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学 位 論 文

Hydroxychloroquine modulates elevated expression of S100 proteins in systemic lupus erythematosus

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Abstract

Objectives: We investigated the effect of hydroxychloroquine (HCQ) on S100A8 and S100A9 serum levels in systemic lupus erythematosus (SLE) patients with low disease activity receiving immunosuppressants.

Methods: SELENA-SLEDAI, Cutaneous Lupus Erythematous Disease Area and Severity Index (CLASI) and serum levels of complement factors, anti-double stranded DNA antibodies, and white blood cell, lymphocyte and platelet counts were used to evaluate disease activity, cutaneous disease activity and immunological activity, respectively. Serum S100A8 and S100A9 were measured at HCQ administration and after 3 or 6 months using ELISA.

Results: S100A8 and S100A9 serum levels were elevated at baseline and the magnitude of decrease from baseline at 3 and 6 months after HCQ administration was greater in patients with renal involvement than in those without (baseline: S100A8, p=0.034; S100A9, p=0.0084; decrease: S100A8, p=0.049; S100A9, p=0.023). S100-modulating was observed in patients with (n=17; S100A8, p=0.0011; S100A9, p=0.0002) and without renal involvement (n=20; S100A8, p=0.0056; S100A9, p=0.0012), and was more apparent in patients with improved CLASI activity scores (improved: S100A8, p=0.013; S100A9, p=0.0032; unimproved: S100A8, p=0.055; S100A9, p=0.055). No associations were observed for immunological biomarkers.

Conclusion: HCQ may improve organ involvement in SLE by modulating S100 protein levels, especially in patients with renal or skin involvement.

Keywords

Systemic lupus erythematosus (SLE), Lupus Nephritis, Skin, Hydroxychloroquine, S100A8, S100A9

Introduction

Lupus nephritis (LN) is the major important organ involved in systemic lupus erythematosus (SLE) and is caused by immune complexes [1]. Macrophage infiltration can be observed histologically in LN and plays an important role in the pathogenesis of glomerulonephritis; however, the details underlying this process remain unclear. Cellular infiltrates in LN may be induced via responses coordinated between the innate and adaptive immune systems.

Little is known about the role of the innate immune response in LN. Myeloid-related proteins (MRPs) such as MRP-8 and -14 have been reported to play important roles in the development of autoimmune nephritis in mice. Both of these proteins are ligands of Toll-like receptor 4 (TLR4) expressed by monocytes, and they also participate in the development of autoreactive CD8+T cells [2]. These proteins have been identified as ‘‘danger signals’’ and are also known as DAMPs (damage-associated molecular patterns). According to Heizmann's group, MRP8 is also known as S100A8, and MRP14 as S100A9 [3]. These proteins are expressed in the cytoplasm of neutrophils and macrophages [4]. Upon activation of these cells through TLR4 and receptor for advanced glycation endproducts (RAGE) stimulation, S100A8 and S100A9 are secreted [5,6]. The secreted MRP-8/14 complex can bind to the activated endothelium, assisting phagocytes in migrating anywhere in the body [7,8]. The heterodimeric MRP-8/14 complex can also induce upregulation of CD11b/CD18 (MAC-1) on neutrophils, which results in enhanced adhesion to the endothelium [9].

Phagocytes expressing MRP-8 and -14 were reported to be involved in the pathogenesis of multiple autoimmune and malignant conditions [10-14]. Serum levels of these protein in SLE patients were positively correlated with disease activity, especially lupus glomerulonephritis [15,16]. Additionally, these proteins were demonstrated histologically to be expressed in renal tissues, with levels proportional to the severity of LN [17,18]. The authors suggested that MRP-8 and -14 can be used as diagnostic and prognostic markers for the progress of LN.

Tydén *et al*. observed that serum levels of S100A8 and S100A9 were associated with disease activity in SLE [19]. Moreover, serum levels of these proteins were reduced by treatment of SLE patients with several immunosuppressants. However, there

have been no reports on the effect of HCQ, which is used as an adjunctive therapy in SLE patients, on expression of these proteins.

On the other hand, HCQ has approved for SLE treatment in Japan since July 2015. After the approval of HCQ treatment, many SLE patients with immunosuppressant treatment, especially for SLE in women of child bearing age, started to be treated with additional HCQ in Japan.

In this study, we investigated the effect of HCQ treatment on serum levels of S100A8 and S100A9 proteins in SLE patients with low disease activity being treated with immunosuppressants.

Patients and Methods

Patients

This was a single-center, retrospective study. All subjects were diagnosed with SLE using the American College of Rheumatology criteria [20] or the SLICC criteria [21], and started additional HCQ treatment for the first time from September 2015 to December 2017. All patients enrolled in this study had been receiving oral HCQ sulfate (Plaquenil; Sanofi-Winthrop, Paris, France) continuously for at least 3 months. There was no need for additional immunosuppressive treatments, including glucocorticoids, in any patient during the 3 months prior to starting HCQ because of sustained low disease activity in these patients. Low disease activity was defined as SELENA-SLEDAI score of 8 or less with no activity in major organ systems such as renal involvement, NPSLE, cardiopulmonary and vasculitis, current prednisolone or equivalent dose of 10mg per day or less and well-tolerated maintenance doses of immunosuppressant. Informed consent was obtained from all participants. The study was approved by the ethical committee of Kagawa university.

