タイトル	Stratum corneum levels of calprotectin proteins S100A8/A9 correlate with disease activity in psoriasis patients
別タイトル	角質中のカルプロテクチンタンパク質S100A8/A9 は乾癬患者の疾患活動性と相関する
作成者（著者）	小林(松永), 由紀子
公開者	東邦大学
発行日	2022.03.16
掲載情報	東邦大学大学院医学研究科 博士論文.
資料種別	学位論文
内容記述	主査 : 樋口哲也 / タイトル : Stratum corneum levels of calprotectin proteins S100A8/A9 correlate with disease activity in psoriasis patients /著者 : Yukiko Matsunaga, Yuki Hashimoto, Akira Ishiko /掲載誌 : The Journal of dermatology /巻号・発行年等 : 48(10): 1518-1525, 2021 /本文ファイル: 査読後原稿 /This is the peer reviewed version of the following article:【The Journal of dermatology,49,10】, which has been published in final form at DOI: 【10.1111/1346-8138.16032】. This article may be used for non-commercial purposes in accordance With Wiley Terms and Conditions for self-archiving.
著者版フラグ	ETD
報告番号	32661甲第1050号
学位記番号	甲第698号
学位授与年月日	2022.03.16
学位授与機関	東邦大学
DOI	info:doi/10.1111/1346-8138.16032
その他資源識別子	https://onlinelibrary.wiley.com/doi/10.1111/1346-8138.16032
メタデータのＵＲＬ	https://mylibrary.toho-u.ac.jp/webopac/TD61362968

Stratum corneum levels of calprotectin proteins S100A8/A9 correlate with disease activity in psoriasis patients

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Short running title: SC S100A8/A9 levels in psoriasis

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ABSTRACT

Background: Psoriasis is an intractable inflammatory skin disorder characterized by scaly erythema and plaques. The Psoriasis Area and Severity Index (PASI) is widely used to score disease severity, but evaluation is subjective, and an objective biomarker would be useful. Stratum corneum (SC), which can be non-invasively harvested, may reflect psoriasis-associated changes in epidermal keratinocytes, such as the upregulation of the calprotectin proteins S100A8 and S100A9.

Objective: To examine the availability of S100A8/A9 protein levels in SC as a biomarker of psoriasis disease activity.

Methods: Fifty-three patients with psoriasis, 30 with psoriasis vulgaris (PsV), and 23 with psoriatic arthritis (PsA) participated. Stratum corneum cells (SC) from lesional and non-lesional skin were collected by tape-stripping. S100A8/A9 levels in serum and in SC were quantified by ELISA and compared with PASI score before and after treatment initiation or switching. Atopic dermatitis (AD) patients and disease-free individuals were used as controls.

Results: Expression of S100A8/A9 in SC of lesional skin of psoriasis patients was significantly higher than in non-lesional skin or AD skin. There was no significant difference of SC S100A8/A9 levels between PsV and PsA patients. The S100A8/A9 levels in SC of psoriasis patients were significantly positively correlated with the PASI

score. When patients' skin lesions cleared (PASI clear) in response to treatment, expression of S100A8/A9 in SC was no longer detectable.

Conclusion: S100A8/A9 protein levels in SC may be available as an objective, non-invasive biomarker of psoriasis activity to complement PASI scoring.

Key words:

Psoriasis, S100A8/A9, biomarker, stratum corneum, Psoriasis Area and Severity Index (PASI) score

INTRODUCTION

Psoriasis is characterized by epidermal hyperproliferation and inflammatory cell infiltration due to immune disorders associated with genetic predisposition and environmental factors. Its clinical manifestations include scaly erythema and plaques over the whole body, which can significantly reduce quality of life, and its incidence appears to be increasing.[1] Activated T cells, such as Th1, Th17, and Th22, are involved in the development of psoriasis, and Th17 cells in particular play a central role, producing multiple inflammatory cytokines, including IL-17A, IL-17F, IL-22, IL-26, and IL-21,[2, 3] which are involved in the onset and progression of psoriasis through their interactions with dendritic cells, neutrophils, and keratinocytes. Formation of Th17 cells from CD4-positive central memory T cells is induced by cytokines such as IL-1β, IL-6, TGF-β, and IL-21.

Most psoriasis patients are treated with topical medication, but a considerable number require additional systemic therapy, such as ultraviolet radiation, retinoids, apremilast, cyclosporin, or biologics. Evaluation of disease activity is important for the appropriate

choice of treatment, and the Psoriasis Area and Severity Index (PASI) is a well-established scoring system. However, the PASI score is a compilation of subjective evaluations by a physician based on visual inspection and palpation, and consequently assessments by different evaluators may differ. Thus, there is a need for an objective biomarker of disease activity.

Schonthaler, *et al*. recently showed that S100A8 and S100A9 proteins are highly upregulated in psoriatic epidermis.[4] The pro-inflammatory S100 family consists of approximately 20 calcium-binding proteins with two EF-hand motifs.[5] S100A8 (also known as MRP8, calgranulin A, and CP-10) and S100A9 (also known as MRP14 and calgranulin B) form a heterodimer called calprotectin that is expressed in neutrophils, monocytes, Schwann cells, and keratinocytes during inflammation.[6-9] Human calprotectin has a molecular weight of approximately 24 kDa (S100A8 11 kDa, S100A9 13 kDa), and is one of the alarmins that induce cell proliferation, inflammation, and angiogenesis through pattern-recognition receptors (PRRs) such as TLR-4 and the receptor for advanced glycation end-products (RAGE).[10, 11] It has antibacterial and antifungal activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[12]

S100A9 was shown to be involved in the pathology of psoriasis via regulation of the complement component C3. Moreover, deletion of S100A9 by double knockout of the *Jun/Junb* genes strongly attenuated psoriasis-like skin disease and inflammation in mice. [4] Thus, epidermal S100A8/A9 is a candidate target for psoriasis diagnosis and treatment, [13] and has been proposed as a biomarker based on its overexpression in the psoriatic epidermis[14] and its decrease in patients treated with biologics such as anti-TNF-α and anti-IL-12/23 p40 antibodies.[14, 15] However, these studies utilized skin biopsies, which are too invasive to perform repeatedly in clinical practice. In this study, therefore, we

aimed to establish whether S100A8/A9 in the stratum corneum (SC), which can be collected by non-invasive tape-stripping, might be available as a biomarker of psoriasis disease activity.

METHODS

This study was approved (no. A19008-A17097-(27126)) by Toho University School of Medicine Ethics Committee, and conducted in accordance with the Declaration of Helsinki. All participants received a full explanation of the methods and aims of the study, and gave their informed consent to participate.

Sampling

A total of 68 patients and 7 healthy controls were enrolled. The psoriasis and AD patients met the diagnostic criteria defined in the guidelines for each disease published by the Japanese Dermatological Association. Among the psoriasis patients, the differential diagnosis of 39 cases was confirmed by biopsy, while 14 cases had previously been diagnosed at the time of admission to our hospital and had already started treatment. The diagnosis of AD was based on the state of expression and distribution of characteristic eruptions, medical history, serum TARC level and IgE antibody level.

