# Autoantibody profile in eosinophilic granulomatosis and polyangiitis: predominance of anti-alpha-enolase antibodies

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*Received on May 14, 2020; accepted in revised form on August 31, 2020.*

*Clin Exp Rheumatol 2021; 39 (Suppl. 129): S83-S87.*



**Key words:** eosinophilic granulomatosis and polyangiitis (EGPA), anti-alpha-enolase antibodies, anti-neutrophil cytoplasmic antibodies (ANCA)

*Competing interests: none declared.*

## ABSTRACT

**Objective.** *To evaluate the autoantibody profile in eosinophilic granulomatosis and polyangiitis (EGPA) patients.*

**Methods.** *33 EGPA patients were tested for anti-neutrophil cytoplasmic antibodies (ANCA), antinuclear antibodies (ANA), rheumatoid factor (RF), anti-alpha-enolase antibodies, and anti-eosinophil peroxidase (EPO) antibodies. Granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), hypereosinophilic syndrome (HES), rheumatoid arthritis (RA), primary biliary cirrhosis (PBC) patients and healthy subjects were tested as a control group.*

**Results.** *Anti-alpha-enolase antibodies were positive in 82% of EGPA patients at high titres. Although a high sensitivity was shown for an anti-alpha-enolase antibody titre above 1/100 (82%), the specificity for EGPA remained low (44%) (AUC=0.653, p=0.008). Anti-alpha-enolase antibodies predominated in males with EGPA (p=0.048) and were associated with skin involvement (p=0.040). Most of the EGPA patients positive for anti-alpha enolase antibodies (20 out of 27) had a negative indirect immunofluorescence test (IFT) for ANCA. ANCA were positive in 8 EGPA patients (24%) with a perinuclear pattern in all but one patient. The ANCA-target antigen was myeloperoxidase (MPO) and/or alpha-enolase. A usually fine-speckled ANA pattern was observed in 42% of the EGPA patients. RF was positive in 1 (6%) of the 18 EGPA patients tested. There was no association between the presence and levels of autoantibodies and EGPA disease activity. None of the patients and controls was positive for anti-EPO antibodies.*

**Conclusion.** *Alpha-enolase may be a target of autoimmunity in EGPA patients and shows usually negative ANCA IFT results.*

## Introduction

Eosinophilic granulomatosis and polyangiitis (EGPA) is a rare systemic necrotising pauci-immune vasculitis of unknown aetiology associated with peripheral and tissue eosinophilia, asthma and/or allergic rhinitis affecting small-sized vessels (1). Anti-neutrophil cytoplasmic antibodies (ANCA) are the most common autoantibodies detected in about 30–48% of patients (2-5). In this study, we evaluated the autoimmune serological profile of EGPA patients and searched for novel autoantibody specificities. Eosinophil peroxidase (EPO) of the eosinophil granulocytes as well as the glycolytic cytoplasmic enzyme alpha-enolase have been identified as autoantigens in patients with autoimmune disorders (6, 7). Besides, previous studies report on ANCA directed either against EPO or alpha-enolase in systemic vasculitis (8, 9). Apparently, the anti-alpha-enolase response does not appear to be restricted to a specific disease, but the fine epitopes have not been examined in the majority of those diseases and could be important in determining the immunopathogenicity (10). We speculate that these antibodies may also initiate inflammation leading to severe necrosis of the endothelium (11). Since both eosinophils and ANCA play a pathogenetic role in EGPA (12), our aim was to investigate if anti-alpha-enolase and anti-EPO antibodies are present in the sera of EGPA patients and estimate their diagnostic value.

## Patients and methods

This study was performed in a cohort of patients with EGPA. Sera from 37 MPA, 23 GPA and 50 RA patients matched for age, sex and disease duration with the main group were tested for ANA, ANCA and/or anti-alpha-enolase antibodies as a control group. As control group for anti-EPO anti-

bodies, sera from 12 GPA, 4 MPA, 8 HES, 5 PBC patients and 100 healthy subjects were used.

