# Elevated Serum BAFF Levels in Patients with Localized Scleroderma in contrast to other organ-specific autoimmune diseases

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**Running title:** BAFF in localized scleroderma

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**Abstract**

Serum levels of B cell activating factor belonging to the tumor necrosis factor family (BAFF), a potent B cell survival factor, are elevated in patients with systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, and systemic sclerosis (SSc). The objective of this study was to determine serum BAFF levels and relate the results to the clinical features in patients with organ-specific autoimmune diseases of the skin, such as localized scleroderma and autoimmune bullous diseases. Serum BAFF levels were examined by ELISA in 44 patients with localized scleroderma, 20 with pemphigus vulgaris/pemphigus foliaceus, 20 with bullous pemphigoid, and 30 healthy controls. Twenty patients with SSc, and 20 with SLE were also examined as disease controls. Serum BAFF levels were elevated in localized scleroderma patients compared to healthy controls. Concerning localized scleroderma subgroups, patients with generalized morphea, the severest form of localized scleroderma, had higher serum BAFF levels than linear scleroderma or morphea patients. The BAFF levels of generalized morphea were comparable with those of SSc or SLE. Furthermore, serum BAFF levels correlated positively with antihistone antibody levels and the severity of skin lesion as well as the number of skin lesions. By contrast, serum BAFF levels were not significantly elevated in patients with pemphigus or pemphigoid. These results suggest that BAFF may be contributing to autoimmunity and disease development in localized scleroderma.

**Keywords:** BAFF, localized scleroderma, autoimmune bullous diseases, B cell, autoantibody

## Introduction

B cell activating factor belonging to the tumor necrosis factor family (BAFF), also known as BLyS, plays a role as a survival and maturation factor for B cells (1). BAFF, which is expressed as a putative type II transmembrane protein, can be shed from the membrane and is detectable in human serum (2-5). BAFF is produced by monocytes, macrophages, neutrophils, dendritic cells, and T lymphocytes (3, 6), and is a ligand for at least three receptors: B-cell maturation antigen (BCMA), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI), and BAFF receptor (BAFF-R) (7, 8). All three receptors are primarily expressed by B cells (1). BAFF/BAFF receptor family appears to span nearly all stages of B-lineage differentiation, ranging from the development, selection, and homeostasis of naive primary B cells to the maintenance of long-lived bone marrow plasma cells (9, 10). In humans, previous reports have shown elevated serum BAFF levels in patients with systemic autoimmune diseases, such as SLE, rheumatoid arthritis (RA), Sjögren's syndrome, and systemic sclerosis (SSc) (2, 4, 5, 11, 12). Furthermore, elevated serum BAFF levels correlated with titer of autoantibodies in these autoimmune disorders (2, 4, 13).

Localized scleroderma is an organ-specific autoimmune disease that involves the skin and the subcutaneous tissues beneath the cutaneous lesions. This disease differs from SSc in that it lacks Raynaud's phenomenon, acrosclerosis, and internal organ involvement. Morphologically, localized scleroderma is classified into three subsets: morphea, linear scleroderma, and generalized morphea (14). Morphea is usually characterized by one or a few circumscribed sclerotic plaques with an ivory-colored center and a surrounding violaceous halo. Linear scleroderma appears in a linear, band-like distribution, and often involves the muscle and bone underlying the skin lesions. Generalized morphea is the severest form of localized scleroderma characterized by widespread skin involvement with multiple lesions. Localized scleroderma, especially generalized morphea, is accompanied by the presence of various autoantibodies, such as antinuclear antibody (Ab) (ANA), antihistone Ab,

anti-single-stranded DNA (ssDNA) Ab, anti-phospholipid Ab, rheumatoid factor, and lupus erythematosus cell phenomenon (15-19). There are ~~a~~utoimmune mechanisms characterized by autoantibody production are considered to play an important role in the induction of localized scleroderma (20).

