The presence of staphylococcal superantigens in nasal swabs and correlation with activity of granulomatosis with polyangiitis in own material

J. Fijolek[1], E. Wiatr[1], V. Petroniec[2], E. Augustynowicz-Kopec[2], M. Bednarek[3], D. Gawryluk[1], M.M. Martusewicz-Boros[1], K. Modrzewska[1], E. Radzikowska[1], K. Roszkowski-Sliz[1]

[1]*The Third Department of Pneumonology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw;*
[2]*Department of Microbiology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw;*
[3]*The Second Department of Pneumonology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw, Poland.*

Justyna Fijolek, MD
Elzbieta Wiatr, Prof.
Violetta Petroniec, ms
Ewa Augustynowicz-Kopec, Prof.
Michal Bednarek, MD
Dariusz Gawryluk, PhD
Magdalena M. Martusewicz-Boros, Assist. Prof.
Katarzyna Modrzewska, PhD
Elzbieta Radzikowska, Assist. Prof.
Kazimerz Roszkowski-Sliz, Prof.

Please address correspondence to:
Dr Justyna Fijolek,
The National Research Institute of Tuberculosis and Lung Diseases,
The Third Department of Pneumonology,
Plocka St 26,
01-138 Warsaw, Poland.
E-mail: jfijolek@op.pl

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ABSTRACT

Objective. *Nasal carriage of Staphylococcus aureus and its superantigens (SAg) seem to be a risk factor disease exacerbation in granulomatosis with polyangiitis (GPA). We investigated the association between the presence of SAg in nasal swabs and activity of disease in GPA patients also taking into account correlation with an antimicrobial treatment.*

Methods. *In a prospective study of a total of 150 GPA patients hospitalised in the period 2009-2016, nasal swabs were examined for the presence of Staphylococcus aureus and SAg. Subsequently, the association with disease activity was assessed.*

Results. *Of 362 Staphylococcus aureus-positive nasal swab cultures from 115 of the 150 patients, the presence of at least one SAg in 126 samples (34.8%) from 56 patients (48.7%) was found. Among the 17 patients with limited to subglottic stenosis (SGS) disease, SAg were detected in 6 cases (35.3%). We did not find a significant correlation between the presence of SAg and disease activity (p=0.986), although when individual SAg were analysed separatively, SED and TSST-1 were more frequently present in active disease. Additionally, the results of the analysis demonstrated a protective effect of trimethoprim/sulfamethoxazole (T/S) treatment (0R 0.52, p<0.0092) in GPA patients. Interestingly, GPA limited to SGS appeared as an unfavourable factor associated with disease activity (0R 1.84, p=0.05).*

Conclusion. *The association between staphylococcal SAg in nasal swabs and GPA activity is not evident. Multiple mechanisms that may lead to disease activation still need to be investigated.*

Introduction

Granulomatosis with polyangiitis (GPA) is an idiopathic small-vessel vasculitis characterised by granulomatous inflammation of the respiratory tract, necrotising small vessel vasculitis and glomerulonephritis. The disease is frequently hallmarked by the presence of autoantibodies ANCA (antineutrophil cytoplasmic antibodies) directed predominantly against the myeloid enzyme proteinase 3 (PR3) in 80% of GPA patients (1). This is a multifactorial disease with numerous contributing genetic and environmental factors (2, 3). Many studies report a relationship between bacterial infections and initiation or relapse of GPA. A special role is suggested for *Staphylococcus aureus* (*S.aureus*), because 60-70% of GPA patients are chronic nasal *S.aureus* carriers in contrast to 20-30% of healthy individuals (3-8).

The mechanism by which *S.aureus* exerts its noxious effect is not fully understood, but the role of *Staphylococcus* superantigens (SAg) is underlined by many authors (8-12). The term "superantigen" describes a group of antigens that differ from typical protein or peptide antigens. In contrast to a conventional peptide that usually stimulates less than 0.01% of naive lymphocytes, a SAg is able to induce global changes in the composition of the lymphocyte repertoire by stimulating more than 5% of the naive lymphocyte pool (13-15). Superantigens are exotoxins with strong capacity to stimulate T cells in a specific non-antigen way (16). Three classes of SAg have been described to date, comprising the staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE), exfoliative toxins (ET) and toxic-shock syndrome toxin 1 (TSST-

1) (8, 11, 12, 16). Penetration of low levels these SAg may stimulate monocytes/macrophages to the secretion of proinflammatory cytokines, resulting in the activation of the vascular endothelium. Subsequently, activated endothelial cells may be damaged by adherent granulocytes that are stimulated by ANCA produced by B cells and stimulated by SAg-activated T cells. Alternatively, SAg may stimulate autoreactive T cells that may result in granulomatous inflammation and induction or reactivation of vasculitis (11, 12, 14, 15).