Patients with psoriasis were recruited before introduction or switching of systemic therapy, including biologics (infliximab, adalimumab, ustekinumab, secukinumab, ixekizumab, brodalmab, guselkumab, risankizumab), apremilast, cyclosporin, methotrexate, and utraviolet therapy. Since our aim was to examine the usefulness of SC S100A8/A9 for the purpose of monitoring local inflammatory conditions, we evaluated the patients' SC S100A8/A9 levels at the time of examination without requiring a

withdrawal period for topical drugs that the patients were receiving. Patients with atopic dermatitis (AD) were included as disease controls, and healthy participants as normal controls. A 10 cm piece of cellophane tape (Nichiban, Japan) was applied to normal or lesional skin, and SC was collected by tape-stripping the skin surface several times. For psoriasis and AD patients, samples were taken from the largest lesion with a typical appearance, usually at the lower back or lower legs, and from nearby non-lesional skin. At the same time, the PASI score for patients with psoriasis and the Eczema Area and Severity Index (EASI) score for patients with AD were calculated according to the standard evaluation criteria. The same physician assessed the PASI and EASI scores throughout this study. Psoriasis patients who consented were re-examined several weeks after the start of systemic treatment at the site of the originally targeted lesion.

Protein extraction and quantification

The first tape strip was discarded to avoid possible environmental contamination, and the second tape strip was used for the evaluation of S100A8/A9 in order to minimize invasiveness for the patient, as described previously.[16, 17] Horii et al. have reported that the amount of amino acids in successive strippings of the SC reached a plateau at the 5th tape, but the 2nd tape had a content of 70-80% of that of the 5th sheet.[18] It was also reported elsewhere that the composition of the 2nd tape is almost the same as that of deeper tapes.[19]

Each strip was cut into small pieces, which were placed in an ultrasonic homogenizer (BIORUPTOR, Sonic Bio Inc., Japan) with 0.1 mol/L Tris-HCl bio buffer solution (pH 8.0) to extract proteins. Total protein in the SC extract was quantified using the DC Protein Assay Reagent (Bio Rad, USA). After appropriate dilution with extraction buffer,

S100A8/A9 was quantified using the Human S100A8/S100A9 Heterodimer Quantikine ELISA Kit (R&D Systems, USA). Amounts of S100A8/A9 are expressed amount per 1 mg protein of the SC sample. All samples were assayed in duplicate.

Statistics

The data are presented as mean values ± standard error. For nonparametric multiple comparison between groups, we employed the Steel-Dwass method. For comparison of lesional skin and non-lesional skin, the Wilcoxon signed rank sum test was used. Spearman correlation coefficients were used to evaluate the association between biomarkers and disease severity, with $p < 0.05$ being considered significant.

RESULTS

Patients' background

SC and sera were sampled from 34 patients with PsV (27 males, 7 females), 24 patients with PsA (21 males, 3 females), 10 patients with AD as disease controls, and 7 healthy participants as normal controls. Finally, data from 5 patients in the psoriasis group were excluded for the following reasons: diagnosis of AD in addition to psoriasis for 2 patients, diagnosis of guttate psoriasis for two patients, and incorrect diagnosis of psoriasis for 1 patient. Two patients from the AD group and 2 from the healthy participant group were excluded as outliers (over 2 standard errors from the mean).

The EASI score of one patient showing mild symptoms in the AD group was not determined, and consequently, data for only 7 patients were included in the correlation calculation. The mean PASI score for psoriasis patients on initial stripping was 12.7, and the mean EASI score for AD patients was 19.7. The number of patients and their average

age by gender for each condition are shown in Table 1.

Increased expression level of SC S100A8/9 in lesional skin of patients with psoriasis

The amounts of S100A8/9 per 1 mg total protein of SC (SC S100A8/A9), determined by ELISA, were compared among psoriasis patients, AD patients, and normal controls (Figure 1). The average expression level in the SC of healthy participants was 0.04±0.02 μg/mg protein, while significantly higher levels of 5.1±2.5 μg/mg in AD lesions and 53.5 ± 9.0 μg/mg in psoriasis lesional skin were seen ($p < 0.01$). The amount of SC S100A8/A9 in the non-lesional skin was slightly elevated at 2.9±0.4 μg/mg. Thus, the level of SC S100A8/A9 in psoriasis lesions was more than 1000 times greater than that of normal controls, and more than 10 times greater than that of the non-lesional skin of patients.

No significant difference in SC S100A8/A9 between PsV and PsA

Patients with PsV showed SC S100A8/A9 levels of 48.1±9.5 μg/mg in lesional skin and 2.9±1.3 μg/mg in non-lesional skin, while patients with PsA showed levels of 41.8±8.9 μg/mg and 2.5±0.6 μg/mg, respectively (Figure 2). There was no significant difference between the two subtypes in either lesional or non-lesional skin.

Correlation of SC S100A8/A9 with PASI score in psoriasis patients

The amounts of SC S100A8/A9 in both lesional and non-lesional skin of patients with psoriasis showed positive correlations with the PASI score (Figure 3). The correlation coefficient in lesional skin was 0.265 ($p = 0.019$) and that in non-lesional skin was 0.355 ($p = 0.0013$). The SC S100A8/A9 level and the EASI score of AD patients showed no

significant correlation ($p = 0.525$ and $p = 0.659$ in lesional and non-lesional skin, respectively).

Change of SC S100A8/A9 in lesional skin of psoriasis patients in response to therapy

Twelve patients with psoriasis treated with biologics all showed considerably reduced PASI scores (Figure 4a). Concomitantly, the amount of SC S100A8/A9 in lesional skin sites decreased to almost zero (Figure 4b). The SC S100A8/A9 levels following resolution of the psoriasis after treatment were comparable to those of normal controls and were also much less than those of non-lesional skin in the active phase of psoriasis.

Figure 5 shows a representative case of successful treatment. This 56-year-old male PsV patient showed a remarkable response to blodalumab (anti-IL-17 receptor A antibody). As shown in Figure 5b, the PASI score fell from 25 to 0 (clear) and the SC S100A8/A9 level fell from 226.3 μg/mg protein to below the detection limit of the ELISA kit (0.000086 μg/mL) after treatment.

Figure 6 shows a representative case of unsatisfactory improvement after treatment. A 37-year-old man with PsV (PASI 12.5) was treated with apremilast (a PDE4 inhibitor). His PASI score gradually decreased until 9 weeks after treatment, but he relapsed after 18 weeks. The SC S100A8/A9 level of the lesional skin initially decreased in line with the PASI score, but then started to increase as early as 9 weeks, i.e., before the PASI score began to increase.

DISCUSSION

Our finding that S100A8/A9 is expressed at a significantly higher level in the SC of lesional skin of psoriasis patients compared with non-lesional skin or AD lesional skin

suggests that SC S100A8/A9 might be a good marker of psoriatic lesions. There was no significant difference between PsA lesions and PsV lesions. However, the fact that SC S100A8/A9 was also detected in AD means that elevation of SC S100A8/A9 is not specific to psoriasis, but nevertheless, the expression level in psoriasis was approximately 10 times higher than in AD. Furthermore, the amount of SC S100A8/A9 correlated well with the PASI score of psoriasis patients, whereas it did not correlate with the EASI score in AD patients. Moreover, SC S100A8/A9 decreased in parallel with the improvement of symptoms during treatment of psoriasis patients, becoming undetectable after successful treatment (i.e., in PASI-clear patients). These results suggest that SC S100A8/S100A9 in SC could serve as an objective biomarker of psoriasis disease severity that would be compatible with the daily practice of dermatology clinics, since tape-strips can easily be frozen and stored until required for protein extraction. The tape-stripping method employed here has already been demonstrated to be useful for biomarker discovery and treatment outcome evaluation in patients with AD.[20]

It is interesting that SC S100A8/A9 levels were elevated in the uninvolved skin of patients with active psoriatic lesions. S100A8/A9 is a type of alarmin locally released during cell stress and is an early amplification product of inflammation. There are two possibilities as to the origin of SC S100A8/A9 in the uninvolved skin. One possibility is that S100A8/A9 is released from normal-appearing keratinocytes just beneath the stripped keratinocytes, and the other is that it originates from psoriasis lesions remote from the stripped area. The former would imply that non-lesional skin might be involved in the systemic inflammatory condition of psoriasis patients. The latter is also plausible, because S100A8/A9 can be secreted extracellularly. Further study will be needed to resolve this issue.

