Involvement of high mobility group box 1 in the auto-inflammatory process in systemic lupus erythematosus
Schaper, Fleur

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CHAPTER

Autoantibodies to High Mobility Group Box 1 in Systemic Lupus Erythematosus

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Departments of Rheumatology and Clinical Immunology¹, Pathology and Medical Biology², Laboratory Medicine³, University Medical Center Groningen, University of Groningen, the Netherlands

SUBMITTED
ABSTRACT

Objective

Auto-antibodies to nuclear structures are a hallmark of systemic lupus erythematosus (SLE) including auto-antibodies to the nuclear protein High Mobility Group Box 1 (HMGB1). However, the clinical relevance of anti-HMGB1 antibodies is unclear and has not been extensively studied. Therefore, levels of anti-HMGB1 and reactivity to different parts of HMGB1, specifically Box A and Box B, were examined in SLE patients in association with disease activity and clinical parameters.

Methods

Eighty-six SLE patients and 44 age- and sex-matched healthy controls (HC) were included. Serum anti-HMGB1 IgM and IgG levels as well as anti-Box A and anti-Box B levels were measured during quiescent disease (SLEDAI ≤ 4, n=47), and active disease (SLEDAI ≥ 5, n=39) by ELISA.

Results

Quiescent and active SLE patients had similar levels of IgG anti-HMGB1, but in both groups levels were increased compared to HC. Anti-Box A and anti-Box B levels in active SLE patients were higher compared to quiescent patients, and were significantly increased compared to HC. Positivity for anti-HMGB1 was associated with arthritis, whereas all patients with neurological involvement were negative for anti-HMGB1. Although no associations between total anti-HMGB1 levels and SLEDAI or anti-doublestranded DNA (dsDNA) levels could be established, anti-Box A levels correlated with SLEDAI and anti-dsDNA levels, and correlated negatively with complement C3 levels.

Conclusions

Although total anti-HMGB1 IgG levels are increased in SLE patients no clear relation with disease activity or clinical parameters was found. In contrast, antibodies reacting specifically with the Box A domain of HMGB1 might be an interesting new biomarker since these were associated with disease activity and its sensitivity in SLE was comparable to the sensitivity of anti-dsDNA.
INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease which can involve multiple organs. It is generally thought that aberrant clearance of apoptotic cells plays an important role in the pathogenesis of the disease. When apoptotic cells are not cleared efficiently, they can turn into necrotic cells which lose their structural integrity and release several potential auto-antigens (1,2). Exposure of these auto-antigens to immune cells may lead to auto-antibody production. The auto-antibodies are used as serological markers in diagnosing SLE and during follow-up to predict disease activity. In SLE, several disease specific auto-antibodies have been documented and especially anti-double stranded DNA (anti-dsDNA) and anti-Sm (anti-Smith), both anti-nuclear antibodies (ANA), are of diagnostic value (1).

High Mobility Group Box 1 (HMGB1) is a nuclear protein which can act as damage-associated molecular pattern when released into the extracellular milieu (3). The molecule consists of three separate domains: Box A, Box B and the acidic tail. Both Box A and B are important for the DNA binding function of the molecule. HMGB1 can be released after cell activation, damage, apoptosis or necrosis and has been implicated in the pathogenesis of several diseases including SLE (4). It has been shown that serum and urinary levels of HMGB1 are increased in SLE, and correlated with disease activity (5-7). Released HMGB1 can have different functions depending on the redox status of HMGB1. For cytokine activity of HMGB1 cysteines C23 and C45 in Box A must form a disulfide bond while cysteine C106 in Box B must be in the thiol state. HMGB1 has chemoattractant properties when all three cysteines are reduced (8). Extracellular HMGB1 can bind to several receptors including Receptor for Advanced Glycation End products (RAGE) and Toll Like Receptor 4 (TLR4), of which binding sites are located in the Box B domain (3). Recombinant Box A can serve as a competitive antagonist for HMGB1 and can inhibit HMGB1 activity (3).

