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The IgG response against *Staphylococcus aureus* is associated with severe atopic dermatitis in children

Running title: IgG against *Staphylococcus aureus* in paediatric atopic dermatitis

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Conflict of interest statement

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What is already known on this topic?

- *Staphylococcus (S.) aureus* plays a major role in the pathogenesis of atopic dermatitis (AD), possibly through the deregulation of immunological pathways.
- *S. aureus* expresses different antigens that could trigger immune responses in AD and contribute to the inflammatory response.

What does this study add?

- IgG responses against a large panel of 55 *S. aureus* antigens were profiled simultaneously.
- In young children, AD disease severity was found associated with IgG responses directed against antigens with mainly immune-modulatory functions.

ABSTRACT

Background: An altered immune response against *Staphylococcus (S.) aureus* might contribute to inflammation and barrier damage in atopic dermatitis (AD).

Objectives: We profiled IgG antibodies against 55 *S. aureus* antigens in sera of children with mild to severe AD using a Luminex assay. Additionally, we evaluated the association between IgG levels and disease severity.

Methods: In this cross-sectional study, we included children with AD of two interventional study cohorts, namely SMA (n= 131) and the older DAVOS cohort (n= 76). AD severity was assessed using the Self Administrated-Eczema Area and Severity Index (SA-EASI) and levels of thymus and activation-regulated chemokine (TARC) in serum. IgG antibody levels against 55 *S. aureus* antigens were quantified simultaneously using a Luminex assay. Pair-wise correlations were calculated between the 55 IgG levels using the Spearman rank correlation test. A linear regression analysis was performed to test for associations between 55 IgG levels and SA-EASI and TARC adjusting for age, sex and S. *aureus* colonisation.

Results: In the SMA cohort 16 antigens were associated with SA-EASI and 12 antigens were associated with TARC (10 overlapping antigens; *P*-values from 0.001 to 0.044). The associated IgG antibodies targeted mainly secreted proteins with immune-modulatory functions. In the DAVOS study, IgG levels against only four and one *S. aureus* antigen(s) were associated with SA-EASI and TARC, respectively (no overlap).

Conclusions: In young children, severity of AD is associated with an IgG response directed against *S. aureus* antigens with mainly immune-modulatory functions. These findings encourage further evaluation of the role of *S. aureus* in AD pathogenesis.

INTRODUCTION

Staphylococcus (S.) aureus is involved in the multifactorial pathogenesis of atopic dermatitis (AD).[1] Approximately 70% of the skin lesions in AD are colonised with *S. aureus*, and bacterial density was found associated with AD severity.[2] The exact mechanisms through which *S. aureus* causes inflammation are not fully understood, but the bacterium expresses different virulence factors that can trigger T-cell immune responses in AD and contribute to the inflammatory response.[3] For example, staphylococcal enterotoxins (SE) have the ability to act as superantigens via direct stimulation of T-cells.[4] Colonisation with staphylococcal strains that produce these virulence factors, including SEA, SEB, SEC and SED, is thought to be related with AD severity.[5,6] The role of other antigens in AD has barely been investigated until now.[7]

There is increasing interest in understanding the immune response against *S. aureus* in AD as an altered immune response might contribute to inflammation and barrier damage. Current literature focuses on IgE antibody titers directed against a few of the *S. aureus* antigens. Increased IgE-specific antibodies against *S. aureus* antigens, mainly SEA and SEB, have been described in AD patients compared to healthy controls. Furthermore, an association between IgE levels and AD severity is confirmed in a few studies.[8-10] Although IgG is known for its involvement in the neutralization and elimination of microbes, little is known about anti-*S. aureus* IgG antibody patterns in patients with AD.[11] Currently available literature measured IgG against two antigens, exfoliative toxin (ET) A and SEB, and reports higher IgG levels in patients compared to controls (significant for SEB).[12,13] Other antigens were not studied. Two studies performed detailed IgG subclass analysis and found an IgG2 deficiency against SEC1 and an elevated IgG4 against SEB in patients with AD.[14,15] Although studies are limited in number and focus on a few single antigens, they emphasize the possible relevance of this immunoglobulin class in the response against *S. aureus* in AD.