The aim of this study was to present the relation between a presence of *S.aureus* and its SAg in nasal swabs and the disease activity in GPA patients also taking into account the correlation with an antimicrobial treatment.

Patients and method

The study is prospective, obviating the need for approval from the local Ethics Committee (examination of nasal swabs is a routine examination in GPA patients). However, all procedures performed in the study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments.

In a total of 150 GPA patients hospitalised in the National Research Institute of Tuberculosis and Lung Diseaes in the period 2009-2016, nasal swabs were examinated for the presence of *S.aureus* and SAg. GPA was diagnosed based on clinical features consistent with vasculitis and/or biopsy consistent with vasculitis and/or a positive ANCA test in accordance with the Chapel Hill Consensus Conference 2012 nomenclature (17). One hundred and fifteen patients with GPA and coexiting *S.aureus* were recruited to the study. Nasal swabs were taken at least once during hospitalisation for control or because of an intensification of syndromes. To assess GPA activity, the Birmingham Vasculitis Activity Score (BVAS) was used (18). Non-active disease was defined as the absence of new or worse clinical BVAS items. In this group were the patients usually after immunosuppressive treatment or during maintenance immunosuppression, in clinical remission of disease. Active disease was recognised in newly-diagnosed patients, during relapse or progression of disease (according to BVAS, clinical and radiological syndromes together with laboratory findings). Cases of slow lung radiological progression without clinical syndromes, have been identified also as an active disease. In patients with GPA limited to subglottic stenosis (SGS), an activity indicator of disease was dyspnea, requiring surgical intervention (intratracheal dilation-injection technique during "rigid" bronchoscopy). The treatment, including corticosteroids (CSs) and cyclofosfamide (CYC) and then – for the maintenance of remission – azathioprine (AZA) or methotrexate (MTX), was given for 18–24 months. Patients with severe and recurrent disease received rituximab, but patients with GPA limited to SGS were mainly locally treated (19). A part of the patients received Trimetophrim/Sulfamethoxazole (T/S) in prophylactic doses (960 mg three times a week), while another part additionally received a local treatment by mupirocin. ANCA by enzyme-linked immunosorbent assay (ELISA) was measured in all the patients.

Nasal cultures and SAg typing

The swabs were taken by firmly rotating a sterile cotton-tipped swab from the anterior nares. Gram stain was first performed to guide the way which should typical gram-positive bacteria, cocci in clusters. Secondly, the organisms were cultured in Mannitol Salt Agar. The swabs were inoculated on 5% sheep-blood agar medium and salt mannitol agar medium for 48h at 35^0C. Isolates were identified as *S.aureus* by the typical appearance of colonies being coagulase-positive by slide coagulase testing and the ability to cleave DNA. Enterotoxicity was tested using the Oxoid SET-RPLA toxin detection kit. The strains were stored frozen at -70^0C. Each time before the next stage of testing, the strains were thawed and then plated onto Brain Heart Infusion (BHI) and Trypton Soya medium and incubated at 37ºC for 18 hours.

Principle of assay: Polystyrene latex particles are sensitised with purified antiserum taken from rabbits, immunised individually with purifed staphylococcal SAg (SEA, SEB, SEC, SED and TSST-1). These latex particles will agglutinate in the presence of the corresponding SAg. A control reagent is provided which consists of latex particles sensitised with non-immune rabbit globulins. The test is performed in V-well microtitre plates. Dilutions of the culture filtrate are made in five rows of wells, a volume of the appropriate latex suspension is added to each well and the contens mixed. If staphylococcal SAg (SEA, SEB, SEC, SED and TSST-1) are present, agglutination occurs, which results in the formation of a lattice structure. If SAg are absent or at a concentration below the assay detection level, no such lattice structure can be formed and, therefore a tight button is observed.

