Spectrum of ANCA-specificities in eosinophilic granulomatosis with polyangiitis. A retrospective multicentre study

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Abstract

Objective

To determine the spectrum of anti-neutrophil cytoplasmic antibody (ANCA) antigen-specificities in eosinophilic granulomatosis with polyangiitis (EGPA), an ANCA-associated vasculitis (AAV) entity.

Methods

We conducted a retrospective analysis of 73 EGPA patients from three German tertiary referral centres for vasculitis. In addition to in-house ANCA testing, pentraxin 3 (PTX3)- and olfactomedin 4 (OLM4)-ANCA were determined using a prototype cell-based assay for research (EUROIMMUN, Lübeck, Germany). Patient characteristics and clinical manifestations were evaluated and compared based on ANCA status.

Results

Myeloperoxidase (MPO)-ANCA positive patients (n=8; 11%) significantly more frequently displayed peripheral nervous system (PNS) and pulmonary involvement and less frequently heart involvement compared to MPO-ANCA negative patients. PTX3-ANCA positive patients (n=5; 6.8%) had a significantly higher prevalence of ear, nose and throat, pulmonary, gastrointestinal and PNS involvement, and a lower prevalence of renal and central nervous system involvement compared to PTX3-ANCA negative patients. Proteinase 3 (PR3)-ANCA and OLM4-ANCA were detected in 2 patients (2.7%) each with multiorgan involvement. One PR3-ANCA positive patient was also positive for bactericidal permeability increasing protein (BPI)-ANCA.

Conclusion

In addition to MPO, the spectrum of ANCA antigen specificities includes various other target antigens such as PR3, BPI, PTX3, and OLM4, potentially segregating further EGPA subgroups. A lower prevalence of MPO-ANCA was detected in this study compared with other studies. OLM4 is reported as novel ANCA antigen-specificity in EGPA, and thus AAV.

Key words

eosinophilic granulomatosis with polyangiitis, anti-neutrophil cytoplasmic antibodies, myeloperoxidase, pentraxin 3, olfactomedin 4

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Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare organ- and life-threatening disease characterised by systemic necrotising vasculitis predominantly affecting small- to medium-sized vessels and extravascular eosinophil-rich and necrotising granulomatous inflammation associated with asthma and hypereosinophilia (1, 2). In Europe, the annual incidence of EGPA is 1.1 cases per million individuals and the prevalence 12.1 per 1 million individuals (3). Thus, EGPA is less common than granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA), the other two anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) entities (2, 3). GPA and MPA are strongly associated with proteinase 3 (PR3)-ANCA and myeloperoxidase (MPO)-specific ANCA, respectively. By contrast, only about 30% of the patients with EGPA are ANCA positive, mostly MPO-ANCA. The remainder is commonly referred to as "ANCA negative" with reference to their MPO-ANCA status (2, 4, 5). Accordingly, two subtypes of EGPA with differing clinical presentations, genetic associations and response to therapy can be distinguished based on the presence or absence of MPO-specific ANCA (2, 4, 6). Whereas MPO-ANCA positive patients more often present with glomerulonephritis and peripheral neuropathy, MPO-ANCA negative patients more frequently present with lung infiltrates and cardiomyopathy (4, 6). Furthermore, a retrospective analysis showed a higher efficacy of anti-CD20 antibody treatment with rituximab in MPO-ANCA positive EGPA patients compared with ANCA negative patients (6).

As yet, few studies reported on the detection of selected single ANCA specificities other than MPO such as PR3, alpha-enolase, lactoferrin (LF) or pentraxin 3 (PTX3) in the group of MPO-ANCA negative EGPA patients (5, 7-12). Only one study addressed the detection of a choice of different ANCA antigen-specificities, *i.e.* MPO, PR3, LF, cathepsin G (CG), bactericidal permeability increasing protein (BPI), and human elastase (HLE), in AAV. However, only few EGPA patients were included in that study with solely one being MPO-ANCA positive (13). Thus, we conducted the present study to determine the spectrum of ANCA antigen-specificities and analyse their association with clinical features in a multicentre cohort of patients with EGPA.