In addition to S100A8/A9, we also quantified BD-2 (beta-defensin-2),[21] which is expressed in the SC and is proposed to be a biomarker candidate for AD. But, although BD-2 tended to be increased in the SC of patients with psoriasis, there was no correlation with disease severity (Supporting information 1). We also quantified IL-1α, an early inflammatory cytokine expressed in the SC, but found no apparent relationship to disease severity (data not shown).

In addition, we quantified S100A8/A9 in patients' serum. S100A8/A9 is elevated in synovial fluid of patients with rheumatoid arthritis and in serum of patients with cardiovascular disease, AD, and PsA,[22-25] and the serum level correlates with body steatosis and white blood cell count.[26] In contrast, a previous study reported no correlation between PASI score and serum S100A8/A9 protein levels in psoriasis patients.[14] Here, we found that serum S100A8/A9 was moderately elevated not only in psoriasis, but also in AD, compared with healthy controls, but the differences were not significant (Supporting information 2). In patients with psoriasis, we found a weak but significant correlation between serum S100A8/A9 level and PASI score (Supporting information 3). Interestingly, however, we found that some patients had elevated serum S100A8/A9 levels despite administration of biologics leading to PASI-clear status. The reason for this may be that serum S100A8/A9 level also reflects systemic inflammatory status associated with other disorders such as rheumatism and carotid atherosclerosis.[27] Thus, it seems likely that the SC S100A8/A9 levels would be preferable for evaluating cutaneous-specific pathological conditions.

Although the PASI score is widely used for quantitative assessment of skin severity based on assessment of redness, infiltration, and desquamation,[28] it is known to vary depending on the assessment skill and clinical experience of the evaluator, so it is not an

ideal index for treatment selection. Furthermore, the PASI scoring scheme may not fully reflect the severity of eruptions individual sites, such as the scalp and upper limbs. This can be seen in the present study, in which we encountered a refractory patient with a particularly severe scalp eruption, who showed a PASI score as low as 5.6, although the SC S100A8/A9 level was as high as 341 μg. This may suggest that the use of SC S100A8/A9 as a diagnostic marker could be valuable as a complement to PASI scoring.

In conclusion, our results suggest that S100A8/A9 in SC could be useful as a non-invasive biomarker of psoriasis disease activity. We believe our findings justify a larger-scale trial to assess whether the combination of SC S100A8/A9 and PASI scoring would be superior to PASI scoring alone as a basis for treatment selection for psoriasis patients. Furthermore, SC S100A8/A9 might also be a useful biomarker for efficacy assessment of new therapeutic candidates.

ACKNOWLEDGMENTS:

This work was supported by a grant from the Private University Research Branding project from the MEXT (Ministry of Education, Culture, Sports, Science and Technology), Japan.

We thank Dr. Toshihiko Hibino (Shiseido Global Innovation Center) for his valuable advice and Ms. Yoko Araki for her technical support.

CONFLICT OF INTEREST:

The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1. The amount of stratum corneum S100A8/A9 in psoriasis is significantly higher than that in atopic dermatitis and healthy subjects.

Protein was extracted from the SC of lesional skin collected by tape stripping, and S100A8/A9 was quantified by means of ELISA. S100A8/A9 is rarely expressed in the normal SC, but is slightly increased in the SC of lesional skin of AD. The amount of SC

S100A8/A9 per mg total protein in psoriasis patients was significantly higher than that in atopic patients. Mean ±se, p*<0.05, p**<0.01, p***<0.001

Figure 2. In both PsV and PsA, the amount of S100A8/A9 in lesional skin is greater than that in non-lesional skin.

The amounts of SC S100A8/A9 in the lesional skin and non-lesional skin of PsV (a) patients and PsA (b) patients were compared. In both conditions, the protein levels of S100A8/A9 in lesional skin were significantly higher than in non-lesional skin. Mean ± se, p***<0.001.

Figure 3. Correlation between PASI score and amount of SC S100A8/9 in lesional skin of psoriasis patients.

PASI score was weakly but significantly correlated with SC S100A8/A9 amount in lesional skin (a) and non-lesional skin (c) of psoriasis patients. These plots include data measured from the second and subsequent samplings after treatment. In patients with atopic dermatitis, the correlations in lesional skin (b) and non-lesional skin (d) were not significant. R; Pearson's correlation coefficient (5% on both sides)

Figure 4. S100A8/A9 in the SC reflects the therapeutic effect of psoriasis treatment.

(a) PASI score before and after treatment with biopharmaceuticals for psoriasis. (b) SC S100A8/A9 levels measured at the same times (b). Changes in both values are well synchronized.

Figure 5. Improving PASI score is associated with reduced amount of SC S100A8/A9 in a psoriasis patient receiving long-term administration of biopharmaceutical.

This is a case of a 56-year-old male with PsV. PASI score at 6 weeks before the administration of brodalumab (IL-17 receptor A antibody) was 27.8, and PASI clear was achieved 52 weeks later. Changes in the appearance of the patient's skin are shown in (a). As shown in (b), SC S100A8/A9 (solid line) in the lesional skin decreased significantly as PASI score (bar graph) decreased.

Figure 6. Increase of SC S100A8/9 levels during treatment in a PsV patient is followed by worsening of the PASI score.

This is a case of a 37-year-old male PsV patient. Oral administration of apuremilast, a PDE4 inhibitor, gradually lowered the PASI score. However, SC S100A8/A9 (solid line) started to increase from 9 weeks, followed by worsening of the PASI score (bar graph) at 18 weeks.

SUPPORTING INFORMATION

Supporting information 1. SC h BD-2 of psoriasis patients showed no correlation with PASI

h (human) BD-2 was also detected at higher levels in the SC of psoriasis patients than in healthy individuals, but there was no correlation with PASI.

Supporting information 2. Serum S100A8/A9 of psoriasis and AD showed a mild increase.

Serum level of S100A8/A9 was mildly elevated in psoriasis and AD. However, there was

no significant difference compared with healthy subjects.

Supporting information 3. Serum S100A8/A9 of psoriasis patients showed a weak positive correlation with PASI.

The correlation coefficient between PASI and serum S100A8/A9 in psoriasis patients was 0.29. However, serum S100A8/A9 was high despite low PASI scores in some patients.

Table 1.

	Male	Age (years)	Female	Age (years)	Total
Psoriasis vulgaris	24	49.4 ± 2.9	6	55.2 ± 6.0	30
Psoriasis arthritis	20	49.3 ± 2.7	3	68.0 ± 11.5	23
Atopic dermatitis	6	41.0 ± 4.8	2	35.5 ± 6.5	8
Healthy volunteers	2	51.5 ± 6.5	3	48 ± 10.4	5
	53		13		66

Values are expressed as mean ± standard error of the mean

Figure 1.