Statistical analyses

Statistical analyses were performed using Statistica v. 10 software (StatSoft, Inc., USA). Tests were considered significant when $p<0.05$. Data distribution was analysed using the Kologomorov-Smirnov test with Lilliefors correction. Homogeneity of variance was assessed using Levene's test. Quantitative data were described using mean ± SD. Between group characteristics were described using *t*-test, if a variable demonstrated normal distribution and homogenous variance.

Categorised, qualitative, between groups analyses were performed using Pearson's chi-square (χ^2) test with appropriate corrections for N.

A logistic regression modelling was used for predictive analyses of qualitative data. A Medical Package v. 2.0 (StatSoft, Inc., USA, 2011) for Statistica was used. The variables were coded using quasi-experimental model with sigma restrictions. Factors were added using stepwise forward and backward method. For data entry and removal, a *p*-value of 0.05 was used. The data were presented as odds ratio (OR), confidence intervals (CI) and *p*.

Results

Among the 150 patients with GPA hospitalised in the National Research

Table I. Characteristics of the 115 study patients.

Patients' characteristics	n. (%)
Male patients	60 (52,2%)
Female patients	55 (47,8%)
Male episodes	168 (46,4%)
Female episodes	194 (53,5%)
Generalised disease patients	98 (85,2%)
Limited disease – SGS patients	17 (14,8%)
Generalised disease active episodes	96 (81,4%)
Generalised disease non-active episodes	217 (88,9%)
Limited disease active episodes	22 (18,6%)
Limited disease non-active episodes	27 (11%)
All active episodes	118 (32,6%)
All non-active episodes	244 (67,4%)
Only one time swab taken patients	42 (36,5%)
Many times swab taken (2–13) patients	73 (63,5%)
S.aureus positive nasal swabs patients	115 (100%)
ANCA (+) episodes	215 (59,4%)
ANCA (-) episodes	147 (40,6%)
T/S treatment patients	
Yes	45 (39%)
No	70 (60,9%)
T/S treatment episodes	
Yes	123 (34%)
No	239 (66%)
All patients	115 (100%)
All episodes (samples)	362 (100%)

SGS: subglottic stenosis; ANCA: antineutrophil cytoplasmic antibodies; T/S: Trimetophrim/Sulfamethoxazole; S. Aureus: Staphylococcus aureus.

Table II. Correlation between S.aureus positive nasal swabs and SAg presence taking into account the number of positive SAg in a single nasal swab and its individual types, T/S treatment and activity of disease in GPA patients (362 episodes).

Patients characteristics/Parameter	Active disease	Non-active disease	*p*
S. aureus positive nasal swabs (n, %)	118 (32.6)	244 (67.4)	---
SAg + episodes (n, %)	41 (34.8)	85 (34.8)	0.986 (ns)
SAg – episodes (n, %)	78 (66.1)	162 (66.4)	
SEA	22 (18.6)	51 (20.9)	0.616 (ns)
SEB	2 (1.7)	7 (2.9)	0.501 (ns)
SEC	8 (6.8)	20 (8.2)	0.636 (ns)
SED	4 (3.4)	3 (1.2)	0.162 (ns)
TSST-1	15 (12.7)	21 (8.6)	0.221 (ns)
The number of positive SAg in a single nasal swab/episode (n, %)			
0	77 (65.3)	159 (65.2)	ns
1	33 (28.0)	72 (29.5)	ns
2	6 (5.1)	9 (3.7)	ns
3	2 (1.7)	4 (1.6)	ns
T/S treatment episodes			
Yes (n, %)	29 (24.6)	94 (38.5)	0.009
No (n, %)	89 (75.4)	150 (61.5)	

SAg: staphylococcal superantigen; SEA: staphylococcal enterotoxin type A; SEB: staphylococcal enterotoxin type B; SEC: staphylococcal enterotoxin type C; SED: staphylococcal enterotoxin type D; TSST-1: toxick-shock syndrome toxin 1; T/S: trimethoprim/sulfamethoxazole; ns: not significant.