Serum samples were collected at a tertiary referral centre for vasculitis in Germany (Klinikum Bad Bramstedt). All patients fulfilling the American College of Rheumatology classification criteria for EGPA (13), followed-up for at least one year, were included in the study. Patients with acute or chronic infections and/or a history of malignancy were excluded. EGPA patients were tested for the presence of ANCA, anti-alpha-enolase antibodies, ANA, RF and anti-EPO antibodies. The design of the work was in accordance with the standards of the Helsinki Declaration of 1975/83.

*Autoantibody detection*

*- ANCA*

Indirect IFT was performed on ethanol-fixed neutrophils in combination with additional tests on formalin-fixed neutrophils and HEp-2 cells to better discriminate between P-ANCA/ atypical-ANCA and ANA, as previously described (14). For the detection of proteinase 3 (PR3)-ANCA, a third generation anti-PR3 hs Enzyme linked Immunosorbent Assay (ELISA) and first generation anti-MPO ELISA (Orgentec, Mainz, Germany) were used (15). To identify the ANCA target antigens, lactoferrin-, human leucocyte elastase-, bacterial permeability increasing protein-, and cathepsin-G-ANCA antigen specific kits from Orgentec were used.

*- Anti-EPO Autoantibodies*

In house ELISA was used. Purified EPO (Calbiochem) was coated to microtitre plates at a protein concentration of 1 microgram/ml. EGPA patients and control sera were used in a 1:50 dilution.

*- Anti-alpha enolase autoantibodies*

Anti-alpha enolase antibodies were detected using ELISA as previously described (16). Falcon microtitre plates were coated with 500 ng/well of purified bovine retinal α-enolase. Two-fold serially diluted patients' serum was added to each well followed by incubation with anti-human IgG (H and L chain) conjugated to peroxidase (Zymed, San Francisco, CA). As positive control, we used a reference human serum containing anti-recoverin antibodies diluted 1:100. As negative control, we omitted serum and applied only a secondary antibody. The results are presented as anti-alpha enolase antibody titres.

Sera of 12 EGPA patients were also tested by Western blotting (16). Retinal _lpha enolase was separated by SDS-gel electrophoresis on a 10% gel and transferred to an Immobilon membrane (Millipore, Bedford, Massachusetts). Individual strips containing alpha enolase were incubated with 1:100 diluted serum followed by 1:5000 diluted anti-human IgG (H and L chain) conjugated to alkaline phosphatase (Sigma, St. Louis, MO).

*- Other autoantibodies*

Indirect IFT on HEp-2 cells (Inova) and ELISAs (Varelisa ANA profile Enzyme-Immunoassay) were used to detect ANA and their main target antigens.

**Table I.** Clinical characteristics of the EGPA patients. Values are expressed either as absolute numbers or as proportions (%) or as median (min-max).

| | | |
|---|---|---|
| Gender | 18 | M:15 F |
| Age at serum testing [years] | 47 yrs. | (23-78) |
| Age at diagnosis [years] | 44 yrs. | (17-75) |
| Disease duration [years] | 2 yrs. | (1-21) |
| Eosinophil count at serum testing [/nl] | 212 | (4-5120) |
| Active disease, patient no. (%) | 24 | (73%) |
| Complete remission, patient no. (%) | 9 | (27%) |
| BVAS score at serum testing | 6 | (0-25) |
| Manifestations during f-u, patient no. (%) | | |
| - General symptoms | 24 | (73%) |
| - ENT/eye involvement | 28 (85%)/7 | (21%) |
| - Lung involvement | 29 | (88%) |
| - Kidney involvement | 5 | (15%) |
| - Arthralgias/arthritis | 15 | (45%) |
| - Skin involvement | 19 | (58%) |
| - Heart involvement | 16 | (48%) |
| - PNS/CNS involvement | 21 | (64%)/5 (15%) |
| - GI involvement | 13 | (39%) |
| - Thrombosis | 2 | (6%) |
| Prednisolone [mg] at serum testing | 8 | (0-28) |
| Treatment at serum testing, patient number | | |
| - CYC | 7 | |
| - IFN | 6 | |
| - MTX/AZA/LEF | 14/2/1 | |
| - None | 3 | |
| Follow up time [years] | 6 yrs. | (1-19) |