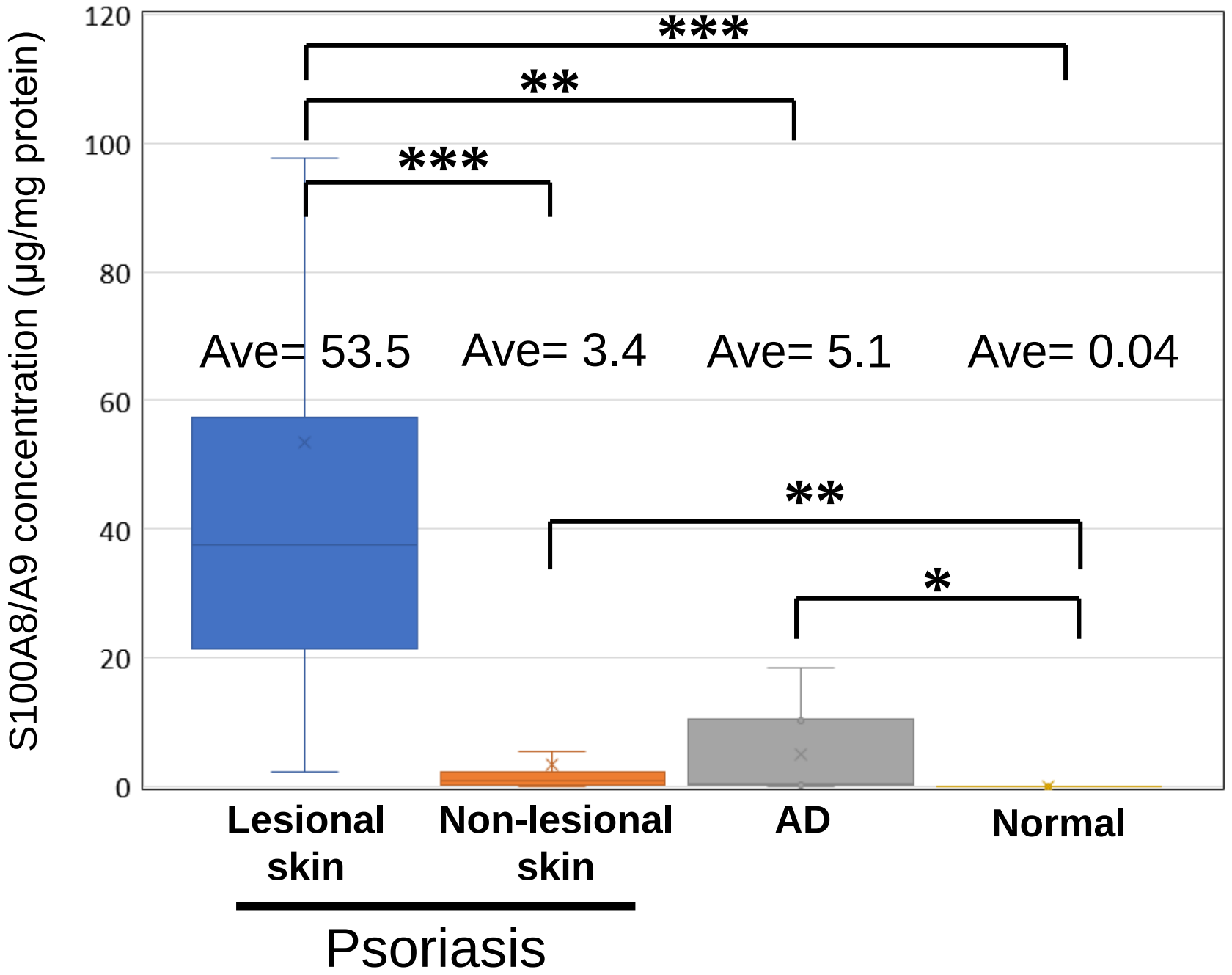


Figure 2.