Auto-antibodies against HMGB1 have been found in patients with several different autoimmune diseases including SLE (5,9-11), Sjögren's Syndrome (12), Systemic Sclerosis (12,13), drug-induced autoimmunity (14), amyotrophic lateral sclerosis (15) and juvenile arthritis (16-18). In general, the clinical and/or pathological relevance of anti-HMGB1 antibodies is unclear but their presence can interfere with the detection of HMGB1 in ELISA systems (5,19). For SLE, we previously reported that anti-HMGB1 antibody levels correlate with serum HMGB1 levels, disease activity, and anti-dsDNA levels suggesting that these autoantibodies may play a role in disease pathogenesis (5). Moreover, beneficial effects of anti-HMGB1 antibody treatment on disease progression were demonstrated in a lupus mouse model as treatment with a monoclonal antibody against HMGB1 attenuated proteinuria, glomerulonephritis and decreased serum levels of pro-inflammatory
cytokines and anti-dsDNA (20). In other disease models, including rheumatoid arthritis, positive effects of anti-HMGB1 treatment on disease progression have been reported as well (20-24).

Commercially available monoclonal or polyclonal antibodies against HMGB1 are usually directed against either Box A or Box B of the molecule. Previously, it has been shown that treatment with a monoclonal antibody against box A as well as recombinant Box A can inhibit cell migration with no influence on the cytokine production induced by HMGB1 (25) suggesting that antibody reactivity to different parts of the molecule may have differential effects.

While increased levels of auto-antibodies to HMGB1 have been detected in SLE patients, the clinical relevance of these auto-antibodies is unclear. Additionally, reactivity to Box A or Box B has not been extensively studied. Therefore, the present study was designed to determine anti-HMGB1 antibody levels and their reactivity to Box A and Box B in SLE patients and healthy controls (HC). Moreover, associations with specific clinical symptoms as defined by American College of Rheumatology (ACR) criteria, disease activity, auto-antibody levels and medication use were explored.

MATERIALS AND METHODS

Patients and controls

Eighty-six SLE patients and forty-four age- and sex-matched HC were included. SLE patients fulfilled the ACR criteria for SLE (26). The study was approved by the Institutional Review Board of the UMCG and informed consent was obtained from all patients and HC. Disease activity at time of blood sampling was assessed by using the SLEDAI (SLE Disease Activity Index). Serum samples were collected between 2001 and 2013 and stored at –20 °C until analysis. Forty-seven consecutive outpatients with SLEDAI ≤ 4 were included in the quiescent patient group. Thirty-nine active patients were retrospectively selected with a SLEDAI ≥ 5. Further clinical data and medication use was obtained from medical records.

Levels of anti-dsDNA were measured by EliA assay (ThermoFisher Scientific, Nieuwegein, the Netherlands). Total IgG, C-reactive protein (CRP), serum creatinine and complement factors (C3, C4) were determined by routine techniques. IgM rheumatoid factor was measured by an in-house ELISA, as described previously (27). Characteristics of all patients and controls are summarized in table 1.
Table 1: Characteristics of the study population.

<table>
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</tr>
<tr>
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<td>38 (20-67)</td>
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<tr>
<td>Female, n (%</td>
<td>32 (72)</td>
</tr>
<tr>
<td>Disease duration, years</td>
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<td>C3, g/l</td>
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<tr>
<td>C4, g/l</td>
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<td>Serum creatinine, µmol/l</td>
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<td>Rheumatoid factor IgM, IU/ml</td>
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<tr>
<td>Antinuclear antibody</td>
<td>47 (100)</td>
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</table>

Data are displayed as median (range), unless stated otherwise.
Detection of anti-HMGB1 antibodies by ELISA

Anti-HMGB1 antibodies were measured using in-house ELISA. Greiner Bio-one plates were coated for at least 48 hours with 1 µg/ml recombinant HMGB1 (Sigma, St. Louis, MO, USA). After blocking with bovine serum albumin (BSA), serum samples were diluted and were added in duplicate (1:100) to both coated and uncoated wells. Detection of antibodies was done with goat anti-human IgG-HRP (Bethyl Labs, A80-104P). Bound antibodies were detected with 3,3',5,5'-tetramethylbenzidine dihydrochloride and H₂O₂; the reaction was stopped with 2M H₂SO₄. Absorbance was measured at 450nm using a micro-plate-spectrophotometer and concentrations were calculated with Softmax software. Levels of anti-HMGB1 were calculated against a standard curve using serum of a SLE patient with high anti-HMGB1 levels, corrected for reactivity to uncoated wells and expressed as Arbitrary Units (AU).