HCQ was administered at a dose based on ideal body weight (IBW) calculated using the modified Broca's method: 200 mg daily for IBW < 46 kg; 200 mg and 400 mg on alternate days for IBW ≥ 46 kg and < 62 kg; and 400 mg daily for IBW ≥ 62 kg. Clinical parameters (age, gender, HCQ dose, immunological biomarkers, disease activity index and skin score) were investigated before and after HCQ treatment. Disease activity was evaluated using the SELENA-SLEDAI 2011 criteria. Cutaneous disease activity was evaluated using the Cutaneous Lupus Erythematous Disease Area and Severity Index (CLASI). According to CLASI improvement criteria reported by Klein R, et al [22], CLASI disease activity was classified by the principle investigator as improved, unchanged, or worse compared to the previous visit, as described above. Those classified as improved were defined as CLASI responders and those classified as unchanged or worse were defined as CLASI non-responders. Immunologic activity was determined via serum levels of complement factors (C3, C4, CH50), anti-double stranded DNA (dsDNA) antibodies, and counts of white blood cells, lymphocytes and platelets. Serum levels of S100A8 and S100A9 were measured at the time of HCQ administration as well as 3 or 6 months later using ELISA (CircuLex ELISA Kit, MBL) according to the manufacturer's instructions. We investigated whether serum levels of S100A8 and S100A9 proteins were associated with disease activity.

Statistical analysis

Immunological biomarker and serum levels of S100A8 and S100A9 were compared using the Student's t test for continuous variables (or Wilcoxon signed-rank test for non-normally distributed data). Comparisons between groups were performed using the Wilcoxon rank sum test. All p values were two-sided, and p values <0.05 were considered significant. For the statistical analyses, we used the following abbreviations: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data were analyzed using JMP® 13 software (SAS Institute Inc., Cary, NC, USA).

Results

Baseline

Pregnant women and patients who started anti-thrombotic therapy or added immunosuppressant after starting HCQ administration were excluded in this study. Thirty-seven SLE patients (33 women, 4 men) with sustained low disease activity for at least 3 months prior to administration of HCQ were enrolled in this study. Their baseline characteristics are shown in Table 1. The mean age was 40.7 ± 13.7 years, the mean disease duration was 14.4 ± 11.5 years, the mean SELENA-SLEDAI score was 3.7 ± 1.9 and the mean CLASI activity score was 3.2 ± 3.2. Seventeen of 39 SLE patients had a history of LN. All 17 patients have been in complete remission for over one year. Serum levels of S100A8 and S100A9 proteins at baseline were significantly higher in SLE patients with a history of renal involvement compared with those without renal involvement (p=0.034 and p=0.0084, respectively; Figure 1). Involvement of other organs such as skin or neuropsychiatric SLE was not associated with serum levels of S100A8 and S100A9 proteins.

No significant correlations between S100 protein levels and CLASI score, SELENA-SLEDAI score, complement levels or anti-dsDNA antibody levels were identified (data not shown).

Effect of HCQ treatment on levels of S100A8 and S100A9 proteins

We analyzed changes in S100A8 and S100A9 protein levels prior to HCQ treatment as well as 3 or 6 months after HCQ treatment in 37 SLE patients enrolled in this study. However three of the 37 subjects were excluded at 6 month because of a lack of data during observation for 6 months after HCQ administration. Serum levels of S100A8 and S100A9 proteins decreased significantly 3 and 6 months after HCQ treatment compared

with those at baseline (Figure 2). Additionally, the modulating effect of HCQ treatment on serum S100A8 and S100A9 levels was observed in both SLE patients with (n=17; S100A8, p=0.0011; S100A9, p=0.0002) and without a history of renal involvement (n=20; S100A8, p=0.0056; S100A9, p=0.0012; Supplementary Material Figure S1). The magnitude of the changes in serum S100A8 and S100A9 levels in SLE patients with renal involvement were significantly higher than in those without renal involvement (S100A8, p=0.049; S100A9, p=0.023; Figure 3).

As for the correlation with skin involvement, there was no significant difference in the magnitude of changes in serum S100A8 and S100A9 levels in SLE patients with or without skin involvement (S100A8, p=0.224; S100A9, p=0.072; Supplementary Material Figure S2). However, the modulating effect of HCQ treatment on serum S100A8 and S100A9 levels was much more apparent in CLASI responders (CLASI responders: S100A8, p=0.013, S100A9, p=0.0032; CLASI non-responders: S100A8, p=0.055, S100A9, p=0.055; Figure 4).

