volume and anti-PTM Abs

Previous analyses were based on the distinction between major and minor NP involvement, of which the diagnosis was based on multidisciplinary assessment. As this has the risk for phenotypic misclassification, we sought to study the correlation between anti-PTM Abs and an objective marker of central nervous system involvement in SLE. For 182 patients (52%), assessment of brain volumes was available (*see Supplementary Table 5*). The strongest correlations were between anti-MAA and white matter volume (WMV) and total brain volume (TBV) (Spearman ρ: –0.20 and –0.18; both $p < 0.02$) and anti-AGE and WMV and TBV (Spearman ρ: –0.16 and –0.15; both $p = 0.03$). Anti-CarP showed a significant association with white matter hyperintensity volume (Spearman ρ: 0.19, $p = 0.03$)

Longitudinal study comparing anti-PTM Ab responses overtime

In order to study anti-PTM Abs over time, serum samples from 54 patients, that were taken within 2 years after the first visit, were analyzed. Levels of anti-MAA, -AGE and -CarP Abs generally showed a decrease (*Figure 3A-C*). This decrease was significant for anti-MAA and anti-AGE ($p \leq 0.0001$), but not for anti-CarP ($p = 0.20$). Change in anti-MAA and anti-AGE Ab levels associated with change in SLEDAI-2K (SR: 0.29 and 0.28 ($p = 0.04$) respectively, but change in anti-CarP Ab level did not (SR: 0.11, ($p = 0.41$)).

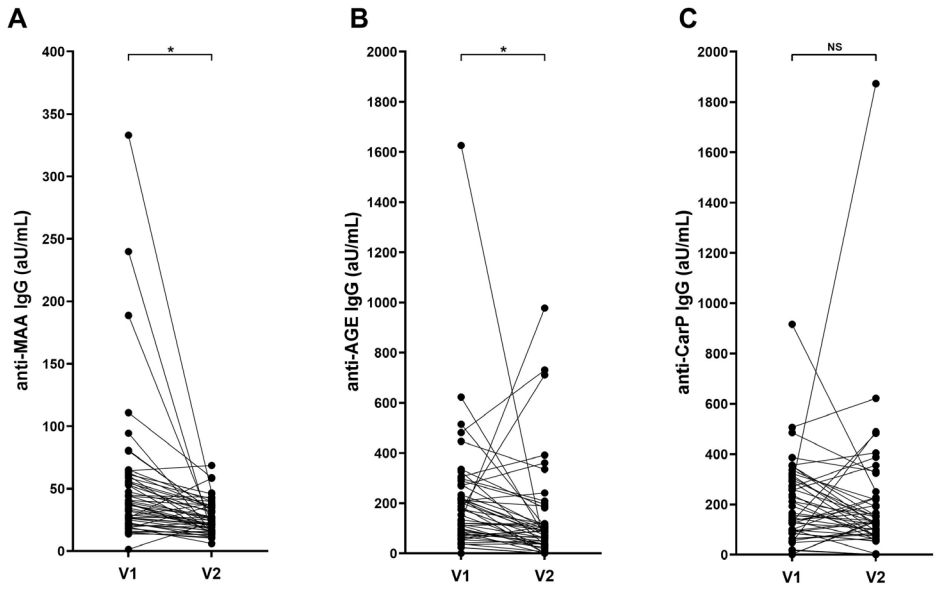

Figure 3: Levels of (A) anti-MAA, (B) anti-AGE and (C) anti-CarP in NPSLE patients (n = 54) overtime within 2 years after first visit. Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls, as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). *p <0.01, NS, not significant. AGE, advanced glycation end-product; CarP, carbamylated protein; NPSLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct; V1, first visit; V2; second visit.

Discussion

We hypothesized that in SLE there is generation of PTMs on relevant antigens and that the presence of anti-PTM autoantibodies may be associated with clinical presentation and/or disease activity. Therefore, we investigated the presence of antibodies against six different PTMs on the same antigen backbone (FCS). We indeed observed that breaking of tolerance in SLE results in production of antibodies against the PTMs, predominantly MAA, AGE and CarP, and less pronounced against Cit, AL and NT. Furthermore, anti-MAA Abs and anti-AGE Abs associated with markers of inflammation. Finally, we searched for antibodies specific for NPSLE and observed that anti-MAA and anti-CarP Abs associated with major NPSLE.