For IgM anti-HMGB1, Costar plates were coated overnight with 1 µg/ml recombinant HMGB1 (Sigma, St. Louis, MO, USA). After blocking with bovine serum albumin (BSA), serum samples were diluted and were added in two dilutions. Detection of antibodies was done with mouse anti-human IgM-HRP (Southern Biotech, Birmingham, AL, USA). Afterwards, measurement of bound antibodies was performed as described above.

Anti-Box A and anti-Box B ELISA

Antibodies directed against Box A and Box B were detected using in-house ELISA. Costar plates were coated for overnight with 1 µg/ml recombinant Box A or Box B (HMGBiotech Srl, Milan, Italy). After blocking with BSA serum samples were diluted in incubation buffer and were added in four dilutions (1:50, 1:150, 1:450 and 1:1350). Detection of antibodies was done with mouse anti-human IgG-HRP (Southern Biotech) for 30 minutes at room temperature. Afterwards, measurement of bound antibodies was performed as described above.

Statistical analysis

Data are presented as median (range) unless stated otherwise. Positivity was determined as upper 95% percentile of the HC values. Statistical calculations were performed using SPSS (version 22, SPSS Inc, Chicago IL, USA). Differences between patients and controls were calculated using Mann-Whitney test. Paired samples were compared using Wilcoxon Signed Rank Test. Kruskal-Wallis test was used for differences between groups larger than two. Spearman rank correlation was used for correlations and for non-continuous variables a Chi-squared test was used. P values < 0.05 were considered significant.
RESULTS

Anti-HMGB1, anti-Box A and anti-Box B levels in SLE and HC

Compared to HC IgG anti-HMGB1 levels were significantly increased in quiescent (p<0.001) and active SLE patients (p<0.001) (figure 1A). No significant differences were found between quiescent and active patients regarding absolute values or after determining the percentage of positive individuals (defined as upper 95% percentile of the HC values) (figure 1A). Sensitivity for IgG anti-HMGB1 in quiescent patients was 40% and 48% in active patients, while specificity was 93% for both. IgM anti-HMGB1 levels did not differ between HC and patients (figure 1B).

Figure 1: Serum levels of IgG and IgM anti-HMGB1 in HC, quiescent and active SLE patients. A: Serum levels of IgG anti-HMGB1, expressed in Arbitrary Units (AU), in HC (n=44), quiescent (n=47) and active (n=39) SLE patients measured by in-house ELISA. B: Serum levels of IgM anti-HMGB1, expressed in Arbitrary Units (AU), in HC (n=44), quiescent (n=47) and active (n=39) SLE patients measured by in-house ELISA. C: Serum levels of anti-Box A expressed in Arbitrary Units (AU), in HC (n=44), quiescent (n=47) and active (n=39) SLE patients measured by in-house ELISA. D: Serum levels of anti-Box B expressed in Arbitrary Units (AU), in HC (n=44), quiescent (n=47) and active (n=39) SLE patients measured by in-house ELISA. HC = Healthy Control and SLE = Systemic lupus erythematosus. Dotted line represents cut-off value for positivity. ** P<0.05 ** P<0.01 and *** P<0.001
To investigate whether anti-HMGB1 antibodies of SLE patients recognize a specific part of the HMGB1 molecule, reactivity to Box A or Box B was measured. Anti-Box A and anti-Box B levels were significantly increased in quiescent and active SLE patients compared to HC (figure 1C-D). Moreover, active patients had higher levels of anti-Box A and anti-Box B compared to quiescent patients (p<0.01 and p<0.05, respectively). Anti-Box A showed a sensitivity of 54% and 72% for quiescent and active patients respectively, with a specificity of 95% for both. Anti-Box B demonstrated a sensitivity of 25% and 41% in quiescent and active patients respectively, with a specificity of 95% for both.