In regard to SLE disease activity, SELENA-SLEDAI scores, CLASI activity scores and anti-dsDNA antibody decreased and serum levels of C3 increased significantly in all patients after 3 months' treatments. However, no association between changes in S100A8 and S100A9 levels and immunological biomarkers or SELENA-SLEDAI scores was identified. Next we focused on the association of autoantibody titers with changes in S100A8 and S100A9 protein levels. The effect of HCQ treatment on serum S100 protein levels differed between patients with and without detectable anti-Sjögren's syndrome type B (SS-B) antibody titers. Serum S100A8 and S100A9 levels decreased significantly during HCQ treatment only in anti-SS-B antibody-negative SLE patients (data not shown). Additionally, changes in serum S100A9 levels in SLE patients positive for lupus anti-coagulant (LAC) were significantly less apparent than in LAC-negative patients (Supplementary Material Figure S3, Figure 5).

Discussion

Several biomarkers, including anti-dsDNA antibodies and/or complement factors, have been suggested to reflect the disease activity of SLE. Recently, it has been suggested that TLRs signaling may play an important role in the pathogenesis of SLE. Additionally, several TLR ligands have been reported to be involved in the pathophysiology of SLE. TLR7 or TLR9 signaling are important for production of the interferons which drive SLE pathogenesis. Additionally, TLR4 has also been reported to participate in the pathogenesis of SLE [23,24]. The S100A8 and S100A9 proteins, which are well-known DAMPs, are also ligands of TLR4. Some reports have indicated that serum levels of S100A8 and S100A9 were related to disease activity or serum titers of anti–dsDNA antibodies [19]. Tantivitayakul *et al*. demonstrated that cells infiltrating the glomeruli and peritubular capillaries in LN expressed S100A8/A9 using immunohistochemical staining [18]. Our study showed that serum S100A8 and S100A9 levels significantly increased in SLE patients with renal involvement at baseline in comparison with patients without renal involvement. However, serum levels of S100A8 and S100A9 proteins were not associated with disease activity indices such as SLEDAI and CLASI (skin lesion activity). The reason for the difference from previous reports is probably related to the disease activity of the study population. The current study analyzed SLE patients with sustained low disease activity, and many other studies examined patient populations with high disease activity. However, our data indicated that elevation of S100 proteins was observed even in SLE with LN remission (data not shown). This suggested the possibility that S100 proteins are produced from renal lesions in SLE with remission of glomerulonephritis. In other words, we hypothesize that many patients, even during clinical remission of LN, retain some LN activity sufficient to increase the expression of S100 proteins, which is related to the pathogenesis of LN.

We also investigated changes of S100A8 and S100A9 protein levels during HCQ treatment. Previous reports demonstrated changes in S100 proteins during immunosuppressive treatments, including glucocorticoid (GC), cyclophosphamide and

mycophenolate mofetil (MMF), in SLE patients with high disease activity. Tydén *et al*. reported that serum S100A8/A9 levels decreased upon treatment of SLE patients with active glomerulonephritis with immunosuppressive drug such as MMF, azathioprine and GC [19]. However, there have been no reports describing the effect of HCQ treatment on S100 protein levels in SLE patients with low disease activity. We demonstrated that HCQ treatment in SLE patients with sustained low disease activity reduced the levels of S100 proteins significantly. The magnitude of changes of serum S100A8 and S100A9 levels in SLE patients with a history of renal involvement was significantly higher than in patients without renal involvement (S100A8, p=0.083; S100A9, p=0.036; Figure 3). This result indicated that HCQ could provide additional benefits for LN through reduction of S100 protein expression at the site of glomerulonephritis. Many reports indicated that HCQ use, combined with conventional immunosuppressive therapy for LN, improved renal prognosis [25]. We speculate that HCQ's modulating effect on S100 protein levels in SLE patients with low disease activity might have a beneficial effect on renal prognosis. However, the modulating effect of HCQ on S100 protein levels was observed to some extent among SLE patients regardless of past renal involvement. That is to say, HCQ reduced the levels of S100 proteins in SLE cases without glomerulonephritis. Furthermore, a significant modulating effect of HCQ on S100 proteins was observed in SLE patients with skin involvement. Recently, Elloumi *et al*. demonstrated that TLR4, a sensor for DAMPs such as S100 proteins, had an important pathogenic role in cutaneous lupus erythematosus inflammation and LN. Upregulation of TLR4 was demonstrated in skin and renal lesions, and played a role in the pathogenesis of cutaneous and renal disorders in SLE [26]. Our data indicated that the mechanism in reducing renal and/or skin lesions may be due to effects on TLR4 signaling through modulation of S100 protein expression. Furthermore, there were some reports that HCQ reduced the expression of TLR4 in several cell types including trophoblastic cells or macrophages [27,28]. According to our data and these previous reports, we suggest that the mechanism of HCQ's effect on skin and or renal lesions in SLE patients may involve modulation of TLR4 signaling by downregulation of both S100 proteins and TLR4 expression.

However, the overall mechanisms through which HCQ modulates S100 protein levels remain unclear.

Taken together, our results indicate that HCQ had a modulating effect on S100 proteins which resulted in inhibition of TLR4 signaling. This represents another example of an effect of HCQ on skin or renal disease in SLE. Furthermore, HCQ reduced the levels of S100 proteins in patients with clinical remission of LN. This may contribute to improved prognosis for LN. However, it remains unclear how HCQ reduced the expression of S100 proteins in SLE. Further studies are needed to clarify this issue.