Many different PTMs occur in both health and disease. Carbamylation and citrullination have been identified in RA patients and antibody responses against these PTMs are nowadays used as a clinical measure (2,3). While it is currently unknown why a subset of the patients produce anti-PTM antibodies, there is substantial insight into the processes

that drive the PTM modification of proteins. Inflammation and oxidative stress can lead to formation of reactive oxygen species (ROS) or induction of enzymes, which may lead to the formation of PTMs. ROS can lead to malondialdehyde (MDA) following peroxidation of lipids from for instance cell walls leading to MAA modification (35). Additionally, the ROS peroxynitrite is the reactive compound for nitration (36). Citrullination is the Peptidyl Arginine Deiminase (PAD) enzyme mediated conversion of arginine (37). The conversion of lysine into homocitrulline is driven by a chemical reaction with cyanate, a compound in equilibrium with urea and induced by smoking and inflammation (38). Excessive glycation is also a response to oxidative stress and inflammation leading to AGEs. AGEs in turn bind to AGE receptor (RAGE) leading to the perpetuation of inflammation (39). Excessive acetylation is a result of dysregulation of acetylation and deacetylation pathways (40). Taken together specific PTMs are a consequence of inflammation and oxidative stress. It is therefore well possible that these modifications occur in SLE patients in which widespread inflammation is going on. We previously observed that the PTM carbamylation is present in the joint of RA patients, but also in the joint of healthy controls, while the anti-CarP Abs are only found in a subset of RA patients (41). Why only a subset of RA and SLE patients produce such anti-PTM antibodies is still unknown. In our study, we demonstrated that three anti-PTM Abs are more prevalent in patients with SLE than in HCs, in increasing prevalence: anti-CarP, anti-AGE and anti-MAA. As only three out of six tested anti-PTMs showed increased reactivity, a specific induction process is implied. MAA, AGE and CarP are all modifications that occur on the lysine residue. However, acetylation (AL) is also a modification of lysine residue, but no difference in reactivity between SLE patients and HCs was observed there. Whether a specific underlying pathological mechanism for reactivity against these three PTMs exists, needs to be further investigated. As SLE patients are known for their global loss of self-tolerance, it is plausible that PTMs, that are persistently or abundantly present, are targeted. However, other factors, such as genetics and environmental triggers might play a role in breaking tolerance towards PTMs (42,43). In order to understand specific anti-PTM reactivities, the location of specific PTMs in different organ tissues in SLE patients needs to be evaluated. Furthermore, studies on monoclonal antibodies obtained from SLE patients are required to further pinpoint specific reactivity.

The three notable anti-PTM antibody responses have been studied to some degree in SLE before. Anti-CarP Abs, prevalent in 8 – 53% of SLE patients in other studies, have been associated with articular involvement, joint damage and disease activity (SLEDAI) (12,15,16). Our study was unable to confirm these findings, possibly because of the low number of patients with arthritis in our cohort. We can however exclude that the observed association between the presence of certain anti-PTM Abs and major NPSLE is driven by the association between anti-PTM Abs and arthritis and the concurrent presence of arthritis and major NPSLE. An increase of anti-CarP Abs was observed in patients

with major NPSLE, but the clinical meaning of this remains to be elucidated as limited correlation with other clinical markers was observed. Anti-AGE Abs have not been studied previously in SLE. However, increased amounts of AGEs have been identified in skin tissue of SLE patients and increased AGEs in blood plasma are described, which correlated with disease activity (44). We demonstrated an association between anti-AGE Abs and different markers of systemic inflammation. In our study, anti-MAA associated most clearly with different markers of inflammation. Few previous studies have investigated the role of malondialdehyde (MDA), the unstable predecessor of MAA in SLE. One study demonstrated that anti-MDA IgG positively correlated with disease activity, ESR and CRP and negatively correlated with complement factors (18). This is in line with the results of our study. In this same study, an association with renal involvement was found, using a different definition for active nephritis (proteinuria > 5 g per day or greater than 3+ by dipstick, and/or cellular casts), whereas our study demonstrated an association with major NPSLE, a disease for which virtually no biomarker exists.