Possible effects of medication were investigated by stratifying patients into the following groups: no medication, immunosuppressive therapy (including prednisolon, mycophenolate mophetil, cyclophosphamide, azathioprine or combinations) and hydroxychloroquine only. No effect of therapy was observed on the absolute levels of IgG anti-HMGB1, IgM anti-HMGB1, anti-Box A or anti-Box B or regarding positivity. Sub-analysis also revealed no difference when patients were further divided based on active or quiescent disease.

**Associations between anti-HMGB1 and serological parameters and ACR criteria**
To assess whether anti-HMGB1 antibodies are associated with disease parameters, anti-HMGB1 levels were analyzed in relation to clinical and serological parameters. There was no significant correlation between IgG anti-HMGB1 levels and SLEDAI (figure 2A rho: 0.13, p=0.22) or anti-dsDNA antibodies (figure 2B rho: 0.20, p=0.08). IgM anti-HMGB1 levels were negatively correlated with both SLEDAI (figure 2C rho: -0.24, p<0.05) and anti-dsDNA levels (figure 2D rho: -0.24, p<0.05). Both anti-Box A and B were positively associated with SLEDAI (figure 2E rho: 0.38, p<0.001, figure 2F rho: 0.31, p<0.01 respectively) and anti-dsDNA levels (figure 2F rho: 0.47, p<0.001, figure 2H rho: 0.36, p<0.01 respectively).

No significant correlations between complement C4, disease duration, CRP, IgM Rheumatoid Factor or creatinine and IgG or IgM anti-HMGB1, anti-Box A or anti-Box B levels were observed (p>0.1). Moreover, there was also no correlation between complement C3 and IgG or IgM anti-HMGB1 levels, but both anti-Box A and anti-Box B were negatively associated with complement C3 (rho: -0.31, p<0.05).

**Figure 2:** Correlations between IgG anti-HMGB1 (AU) levels with SLEDAI and anti-dsDNA (IU/ml) in SLE patients.
A: No correlation between IgG anti-HMGB1 (AU) levels and SLE Disease Activity Index (SLEDAI) in 86 SLE patients (rho: 0.13, p=0.22). B: No correlation between IgG anti-HMGB1 (AU) and anti-dsDNA (IU/ml), as measured by EliA assay, in 71 SLE patients (rho: 0.20, p=0.08).
C: Negative correlation between IgM anti-HMGB1 (AU) levels and SLE Disease Activity Index (SLEDAI) in 86 SLE patients (rho: -0.24, p<0.05). D: Negative correlation between IgM anti-HMGB1 (AU) and anti-dsDNA (IU/ml), as measured by EliA assay, in 71 SLE patients (rho: -0.24, p<0.05). E: Positive correlation between anti-HMGB1 Box A antibody (AU) levels and SLE Disease Activity Index (SLEDAI) in 86 SLE patients (rho: 0.38, p<0.001). F: Positive correlation between anti-HMGB1 Box A antibody (AU) levels and anti-dsDNA (IU/ml), as measured by EliA assay, in 71 SLE patients (rho: 0.47, p<0.001). G: Positive correlation between anti-HMGB1 Box B antibody (AU) levels and SLE Disease Activity Index (SLEDAI) in 86 SLE patients (rho: 0.31, p<0.01). H: Positive correlation between anti-HMGB1 Box B antibody (AU) levels and anti-dsDNA (IU/ml), as measured by EliA assay, in 71 SLE patients (rho: 0.36, p<0.01).
and rho: -0.34, p<0.01). Total IgG levels were not associated with IgG anti-HMGB1 or anti-Box B, but were weakly associated with anti-Box A (rho: 0.25, p<0.05).