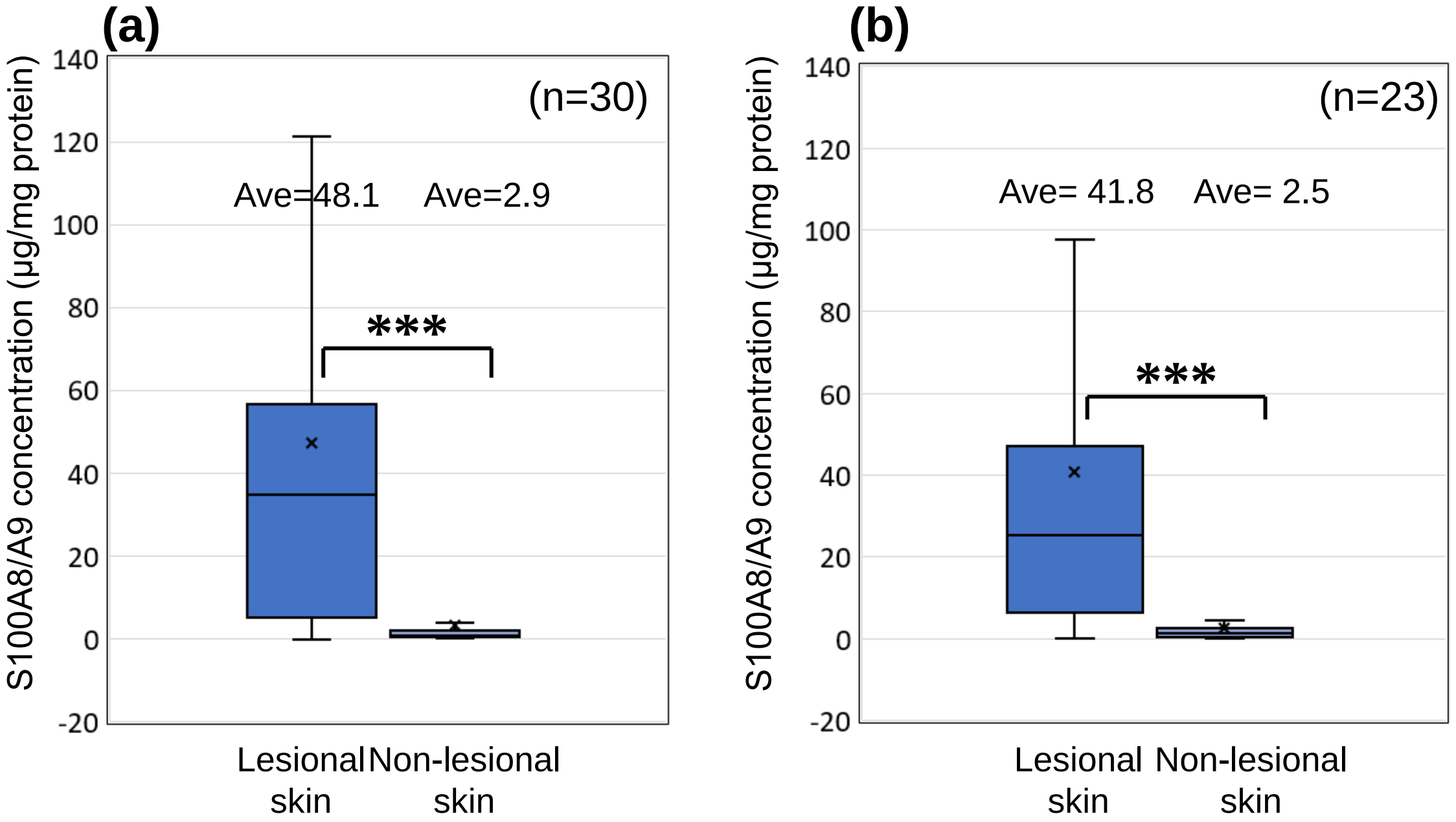
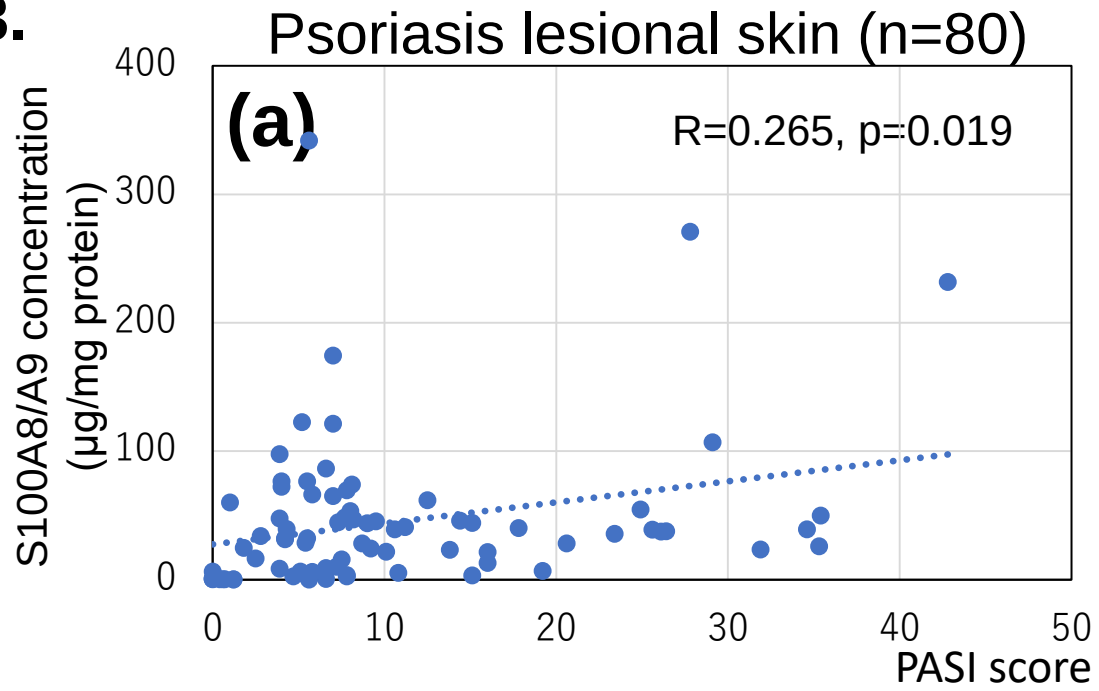
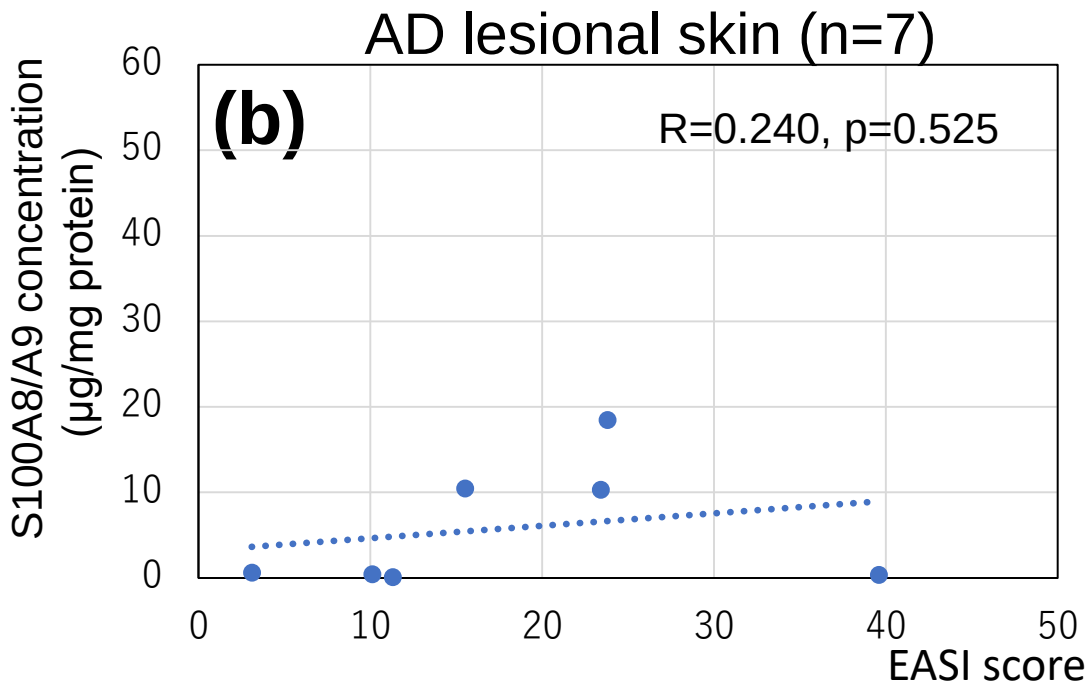
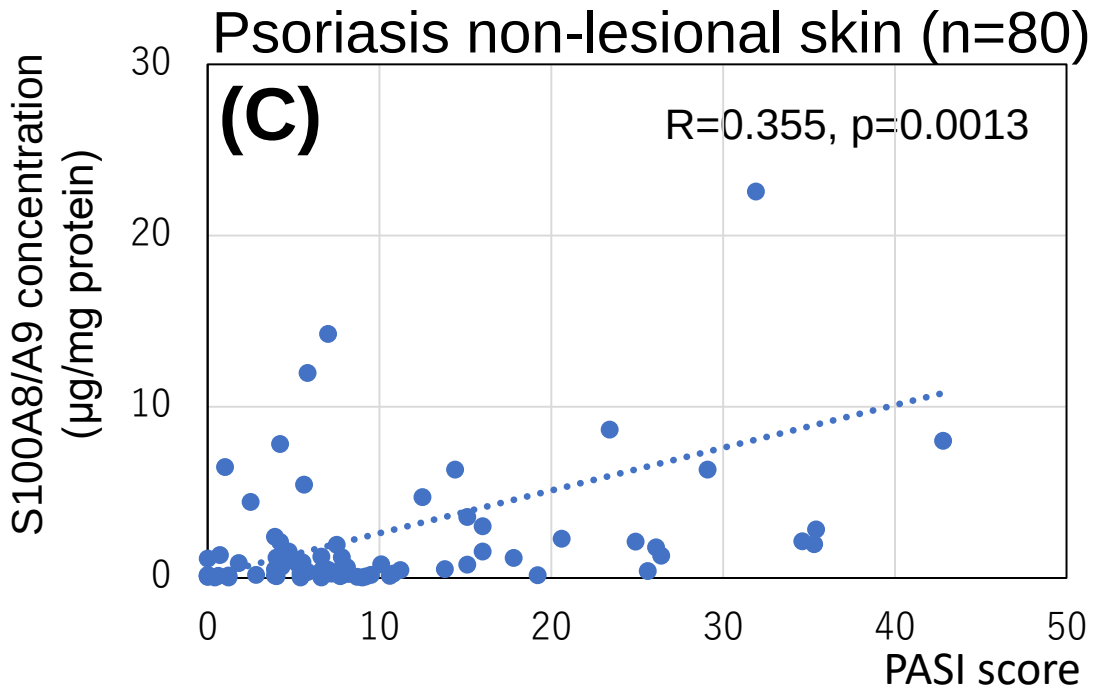


Figure 3.