Materials and methods

This study was conducted as a retrospective analysis of 73 EGPA patients treated between 2015 and 2020 in three tertiary referral centres for vasculitis in Germany. The diagnosis of EGPA was based on the 2012 revised International Chapel Hill Consensus Conference (CHCC) definitions, 1990 American College of Rheumatology (ACR) and 2022 American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) and Mepolizumab in Relapsing and Refractory EGPA (MIRRA) criteria (1, 14-16). At least one of the respective criteria had to be met to be included in the study. The study complies with the Declaration of Helsinki and was approved by the regional ethics review board of the University of Lübeck (Az 16-199). All participants gave their informed consent. Patient characteristics, clinical manifestations and laboratory findings were evaluated based on the medical charts. Disease activity and vasculitis damage were assessed using the Birmingham Vasculitis Activity Score (BVAS) V3.0 and Vasculitis Damage Index (VDI) (17, 18). Calculations regarding clinical manifestations refer to the cumulative organ involvement. Serum samples were collected at all three tertiary referral centres. In-house ANCA testing was performed using indirect immunofluorescence (IFT) on ethanol- and formalin-fixed human neutrophils and antigen-specific immunoassays (enzyme-linked immunosorbent assay, ELISA, and chemiluminescence immunoassay, CLIA) as described previously (19, 20). In addition to in-house ANCA testing covering MPO-, PR3-, BPI-, LF-, CG-, and HLE-specificity, PTX3- and olfactomedin 4 (OLM4)-ANCA were determined using a prototype

cell-based assay (CBA) for research (EUROIMMUN, Lübeck, Germany). Statistical analysis was performed using GraphPad Prism software. Fisher's exact test was employed for comparison of patient groups. A p-value ≤0.05 was considered significant.

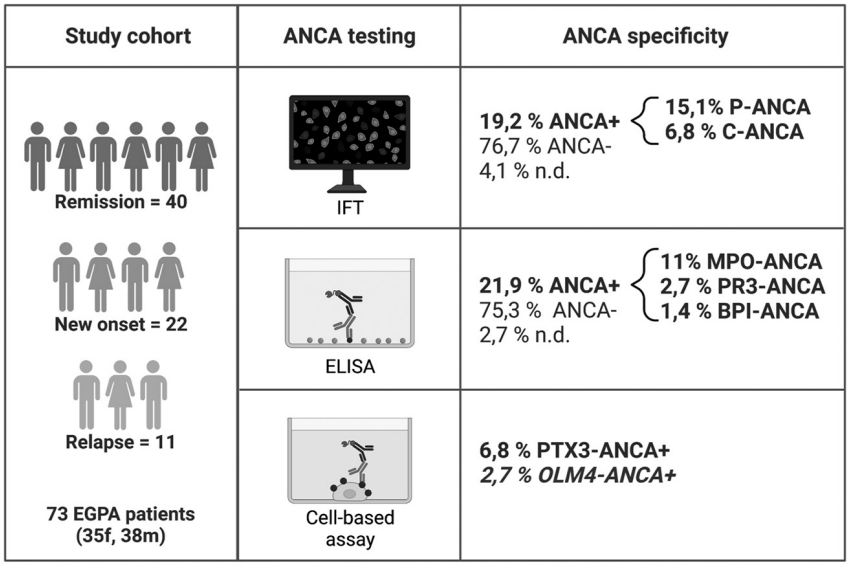
Results

Overall, 73 EGPA patients were included, hereof 35 females and 38 males with a median age of 58 years [46-65 years, IQR]. All patients met the 2012 CHCC definitions, and ACR 1990 and ACR/EULAR 2022 criteria. No asthma was found in 3 of 73 patients, who therefore did not meet the MIRRA criteria, which classify asthma as an essential feature. However, history of allergy other than asthma, hypereosinophilia, multi-organ involvement including mononeuritis multiplex, and biopsy-proven necrotising eosinophilic granulomatosis and vasculitis were shown in these 3 patients, therefore also matching 2012 CHCC definitions, and ACR 1990 and ACR/EULAR 2022 criteria. At the time of serum testing, most patients were in remission (n=40). Twenty-two patients presented with new onset EGPA, 11 patients had a relapse. In this group of patients, pulmonary involvement was the most common organ manifestation (n=28), followed by ear, nose, and throat (ENT) (n=25), peripheral nervous system (PNS; polyneuropathy and mononeuritis multiplex; n=17), heart (n=15), general symptoms (n=14), gastrointestinal tract (n=10), arthralgia/arthritis (n=10), kidney (n=5), skin (n=5) and central nervous system (CNS) (n=3). With regard to specific lung manifestations, 70 patients (95.9%) suffered from asthma and 50 (68.5%) had pulmonary infiltrates. Alveolar haemorrhage was reported in 3 patients (4.1%). A closer look at ENT manifestations showed that 33 patients (45.2%) suffered from polyposis nasi and 65 (89.0%) had rhinosinusitis.