To investigate whether positivity of IgG anti-HMGB1, anti-Box A or anti-box B antibodies were associated with specific organ involvement, patients were divided according to ACR criteria. Skin involvement was defined as positivity for either malar rash, discoid rash, photosensitivity or oral ulcers. There was no relation with nephritis or skin involvement for IgG anti-HMGB1, anti -Box A or anti-Box B. However, there were more patients positive for IgG anti-HMGB1 who had arthritis (p<0.01). Furthermore, 8 patients with neurological involvement were all negative for IgG anti-HMGB1 (p<0.01). Positivity for either anti-Box A or anti-Box B was not associated with any of the ACR criteria. No differences in positivity for IgG anti-HMGB1, anti-Box A or anti-Box B were observed when comparing patients with active nephritis (n=22) versus non-nephritic active disease (n=17). Sensitivity for nephritis compared to non-nephritic exacerbations was 45.5% vs 41.2% for IgG anti-HMGB1, 72.7% vs 70.6% for anti-Box A and 40.9% vs 41.2% for anti-Box B respectively.

As IgM anti-HMGB1 levels were similar between patients and controls it was not possible to determine positivity, therefore absolute values of IgM anti-HMGB1 were investigated for relation with specific organ involvement. IgM anti-HMGB1 levels were higher in patients without nephritis (929 (10-18444) AU) compared to patients with nephritis (362 (19-16458) AU) (p<0.01). On the contrary, levels of IgM anti-HMGB1 were higher in patients with arthritis (875 (10-18444) AU) compared to patients without arthritis (460 (19-3323) AU) (p<0.05). There were no correlations between IgM anti-HMGB1 and other ACR criteria.

**Levels of anti-HMGB1 antibodies during and after active disease**

Anti-HMGB1 levels were compared during active and subsequent quiescent disease (4 to 8 months later) in 23 SLE patients. No significant increase or decrease could be observed in anti-HMGB1 levels in these 23 patients (p=0.6) (figure 3A). Further analysis showed that in 13 patients anti-HMGB1 levels decreased after exacerbation, while in 10 patients levels increased. These two patient groups were compared for differences at exacerbation and during remission. No differences in SLEDAI, C3, C4 or anti-dsDNA were observed at both time points (table 2). Furthermore, in both groups similar numbers of renal active patients were present (n=6 vs n=7) and medication (including no medication, immunosuppressive therapy or hydroxychloroquine only) was not different.

Anti-Box A and anti-Box B levels were significantly decreased after active disease (p<0.01) (figure 3B-C). In only 3 patients anti-Box A levels increased after exacerbation but two of these patients were noncompliant with the treatment regime.
Figure 3: Levels of IgG anti-HMGB1, anti-Box A and anti-Box B in active disease and subsequent remission in SLE patients.
Levels of IgG anti-HMGB1, anti-Box and anti-Box were measured by in-house ELISA, and expressed in Arbitrary Units (AU), in 23 Systemic lupus erythematosus (SLE) patients during active and subsequent remission (4 to 8 months later).
A: No clear pattern of increase or decrease of IgG anti-HMGB1 (AU) levels after active disease (p=0.6) B: Anti-Box A levels (AU) decreased after active disease. C: Anti-Box B levels (AU) decreased after active disease.
** P<0.01

DISCUSSION

In this study, we demonstrated that patients with SLE have increased levels of IgG anti-HMGB1 but not of IgM anti-HMGB1 irrespective of current treatment. Total IgG anti-HMGB1 levels are not associated with disease activity. However, anti-Box A antibodies are associated with SLEDAI, anti-dsDNA and negatively associated with complement C3 levels. Moreover, anti-Box A antibodies displayed higher sensitivity for active SLE compared to IgG anti-HMGB1 and anti-Box B.

Previous studies have also demonstrated increased anti-HMGB1 levels in SLE patients (5,9-11). In a previous study, we showed a relation with disease activity as anti-HMGB1 levels were weakly correlated to SLEDAI and anti-dsDNA (5). In the present study in another patient cohort, no correlation was found between disease activity and IgG anti-HMGB1 levels. However, we did find a significant correlation between disease activity and anti-Box A levels. The differences between the present and previous studies may be explained by a modification of the ELISA assay for anti-HMGB1, which currently includes a correction for unspecific binding. The difference between the anti-HMGB1 assay and the anti-Box A assay might be explained by the fact that HMGB1 is a sticky protein that can also bind several other serum factors non-specifically, which is not the case for the Box A subunit.