There were some limitations in this study. First, there was no monitoring of HCQ adherence through pharmacological dosage of blood HCQ levels. However, disease activity and immunological biomarker were improved significantly 3 months after HCQ treatment compared with those at baseline. Second, sample size was too small in this study since pregnant women and patients who started anti-thrombotic therapy or added immunosuppressant after starting HCQ administration were excluded in this study.

In conclusion, S100 protein levels have been reported to correlate with the pathogenesis of organ involvement in SLE patients. Our findings suggest that HCQ improves organ involvement in SLE through modulation of S100 protein levels, especially in patients with renal or skin involvement. Further investigation is needed to clarify the mechanisms underlying S100 protein modulation by HCQ.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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Figure legend.

Figure 1. Association between serum S100 protein levels and renal involvement in SLE patients prior to HCQ treatment.

S100A8 and S100A9 serum levels were increased in SLE patients with renal involvement.

For statistical analyses *$p<0.05$, **$p<0.01$

P value: Wilcoxon rank sum test

Figure 2. The change of serum level S100 proteins by HCQ treatment.

Serum S100A8 and S100A9 levels at baseline were compared with levels after 3 and 6 months of HCQ treatment with Bonferroni correction. Serum S100A8 and S100A9 levels significantly decreasing during HCQ treatment in SLE patients.

For statistical analyses **** $p<0.0001$, ** $p<0.015$, NS: Not significant P value: t test

Figure 3. Degree of change in serum S100 protein levels in SLE patients with renal involvement (n=17) or without renal involvement (n=20).

The magnitude of changes in serum S100A8 and S100A9 levels in SLE patients with renal involvement were significantly higher than in patients without renal involvement.

For statistical analyses * $p<0.05$.

P value: Wilcoxon rank sum test

Figure 4. Changes of serum S100 protein levels in CLASI responders (n=17) and CLASI non-responders (n=8).

Serum S100A8 and S100A9 levels at baseline were compared with levels after 3 months of HCQ treatment in patients classified into CLASI responders and CLASI non-responders. Decreases in serum S100A8 and S100A9 levels were much more apparent in CLASI responders.

For statistical analyses * $p<0.05$, * * $p<0.01$. Wilcoxon signed-rank test

Figure 5. Changes in serum S100 protein levels during HCQ treatment in SLE patients positive (n=6) or negative (n=31) for lupus anticoagulant(LAC).

Changes in serum S100A9 levels in LAC-positive SLE patients were significantly lower than in LAC-negative patients.

For statistical analyses * $p<0.05$.

lupus anticoagulant: LAC.

P value: Wilcoxon rank sum test

Table 1　Characteristics of SLE patients enrolled in this study

Characteristics	n=37
Female, no(%)	33(89)
Age, years, mean±SD	40.7±13.7
Disease duration, years, mean±SD	14.4±11.5
Complication	
APS	4 (11)
Past involvement	
Skin involvement	31 (84)
Renal involvement	17 (46)
Duration of CR free, years	5.2±3.3
NPSLE	2 (5)
Concomitant immunosuppressive treatments	
Prednisone	32 (86)
No.(%)	4.8 (1-10)
Median Dosage, mg/day (range)	24 (62)
Other immunosuppressant*[1]	
Tacrolimus	13 (35)
Mycophenolate mofetil	6 (16)
Cyclosporine A	2 (5)
Mizoribine	1 (3)
Methotrexate	1 (3)
Azathioprine	1 (3)
Anti-thrombotic therapy	
Anti-thrombotic therapy*[2]	18 (49)
Antiplatelet agent	12 (32)
Anticoagulant agent	7 (19)
Positive rate of autoantibody	
Anti-Sm	8 (22)
Anti-RNP	17 (46)
Anti-SS-A	18 (49)

Anti-SS-B	7 (19)
Lupus anticoagulant	6 (16)
Anti-cardiolipin	15 (41)
Anti-β2GPI	2 (5)
Disease activity	
SELENA-SLEDAI score	3.7±1.9
CLASI activity score	3.2±3.2 (n=25)
CLASI damage score	0.6±1.3 (n=25)
anti-dsDNA positive, no(%) *3	14 (38)
anti-dsDNA (IU/mL)	16.2±19.1
C3 (mg/dL)	81.5±21.1
C4 (mg/dL)	16.4±8.1
CH50 (U/mL)	35.6±8.9
low complement, no(%) *4	18 (49)
White Blood Cell (/μL)	4978.1±1639.5
Lymphocytes (/μL)	1183.9±649.7
Platelet ($\times 10^4$/μL)	22.1±7.2

*1 Two patients received multiple immunosuppressants.

*2 One patient received antiplatelet agent and anticoagulant agent.

*3 Anti-dsDNA positive means anti ds-DNA titer increases over 12 IU/ml

*4 Low complement means any of C3, C4 and CH50 decreases to less 68mg/dl, less 12mg/dl, 30U/ml.