Patients in remission had no clinical manifestations at the time of serum testing. Most of the patients were treated with azathioprine (n=21), followed by methotrexate (n=15), cyclophosphamide (n=7), a combination of azathioprine and mepolizumab (n=4), mepolizumab (n=3), mycophenolate (n=3), rituximab (n=2), and leflunomide (n=1). Sixteen patients were not on immunosuppressive therapy except for glucocorticoid therapy. In total, 58 patients received glucocorticoid therapy at the time of serum testing. The median prednisolone dose was 5 mg (2-8 mg, IQR). The median BVAS V3.0 was 0 (0-12, IQR) and median VDI 1 (0-3, IQR).

The median eosinophil count was 290/µl (80–630/µl, IQR), median leukocyte count 8360/µl (6775–10650/µl, IQR), and median CRP concentration 3.2 mg/l (2.6-7.7 mg/l, IQR). ANCA IFT was positive in 14 patients (P-ANCA, n=11; C-ANCA, n=5; double-positive, n=2). At diagnosis 56 patients were ANCA negative on IFT, and no data were available for 3 patients. There were two ANCA positive patients with both perinuclear and cytoplasmatic staining patterns on IFT with one being MPO-ANCA positive and the other being PR3-ANCA positive on ELISA. The PR3-ANCA positive patient with perinuclear and cytoplasmatic staining pattern on IFT displayed an additional BPI-ANCA on ELISA. Overall, at diagnosis ten patients had a positive ANCA ELISA (MPO-ANCA, n=8; PR3-ANCA, n=2; BPI-ANCA, n=1; double-positive, n=1), 61 patients were negative in ELISA, and no data were available for 2 patients. At the time of serum testing ANCA IFT was positive in 7 patients (P-ANCA, n=6; C-ANCA, n=1), 63 patients were ANCA negative on IFT, and no data were available for 3 patients. Two of the P-ANCA positive patients were MPO-ANCA positive on ELISA. Another patient that was ANCA negative on IFT had a positive MPO-ANCA on

A



B

ANCA specificity	Clinical manifestations
MPO-ANCA+	⇧PNS, pulmonary involvement ⇩heart involvement
PTX3-ANCA+	⇧ENT, pulmonary, GI, PNS involvement ⇩renal, CNS involvement
PR3-ANCA+	multiorgan involvement
OLM4-ANCA+	multiorgan involvement

Fig. 1. Graphical abstract of the study. Retrospective analysis of ANCA specificities in 73 EGPA patients collected from three German tertiary referral centres for vasculitis using different ANCA testing strategies. Frequencies of ANCA specificities are displayed in percent. Novel ANCA specificity OLM4 is highlighted in italics (**A**). Clinical manifestations according to the ANCA status are indicated with an arrow up for significantly higher prevalence and an arrow down for significantly lower prevalence compared to the corresponding patients with negative ANCA status (**B**).
BPI: bactericidal permeability increasing protein; CNS: central nervous system; ELISA: enzyme-linked immunosorbent assay; ENT: ear nose throat; GI: gastrointestinal; IFT: indirect immunofluorescence; MPO: myeloperoxidase; n.d.: no data; OLM4: olfactomedin 4; PNS: peripheral nervous system; PR3: proteinase 3; PTX3: pentraxin 3.
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ELISA. The C-ANCA positive patient was negative on ELISA. One patient was PR3-ANCA positive being ANCA negative on IFT. Using CBA panel (EUROIMMUN, Lübeck, Germany), PTX3-ANCA were detected in 5 patients, OLM4-ANCA in 2 patients at the time of serum testing. Three of the 5 PTX3-ANCA positive patients had a negative IFT and ELISA. At the time of serum testing 2 of them, however, were P-ANCA positive, one showing a negative ANCA ELISA while the other was MPO-ANCA positive. The two OLM4-ANCA positive patients showed a negative IFT and ELISA (Table I). Other ANCA antigen-specificities of our panel were not detected.