ENT: ear nose throat; PNS: peripheral nervous system; CNS: central nervous system; GI: gastrointestinal; CYC: cyclophosphamide; IFN: interferon; MTX: methotrexate; AZA: azathioprine; LEF: leflunomide.

*Statistical analysis*

Statistical analysis was performed using the SPSS program. The Mann-Whitney U and the $\chi^2$ tests were used as appropriate. Correlations between continuous variables were determined by the Pearson's correlation coefficient. A $p$-value $\leq 0.05$ was considered significant. Receiver operating characteristics (ROC) analysis was applied in order to define specificity and sensitivity of anti-alpha-enolase antibodies. In order to assess diagnostic performance, the area under the ROC curve (AUC) was calculated.

## Results

A total of 33 EGPA patients were evaluated. The clinical and laboratory patient characteristics and treatment details are shown in Table I. Twenty-four patients had active disease (Birmingham Vasculitis Activity Score, BVAS≥1) (17) and 9 patients had inactive disease (BVAS: 0 and prednisolone dose ≤7.5 mg/day). Anti-alpha-enolase antibodies were detected at high titres in 82% of EGPA patients. The results were confirmed

**Table II.** Frequency and titre of autoantibodies (Abs) in EGPA and controls.

| Abs | EGPA n=33 (active/inactive n=24/9) | MPA n=37 (active/inactive n=23/14) | GPA n=23 (active/inactive n=21/2) | RA n=50 |
|---|---|---|---|---|
| C-/P-ANCA (%) | 8 (24) | 23 (62) | 18 (78) | 2 (4) |
| Titre [Median (Min-Max)] | 1/128 (1/128-1024) | 1/256 (1/128-2048) | 1/512 (1/128-4096) | 1/1024 both |
| P-ANCA [target antigen] | 7 [2 MPO and a-enolase, 5 a-enolase] | 23 [6 MPO, 12 MPO and a-enolase, 1 PR3 and a-enolase, 4 a-enolase] | 3 [2 MPO, 1 PR3 and a-enolase] | 2 [1 LF, 1 a-enolase] |
| C-ANCA [target antigen] | 1 [MPO] | - | 15 [11 PR3, 3 PR3 and a-enolase, 1 unknown] | - |
| Anti-alpha-enolase Abs* (%) | 2‡ (82) | 27† (73) | 5** (22) | 30 (60) |
| Titre [Median (Min-Max)] | 1/1600 (1/100-6400) | 1/3200 (1/200-25600) | 1/400 (1/100-12800) | 1/200 (1/200-6400) |
| ≤1/200 | 6 | 4 | 1 | 16 |
| 1/400-800 | 8 | 6 | 3 | 10 |
| 1/1600-3200 | 2 | 8 | - | 3 |
| ≥1/6400 | 11 | 9 | 1 | 2 |
| ANA (%) | 15 (45) | 17 (46) | 7 (30) | 32 (64) |
| Titre [Median (Min-Max)] | 128 (64-1024) | 128 (64-2048) | 128 (64-640) | 256 (64-2048) |
| RF (%) | 1/18 (6) | nd | nd | 35 (70) |
| Anti-EPO Abs | 0 | 0 | 0 | 0 |