Institute of Tuberculosis and Lung Diseases in Warsaw in the period 2009-2016, coagulase-positive *S.aureus* was found in 115 (76.6%) nasal swabs. There were 55 women (47.8%) and 60 men (52.2%), with a mean age during the first episode of 45.0±17.0 and 44.5±13.6 years, respectively, without any statistical significant difference depending on the sex (p=0.85). In 98 patients (85.2%) the disease was generalised, while in 17 cases the disease was limited to SGS (14.8%). A single episode of staphylococcal-positive nasal swab occurred in 42 patients, in 73 others positive nasal swabs were frequently identified (2–13 episodes). In total there were 362 episodes analysed in the study. Active disease was found only in 32.6% (118 episodes) compared with 67.4% (244 episodes) of non-active disease. The characteristics of the study patients are shown in Table I.

The presence of at least one SAg was found in 126 samples (34.8%) from 56 patients (48.7%), Among the 17 patients with limited to subglottic stenosis (SGS) disease, SAg were detected in 6 cases (35.3%). A single SEA was found in 15 patients, only SEB in 2 cases, only SEC in 12, only SED in 3, only TSST-1 in 6, and in 18 cases more than one SAg was detected. In all samples the most commonly found SAg was SEA (73/126 episodes, 60%), but the rarest was SEB (8/126 episodes, 6.3%). Based on the statistical analysis, a significant relationship between the presence of SAg and disease activity was not found. The frequency of occurrence of SAg in nasal samples was similar both in active and non-active disease episodes (34.7% *vs*. 34.8%, p=0.986). Similarly, no significant correlation between the presence of individual types of SAg and the number of positive SAg in one nasal swab and activity of disease was detected (Table II).

Among the all 362 positive nasal swab episodes, 142 occurred during chronic prophylactic antimicrobial therapy by T/S and/or mupirocin (39.2%). A single T/S therapy applied was for 86 episodes (23.8%), single mupirocin for 19 episodes (5.2%) and a double antimicrobial treatment (T/S with mupirocin) for 37 episodes (60.8%). Chronic antimicrobial therapy was not carried out for 220 episodes (60.8%). Comparative analysis of these two groups demonstrated a significantly less frequency of the active disease in the treatment group compared with untreated group (24.6% *vs*. 75.4%, p=0.009), which indicates a protective effect of T/S prophylaxis in GPA patients (Table II). Monotherapy by mupirocin did not affect the frequency of active disease, but in combined therapy the effect depended on the T/S application.

Based on the logistic regression model, we assessed the influence of individual qualitative factors on disease activity (Fig. 1). The results of the analysis confirmed a protective effect of T/S treatment (0R 0.52, p<0.0092) in GPA patients. Interestingly, GPA limited to SGS appeared as an unfavourable factor associated with disease activity (OR 1.84, p=0.05). The analysis confirmed the lack of significant correlation between the presence of staphylococcal SAg and GPA activity, which was previously demonstrated, but when individual types of SAg were analysed separately, SED and TSST-1 were more frequently found in active episodes of disease, but SEA, SEB and SEC were slightly more often present in non-active episodes. These differences were without statistical significance and may only be in the trend category considered. Additionally, the