The presence of a multitude of auto-antibodies is a hallmark of SLE. In particular, auto-antibodies directed to dsDNA are very specific for the disease. These antibodies are important for diagnosis, as well as in monitoring disease activity (1,28). Although we observed a high specificity for anti-HMGB1 antibodies,
<table>
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<th>Patients with decreasing anti-HMGB1 levels</th>
<th>Patients with increasing anti-HMGB1 levels</th>
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<td>Active disease</td>
<td>Remission</td>
<td>Active disease</td>
</tr>
<tr>
<td>N</td>
<td>23</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Anti-HMGB1 (AU)</td>
<td>215 (9-1310)</td>
<td>212 (0-1139)</td>
<td>393 (49-1310)</td>
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<td>SLEDAI</td>
<td>9 (5-13)</td>
<td>2 (0-4)</td>
<td>9 (5-13)</td>
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<td>Renal active/not renal active</td>
<td>13/10</td>
<td>6/7</td>
<td>9 (7-12)</td>
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<tr>
<td>anti-dsDNA, IU/ml</td>
<td>176 (1.7-439)</td>
<td>10 (0-111)</td>
<td>194 (1.7-379)</td>
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<td>complement C3, g/l</td>
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<td>0.75 (0.2-1.82)</td>
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<td>complement C4, g/l</td>
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<td>3</td>
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Data are represented as median (range).
we only compared anti-HMGB1 antibodies in SLE patients to HC. Earlier studies have shown that anti-HMGB1 antibodies are also present in other auto-immune diseases (12,13,18), however these have not studied the presence of anti-Box A or anti-Box B. Therefore, to demonstrate the specificity of anti-Box A and anti-Box B for SLE these antibodies should be studied in patients with other autoimmune diseases. Our data indicates that sensitivity of antibodies directed to anti-Box A was similar to the sensitivity of anti-dsDNA in active and quiescent SLE patients (28,29). Moreover, sensitivity of anti-Box A during nephritis was equal to the sensitivity during non-nephritic exacerbations, while anti-dsDNA levels are more often increased during nephritis compared to non-nephritic exacerbations. These results should be validated in a larger cohort of SLE patients. Furthermore, whether an increase in anti-Box A will predict an exacerbation and whether anti-Box A might serve as an additional biomarker next to anti-dsDNA has to be further investigated prospectively.

While IgG anti-HMGB1 levels were increased in SLE, IgM anti-HMGB1 levels were similar between patients and controls. The presence of IgM anti-HMGB1 in HC might indicate that IgM anti-HMGB1 is a natural autoantibody. This could also explain why negative correlations with SLEDAI and anti-dsDNA antibodies were found. In general, IgG and not IgM auto-antibodies are considered to be more pathogenic in SLE (30-34). In an animal model of lupus nephritis, administration of IgM anti-dsDNA antibodies has been found to reduce renal pathology (32). Furthermore in patients, it has been postulated that progression to or worsening of SLE may be preceded by a switch from IgM to IgG antibodies (33,34). As such it has been demonstrated that IgM anti-dsDNA levels are significantly higher in SLE patients without renal disease (31), which we also observed for IgM anti-HMGB1.

In a recent study IgG and IgM anti-HMGB1 antibodies in SLE patients with skin involvement were investigated (9). Similar levels of antibodies in patients with and without skin involvement and other clinical parameters were shown. This is in line with our data, as we also did not find a correlation between anti-HMGB1 antibodies and skin involvement in our cohort. In our study both IgG and IgM anti-HMGB1 antibodies were found to be associated with arthritis. This is interesting as earlier studies have shown that anti-HMGB1 antibodies are also present in patients with rheumatoid arthritis (12,16). However, IgM rheumatoid factor levels were similar in SLE patients with and without arthritis and no correlation was found between rheumatoid factor and anti-HMGB1 levels.