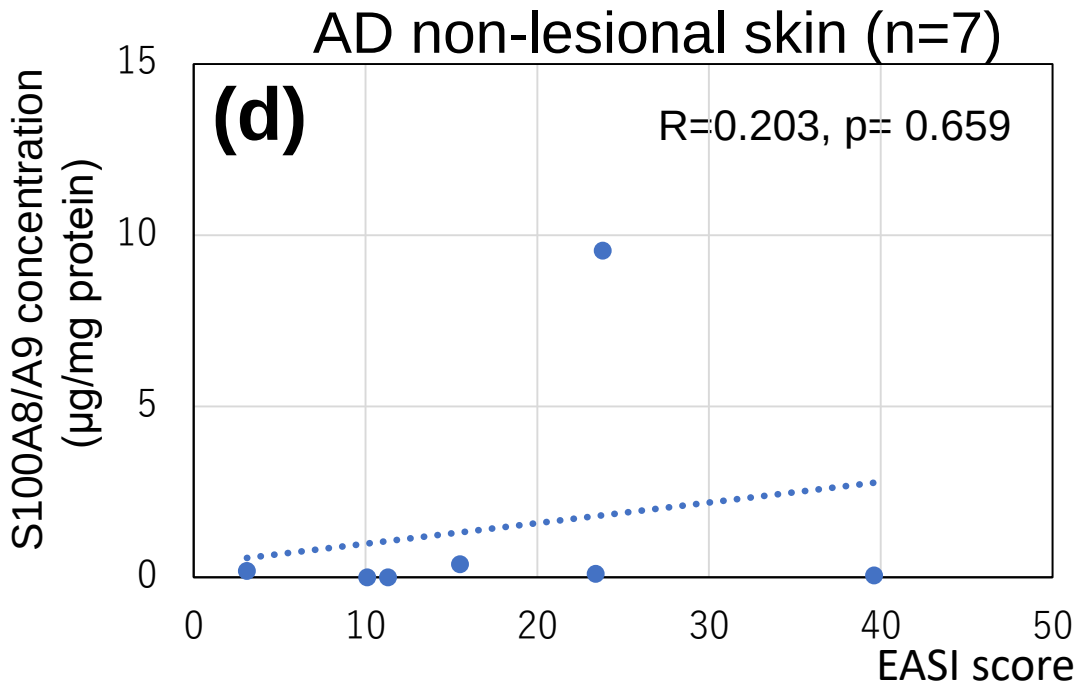


Figure 4.

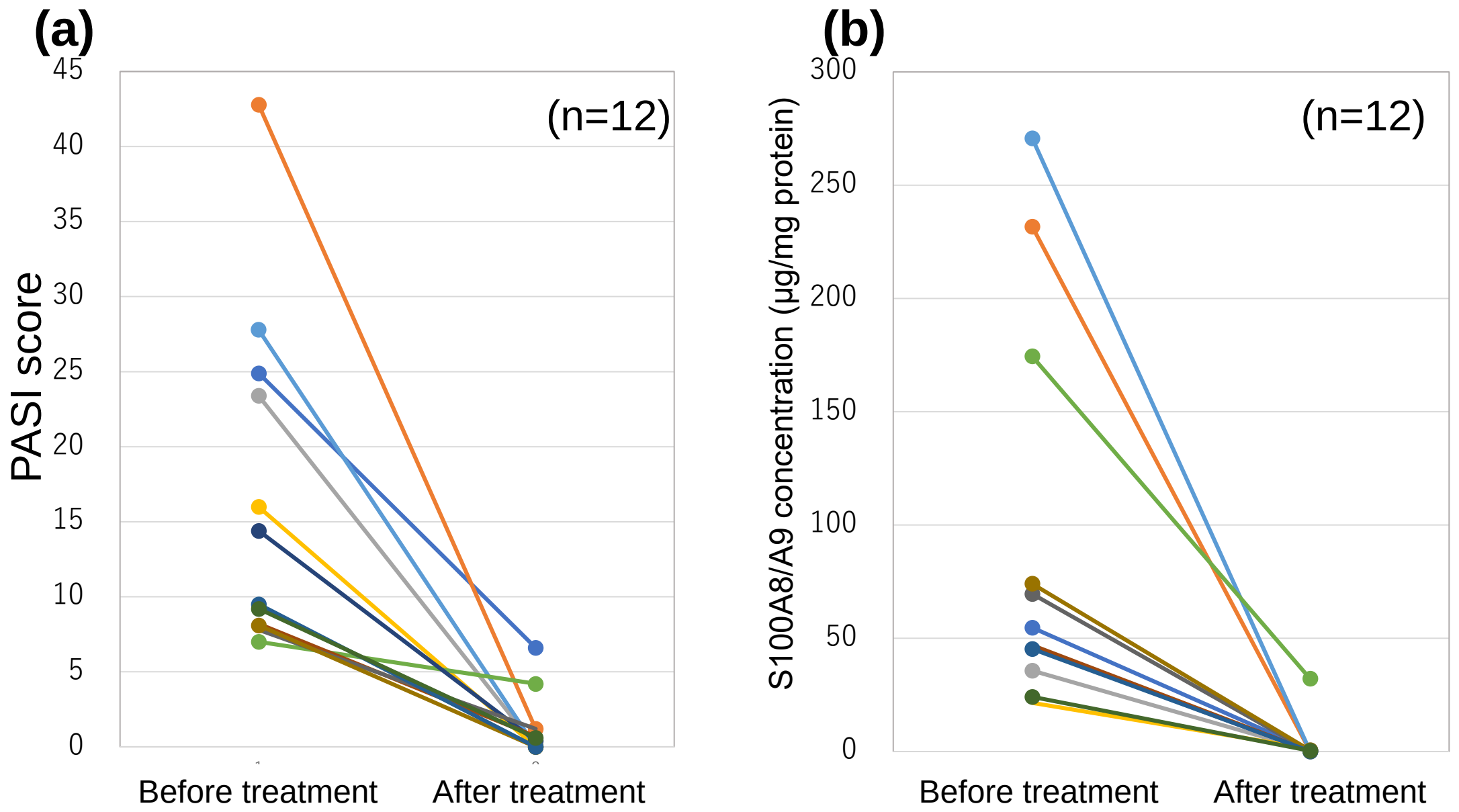


Figure 5.