To gain more insights into the IgG mediated immune response against *S. aureus* in patients with AD, we profiled IgG antibodies against 55 *S. aureus* antigens in sera of children with mild to severe AD using a Luminex assay.[16] Additionally, we evaluated the association between IgG levels and disease severity.

MATERIALS AND METHODS

Study design and population

This cross-sectional study was embedded in two interventional studies: the Shared Medical Appointment (SMA) study and the DAVOS study.[17,18] SMA included patients with mild to severe AD, aged between 0 and 18, between November 2009 and December 2011. DAVOS included children with difficult to treat eczema from 8 to 18 years, between January 2011 and June 2015. Both studies were conducted at the Wilhelmina Children's Hospital in The Netherlands and were approved by the University Medical Centre Utrecht medical and ethical review board (09-192/K, 08-368/K). Written informed consent was obtained from all patients. Serum samples, microbial swabs, eczema severity scores and patient characteristics, obtained at baseline in both the SMA and DAVOS study, were analysed in this study. In both studies, AD was diagnosed according to the UK Working Party criteria.[19-21] Severity was assessed by the parents using the Self-Administrated Eczema Area and Severity Index (SA-EASI).[22] Apart from the SA-EASI, the levels of thymus and activation-regulated chemokine (TARC) in serum were used as a marker for AD severity.[23,24] Age of AD onset, treatment history and diagnosis of asthma and allergic rhinitis were based on clinical history. Food allergy was diagnosed based on convincing clinical history in SMA and/or double-blind food provocation test in DAVOS.

Microbial samples

Skin microbial samples were taken from the nose and lesional skin according to a standardised procedure using a sterile swab (Sterile Dryswab™) moistened with NaCl 0.9%. Skin samples were collected from lesions at the antecubital fold or the popliteal fossa. Bacterial cultures for *S. aureus* were performed using routine diagnostic culture procedures using *S. aureus* specific Mannitol salt agar plates.

Measurement of antibodies against S. aureus

Antigens and coupling procedure

In a pilot experiment, the IgG antibody titers against a set of 57 *S. aureus* antigens, were measured in sera of 23 AD patients. It was decided to include 55 antigens in the current study as the signals for two antibodies (ESAT-6 secretion system extracellular (Esx) A and SA2097) were very low in the pilot (data not shown). The 55 antigens used in this study were divided into four categories based on their

biological function: household enzymes, immune modulators (superantigens and non-superantigens), cell membrane damaging molecules and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Table E1). All used antigens were recombinant proteins, expressed with a His-tag in Escherichia coli XL1-blue strain and purified under denaturing conditions with nickelnitrilotriacetic acid agarose. They were coupled to the SeroMAP carboxylated beads (Luminex Corporation) as described previously.[25,26] The final bead concentration was adjusted to 3000 beads/µL and they were stored at 6 °C in the dark. As a negative control, the coupling procedure was performed in absence of any antigen.

Luminex assay

Serum samples were stored at -80°C until use before quantification of IgG antibody levels against *S. aureus* antigens with a fluorescent bead-based flow cytometry technique (xMap®, Luminex Corporation).[16] In wells of a 96-well filter microtiter plate (Millipore Corporation), 50 µL bead mix (containing the different antigen-coupled beads each at a concentration of 3000 beads/µL) was mixed with 50 µL of 1:100 diluted patient serum. Follow-up steps were described in detail previously.[27] Each measurement lasted 1 minute, during this time for each antigen-coupled bead a minimum of 100 beads had to be analysed otherwise the data were expelled from further analysis. IgG antibody levels against *S. aureus* antigens were expressed as median fluorescence intensity (MFI) values. A control bead, without protein coupled, was included to determine non-specific antibody binding. The non-specific MFI values were subtracted from the results.[26] MFI values of the two independent experiments, reflecting semi-quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for the duplicate experiments. Measurements were excluded if the CV value was >25% and average MFI values were >1000. Failures of the luminex per well were defined as missing values.