Autoimmune bullous diseases are also known as organ-specific autoimmune disorders presenting with blisters on the skin and/or mucous membranes. Pemphigus vulgaris/ pemphigus foliaceus (PV/PF) are an autoimmune intraepidermal blistering disease of the skin and mucous membranes (21). Patients with PV/PF develop immunoglobulin G (IgG) autoantibodies against desmoglein 1/3, which is a desmosomal transmembrane glycoprotein that belongs to the cadherin superfamily of cell-cell adhesion molecules (22). Bullous pemphigoid (BP) is also an autoimmune subepidermal skin blistering disease characterized by the linear deposition of IgG and/or C3 along the epidermal basement membrane zone on direct immunofluorescence (23). IgG autoantibody specific for the hemidesmosomal BP antigens BP180 is thought to be crucial for the initiation of the disease (24). B cell abnormalities are likely to contribute to the development of these autoimmune bullous diseases (21, 23, 25)

Despite distinct B cell abnormalities in localized scleroderma and autoimmune bullous diseases, serum BAFF levels were not previously investigated in these diseases. In this study, we examined serum BAFF levels in patients with localized scleroderma and autoimmune bullous diseases.

## Methods

### *Patients*

Serum samples were obtained from 44 Japanese patients with localized scleroderma (31 females and 13 males). Patients were classified into the following 3 subgroups as described previously (14): 17 patients with generalized morphea (13 females and 4 males), 16 patients with linear scleroderma (11 females and 5 males), and 11 patients with morphea (7 females and 4 males). Their median (range) age of patients with localized scleroderma was 16 (1.0-68) years old [generalized morphea, 19.0 (2.0-68); linear scleroderma, 11.5 (1.7-50); and morphea, 24.0 (1.0-67) years old]. The median (range) disease duration of patients with localized scleroderma was 2.0 (0.1-30) years [generalized morphea, 2.0 (0.5-20); linear scleroderma, 2.0 (0.5-30); and morphea, 0.5 (0.1-24) years]. In patients with typical symptoms, the diagnosis was made without skin biopsy. Histological examination was performed to confirm the diagnosis in other patients. None of the localized scleroderma patients were treated with corticosteroids or immunosuppressive therapy. None of the patients had a recent history of infection and abnormal liver function. The number of sclerotic lesions more than 3 cm in diameter was counted in each patient with localized scleroderma when the serum samples were obtained. The sclerotic lesions were morphologically classified into plaque and linear lesions. We divided the whole body into the following 7 areas: head and neck; right upper extremity; left upper extremity; anterior trunk; posterior trunk; right lower extremity; and left lower extremity, then we counted the number of involved areas as described previously (14).

In this study, 20 patients with SSc [15 females and 5 males; age, 45 (14-62) years old], 20 with SLE [16 females and 4 males; 42 (16-58) years old], 20 with PV (n=10)/PF (n=10) [13 females and 7 males; age, 55 (42-83) years old], and 20 with BP [11 females and 9 males; age, 68 (29-85) years old] were also examined as disease control. SSc and SLE were diagnosed according to the criteria proposed by the American College of Rheumatology (26, 27). PV/PF and BP were diagnosed by clinical, pathological, and immunological features

typical for each disease. Direct immunofluorescence showed IgG deposition at the intercellular spaces of epidermal cells in all patients with PV/PF, and linear deposition of IgG and/or C3 along the epidermal basement membrane zone of peribullous skin from BP patients. Thirty Japanese healthy persons [22 females and 8 males; age, 24 (20-35) years old] that were age- and sex-matched to patients with localized scleroderma were used as healthy controls. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C prior to use. All study participants provided written informed consent. The protocol was approved by the Kanazawa University Graduate School of Medical Science and Kanazawa University Hospital.

***ELISA***

Specific enzyme-linked immunosorbent assay (ELISA) kits were used for measuring serum BAFF levels (R&D systems, Minneapolis, MN, U.S.A), according to the manufacturer's protocol. All sera were preabsorbed with protein A (Amersham Biosciences, Piscataway, NJ, U.S.A) to deplete Igs. Each sample was tested in duplicate. The detection limit of this assay was 3.38 pg/ml.