Several antibodies have been suggested as biomarker for NPSLE (45). In particular, anti-ribosomal P and anti-N-methyl-D-aspartate receptor antibodies which have been implicated in the pathogenesis of NPSLE, although their exact role remains inconclusive and their clinical value limited (46-48). It is thought that breaches of the neuroimmune interface (amongst others, the blood-brain barrier) might enable neuropathic antibodies in the serum of patients to enter the central nervous system (49). Interestingly, we demonstrated that several anti-PTM Abs are more prevalent in patients with major NPSLE. Discrimination between the presence and absence of NPSLE is important, as it influences treatment. The diagnosis is based on multiple clinical factors, and the presence or absence of anti-PTM Abs could give further direction. Additional studies need to clarify whether specific anti-PTM responses are specific enough for NPSLE or should be interpreted as part of a series of markers to point towards specific subgroups. The observed association between anti-PTM Abs and NPSLE is supported by the correlation between specific anti-PTM Abs and white matter and white matter hyperintensity brain volume, objective measures of CNS involvement linked to NPSLE. MAA has previously been linked to brain injury and neurodegenerative diseases (50,51). Furthermore, in aging individuals, increased levels of MDA are seen in the temporal lobes, occipital lobes and hippocampus, underlying the potential relevance of anti-MAA Abs (52). We demonstrated that all three anti-PTM Abs (anti-MAA, -AGE and -CarP) showed a decrease in reactivity over time, of which anti-MAA and anti-AGE correlated with disease activity. To further uncover the role of anti-PTM Abs in NPSLE, future studies should assess the presence of anti-PTMs in cerebrospinal fluid.

Our study has several strengths: we used a standardized controlled ELISA set-up with one antigen backbone for each of the PTMs and a well-defined cohort of patients with

SLE and specifically NPSLE.

There are also several limitations to our study. A relatively large number of patients were negative or weakly positive for ANA at inclusion, which was tested at a dilution of 1:40. Therefore, we repeated the analyses in the patients positive for ANA (see *Supplementary files part III*), which led to similar results as the main analyses. Furthermore, as this study cohort is part of a tertiary referral for NPSLE, other clinical subsets (such as arthritis) are less prevalent. In this exploratory study within a well-defined cohort we found anti-PTM antibodies as potential biomarker for NPSLE and now additional studies need to be performed to determine the discriminative value of anti-PTM in different clinical settings, such as the outpatient clinic of a non-academic hospital. In addition, the clinical correlations identified in this study were modest and need further investigation. Lastly, the diagnosis of major NPSLE is made on clinical grounds. Although this is a clinically relevant phenotype, there still might be different underlying biological processes. Therefore, we used an objective marker (brain volumes) for central nervous system involvement and found an association with anti-PTM antibodies.

In conclusion, we identified three anti-PTM Abs (anti-MAA, -AGE and -CarP) that are present more frequently in patients with SLE, of which anti-MAA and anti-AGE correlate with measurements of systemic inflammation. Furthermore, several anti-PTMs Abs (anti-MAA and -CarP) were more prevalent in patients with major NPSLE, a disease manifestation currently lacking a suitable biomarker. In addition, all three anti-PTM Abs also correlated with brain volumes. Further research should confirm the role of anti-PTM Abs as well as its discriminative value for (NP)SLE.