Statistical analysis

For this study, a convenience sample was obtained from the SMA and the DAVOS study. Because the DAVOS and SMA studies used different inclusion criteria regarding AD severity and age, the study cohorts were analysed separately. For further analysis the IgG data were pre-processed by replacing negative and zero MFI values (result from correction for non-specific binding) by one. Absolute IgG levels per antigens were presented using median and interquartile range (IQR). The IgG data were log transformed to obtain a parametric distribution, and standardised using a zero-mean-unit-

variance method. Pair-wise correlations were calculated between the 55 IgG levels using the Spearman rank correlation tests. A hierarchical clustering analysis with the 55 antigens was carried-out to identify main antibody clusters but no robust clusters were identified (data not shown). Therefore, for further analysis the 55 antibodies were analysed separately.

Linear regression analysis

Severity scores and patient age were tested for normal distributions with the one-sample Kolmogorov–Smirnov test and transformed when necessary, to obtain a normal distribution. Multivariable linear regression analyses were carried out using the standardised IgG levels (described above) for each of the 55 *S. aureus* antigens against the SA-EASI score as a main predictor, adjusted for age, sex and colonisation with *S. aureus* on skin and/or in nose (*S. aureus* present yes or no). The multivariable linear regression was repeated in a separate model using TARC as a main predictor instead of SA-EASI to validate our results with an intrinsic marker for AD severity. For antibodies that did not follow linear distribution after transformation, bootstrapping (iteration 1000) was used to obtain regression coefficients and 95% confidence intervals (CI). Given the exploratory nature of this study and the observed correlations between the 55 tested antibody levels (Fig. 2), we used a p-value <=0.05 to claim for significant associations between the antibody levels and SA-EASI/ TARC. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 23.0 for Windows (IBM Corporation, Armonk, NY).

RESULTS

Population characteristics

We included 136 and 76 children of the SMA and DAVOS study, respectively (Figure E1). The median age of the children of the SMA cohort was 2 years (IQR 1-5). The DAVOS population consisted of older children with a mean age of 13 (IQR 11-15) years. Eczema severity measured with the SA-EASI showed medians of 27 (IQR 16-42) in the SMA group and 24 (IQR 12-42) in the DAVOS group. Median TARC values in pg/ml were also higher in the SMA group, 1441 (IQR 713-2794) versus 1119 (IQR 696-2400). Skin and nasal colonisation with *S. aureus* was found in 36% and 34% of the children in de SMA group and in 47% and 67% of the children in the DAVOS group. Detailed baseline characteristics, including use of medication, are presented in Table 1.

Antibody characteristics

Median IgG levels against 55 *S. aureus* antigens measured in the serum of the children of both study cohorts are presented in Table E1. In the DAVOS cohort, the medians of the absolute antibody levels were higher and showed less variation, compared to the SMA study population. Figure 1 shows that the IgG antibody responses do not clearly differ between the four main biological groups of antigens (immune modulators (superantigens and non-superantigens), household enzymes, cell membrane damaging molecules and MSCRAMMs). A Spearman correlation test showed correlations between the IgG levels of the Staphylococcal Superantigen Like-proteins (SSL) 3, 5, 9 and 10 (coefficients > 0.7). High correlations (>0.7) were also identified between Leukotoxin (Luk) E, LukD, LukS and between Extracellular fibrinogen-binding protein (EfB) and Alt2. Additionally, some enterotoxins were correlated; SEB with SEC (>0.7), SEI with SEM (>0.8), SEN with SEI (0.69) and SEA with SEE (0.69) (Fig. 2).

Association between anti-staphylococcal IgG levels and AD severity measured with SA-EASI and TARC

We found significant associations between IgG levels and AD severity in the SMA cohort. Sixteen antigens were associated with SA-EASI and 12 antigens were associated with TARC (Table 2). Ten of the 12 antigens that were associated with TARC were also associated with SA-EASI (*P*-values ranging from 0.001 to 0.044). The associated IgG antibodies targeted mainly secreted proteins with immune-modulatory functions (e.g.: Leukotoxin (Luk) D and E, Table 2). The described associations between antigen levels and AD severity were independent of age, sex and colonisation of the skin and/or nose with *S. aureus.* In the DAVOS study, IgG levels against only four and one *S. aureus* antigen(s) were associated with SA-EASI and TARC, respectively and there was no overlap between the two markers for AD (Tables E2a-d).