***Detection of ANA, antihistone Ab, anti-ssDNA Ab, and rheumatoid factor***

ANA was detected by indirect immunofluorescence using HEp-2 cells as substrate (19). ELISA for antihistone Ab was performed as described previously (19). Briefly, 96-well microtiter plates were coated with total histones (Sigma-Aldrich, St. Louis, MO, U.S.A) at 5 μg/ml. The wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris-buffered saline for 1 hour at 37°C. The serum samples (100 μl) diluted to 1:100 were added to triplicate wells for 90 min at 20°C. After washing four times, the bound Abs were detected with alkaline phosphatase-conjugated goat anti-human IgG or IgM Abs (Cappel, Durham, NC, U.S.A), using p-nitrophenyl phosphate (Sigma-Aldrich) as substrate. The optical density of the wells was subsequently determined. For anti-ssDNA Ab, wells were pretreated for 1 hour with 0.1% protamine sulfate (grade X; Sigma-Aldrich). After rinsing, calf thymus ssDNA

(Sigma-Aldrich) was added at 1 μg/ml. ELISA was then performed as described above. IgM rheumatoid factor was measured using a latex agglutination slide test (Eiken, Tokyo, Japan).

***Statistical Analysis***

Statistical analysis was performed using Mann-Whitney U test for comparison of values, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. *P* values less than 0.05 were considered statistically significant. The data were shown as the median (range) unless otherwise indicated.

## Results

### *Serum BAFF levels in autoimmune diseases*

BAFF levels in serum samples from patients with autoimmune diseases and healthy controls were assessed by ELISA. Serum BAFF levels were significantly higher in patients with localized scleroderma [0.87 (0.35-1.58) ng/ml] than healthy controls [0.42 (0.24-1.09) ng/ml, $P<0.005$; Figure 1]. By contrast, serum BAFF levels in patients with PV/PF and BP, categorized as autoimmune bullous diseases, were comparable to healthy controls. Patients with SSc [1.12 (0.38-2.90) ng/ml] or SLE [1.37 (0.49-3.85) ng/ml] exhibited significantly higher serum BAFF levels than did healthy controls ($P<0.001$ and $P<0.0001$, respectively). Serum BAFF level in patients with localized scleroderma was lower than patients with SSc or SLE ($P<0.001$ and $P<0.0001$, respectively). Concerning localized scleroderma subgroups, patients with generalized morphea [1.08 (0.41-1.58) ng/ml], linear scleroderma [0.79 (0.49-1.14) ng/ml], and morphea [0.71 (0.32-1.28) ng/ml] exhibited increased BAFF levels compared to healthy controls ($P<0.0001$, $P<0.005$, and $P<0.001$, respectively; Figure 2). Especially, serum BAFF levels in patients with generalized morphea, the severest form of localized scleroderma, were significantly higher than other two forms of localized scleroderma (linear scleroderma and morphea; $P<0.05$, Figure 2) and comparable with SSc or SLE (Figures 1 and 2). Thus, serum BAFF levels were significantly elevated in localized scleroderma but not in autoimmune bullous diseases.

### *Frequency of elevated serum BAFF levels in autoimmune diseases*

Values higher than the mean + 2SD (1.02 ng/ml) of the control serum samples were considered to be elevated in this study. Elevated serum BAFF levels were found in 32% (14/44) of patients with localized scleroderma (Table 1). In the subgroups of localized scleroderma, patients with generalized morphea (53%) had more frequently elevated serum BAFF levels than linear scleroderma (13%) and morphea (27%). Frequency of elevated serum BAFF levels in patients with generalized morphea was similar to these of SSc (55%) and SLE (55%). By contrast,

elevated serum BAFF levels were found in only 10% of patients with PV/PF and 15% of patients with BP. One (3%) of 30 healthy controls had elevated serum BAFF level. Thus, elevated serum BAFF levels were frequently detected in patients with generalized morphea.