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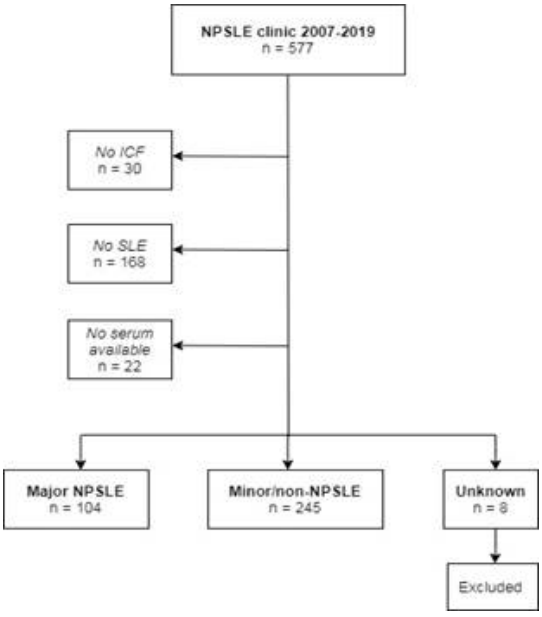
Supplementary files

Part I: Additional cohort information

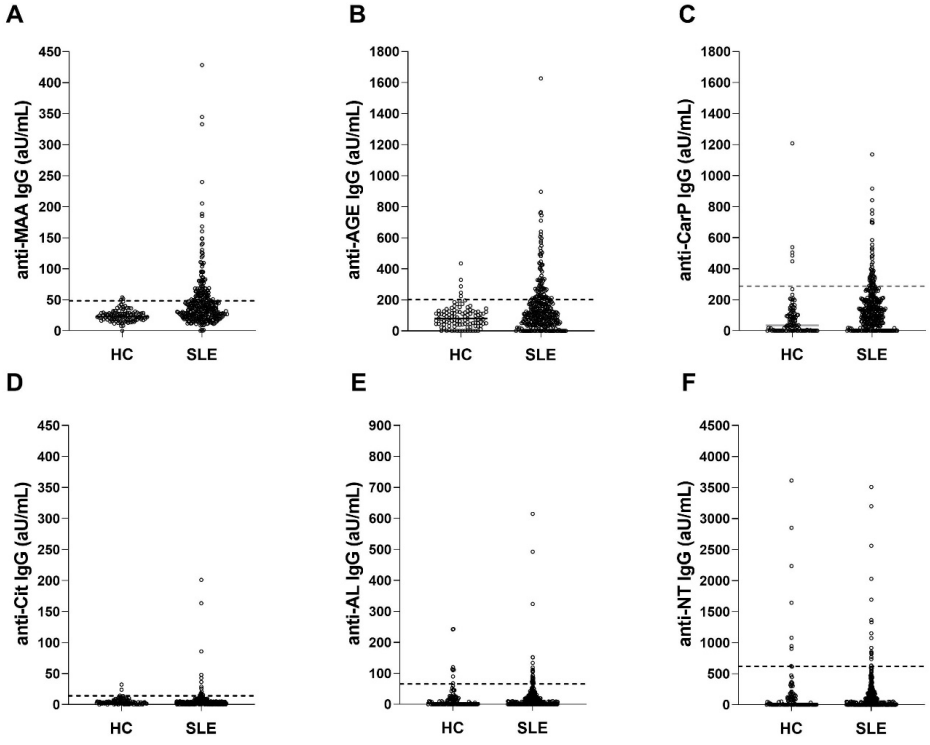


Supplementary Figure 1: Patient inclusion. The diagram illustrates the number of patients that were included and excluded from this study. The resulting number of patients include patients from whom SLE has been confirmed and required data was available. Namely, 104 patients with major NPSLE and 245 minor/non-NPSLE, making a total of 349 patients.
ICF = informed consent form; *NPSLE* = neuropsychiatric systemic lupus erythematosus

Supplementary Table 1: NPSLE syndromes (n = 176) according to 1999 ACR criteria of patients with major NPSLE (n = 104)

NPSLE syndrome (n, % of patients with major NPSLE)	
Aseptic meningitis	1 (1)
Cerebrovascular disease	45 (43)
Demyelinating syndrome	0 (0)
Headache	8 (8)
Movement disorder (chorea)	3 (1)
Myelopathy	9 (9)
Seizure disorders	9 (9
Acute confusional state	8 (8)
Anxiety disorder	1 (1)
Cognitive dysfunction	39 (38)
Mood disorder	13 (13)
Psychosis	7 (7)
AIDP[b]	0 (0)
Autonomic disorder	0 (0)
Mononeuropathy	0 (0)
Myasthenia gravis	0 (0)
Neuropathy, cranial	6 (2)
Plexopathy	0 (0)
Polyneuropathy	4 (4)
Other[c]	31 (30)