DISCUSSION

For the first time IgG immune responses against a large panel of 55 *S. aureus* antigens were profiled in children with AD, showing that the children are exposed to the antigens and develop an IgG mediated humoral immune response towards them. Additionally, severity of the AD was found associated with IgG antibodies directed against *S. aureus* antigens with mainly immune-modulatory functions. Leukotoxins D and E, commonly expressed by *S. aureus* strains and are involved in cell lysis of neutrophils.[28] Staphylococcal superantigen like proteins (SSL) 3, 5, 9 and 10 are variably expressed and are all involved in immune modulation, for example by inhibiting complement

activation.[29] Iron-responsive surface determinants A (IsdA) is a cell surface protein that may function both in iron acquisition and adhesion.[30] SEA is more rarely expressed and has a strong immuno-stimulatory function. As a superantigen it can cause cytokine release and epithelial damage but literature also describes (anti-inflammatory) cytokine downregulating functions (IL-4).[28,31] *S. aureus* formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcγR antagonists that inhibit IgG-mediated effector functions.[32]

Only Sohn et al. studied the Staphylococcal IgG response in relation to AD severity before. They solely measured SEB and found no correlation with AD severity which corresponds with our findings (Table 2).[13] The finding that the antibodies that were associated with AD severity in this study are known to target antigens with an immune-modulatory function, suggests that *S. aureus* down regulates the immune system locally to help it maintain its colonisation on the skin, a theory that was recently suggested by Biedermann et al.[33]

In contrast to the associations between AD severity and IgG levels for specific antigens found in the SMA study, few associations were found in the DAVOS cohort. In addition, there was no overlap between the antibodies associated using the EASI and these associated with TARC in the DAVOS cohort, which suggests that these associations were false positives. The lack of associations in the DAVOS cohort could be the result of the older age of the DAVOS participants who may have been more chronically exposed to *S. aureus* (see Table 1 and Table E1) and therefore exhibited higher levels of IgG.[12,34] Furthermore, IgG antibody levels are known to increase by age, also seen in AD patients, reaching a plateau level around adulthood.[34-36] Indeed, a comparison between IgG levels of the SMA cohort and a sample of healthy adult pooled serum (Supplementary methods), showed higher levels for most of the tested IgG antibodies in the HPS (Supplementary Figure1). The DAVOS cohort had higher values than the healthy adult sample for a large part of the antibodies (Supplementary Figure1). It could be argued that a plateau could have been reached in the older patients of our DAVOS cohort causing the lack of associations. On the other hand, the DAVOS study included patients with difficult to treat (severe) eczema, most of whom were treated with topical corticosteroids or even systemic immunosupressive therapy (Table 1). Hence, the SA-EASI scores at baseline may have been biased towards lower scores, which could have hampered the associations. Most likely the lack of association is a combination of both biased scores and older age in the cohort.

IgG levels against specific antigens showed correlations using a Spearmen correlation test. SSL antigens and the superantigens SEI, SEM and SEN are known to be co-produced by *S. aureus.*[37,38] The combined presentation of these antigens to the immune system probably also contributes to the observed correlations between IgG responses. However, other factors probably also drive the height and correlations of the IgG specific immune responses.

This study has several limitations. First, a control group of children without AD that would allow investigating the normal range of IgG antibodies was not available. Therefore, our conclusion focusses on a comparison between different severity phenotypes in a well characterized cohort of children with AD. Due to the cross-sectional design we cannot conclude whether AD severity is the result of an altered IgG response. In addition, due to the small sample size of this study, we could not perform multivariate analysis such as unsupervised clustering that would allowed us to understand better the correlations found in different antibodies. Although we tested 55 different associations, we did not correct for multiple testing. Due to the high correlations between some of the antibodies multiple testing correction is rather conservative. Because of this, and the hypothesis generating character of this study our associations were kept nominally significant. Although cross-reactivity with other Staphylococcal antigens cannot be completely ruled out, it is highly unlikely as *S. aureus* produces species specific virulence factors that have not been found in *S. epidermidis.*[39] Despite the above limitations, this paper is the first to evaluate antibody responses against a broad panel of *S. aureus* antigens. Our study sheds light into IgG mediated immune response to *S. aureus* children with AD and it highlights the relevance of other antigens (adhesins and immune modulators) next to the often studied superantigens. Further studies need to be conducted to validate the associations we found.