***Clinical correlation of serum BAFF levels in localized scleroderma***

Clinical and laboratory parameters obtained at the first evaluation were compared between localized scleroderma patients with elevated serum BAFF levels and those with normal levels. There was no significant difference in age at onset, sex, and disease duration between patients with elevated BAFF levels and those with normal levels (Table 2). Localized scleroderma patients with elevated BAFF levels exhibited significantly higher numbers of plaque lesions and total lesions than those with normal levels ($P<0.05$). IgM and IgG antihistone Ab levels were significantly increased in localized scleroderma patients with elevated BAFF levels compared to patients with normal BAFF levels ($P<0.05$ and $P<0.01$, respectively). Although the level of IgG anti-ssDNA Ab and prevalence of IgM rheumatoid factor tended to be increased in localized scleroderma patients with elevated BAFF levels compared to those with normal levels, there was no statistical significance. In addition, serum BAFF levels in patients with localized scleroderma correlated positively with IgM and IgG antihistone Ab levels ($r=0.47$, $P<0.05$, and $r=0.42$, $P<0.05$, respectively; Figure 3), but did not correlated with IgG anti-ssDNA Ab levels. By contrast, no significant association was detected between clinical or laboratory features and serum BAFF levels in patients with autoimmune bullous diseases (data not shown). Thus, serum BAFF levels correlated with the disease severity as well as antihistone Ab levels in patients with localized scleroderma.

## Discussion

As far as we know, this is the first report to reveal elevated serum BAFF levels in organ-specific autoimmune diseases. Among skin-specific autoimmune disorders, localized scleroderma but not PV/PF or BP showed significantly elevated serum BAFF levels compared to healthy controls (Figure 1). However, serum BAFF levels in patients with localized scleroderma were significantly lower than in patients with SSc or SLE. Furthermore, serum BAFF levels in patients with localized scleroderma were not significantly higher than in patients with PV/PF or BP. In the 3 subgroups of localized scleroderma, serum BAFF levels in patients with generalized morphea, the severest form of localized scleroderma, were significantly higher than other two forms of localized scleroderma and comparable with SSc or SLE. Elevated BAFF levels were associated with antihistone Ab levels and the severity of skin lesion in patients with localized scleroderma.

BAFF is an essential component of B cell homeostasis and a potent B cell survival factor associated with systemic autoimmune diseases in animals (8, 28, 29). Excess BAFF rescues self-reactive B cells from anergy, which may play a crucial role of autoimmune induction and development (30). Previous studies have provided strong evidence that constitutive BAFF overproduction in mice leads not only to polyclonal hyper-γ–globulinemia, but also to spontaneous production of multiple autoantibodies, circulating immune complexes, and renal Ig deposits (8, 28, 29). In humans, elevated serum BAFF levels have been found in patients with systemic autoimmune diseases, including SLE, RA, Sjögren's syndrome, and SSc (2, 4, 5, 11, 12). Elevated BAFF levels are associated with the disease activity in SSc, while there is no association in SLE, RA, or Sjögren's syndrome. In addition, elevated serum BAFF levels correlate with titer of autoantibodies, such as anti-double-stranded DNA Ab, rheumatoid factor, and anti-Ro/La Ab, in SLE, RA, and Sjögren's syndrome, respectively (2, 4, 13). Similarly, our results demonstrated that elevated BAFF levels were associated with antihistone Ab levels in patients with localized scleroderma. BAFF

Localized scleroderma exhibits autoimmunity as a central feature of the disease, as ANA is detected in 46-80% of patients (16, 19, 31, 32). One of the major autoantigens for ANA in localized scleroderma is nuclear histone (14, 19, 33). The major antigen components recognized by antihistone Ab are histones H1, H2A, and H2B in this disease (18). Since histones H1, H2A, and H2B are located on the outer side of the nucleosome and are relatively more accessible for Ab binding (34), it has been hypothesized that antihistone Ab is induced by nucleosome or native chromatin as immunogens in localized scleroderma (18). We have detected antihistone Ab in 47% of patients with localized scleroderma (19). The frequency of positivity for antihistone Ab was 87% in patients with generalized morphea, which was much higher than that in patients with linear scleroderma (32%) or morphea (25%) (19). Similarly, patients with generalized morphea had higher serum BAFF levels than linear scleroderma and morphea, milder forms of localized scleroderma in this study. Furthermore, serum BAFF levels in patients with localized scleroderma correlated positively with IgM and IgG antihistone Ab levels. Localized scleroderma patients with elevated BAFF levels exhibited significantly higher numbers of plaque lesions and total lesions than those with normal levels. Therefore, excessive BAFF rescues self-reactive B cells from anergy, which may contribute to the induction of autoimmunity, the production of autoantibodies in localized scleroderma. Although it is unclear that augmented BAFF production induces the disease expression in localized scleroderma, our previous findings indicated that BAFF expression may be related to the development of skin fibrosis via cytokine production from B cells in SSc (12). Taken together, BAFF may play a crucial role for autoimmune induction and the development of skin sclerosis in localized scleroderma.