MPO: myeloperoxidase; PR-3: proteinase 3; LF: lactoferrin; nd: not done.
ANCA (IFT) positive ‡ 7/27; † 17/27; **4/5.
*10% of healthy subjects were positive for anti-alpha enolase antibodies.
Statistically significant comparisons:
ANA frequency in RA *vs*. EGPA ($p$=0.04), RA *vs*. GPA ($p$=0.02); ANA titre in RA *vs*. EGPA ($p$=0.025), RA *vs*. GPA ($p$=0.005).
ANCA frequency in GPA *vs*. EGPA ($p$=0.01), MPA *vs*. EGPA ($p$=0.001), EGPA *vs*. RA ($p$=0.026); ANCA titre in MPA *vs*. RA, GPA *vs*. RA (both $p$<0.001).
Anti-alpha-enolase frequency in EGPA *vs*. GPA ($p$<0.001), EGPA *vs*. RA ($p$=0.033), MPA *vs*. GPA ($p$<0.001), RA *vs*. GPA ($p$=0.002). *No significant difference between EGPA and MPA (p=0.403)*; Anti-alpha-enolase Ab titre in EGPA *vs*. RA ($p$=0.007), MPA *vs*. RA ($p$<0.001). *No significant difference between EGPA and MPA (p=0.264) or GPA (p=0.509).*

in all 12 sera tested by Western blotting (6 positive and 6 negative sera for anti-alpha-enolase antibodies in ELISA). Anti-alpha-enolase antibodies were also found in control patients, but their frequency was higher in EGPA as compared to GPA and RA patients and their titre higher in EGPA than RA patients (Table II, Fig. 1A). ROC analysis showed a high sensitivity for autoantibody titres above 1/100 (82%), however, the specificity for EGPA remained low (44%) (AUC=0.653, $p$=0.008). There was no association between the presence and levels of anti-alpha-enolase antibodies and disease activity ($p$>0.40). Anti-alpha-enolase antibodies were detected more often in males ($p$=0.048) and were associated with skin involvement (all 19 EGPA patients with infarcts, purpura, ulcers and/or gangrene were positive for anti-alpha-enolase antibodies) ($p$=0.040). Most of the EGPA patients were positive for anti-alpha enolase antibodies but were negative in IFT ANCA (20/27) in contrast to MPA (10/27) and GPA (1/5).

ANCA were detected by IFT in 24% of EGPA patients showing a perinuclear pattern in the majority of cases. The ANCA target antigens were MPO and/or alpha-enolase (Table II). ANCA were more common in EGPA as compared to RA and less common in EGPA than GPA and MPA patients, but with comparable titres ($p$>0.05) (Table II). There was no association between the ANCA presence ($p$=0.653) or antibody levels ($p$=0.543) and disease activity and other EGPA patient characteristics ($p$>0.05). When EGPA and MPA patients were evaluated together, MPO-specificity in the ANCA-ELISA was associated with lower frequency ($p$=0.047) and titre ($p$=0.09) of anti-alpha-enolase antibodies (Fig. 1B).

ANA, with a usually fine-speckled pattern, occurred at lower titres and less frequently in EGPA (45%) than RA patients (Table II). They were not associated with disease activity or other clinical characteristics ($p$>0.05). EGPA patients were seronegative for ENA. RF was found positive in 1/18 (6%) of EGPA patients tested. Also, all patients and controls were negative for anti-EPO antibodies.

## Discussion

EGPA has been classified alongside MPA and GPA as an ANCA-associated vasculitis (AAV), despite ANCA being found in the minority of cases. The frequency of ANCA was observed in only 24% of our EGPA patients with the most common perinuclear pattern, which is in an agreement with previous studies (2-5). Lyons *et al*. suggest that EGPA might be comprised of two distinct diseases defined by ANCA status, since they had revealed genetic and clinical differences between the EGPA MPO-positive subset and the larger ANCA-negative subset (18). This distinction may suggest an important het-