Interestingly, the association between IgG against *S. aureus* antigens and severity was independent of skin and nasal colonisation with *S. aureus*. This suggests that the immune response against *S. aureus* might be altered, irrespective of the bacterium being present on the skin at that moment, and raises the question whether the decision for anti-*S. aureus* treatment should be guided by a positive culture. To further explore the significance of our findings, future studies should relate our findings to IgE and IgG subclass responses towards *S. aureus* antigens in AD. These findings could also be related to *S. aureus* strain differences as different strains might have different ability to elicit

immunologic alterations is the host.[40] In vitro experiments should reveal the functional effect of the relevant *S. aureus* antigens on T-cell differentiation.

Conclusion

In a cohort of young children with AD, we identified significant associations between disease severity and IgG antibodies directed against *S. aureus* antigens with mainly immune-modulatory functions. The results of this study encourage more detailed evaluation of the role of *S. aureus* in the pathogenesis of AD.

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TABLES

Table 1: Baseline characteristics

	SMA study (n=131)	DAVOS study (n=76)
Age in years; median (IQR)	2 (1-5)	13 (11-15)
Sex (male); N (%)	63 (48.1)	39 (51.3)
Ethnicity		
Dutch; N (%)	95 (72.5)	54 (71.1)
Other ethnicity; N (%)	19 (14.5)	22 (28.9)
Missing; N (%)	17 (13.0)	0 (0)
Age of onset AD		
0-<2 years; N (%)	106 (80.9)	66 (86.8)
2-<6 years; N (%)	6 (4.6)	8 (10.5)
Missing; N (%)	19 (14.5)	2 (2.6)
Atopy		
Food allergy; N (%)	53 (40.4)[1]	49 (64.5)[1]
Allergic asthma; N (%)	40 (30.5)[2]	59 (77.6)
Allergic rhinoconjunctivitis; N (%)	36 (27.5)[2]	67 (88.2)
SA-EASI; median (IQR)	27.00 (16.00-42.20)[3]	24.00 (11.95-41.75)[2]
TARC pg/mL; median (IQR)	1441 (713-2794)	1119 (696-2400)[2]
Corticosteroid treatment		
Topical corticosteroid; N (%)	101 (77.1)	70 (92.1)
Systemic corticosteroid; N (%)	0 (0)	3 (3.9)
Neoral	0 (0)	7 (9.2)
Antibiotic treatment		
Topical antibiotic; N (%)	11 (8.4)	2 (2.6)
Systemic antibiotic; (%)	3 (2.3)	1 (1.3)
***S. aureus* positive** (> 10 CFU)		
Skin; N (%)	47 (35.9)[4]	36 (47.4)[3]
Nose; N (%)	45 (34.4)[2]	51 (67.1)[3]

Missings SMA: [1] = 4 (3.1%); [2] = 2 (1.5%); [3] = 41 (31.3%); [4] = 3 (2.3%)
Missings DAVOS: [1] = 2 (2.6%); [2] = 3 (3.9%); [3] = 6 (7.9%)

Table 2: List of *S. aureus* antigens of which the IgG levels were significantly associated with patient eczema severity, according to SA-EASI and TARC, *P*-value < 0.05)

Antigens	SMA study (n=131)						DAVOS study (n=76)					
	SA-EASI ^			TARC			SA-EASI			TARC		
	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value
LukD	0.134 (0.042)*	0.054-0.219	0.003	0.379 (0.160)*	0.080-0.714	0.018	-	-	-	-	-	-
LukE	0.111 (0.048)*	0.016-0.213	0.033	0.396 (0.141)*	0.108-0.672	0.005	-	-	-	-	-	-
SSL-3	0.145 (0.052)*	0.045-0.258	0.008	0.404 (0.144)*	0.150-0.700	0.006	-	-	-	-	-	-
SSL-5	0.153 (0.044)*	0.070-0.242	0.001	0.379 (0.179)	0.024-0.734	0.036	-	-	-	-	-	-
SSL-9	0.149 (0.048)*	0.053-0.243	0.004	0.309 (0.143)*	0.036-0.617	0.035	-	-	-	-	-	-
SSL-10	0.127 (0.047)*	0.036-0.224	0.009	0.432 (0.151)*	0.162-0.747	0.004	-	-	-	-	-	-
FlipRL	0.120 (0.057)*	0.018-0.243	0.043	0.398 (0.183)*	0.047-0.768	0.036	-	-	-	-	-	-
SEA	0.177 (0.057)*	0.062-0.292	0.002	0.613 (0.194)	0.230-0.997	0.002	-	-	-	-	-	-
IsdA	0.203 (0.045)*	0.115-0.294	0.001	0.346 (0.135)*	0.094-0.624	0.022	-	-	-	-	-	-
Eap	0.146 (0.048)*	0.047-0.236	0.002	0.286 (0.142)*	0.018-0.582	0.044	-	-	-	-	-	-