[a] Patients with neuropsychiatric symptoms attributed to SLE
[b] Acute inflammatory demyelinating polyneuropathy.
[c] Other NPSLE symptoms: cerebral vasculitis (n = 15), organic brain syndrome (n = 3), lethargia (n = 1) visual disturbance other than optic neuritis (n =2), apraxia (n =2), apathy (n = 2) walking disorder (n =2), motor disorder left arm (n =1), paresis left arm and dysarthria (n = 1), increased intracranial pressure (n =1), mononeuritis multiplex (n =1).



Supplementary Figure 2: Levels of different IgG antibodies against specific post-translational modifications (A) MAA, (B) AGE, (C) CarP, (D) Cit, (E) AL and (F) NT in healthy controls (HC) and patients with systemic lupus erythematosus (SLE). Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls (dashed line), as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

Supplementary Table 2: Details regarding correlation coefficients (including 95% confidence intervals) provided in Figure 2

	Anti-MAA		Anti-AGE		Anti-CarP	
		95% CI*	corr	95% CI	corr	95% CI
Demographics						
Age	-0.09	-0.19; 0.02	-0.02	-0.13; 0.09	0.13	0.03; 0.23
Disease duration	-0.17	-0.27; -0.07	-0.10	-0.20; 0.01	-0.02	-0.12; 0.09
Disease activity and damage						
SLEDAI	0.12	0.01; 0.22	0.18	0.08; 0.28	0.09	-0.02; 0.19
SDI	-0.02	-0.12; 0.09	0.02	-0.09; 0.12	0.05	-0.15; 0.06
Complement factors						
C1q	-0.08	-0.19; 0.02	-0.09	-0.19; 0.02	0.05	-0.06; 0.15
C3	-0.20	-0.30; -0.09	-0.17	-0.28; -0.06	-0.04	-0.15; 0.07
C4	-0.24	-0.35; -0.13	-0.19	-0.30; -0.08	-0.07	-0.18; 0.04
Inflammation						
ESR	0.30	0.20; 0.39	0.19	0.08; 0.28	0.10	0.00; 0.20
CRP	0.19	0.09; 0.30	0.10	0.00; 0.20	0.07	-0.04; 0.17
Antibodies						
ANA	0.12	0.02; 0.22	0.12	-0.01; 0.22	0.04	-0.06; 0.15
Anti-dsDNA	0.25	0.15; 0.35	0.15	0.05; 0.25	0.05	-0.06; 0.15
Anti-SSA	0.02	-0.09; 0.12	0.04	-0.07; 0.15	0.09	-0.02; 0.19
Anti-SSB	0.05	-0.09; 0.12	0.04	-0.07; 0.14	0.04	-0.07; 0.14
Anti-SM	0.13	-0.02; 0.23	0.12	0.02; 0.23	0.06	-0.04; 0.17
Anti-RNP	0.08	-0.03; 0.18	0.05	-0.06; 0.15	0.00	-0.11; 0.10
Anti-cardiolipin	0.19	0.09; 0.29	0.07	-0.03; 0.18	-0.02	-0.12; 0.09
Anti-B2-GP1	0.02	-0.10; 0.14	0.02	-0.10; 0.14	0.01	-0.11; 0.12
LAC	0.09	-0.02; 0.19	0.03	-0.07; 0.14	-0.03	-0.13; 0.08

All confidence intervals were calculated using Fisher's transformation

*Correlation between anti-MAA IgG, anti-AGE IgG and anti-CarP IgG and clinical and laboratory markers in patients with systemic lupus erythematosus. Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies).

AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.