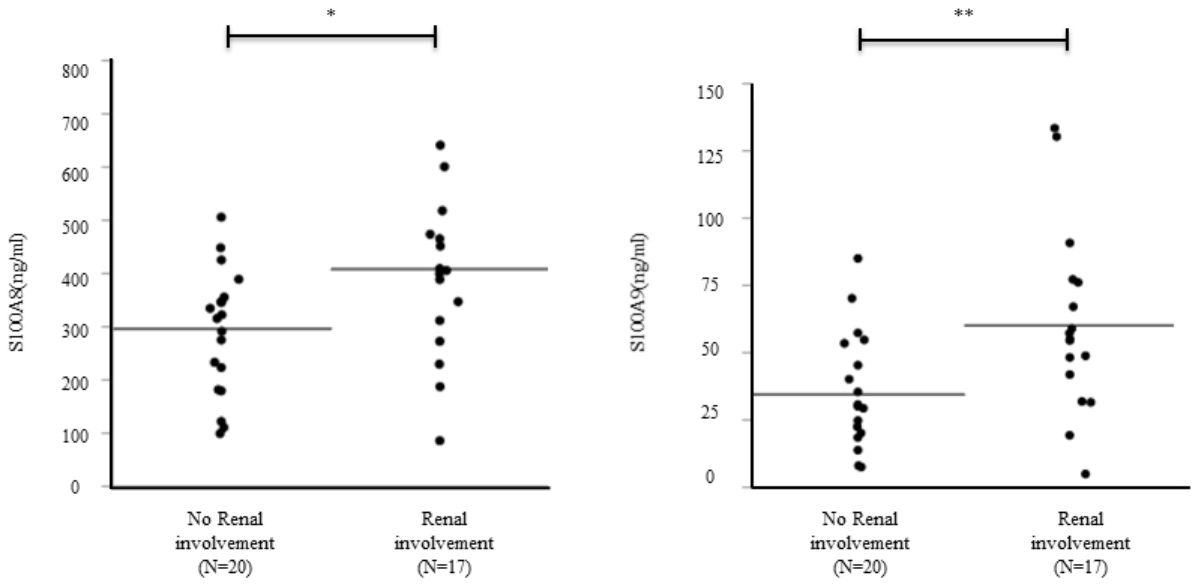
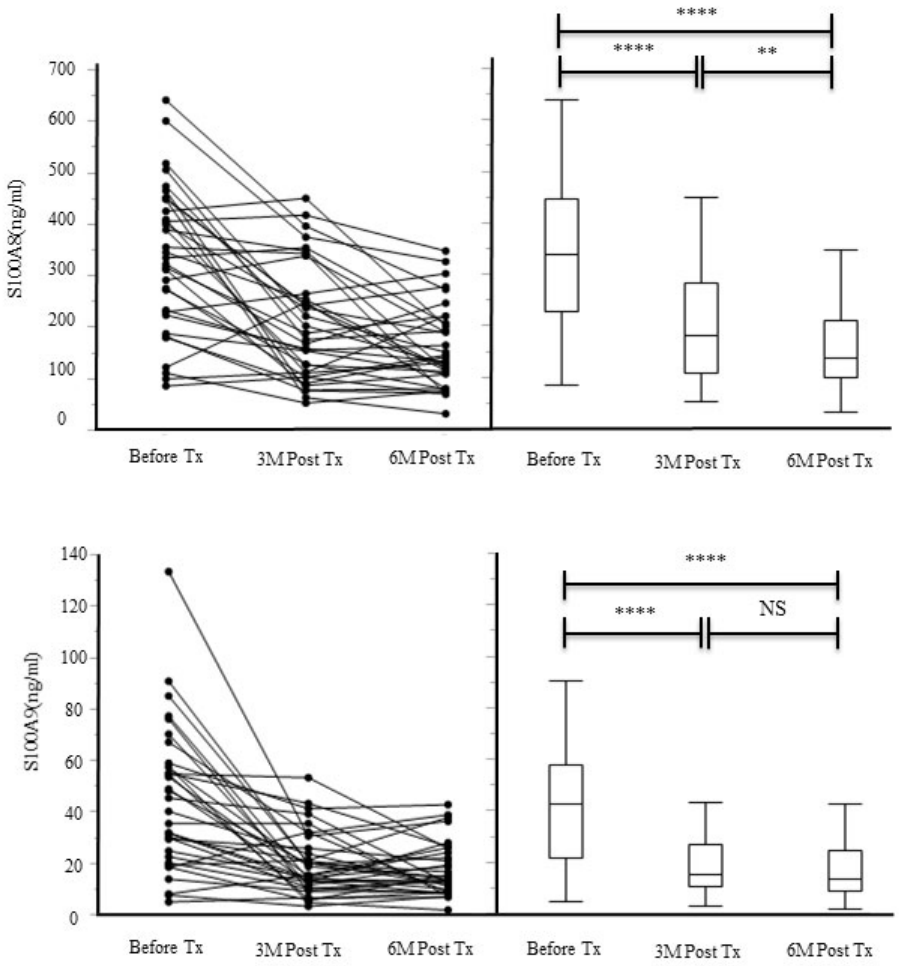

*
S100A8(ng/ml)
800
700
600
500
400
300
200
100
0
No Renal involvement (N=20)
Renal involvement (N=17)
**
S100A9(ng/ml)
150
125
100
75
50
25
0
No Renal involvement (N=20)
Renal involvement (N=17)