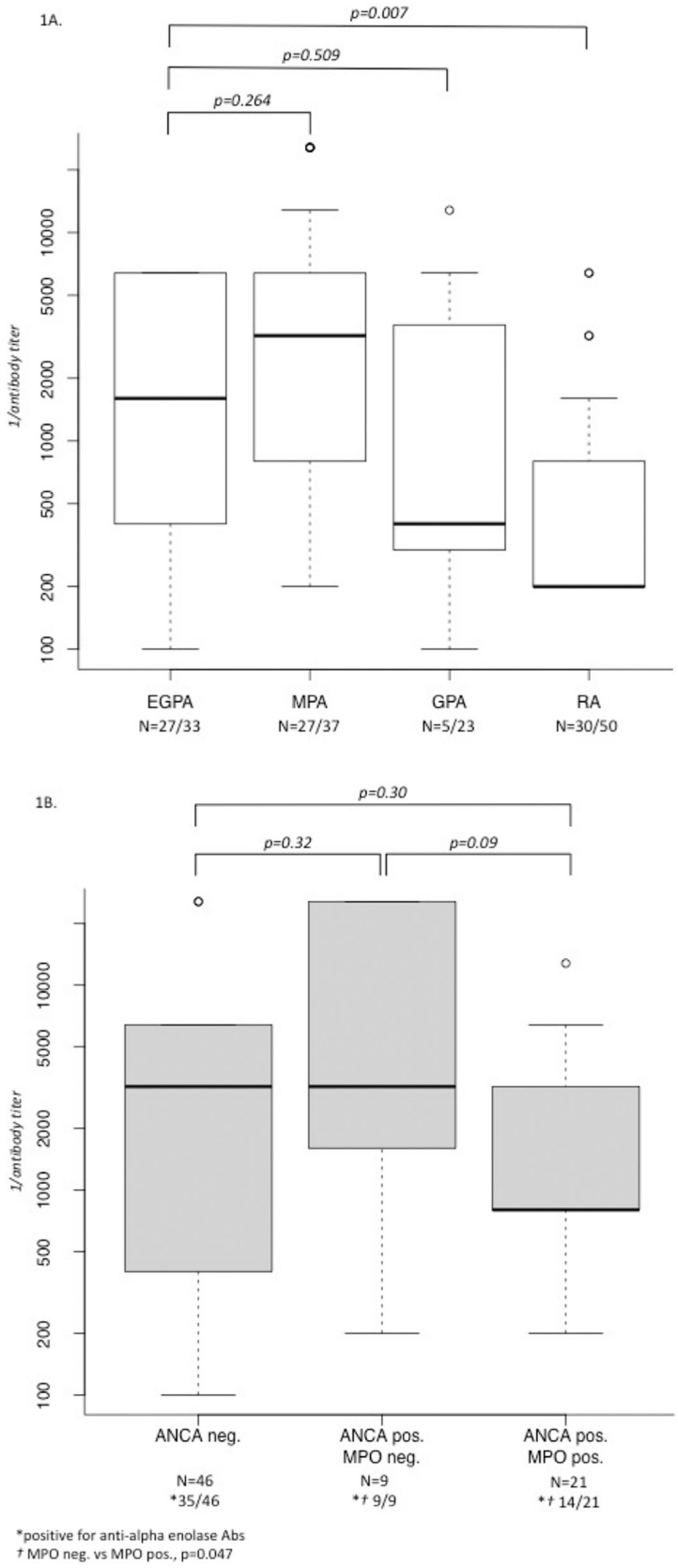


**Fig. 1. A**: Box plot of the antibody titre in EGPA patients and controls positive for anti-alpha enolase antibodies. **B**: Box plot of the anti-alpha enolase antibody titre in EGPA/MPA patients without ANCA and patients with ANCA with and without MPO specificity.
The median value in each category is shown by the horizontal line inside the box.
MPO: myeloperoxidase; Abs: antibodies.

erogeneity in the pathogenesis of the clinical syndrome. The present data support an idea that alpha-enolase can be considered as potential target autoantigen for ANCA in EGPA patients.