Both autoimmune bullous diseases and localized scleroderma are categorized as organ-specific autoimmune diseases involving cutaneous lesion. Before this study, we assumed that serum BAFF levels in patients with autoimmune bullous diseases might be elevated as well as localized scleroderma. B cell depletion therapy by chimeric anti-human CD20 Ab is

reported to be effective in these autoimmune bullous diseases (35, 36). B cell abnormalities characterized by self-reactive B cell and disease-causing autoantibody production are likely to contribute to the development of these autoimmune bullous diseases (21, 23, 25). Nonetheless, serum BAFF levels were not elevated in patients with autoimmune bullous diseases. Although the reasons why serum BAFF levels are different between autoimmune bullous diseases and localized scleroderma are unknown, it may be due to the mechanical difference for inducing autoimmunity. As previous studies demonstrated, serum BAFF levels are generally elevated in systemic autoimmune diseases accompanied by ANA or rheumatoid factor (2, 4, 5, 11, 12). Although the lesions of localized scleroderma are limited to the skin and subcutaneous tissues, patients with this disease frequently produce ANA, including antihistone Ab and anti-ssDNA Ab as well as rheumatoid factor (15-17, 19). Therefore, autoimmunity in localized scleroderma, especially generalized morphea, may be similar to that of systemic autoimmune diseases. By contrast, autoimmune bullous diseases develop autoantibodies against restricted antigens expressed in the skin or mucosal tissues.

Previous reports indicated that there are disease monitoring markers, such as anti-ss-DNA Ab, antihistone Ab, anti-topoisomerase II Ab, and serum manganese superoxide dismutase, in localized scleroderma (19, 20, 37, 38). Our finding suggests that serum BAFF levels may also be a useful serological marker for the disease severity. Since there are a few established basic therapies for skin sclerosis in localized scleroderma, new therapeutic agents have been researched. B cells have been recently recognized as one of the therapeutic targets for systemic autoimmune diseases. Especially, BAFF has been shown to be a therapeutic target in SLE (39). Inhibition of BAFF by TACI-Ig and BAFFR-Ig is successful in treating a murine model of SLE (8, 40). Moreover, treatment with BAFF antagonists, such as human anti-BAFF monoclonal Ab, was already started in SLE patients and showed safety (41, 42). Although the efficacy of B cell-targeted therapy remains unknown in localized scleroderma, our finding that elevated serum BAFF levels were associated with the disease severity in localized scleroderma

suggests that BAFF inhibition may be a potential therapeutic target of severe localized scleroderma as well as SLE.

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**Table 1.** Frequency of elevated serum BAFF levels in autoimmune diseases.

| | Elevated BAFF<br>n (%) |
|---|---|
| Localized scleroderma (n = 44) | 14 (32) |
| Generalized morphea (n = 17) | 9 (53) |
| Linear scleroderma (n = 16) | 2 (13) |
| Morphea (n = 11) | 3 (27) |
| PV/PF (n = 20) | 2 (10) |
| BP (n = 20) | 3 (15) |
| SSc (n = 20) | 11 (55) |
| SLE (n = 20) | 11 (55) |

Values are the number (%) of patients with elavated serum BAFF levels.

PV/PF = pemphigus vulgaris/pemphigus foliaceus, BP = bullous pemphigoid, SSc = systemic sclerosis, SLE = systemic lupus erythematosus.