Figure 1


S100A8(ng/ml)
700
600
500
400
300
200
100
0
Before Tx
3M Post Tx
6M Post Tx
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S100A9(ng/ml)
140
120
100
80
60
40
20
0
NS

Figure 2

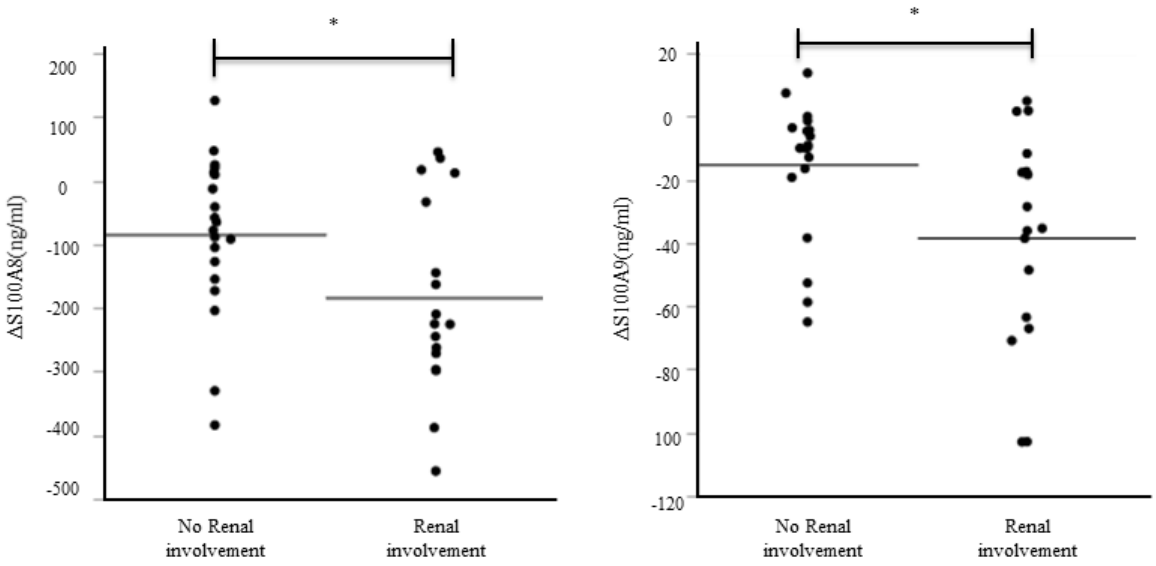

*
200
100
0
-100
-200
-300
-400
-500
ΔS100A8(ng/ml)
No Renal involvement
Renal involvement
*
20
0
-20
-40
-60
-80
100
-120
ΔS100A9(ng/ml)
No Renal involvement
Renal involvement