Supplementary Table 3: The association between anti-PTMs (IgG) and major organ manifestations according to the 1997 ACR criteria for SLE[53]

	Renal disease		Arthritis		Serositis	
	Yes *n = 94*	No *n = 255*	Yes *n = 206*	No *n = 143*	Yes *n = 90*	No *n = 259*
Anti-MAA						
aU/mL	31 [23-45]	37 [23-56]	38 [24-56]	31 [21-50]	38 [24-56]	32 [22-51]
Positive	18 (19)	83 (33)	63 (31)	38 (27)	27 (30)	74 (29)
Anti-AGE						
aU/mL	104 [47-190]	115 [52-102]	112 [46-209]	114 [55-192]	128 [59-215]	110 [47-187]
Positive	14 (15)	49 (19)	22 (15)	41 (20)	20 (22)	43 (17)
Anti-CarP						
aU/mL	133 [70-186]	125 [44-212]	123 [50-204]	130 [50-212]	138 [68-209]	122 [50-205]
Positive	11 (12)	38 (15)	18 (13)	31 (15)	16 (18)	33 (13)

Results are presented as n (%) or median [IQR]. Levels are AU/mL
AGE, advanced glycation end-product; CarP, carbamylated protein; MAA, malondialdehyde-acetaldehyde adduct.

Supplementary Table 4: The association between anti-PTMs (IgG) and specific NPSLE phenotypes

	Major NPSLE		
	Ischemic *n = 28*	Inflammatory *n = 51*	Combined phenotype *n = 25*
Anti-MAA			
aU/mL	29 [20 – 64]	44 [27 – 57]	39 [24 – 65]
Positive	10 (36)	21 (41)	10 (40)
Anti-AGE			
aU/mL	111 [37 – 84]	154 [70 – 253]	125 [44 – 260]
Positive	3 (11)	14 (27)	7 (28)
Anti-CarP			
aU/mL	90 [41 – 245]	137 [55 – 209]	182 [54 – 323]
Positive	6 (21)	7 (14)	8 (32)

Results are presented as n (%) or median [IQR].
AGE, advanced glycation end-product; CarP, carbamylated protein; NPSLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct.

Supplementary Table 5: The association between anti-PTMs (IgG) and brain volumes (n = 182)

	Grey matter	White matter	Total	White matter hyperintensity
	r_s	r_s	r_s	r_s
Anti-MAA	-0.13 (-0.27; 0.01)	-0.20 (-0.34; -0.06)	-0.18 (-0.31; -0.03)	-0.06 (-0.20; 0.09)
Anti-AGE	-0.13 (-0.27; 0.02)	-0.16 (-0.30; -0.02)	-0.16 (-0.29; -0.01)	0.06 (-0.09; 0.20)
Anti-CarP	-0.13 (-0.27; 0.01)	-0.14 (-0.28; 0.01)	-0.14 (-0.28; 0.01)	0.19 (0.05; 0.33)

Results are presented as Spearman R correlation coefficients (95% CI), as calculated by Spearman rank-order correlation.
AGE, advanced glycation end-product; CarP, carbamylated protein; MAA, malondialdehyde-acetaldehyde adduct; $r_{s,}$ spearman rank coefficient.

Supplementary files

Part II: Methods

Routine laboratory assessment

IgG anti-dsDNA antibodies were detected using the *Crithidia luciliae* indirect immune fluorescence technique (Immuno Concepts, Sacramento, CA, USA). IgG antibodies against SS-A/Ro-52, SS-B/La, Sm, RNP and IgG and IgM anticardiolipin (aCL) and anti-β2 glycoprotein 1 (anti-β2GP1) antibodies were determined using a Phadia® 250 EliA fluorescence enzyme immunoassay (FEIA) (Thermo Scientific, Freiburg, Germany). Anti-β2GP1 (IgG/IgM) and IgG antibodies against SS-A, SS-B, SM and RNP were considered positive if levels were >10 U/ml. aCL (IgG/IgM) was considered positive if levels were >30 GPL/U/ml. Lupus anticoagulant (LAC) was determined using STA-Rack and STA Evolution coagulation analyzers (Stago, Parsippany, NJ, USA). ANA analysis was performed with an immunofluorescence assay test on Hep-2 cells using a dilution of 1:40. ANA was defined as positive according to the normal limits of the LUMC laboratory (positive, 74% or weakly positive, 15%).