All patients with neurological involvement were negative for anti-HMGB1. There were however only 8 patients with neurological involvement in our cohort. Therefore it is difficult to draw a definitive conclusion. Several studies did describe that neurological SLE has a very specific set of auto-antibodies, which might explain
the low anti-HMGB1 levels (35). As both anti-Box A and anti-Box B positivity were not associated with any clinical parameters, and the associations for IgG anti-HMGB1 were inconsistent, this indicates that anti-HMGB1 is not a sensitive measure for organ involvement.

In this study, total anti-HMGB1 antibodies were not associated with disease activity. However, there might be a role for more specific anti-HMGB1 antibodies. These antibodies might interfere in different processes since each domain of HMGB1 has its own function and effect. We found that antibodies from SLE patients (62%) predominantly recognized Box A, which is in line with previous observations (10). Box A is the DNA binding domain of the molecule, but can also serve as a competitive antagonist of HMGB1 and several studies in animal models have shown beneficial effects of Box A on disease progression (36,37). As previously mentioned, HMGB1 consists of Box A and B, and also of a C-acidic tail and a linker region which plays a role in binding RAGE. Previously, it was demonstrated that auto-antibodies against the C-acidic tail and linker region are also present in SLE patients (10). We confirmed the presence of anti-Box A and B antibodies in SLE. Furthermore, our results indicate that anti-Box A antibody levels are a more sensitive marker of active disease in SLE when compared to IgG anti-HMGB1 or anti-Box B. Moreover, anti-Box A levels significantly decreased after active disease. As mentioned above, the role of anti-Box A as a marker of active disease should be validated in prospective longitudinal studies.

Why specifically anti-Box A levels are increased during active disease is unclear, but this may relate to the various posttranslational modifications that HMGB1 can undergo influencing its function. HMGB1 exists in three different isoforms which have mutually exclusive functions; cytokine inducing, chemo-attracting or immunologically silent (25). These isoforms are distinguished by differences in redox status, resulting in different conformations. Cytokine activity of HMGB1 is dependent on the disulfide bond of cysteines C23 and C45 in Box A while cysteine C106 in Box B must be in the thiol state (25,38). Antibodies to Box A can inhibit the cytokine activity of HMGB1 as has been demonstrated before (8). In active SLE, an increase in circulating HMGB1 has been demonstrated (5), and in a small study it has been observed that in active SLE oxidized HMGB1 is present (39). Since oxidation may generate neo-epitopes in the HMGB1 molecule, this may promote autoimmune responses. The disulfide bond on which cytokine activity is dependent is located in Box A, therefore it could be that an increase in the cytokine form of HMGB1 subsequently leads to an increase in auto-antibodies that recognize Box A.

In conclusion, although we observed that total anti-HMGB1 antibodies are increased in SLE, no association was found with disease activity. However, antibodies to Box A had a higher sensitivity in SLE and especially during active
disease. Moreover, anti-Box A was positively associated with SLEDAI, anti-dsDNA and negatively with complement C3. Anti-HMGB1, anti-Box A or anti-Box B positivity does not seem to be a useful marker in evaluating organ involvement in SLE patients, as defined by ACR criteria. Taken together, these results indicate that anti-HMGB1 antibodies in SLE patients cannot be regarded as a cross-sectional biomarker of clinical parameters or disease activity, but the role of antibodies directed to Box A warrants further investigation.

AUTHOR CONTRIBUTIONS
FS took part in the study design, serum anti-HMGB1 measurement, statistical analysis, interpretation of results and manuscript preparation. KL, PCL, and JW participated in study design, interpretation of results and critically revised the manuscript. GH participated in serum anti-HMGB1 measurement, interpretation of results and critically revised the manuscript. FM contributed to the statistical analysis and critically revised the manuscript. HB acquired data and critically revised the manuscript. PH contributed to the analysis and interpretation of the data and critically revised the manuscript. All authors read and approved the manuscript.

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