**Table 2.** Clinical and laboratory data of patients with localized scleroderma showing elevated serum BAFF level.

| | Localized scleroderma | |
|---|---|---|
| | Elevated BAFF (n = 14) | Normal BAFF (n = 30) |
| Age at onset (yr) | 15 (2-25) | 13.5 (0.5-64) |
| Sex (female/male) | 11/3 | 20/10 |
| Disease duration (yr) | 1 (0.4-20) | 2 (0.1-30) |
| Clinical features | | |
| No. of linear lesions | 1 (0-3) | 1 (0-4) |
| No. of plaque lesions | 4.5 (0-10)* | 2 (0-6) |
| Total No. of lesions | 5.5 (1-11)* | 2.5 (1-8) |
| No. of body areas affected | 2 (1-6) | 2 (1-7) |
| Bilateral distribution | 36% | 30% |
| Muscle involvement | 29% | 17% |
| Laboratory findings | | |
| Antinuclear Ab | 57% | 33% |
| IgM antihistone Ab (relative OD) | 0.83 (0.03-2.71) * | 0.24 (0.04-2.00) |
| IgG antihistone Ab (relative OD) | 0.65 (0.19-1.48)** | 0.41 (0.19-1.23) |
| IgG anti-ssDNA Ab (relative OD) | 0.36 (0.16-0.88) | 0.21 (0.10-2.92) |
| IgM rheumatoid factor | 43% | 18% |

All the clinical and laboratory parameters and serum BAFF levels were obtained at the first evaluation.

Unless otherwise indicated, values are median (range). Antihistone Ab and anti-ssDNA Ab levels as the relative optical density (OD) were determined by specific ELISA.

*$P$<0.05 or **$P$<0.01 vs. localized scleroderma patients with normal BAFF levels.

## Legends for illustrations

**Figure 1.** Serum BAFF levels in patients with autoimmune diseases at the first evaluation. BAFF levels were determined by a specific ELISA in serum samples from patients with localized scleroderma (LSc), pemphigus vulgaris/pemphigus foliaceus (PV/PF), bullous pemphigoid (BP), systemic sclerosis (SSc), or systemic lupus erythematosus (SLE) and from healthy controls (Control). The dashed lines indicate the cut-off value (mean + 2SD of the control samples). The lines inside the boxes indicate the median; the outer borders of the boxes indicate 25th and 75th percentiles; the bars extending from the boxes indicate the 10th and 90th percentiles.

**Figure 2.** Serum BAFF levels in the 3 subgroups of localized scleroderma patients. BAFF levels were determined by a specific ELISA in serum samples from patients with generalized morphea (GM), linear scleroderma (Linear Scl.), or morphea and from healthy controls (Control). The dashed lines indicate the cut-off value (mean + 2SD of the control samples). The lines inside the boxes indicate the median; the outer borders of the boxes indicate 25th and 75th percentiles; the bars extending from the boxes indicate the 10th and 90th percentiles.

**Figure 3.** The correlation of serum BAFF levels against antihistone Ab and anti-ssDNA Ab levels in patients with localized scleroderma at the first evaluation. Serum BAFF, antihistone Ab, and anti-ssDNA Ab levels were determined by specific ELISAs.