C1q, C3 and C4 were measured in serum using laser nephelometry. Based on the normal limits for our laboratory, C1q, C3 and C4 were defined as low or normal/high.

Erythrocyte sedimentation rate (ESR) was measured using the Westergren-method (StaRRsed Compact). C-reactive protein (CRP) was measured with turbidimetric assays using Roche COBAS 8000 Modular - c702. CRP <3 was under the detection limit until 2017, thereafter high sensitivity CRP was reported (hsCRP). The values of 2017 with CRP < 3 (no exact measurement) were imputed with the median of CRP < 3 after 2017 (exact measurement, median 0.8, range 0.3 – 2.5).

Generation of antigens

For all modifications backbone Fetal Calf Serum (FCS, Bodinco, Alkmaar, the Netherlands) was used. For malondialdehyde acetaldehyde adducts (MAA), first an MDA solution was prepared using 0.5M 1,1,3,3,-Tetramethoxypropane (108383, Sigma) and 0.3% hydrochloric acid (1003171000, EMSURE) and incubated for 12 minutes at 37C in a water bath. [29] Meanwhile 20mg/mL protein was prepared and diluted 1:5 with 20% MilliQ, 20% freshly prepared MDA solution, and 4% acetaldehyde (402788, Sigma), pH was adjusted to pH=4.8 and MilliQ was added till 5 times volume of protein was reached. The solution was then incubated for 2hr at 37C in a water bath. Advanced glycation end-products (AGE) were created by incubating 2mg/mL protein with 1:100 diluted 3.3M glycolaldehyde (23147-58-

2, Sigma). [30] The solution was filtered using a 0.2um whatmann filter (10462200, GE Healthcare) and incubated for 10 days at 37C while shaking. For carbamylation (CarP) of proteins, first a 2M potassium cyanate (KOCN, 215074, Sigma-Aldrich) solution in PBS was prepared. [6] 4mg/mL protein backbone was incubated to a 1:1 volume-to-volume ratio and incubated for 12hr at 37C. Citrullinated (Cit) proteins were created by incubating 1mg protein in a volume of 1mL containing 0.1M TRIS-HCl pH 7.6, 5mM DL-DTT (D0632, Sigma), 1mg/mL protein, 1U Peptidyl Arginine Deiminase 4 enzyme/100uL (PAD enzyme, 1584, Sigma) and CaCl2 10mM (21097, Sigma), volume was supplemented with MilliQ. [27] The mixture was incubated for 6hr at 53C. After incubation citrullinated protein was spun down and stored at -80C. Acetylation (AL) was performed using protein at a concentration of 1mg/mL in 0.1M Na2CO3. 50uL of acetic anhydride (100042, Merck) and 200uL of pyridine (109728, Merck) was added per 10mL of protein solution. [28] Proteins were incubated for 5hr at 30C. After incubation, the reaction was stopped using 200uL (per 10mL solution) of 1M TRIS. Samples were concentrated using Amicon Ultra – 15 centrifugal filter unit (10kDa, UFC901024, Sigma) and buffer was exchanged to PBS using Zeba Spin Desalting columns (89890, Thermofisher). For nitration (NT) 6 equal shots of peroxynitrite (14042-01-4, Cayman Chem) were directly used from stock solution to create an end concentration of 6mM. [26] After every shot the mix was directly vortexed for 30 seconds and left on ice for one minute before adding the next shot. After the last shot the samples were incubated for 1hr at 37C. Following incubation, all modifications (except for Cit, which was spun down) and their non-modified counterpart were extensively dialyzed against PBS using 10kDa cut-off SnakeSkin dialyzing tube (88243, Thermofisher). All modifications were stored at -20C until used. Protein concentrations were measured using Bradford solution (500-0006, Biorad) or Nanodrop. Modifications were verified using an ELISA based assay using anti-PTM specific antibodies: goat anti-MAA-HRP, ab20703, abcam; mouse anti-AGE 4B5, kindly provided by prof. H de Boer; rabbit anti-CBL, STA-078, biolabs; mouse anti-citrulline F95, MABN328, MilliPore; rabbit anti-acetyllysine, ADI-KAP-TF120-E, Enzo Lifesciences; mouse anti-nitrotyrosine, ab61392, abcam.