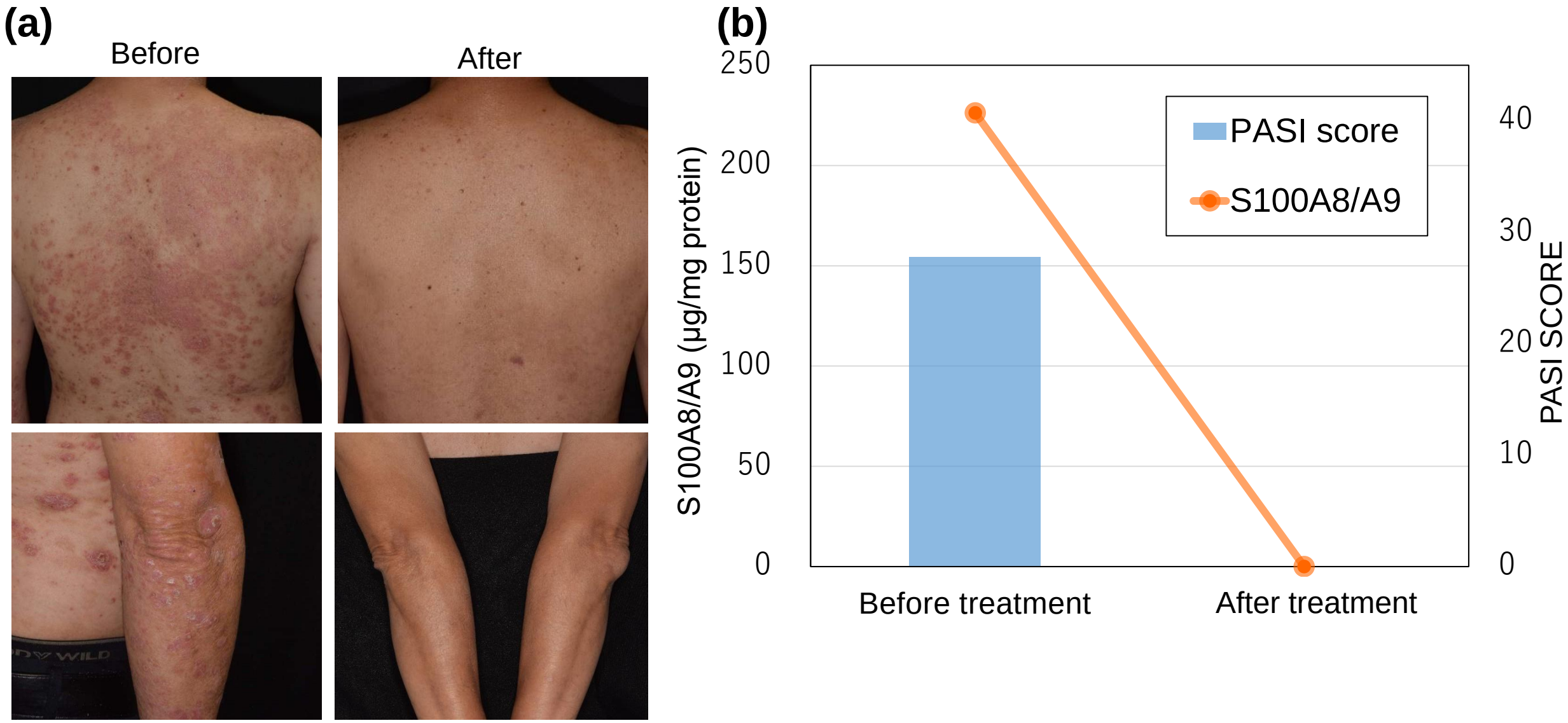
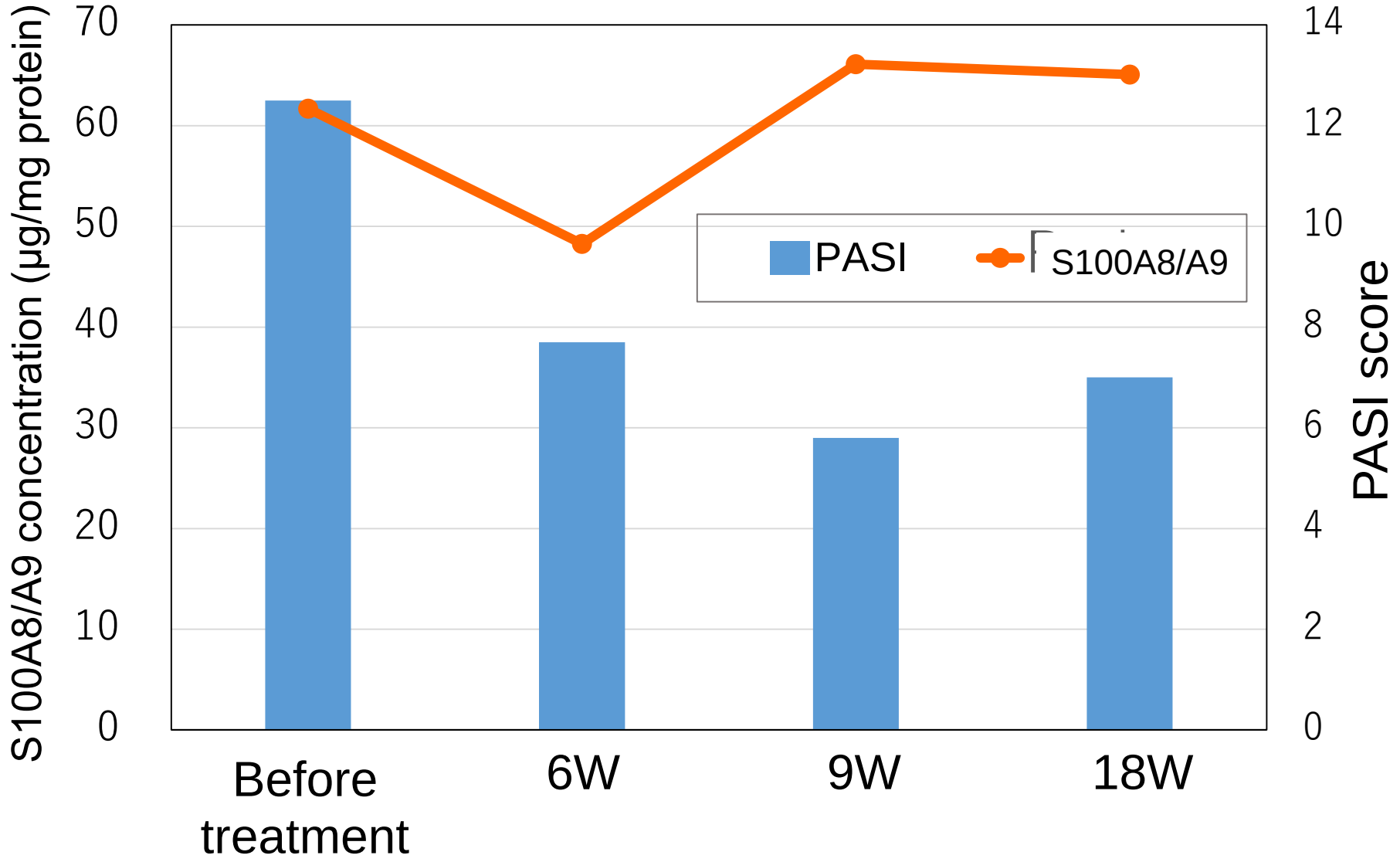
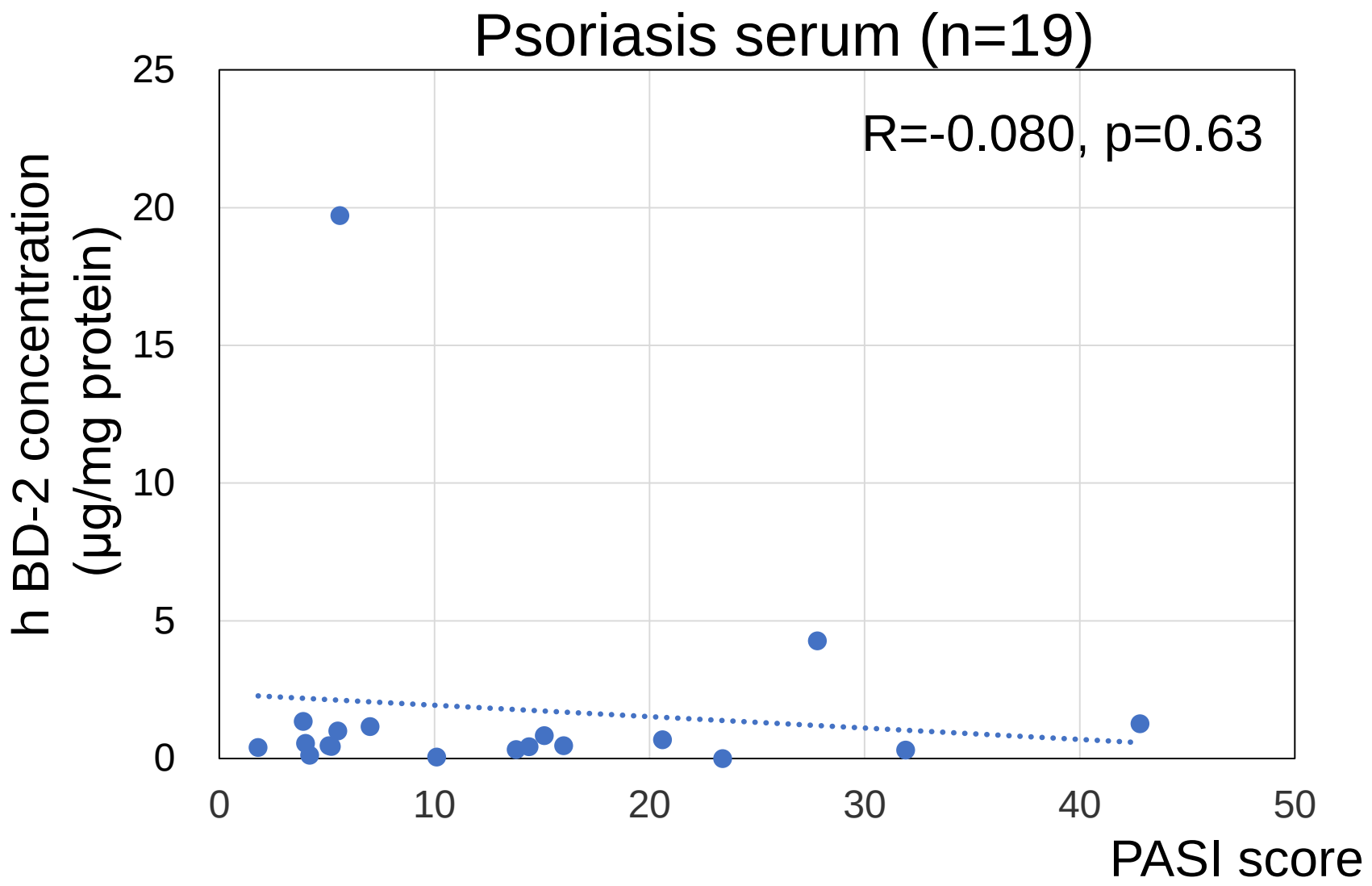