Figure 3

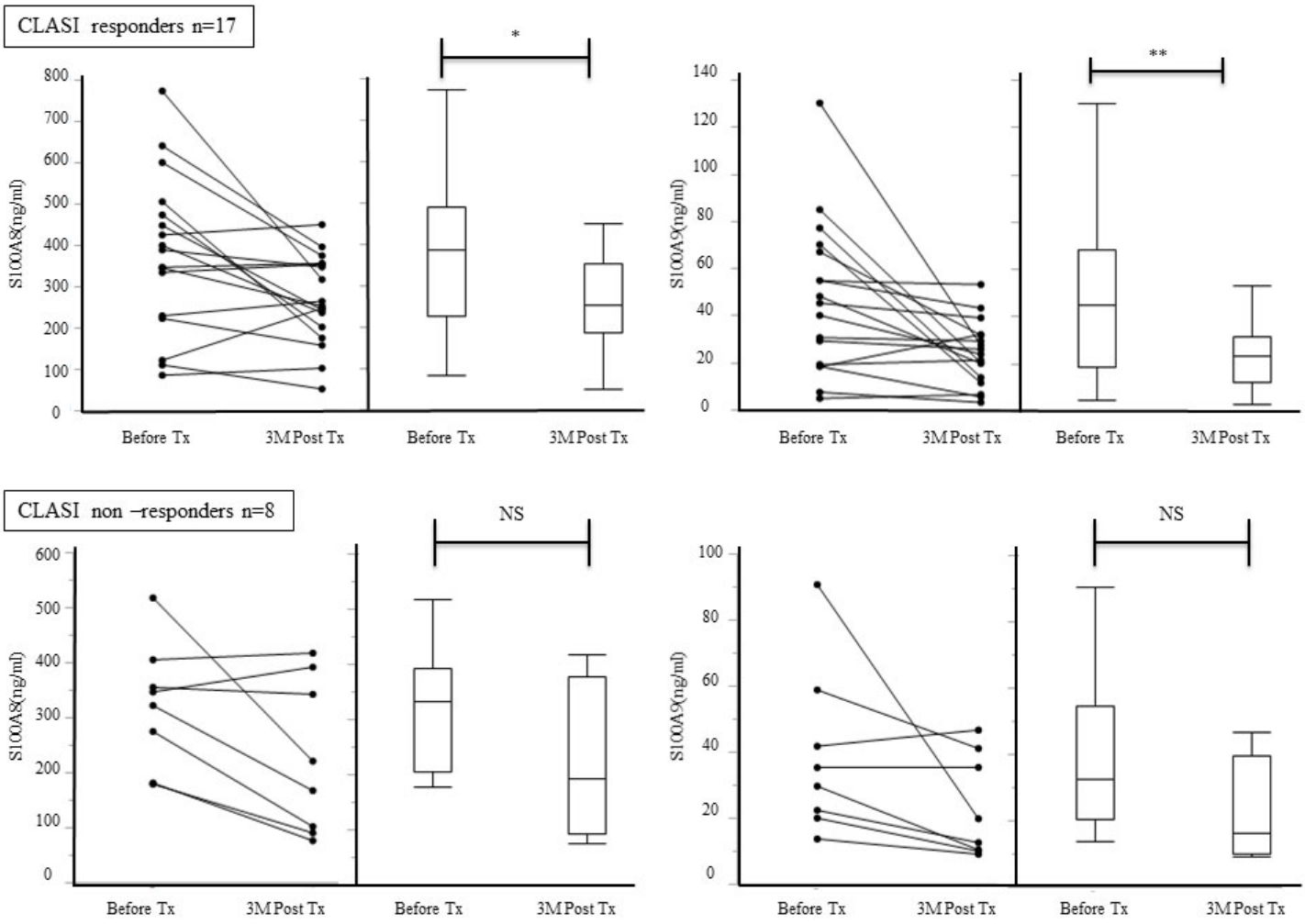

CLASI responders n=17
*
**
S100A8(ng/ml)
S100A9(ng/ml)
Before Tx
3M Post Tx
CLASI non –responders n=8
NS