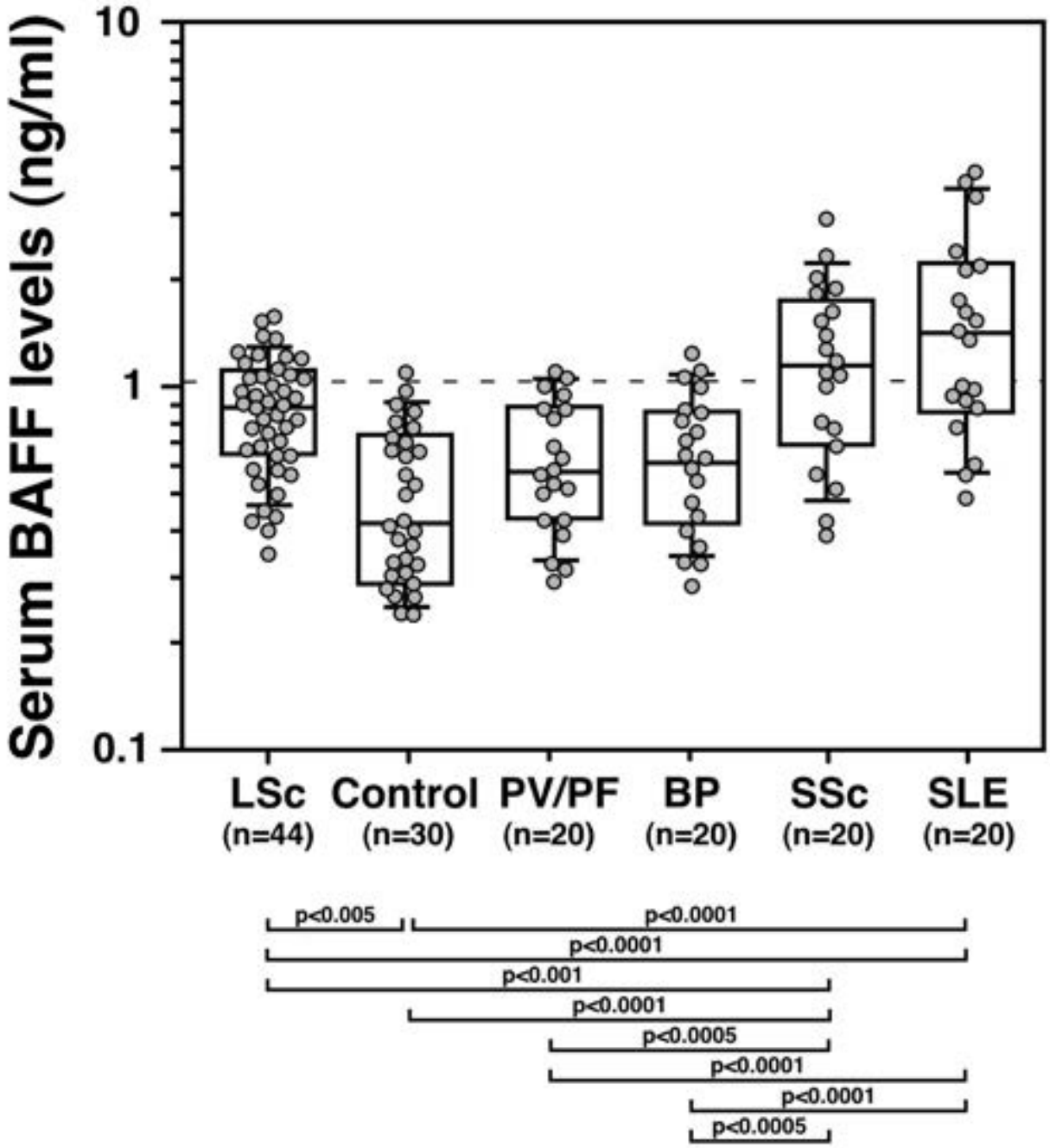


Figure 1
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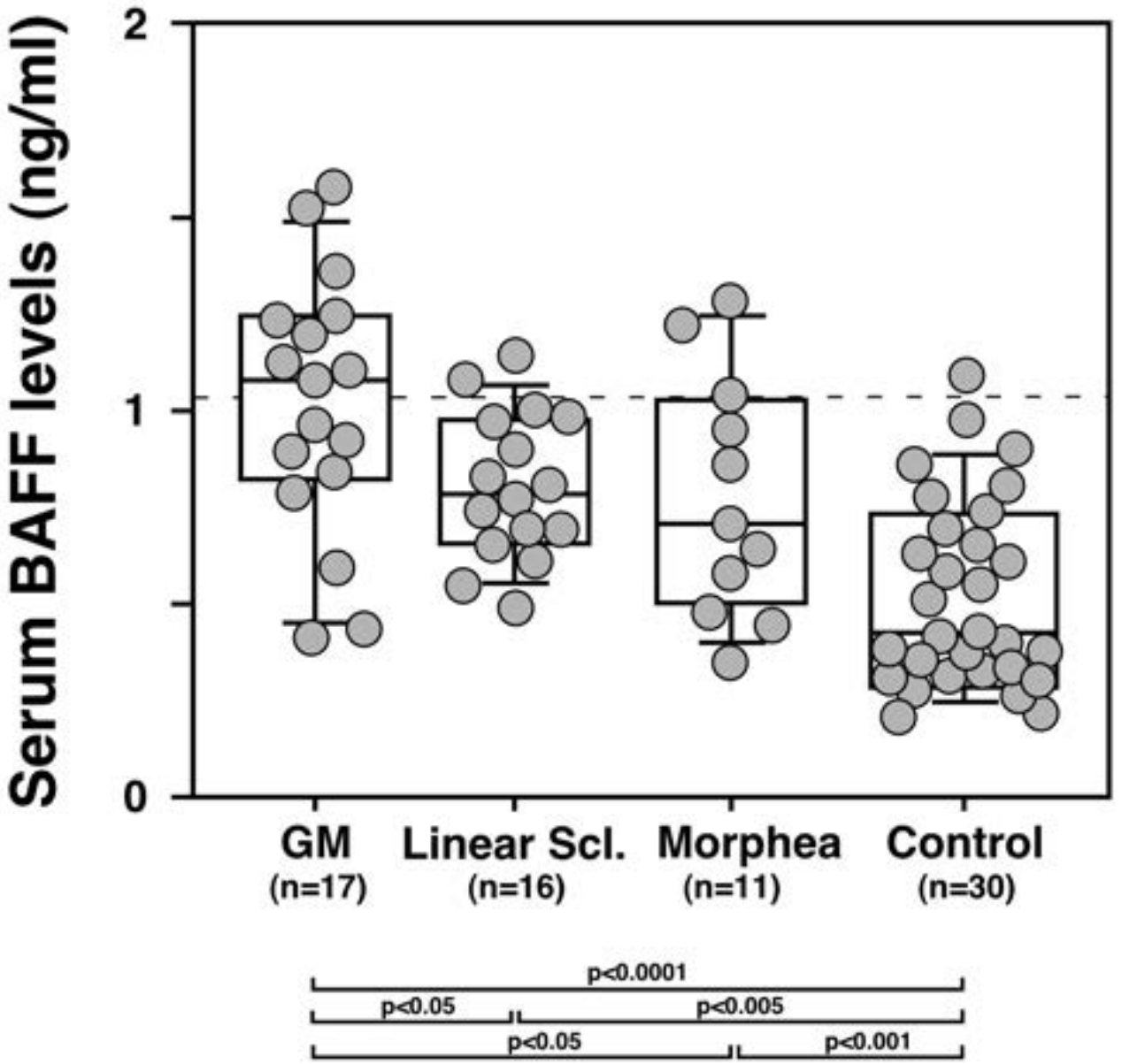


Figure 2