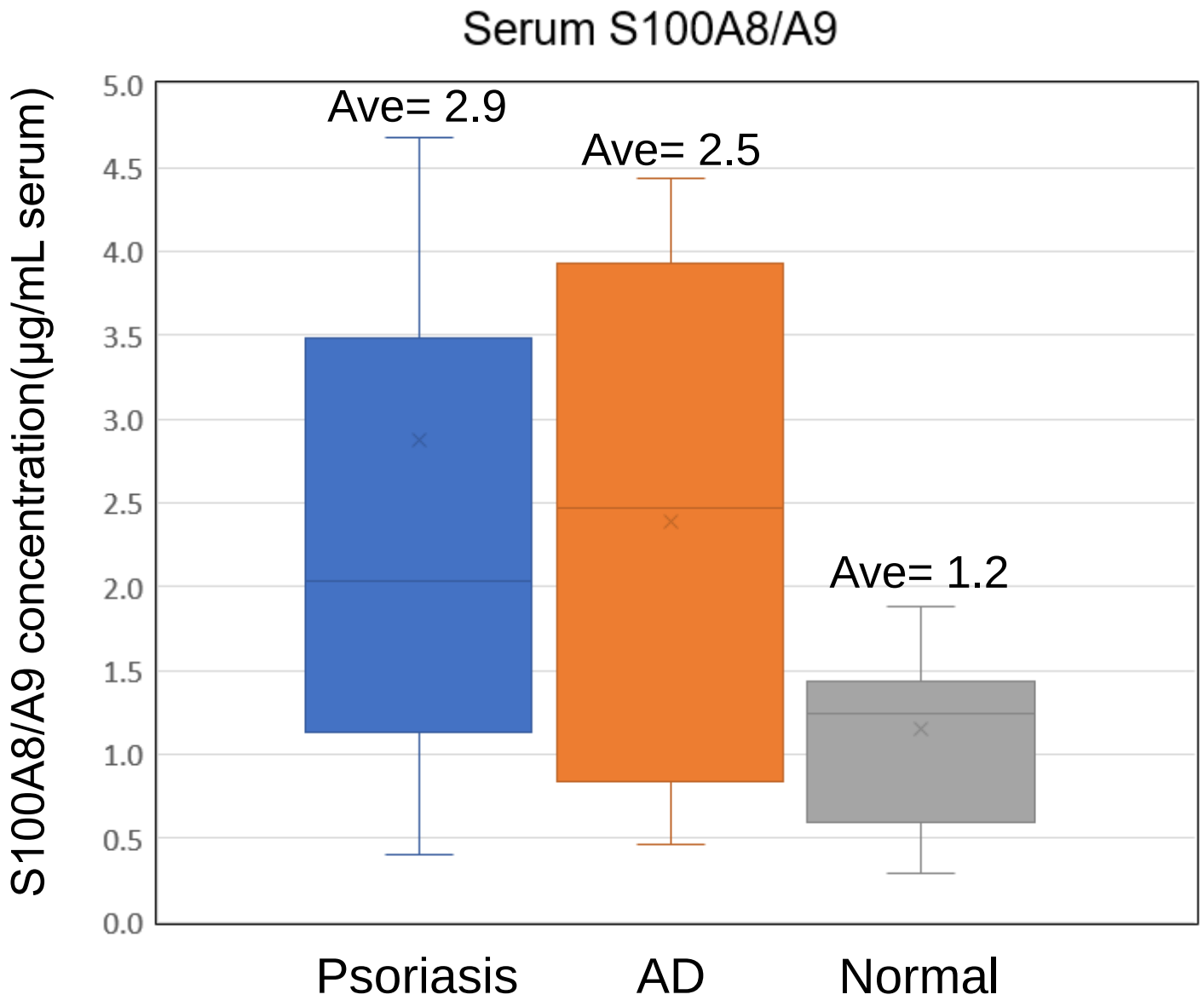
Figure 6.



Supporting information 1.



Supporting information 2.



Supporting information 3.