Anti-alpha-enolase antibodies have been previously reported in AAV at a lower frequency as compared to the present data (40% *vs*. 64%) (9). Our study highlights a high frequency and titre of anti-alpha-enolase antibodies, particularly in EGPA patients (82%). In spite of the high sensitivity, we failed to demonstrate a high specificity of these autoantibodies for EGPA. However, further research on the identification of epitopes for alpha-enolase antibodies could reveal a subset of autoantibodies specific for the disease. Neither the presence nor the titre of anti-alpha enolase antibodies was associated with disease activity in both EGPA patients and controls.

Alpha-enolase has been identified as an ANCA autoantigen in the past (9) and has been shown on the surface of neutrophils (11). It is of note that, although MPO-specificity is most commonly detected in ELISA, in some EGPA cases the ANCA target antigen remains unknown (4, 5). The present study supports alpha-enolase to be another autoantigen in cases with unknown ANCA specificity and sometimes patients with negative ANCA IFT. Among our AAV patients, 61% of those positive for anti-alpha-enolase antibodies were also positive for ANCA (IFT). In particular, among EGPA patients, only 26% of those positive for anti-alpha-enolase antibodies had a positive IFT for ANCA. It is of interest that patients without ANCA or without MPO-specificity in ANCA-ELISA had more frequently and at higher titres anti-alpha enolase antibodies.

Alpha-enolase is not exclusively expressed in neutrophils but also in other cells (11). Indeed, the detection of alpha-enolase in keratinocytes (19) supports the hypothesis that anti-alpha-enolase antibodies may be a causative factor for the development of skin lesions in autoimmune patients. In fact, in the present study all EGPA patients with skin lesions were positive for anti-alpha-enolase antibodies. Skin involve-

ment is a common complication occurring in 40%-70% of EGPA patients (12). The histopathologic spectrum of dermal small-vessel vasculitis ranges from eosinophilic vasculitis with negative MPO-ANCA to neutrophilic vasculitis with positive MPO-ANCA (20). Thus, in patients with cutaneous vasculitic lesions without ANCA, the detection of anti-alpha-enolase antibodies may help in the diagnosis of EGPA.

Finally, in agreement with our results, low levels of circulating RF (21) and ANA (2) have occasionally been described in EGPA but appeared to be nonspecific. Regarding anti-EPO antibodies, our results argue against the presence of these antibodies in health and disease. We also consider that differences in techniques may explain inconsistency in results when compared to previously published studies (8).

Summarising, the detection of autoantibodies in the sera of EGPA patients supports an evidence for the role of autoimmune mechanisms in the development of the disease. Anti-alpha-enolase antibodies occurred in a high percentage of EGPA patients at high titres with usually negative IFT for ANCA and were associated with skin involvement. We believe that enolase could serve as a new antigenic target in the disease pathogenetic mechanisms. Further studies with a greater number of patients will reassure our results and test for autoantibodies directed against unique epitopes of alpha-enolase specific for EGPA with a potential diagnostic value and scientific rationale for targeted therapy.