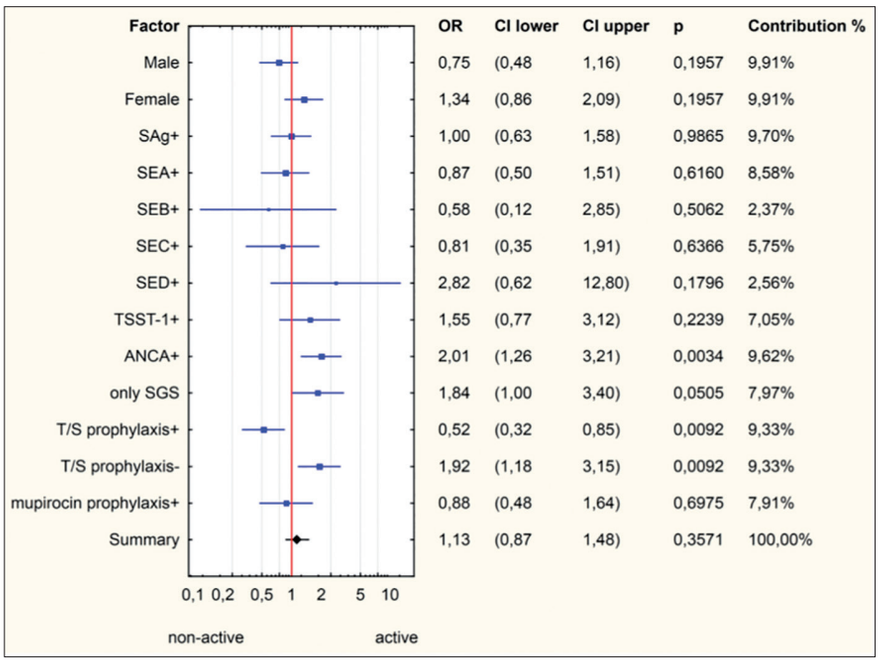

Fig. 1. The influence of selected quality parameters on the GPA activity in the logistic regression modelling in own material.
SAg: staphylococcal suprantigens; SEA: staphylococcal enterotoxin type A; SEB: staphylococcal enterotoxin type B; SEC: staphylococcal enterotoxin type C; SED: staphylococcal enterotoxin type D; TSST-1: toxick-shock syndrome toxin 1; ANCA: antineutrophil cytoplasmic antibodies; SGS: subglottic stenosis; T/S: trimethoprim/sulfamethoxazole.

analysis showed significant correlation between ANCA and GPA activity (OR 1.42, p=0.0034). Significant impact on disease activity was not detected for other factors.

Discussion

An important role for an infectious agent or agents in the induction and activity of GPA was first suggested by Friedrich Wegener in 1936 in the original description of the disease (20). He supposed that the disease is triggered by an infection of the upper respiratory tract and that all the ensuing sequelae are an allergic reaction to this infection. From 1980 and thereafter, it was suggested that *S.aureus* could be a trigger of disease activity in GPA (1, 4, 8, 9). Then, it was shown that chronic nasal carriage of *S.aureus* is approximately three times higher in GPA patients than in healthy individuals. Laudien *et al*. (21) found a nasal colonisation with *S.aureus* in 72% of GPA patients compared to 28% in chronic rhinosinusitis patients and to 25% in healthy subjects. In our study a percentage of GPA patients with positive *S.aureus* nasal swabs was similar and equalled 76.6%. Popa *et al*. (22) had other results. In his study of 1718 swab cultures analysed, 41.2% were *S.aureus*-positive.

Stegeman *et al*. (8) first described the association of nasal colonisation of *S.aureus* and increased risk for relapse of disease in GPA patients, together with creatinine clearance above 60 ml/min and a history of previous relapses of diseases. Then, Zycinska *et al*. (5) identified nasal carriage of *S.aureus* as an independent risk factor for relapse in 17 patients with limited GPA. In turn, Salmela *et al*. (23), in a multicentre prospective study including 200 newly diagnosed ANCA-associated vasculitis (AAV) patients demonstrated that in patients with generalised GPA (73 patients) and with early systemic GPA during immunosuppression (78 patients) relapse rates were higher for chronic *S.aureus* carriers. Interestingly, for all AAV patients in this study nasal *S.aureus* carriage was not associated with an increased relapse risk. In our study analysing patients with *S.aureus*-positive swab cultures, among the 362 sampling episodes active disease was found only in 32.6% compared with 67.4% of non-active disease, which suggests that the role of nasal colonisation of *S.aureus* is not evident and does not always correlate with GPA activity.

The main influence of *S.aureus* on GPA activity and relapses is attributed to its superantigens (SAg) because of its role as extremely potent immunostimulatory molecules (8, 14, 15, 24, 25), but, so far, results of clinical studies evaluating a critical role of SAg for GPA activity have not been unequivocal. Cohen Tervaert *et al*. (26) investigated 42 nasal-positive *S.aureus* cultures from GPA patients and 39 *S.aureus* cultures from diseased or healthy controls for the presence of genes of TSST-1, SEA, SEB, SEC, SED, SEE and exfoliative toxin A and B. They found, that the frequency of the different SAg genes in *S.aureus* cultures from patients with GPA was not different from the frequency of these genes in *S.aureus* cultures from controls, but patients with GPA and SAg-positive strain had a significantly greater risk for the development of a relapse compared with patients with an SAg-negative strain. Popa *et al*. (22) found that the presence of *S.aureus* was associated with relapses of GPA, but the risk was modulated by the presence and type of SAg, with TSST-1 being associated with an increased risk of relapse. In this study, of the 709 *S.aureus*-positive cultures, 46% contained at least one SAg, but only the carriage of TSST-1 represented 16.5% of the GPA isolates. On the other hand, the same author contests *S.aureus* superantigenic activity: despite the production of superantigenic toxins by nasal carriage of *S.aureus*, no peripheral T-lymphocyte repertoire bias was found (11). In turn, in a recent study from Norway, the authors reported only a trend for a higher relapse rate in GPA patients with chronic nasal *S.aureus* carriage, without statistical significance (27). In our study we did not find a relevant relationship between *S.aureus* SAg and GPA activity. The frequency of occurence of SAg in nasal samples was similar both in active and non-active disease episodes (34.7% *vs*. 34.8%, p=0.986). Nor did we find a significant correlation between individual types of SAg presence and GPA activity, although TSST-1 and SED were slightly