LukS	0.113 (0.058)*	0.002-0.223	0.047	-	-	-	-	-	-	-	-	-
HlgB	0.116 (0.047)*	0.022-0.214	0.019	-	-	-	-	-	-	-	-	-
SSL-1	0.134 (0.050)*	0.045-0.238	0.004	-	-	-	-	-	-	-	-	-
FlipR	0.144 (0.059)*	0.026-0.262	0.020	-	-	-	-	-	-	-	-	-
SdrE	-0.140 (0.062)*	-0.271-(-0.022)	0.036	-	-	-	-	-	-	-	-	-
IsaA	0.125 (0.047)*	0.023-0.215	0.008	-	-	-	-	-	-	-	-	-
SEE	-	-	-	0.550 (0.212)*	0.135-0.980	0.011	-	-	-	-	-	-
EfB	-	-	-	0.450 (0.178)	0.097-0.803	0.013	-	-	-	-	-	-
Hlb	-	-	-	-	-	-	0.155 (0.058)*	0.041-0.262	0.010	-	-	-
SEG	-	-	-	-	-	-	0.165 (0.069)*	0.050-0.326	0.019	-	-	-
FnbpB	-	-	-	-	-	-	0.151 (0.060)*	0.029-0.261	0.015	-	-	-
ClfA	-	-	-	-	-	-	0.142 (0.067)*	0.023-0.291	0.040	-	-	-
SAK	-	-	-	-	-	-	-	-	-	0.695 (0.302)*	0.084-1.285	0.027

^ N = 90 for SA-EASI analysis due to missing SA-EASI scores

** Regression coefficients and CI were obtained using bootstrapping iter 1000*

SE = standard error

- = no significant association between the antigen and the severity parameter.

FIGURE LEDGENDS

Figure 1. (color)

Boxplots showing the levels of IgG against 55 antigens in the SMA study (normalised data)

Blue = MSCRAMMs

Green = membrane damaging molecules

Orange = housekeeping antigens

Red = superantigens

Yellow = immune modulating proteins

Figure 2. (color)

Spearman's rank correlation coefficients of the IgG values (MFI) against 55 antigens (SMA-study).

The size and intensity of the red dots reflects the height of the correlation coefficients, identifying high correlations for example between the SSL 3, 5, 9 and 10 antigens.

Table 1.

Baseline characteristics, SMA study and DAVOS study

Missings SMA: [1] = 4 (3.1%), [2] = 2 (1.5%), [3] = 41(31.3%), [4] = 3(2.3%)

Missings DAVOS: [1] = 2 (2.6%), [2] = 3 (3.9%), [3] = 6 (7.9%)

Table 2.

Summary of *S. aureus* antigens; IgG levels associated with patient eczema severity according to SA-EASI and TARC in SMA and DAVOS study (*P*-value <0.05).

^ N = 90 due to missing SA-EASI scores

* Regression coefficients and CI were obtained using bootstrapping iter 1000

SE = standard error

Figure E1. Flowchart of the study population, SMA study and DAVOS study

Table E1.

Overview of 55 *S. aureus* antigens with function and MFI values

Table E2a-d.

a. Results linear regression analysis DAVOS study – association with SA-EASI
   * = significant *P*-value
   Bootstrapping iter 1000 for all antigens
b. Results linear regression analysis DAVOS study – association with TARC
   * = significant *P*-value
   Bootstrapping iter 1000 for all antigens
c. Results linear regression analysis GMA study – association with SA-EASI
   Bootstrapping iter 1000 for all antigens, except SEN
d. Results linear regression analysis GMA study – association with TARC
   Bootstrapping iter 1000 for all antigens, except SEN