## References


1. MONTI S, BOND M, FELICETTI M *et al*.: One year in review 2019: vasculitis. *Clin Exp Rheumatol* 2019; 37 (Suppl. 117): S3-19.
2. GUILLEVIN L, COHEN P, GAYRAUD M *et al*.: Churg-Strauss syndrome. Clinical study and long-term follow-up of 96 patients. *Medicine* (Baltimore) 1999; 78: 26-37.
3. SINICO RA, DI TOMA L, MAGGIORE U *et al*.: Prevalence and clinical significance of antineutrophil cytoplasmic antibodies in Churg-Strauss syndrome. *Arthritis Rheum* 2005; 52: 2926-35.
4. COMARMOND C, PAGNOUX C, KHELLAF M *et al*.: Eosinophilic granulomatosis with polyangiitis (Churg-Strauss): clinical characteristics and long-term followup of the 383 patients enrolled in the French Vasculitis Study Group cohort. *Arthritis Rheum* 2013; 65: 270-81.
5. MOOSIG F, BREMER JP, HELLMICH B *et al*.: A vasculitis centre based management strategy leads to improved outcome in eosinophilic granulomatosis and polyangiitis (Churg-Strauss, EGPA): monocentric experiences in 150 patients. *Ann Rheum Dis* 2013; 72: 1011-7.
6. PANCHOLI V: Multifunctional a-enolase: its role in diseases. *Cell Mol Life Sci* 2001; 58: 902-20.
7. TAKIGUCHI J, OHIRA H, RAI T *et al*.: Anti-eosinophil peroxidase antibodies detected in patients with primary biliary cirrhosis. *Hepatol Res* 2005; 32: 33-7.
8. DOLMAN KM, DAMSMA I, TOOL ATJ *et al*.: A novel specificity of anticytoplasmic autoantibodies directed against eosinophil peroxidase. *Clin Exp Immunol* 1993; 92: 58-64.
9. MOODIE FD, LEAKER B, CAMBRIDGE G *et al*.: Alpha-enolase: a novel cytosolic autoantigen in ANCA positive vasculitis. *Kidney Int* 1993; 43: 675-81.
10. ADAMUS G, AMUNDSON D, SEIGEL GM, MACHNICKI M: Anti-enolase-alpha autoantibodies in cancer-associated retinopathy: epitope mapping and cytotoxicity on retinal cells. *J Autoimmun* 1998; 11: 671-77.
11. ADAMUS G: Impact of Autoantibodies against Glycolytic Enzymes on Pathogenicity of Autoimmune Retinopathy and Other Autoimmune Disorders. *Front Immunol* 2017; 8: 505.
12. NGUYEN Y, GUILLEVIN L: Eosinophilic Granulomatosis with Polyangiitis (Churg-Strauss). *Semin Respir Crit Care Med* 2018; 39: 471-81.
13. MASI AT, HUNDER GG, LIE JT *et al*.: The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). *Arthritis Rheum* 1990; 33: 1094-100.
14. CSERNOK E, MOOSIG F: Current and emerging techniques for ANCA detection in vasculitis. *Nat Rev Rheumatol* 2014; 10: 494-501.
15. HELLMICH B, CSERNOK E, FREDENHAGEN G, GROSS WL: A novel high sensitivity ELISA for detection of antineutrophil cytoplasm antibodies against proteinase-3. *Clin Exp Rheumatol* 2007; 25 (Suppl. 44): S1-5.
16. ADAMUS G, APTSIAURI N, GUY J *et al*.: The occurrence of serum autoantibodies against enolase in cancer-associated retinopathy. *Clin Immunol Immunopathol* 1996; 78: 120-9.
17. LUQMANI RA, BACON PA, MOOTS BA *et al*.: Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. *Q J Med* 1994; 87: 1-8.
18. LYONS PA, PETERS JE, ALBERICI F *et al*.: Genome-wide association study of eosinophilic granulomatosis with polyangiitis reveals genomic loci stratified by ANCA status. *Nat Commun* 2019; 10: 5120.
19. OLSEN E, RASMUSSEN HH, CELIS JE: Identification of proteins that are abnormally regulated in differentiated cultured human keratinocytes. *Electrophoresis* 1995; 16: 2241-8.
20. SHIIYAMA R, CHEN KR, ISHIBASHI M: A Case of Cutaneous Arteritis Presenting as Infiltrated Erythema in Eosinophilic Granulomatosis With Polyangiitis: Features of the Unique Morphological Evolution of Arteritis as a Diagnostic Clue. *Am J Dermatopathol* 2019; 41: 832-34.
21. LANGHAM JG, ELKON KB, PUSEY CD, HUGHES GR: Systemic vasculitis with asthma and eosinophilia: a clinical approach to the Churg-Strauss Syndrome. *Medicine* (Baltimore) 1984; 63: 65-81.