more frequently found in active episodes of disease (12.7% *vs*. 8.6% and 3.4% *vs*. 1.2%), while other SAg (SEA, SEB, SEC) in non-active disease were slightly more present. These differences were without statistical significance and may be only in the trend category considered, but comparative analysis of these two groups showed that patients from the first group were characterised by a more aggressive course of disease with life-threatening organ involvement (lung, kidney and central nervous system), many relapses and the long disease duration (even up to 18 years). Three of them died, one had initially refractory disease to first-line standard immunosuppressive therapy, five received rituximab and two had GPA limited to SGS characterised by a tumultuous disease course. In our study, compared to the work of Popa *et al*., the frequency of occurence of SAg was slightly less: in a total of 362 positive cultures, 34.8% (126/362) contained at least one SAg, similarly, the presence of TSST-1 (a single or co-occurring with other SAg) was rarely found (in 36/362 episodes, 10%), while the presence of SED only in 7/362 (2%) episodes was detected.

The benefit from the T/S treatment in GPA patients has long been raised in the literature. Back in 1985, DeRemee *et al*. (28) had observed a clinical improvement of 11 of 12 patients with GPA treated with T/S, raising the possibility of a microbial infection as an inciting cause of GPA. Then, in 1990, Roberts *et al*. (29) noted that sulfonamides may also function as anti-inflammatory agents. Following these observations, a number of publications confirmed the beneficial effect of T/S in GPA (30-32). When T/S was given at a high dose (960 mg twice daily) combined with the maintenance treatment of GPA, it could further reduce the relapse rate by 66% (32). Importantly, T/S cannot substitut immunosuppressive therapy, being clearly not as effective (33), although Tuin *et al*. (34) demonstrated that T/S monotherapy induces remission in a substantial number of GPA patients with localised disease. According to current guidelines (35), T/S should be prescribed in low doses (480 mg daily or 960 mg three times per week) as a prophylaxis against Pneumocystis jiroveci pneumonia in these patients. A controlled study by Zycinska *et al*. (5) on the prophylactic use of T/S in maintaining remission in 31 patients with GPA, showed a small but significant effect of T/S: 75% of patients in the T/S treated group *versus* 55% in the placebo group maintained remission during the 18-month study period. Our study confirmed these results. Comparative analysis of the two groups: receiving a prophylactic antimicrobial therapy by T/S and not receiving this kind of treatment showed a significantly less frequency of the active disease in the treated group compared with the untreated group (24.6% *vs*. 75.4%, p=0.009), which confirms the protective effect of T/S prophylaxix in GPA patients. Monotherapy by mupirocin did not affect the frequency of active disease episodes, and in combined therapy (mupirocin and T/S) the effect was dependent on T/S.

In conclusion, the association betwen staphylococcal SAg in nasal swabs and GPA activity is not evident and multiple mechanisms that may lead to disease activation still need to be investigated. In this prospective study we present the results of microbiological examination of nasal swabs of all consecutive hospitalised GPA patients in the period 2009–2016. Among the 150 patients, coagulase-positive *S.aureus* was found in 115 cases (76.6%), of which staphylococcal SAg was detected in 56 cases (48.7%). First, we did not find a correlation between staphylococcal SAg in nasal swabs and GPA activity, although in the group of active disease SED and TSST-1 were detected slightly more often in contrast to SEA, SEB and SEC, which were found a little more frequently in non-active disease episodes. Second, in maintaining remission of GPA, an important role is played by T/S therapy, which can be protective even when prohylactic doses are used.

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