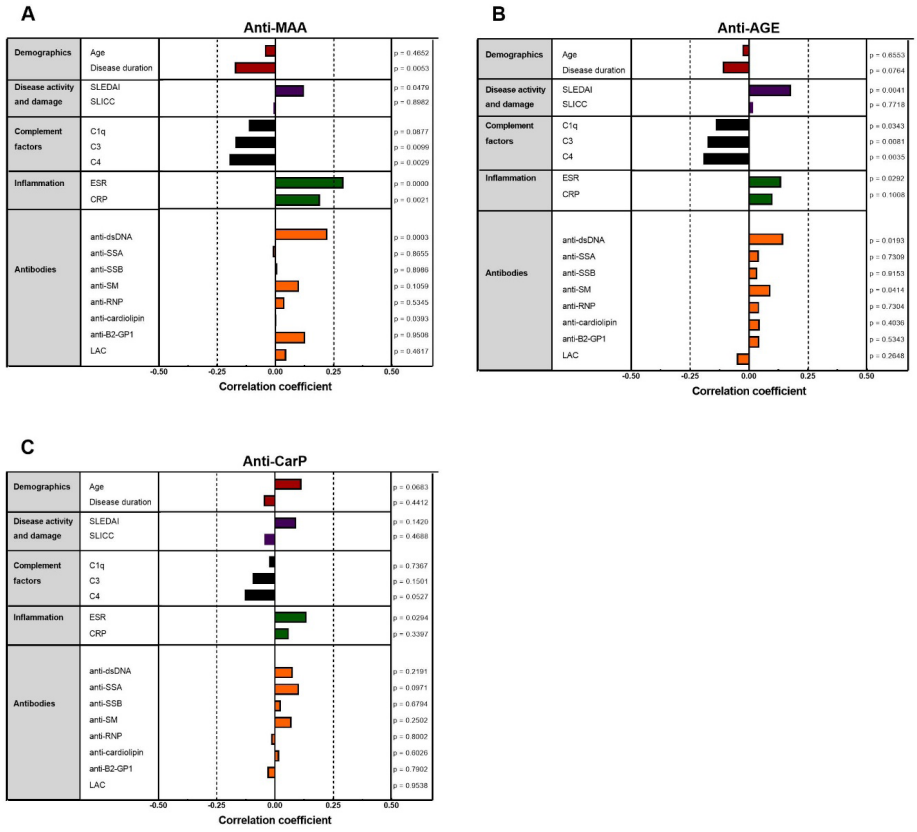
Supplementary files

Part III: Results repeated for patients with positive anti-nuclear antibodies (ANA) at time of visit

Supplementary Table 6: Prevalence of antibodies against specific post-translational modifications in healthy control and patients with systemic lupus erythematosus with positive ANA

	SLE patients (n = 258)		Healthy controls (n = 108)		Median difference (95% CI)
	aU/mL	*n,% positive*		*n,% positive*	
Anti-MAA	39 [1 - 57]*	91 (35)	23 [18 - 29]	3 (3)	16 (10; 22)
Anti-AGE	125 [56 – 215]*	56 (22)	80 [41 – 122]	4 (4)	45 (11; 79)
Anti-CarP	126 [50 – 220]*	41 (16)	35 [0 – 11]	5 (5)	91 (55; 128)
Anti-Cit	3 [2 – 6]	14 (5)	3 [2 – 6]	3 (3)	0 (-1; 1)
Anti-AL	8 [0 – 26]	23 (9)	4 [0 – 19]	8 (7)	4 (-3; 10)
Anti-NT	46 [0 – 180]	10 (4)	10 [0 – 132]	8 (7)	36 (0; 72)

Results are presented as n (%) or median [IQR]. * $p \leq 0.0001$
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.



Supplementary Figure 3: Correlation between (A) anti-MAA IgG (B) anti-AGE IgG and (C) anti-CarP IgG and clinical and laboratory markers in ANA positive patients. Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies). AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.