Figure 4

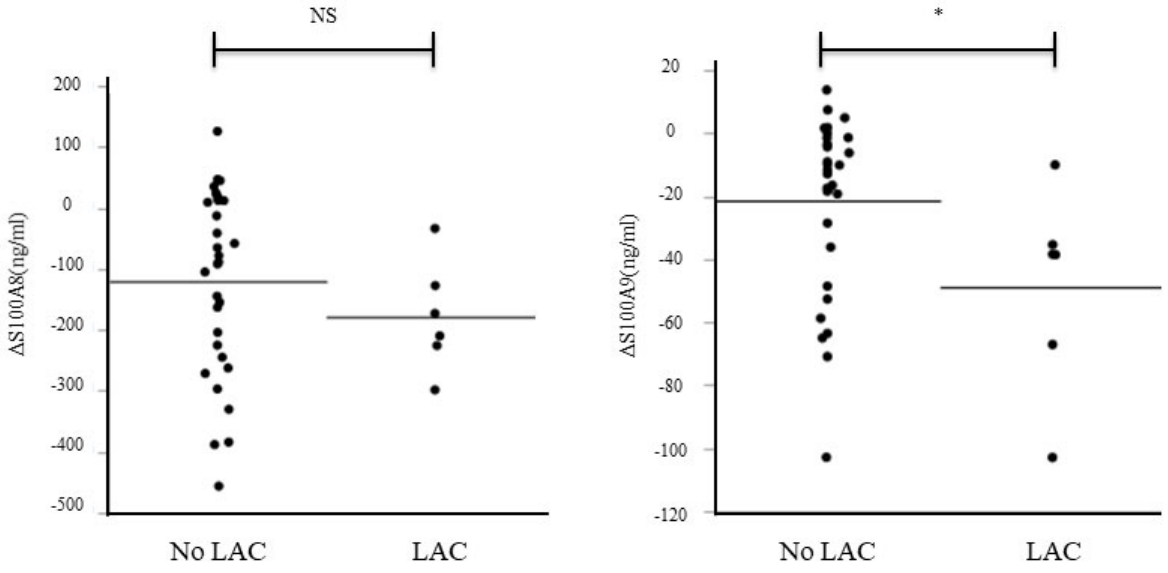
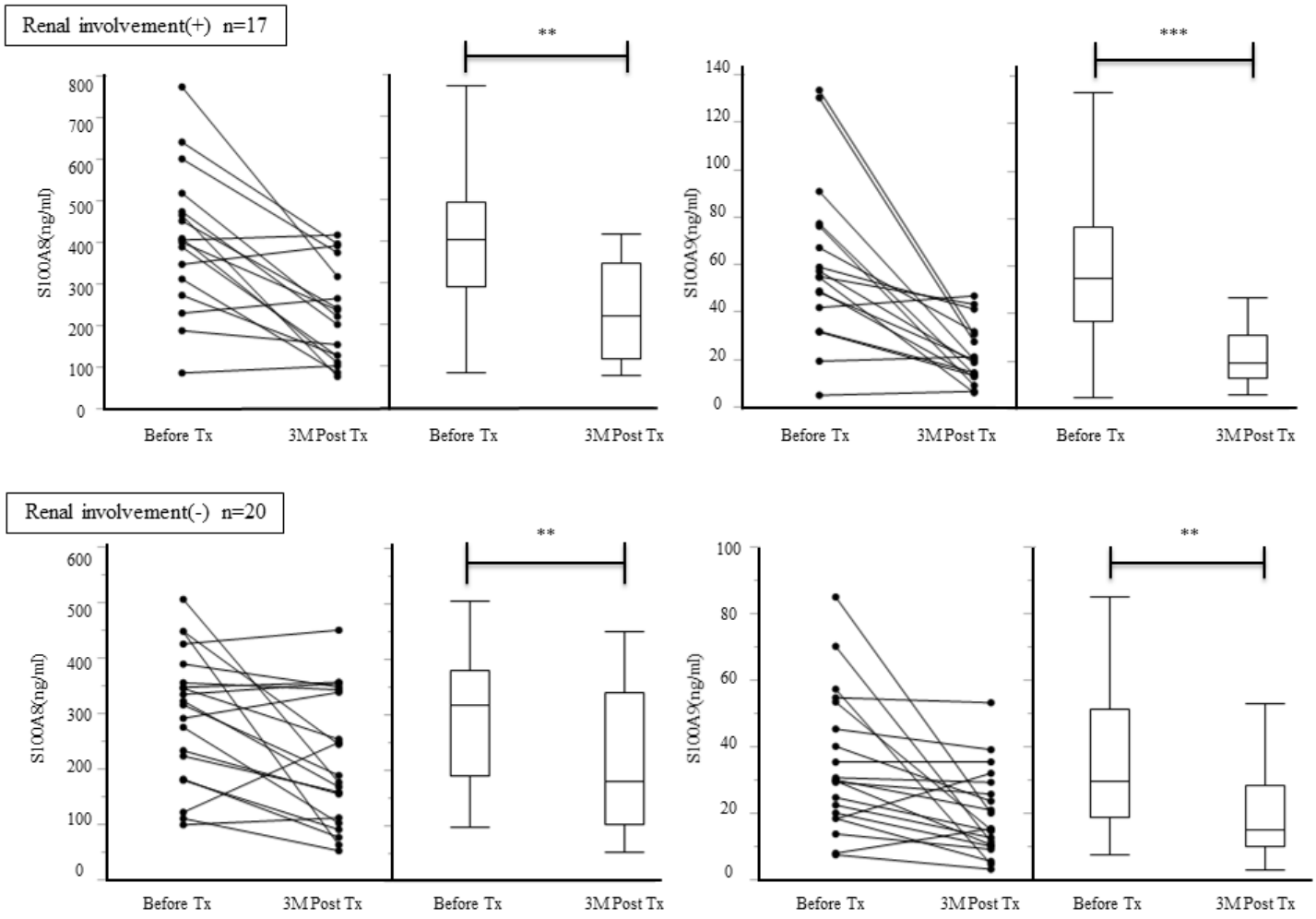
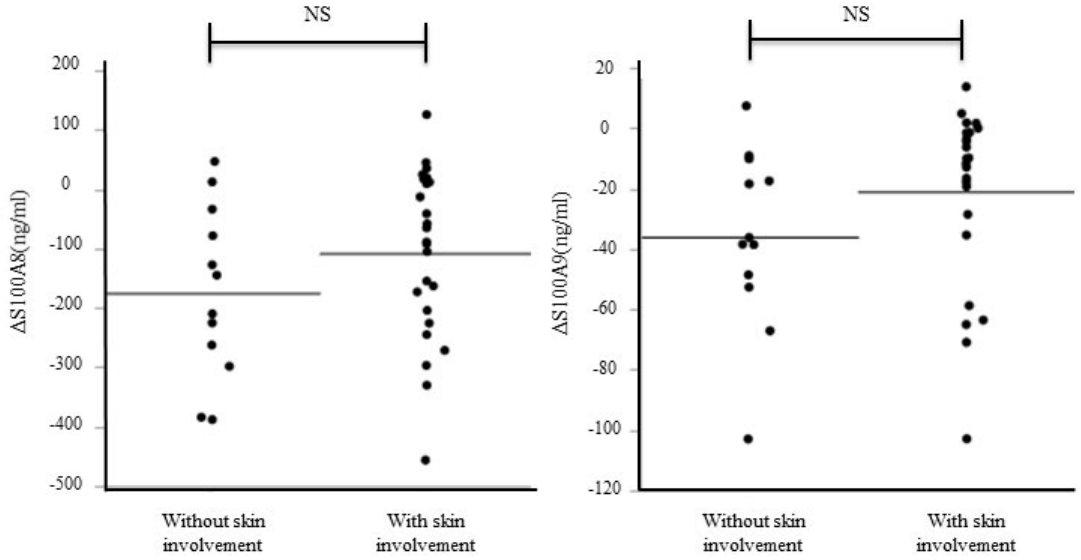

NS
200
100
0
-100
-200
-300
-400
-500
ΔS100A8(ng/ml)
No LAC
LAC
*
20
0
-20
-40
-60
-80
-100
-120
ΔS100A9(ng/ml)
No LAC
LAC

Figure 5

Supplementary Material Figure S1



Supplementary Material Figure S2



Supplementary Material Figure S3