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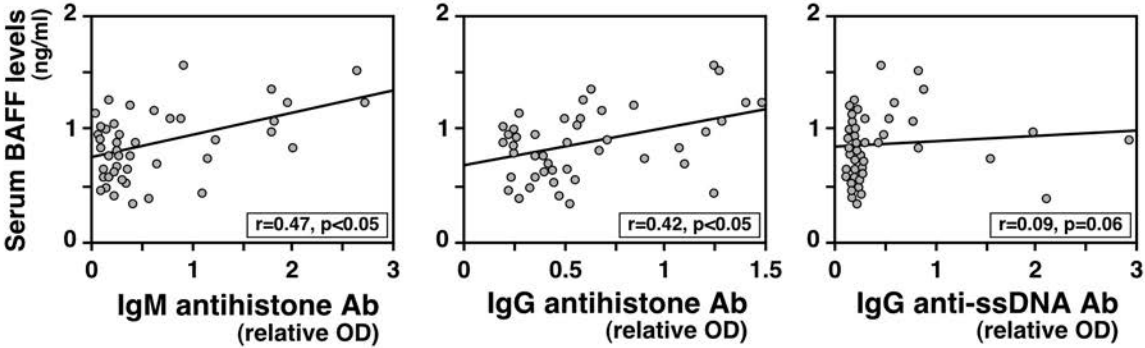


Figure 3
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