Compared to MPO-ANCA negative EGPA patients, the group of patients being MPO-ANCA positive had a higher prevalence of PNS (75% *vs*. 47.7%, p=0.0001) and lung (100% *vs*. 89.2%, p=0.0007) involvement. MPO-ANCA positive patients also more frequently had skin involvement, but this difference did not reach statistically significant difference (37.5% *vs*. 23.1%, respectively). MPO-ANCA negative patients had a higher prevalence of heart (44.6% *vs*. 12.5%, p<0.0001), CNS (13.8% *vs*. 0%, p<0.0001) and ENT (87.7% *vs*. 75%, p=0.0279) involvement. MPO-ANCA negative patients also presented more frequently with gastrointestinal involvement, but this difference was not statistically significant (21.5% *vs*. 12.5%, respectively). Pulmonal infiltrates occurred significantly more frequently in MPO-ANCA negative patients compared to MPO-ANCA positive patients (p<0.0001). Of the 3 patients with alveolar haemorrhage, 2 were ANCA negative, one had a positive C-ANCA in immunofluorescence, but ELISA testing for ANCA-specificity was negative. No significant difference was seen when comparing these two groups with respect to asthma and alveolar haemorrhage. Regarding ENT manifestations, no significant difference could be found in the comparison of MPO-ANCA positive and MPO-ANCA negative patients.

PTX3-ANCA positive patients had a higher prevalence of ENT (100% *vs*. 85.3%, p<0.0001), pulmonary (100%

Table I. Clinical characteristics and ANCA status of EGPA patients.

Number of patients, n	73
Gender, n (%)	
Male	38 (52.1%)
Female	35 (47.9%)
Age at serum testing (years); median, [IQR]	58 [46–65]
ANCA IFT, n (%)	
ANCA positive	14 (19.2%)
ANCA negative	56 (76.7%)
Perinuclear ANCA-pattern (P-ANCA)	11 (15.1%)
Cytoplasmatic ANCA-pattern (C-ANCA)	5 (6.8%)
Double-positive	2 (2.7%)
no data	3 (4.1%)
ANCA IgG-ELISA/CBA, n (%)	
ANCA positive	16 (21.9%)*
ANCA negative	55 (75.3%)
Anti-MPO	8 (11%)
Anti-PTX3	5 (6.8%)
Anti-PR3	2 (2.7%)
Anti-OLM4	2 (2.7%)
Anti-BPI	1 (1.4%)
no data	2 (2.7%)
Disease activity, n (%)	
New onset	22 (30.1%)
Relapse	11 (15.1%)
Remission	40 (54.8%)
BVAS V3.0 at serum testing; median, [IQR]	0 [0–12]
VDI at serum testing; median, [IQR]	1 [0–3]
Clinical features at serum testing, n (%)	
General symptoms (fever, night sweats, weight loss)	14 (23.3%)
ENT	25 (34.2%)
Lung**	28 (38.4%)
Kidney	5 (6.9%)
Arthralgia/arthritis	10 (13.7%)
Myalgia/myositis	0 (0%)
Skin	5 (6.9%)
Heart	15 (20.5%)
PNS	17 (23.3%)
CNS	3 (4.1%)
GI	10 (13.7 %)
No clinical manifestation at serum testing	40 (54.8%)
Cumulative clinical features, n (%)	
General symptoms (fever, night sweats, weight loss)	31 (42.5%)
ENT	63 (86.3%)
Lung**	66 (90.4%)
Kidney	11 (15.1%)
Arthralgia/arthritis	19 (26.0%)
Myalgia/myositis	4 (5.5%)
Skin	18 (24.7%)
Heart	30 (41.1%)
PNS	37 (50.7%)
CNS	9 (12.3%)
GI	15 (20.5%)
Eosinophil count at serum testing (/µl); median, [IQR]	290 [80–630]
Leucocyte count at serum testing (/µl); median, [IQR]	8360 [6775–10650]
CRP at serum testing (mg/l); median, [IQR]	3.2 [2.6–7.7]
Prednisolone dose at serum testing (mg); median, [IQR]	5 [2–8]
Immunosuppressive therapy at serum testing, n (%)	
Azathioprine	21 (28.8%)
Methotrexate	15 (20.5%)
Mepolizumab	3 (4.1%)
Cyclophosphamide	7 (9.6%)
Mycophenolate	3 (4.1%)
Azathioprine + mepolizumab	4 (5.5%)
Leflunomide	1 (1.4%)
Rituximab	2 (2.7%)
No therapy	16 (21.9%)

BPI: bactericidal permeability increasing protein; CBA: cell-based assay; CNS: central nervous system; ELISA: enzyme-linked immunosorbent assay; ENT: ear nose throat; GI: gastrointestinal; IFT: indirect immunofluorescence; MPO: myeloperoxidase; OLM4: olfactomedin 4; PNS: peripheral nervous system; PR3: proteinase 3; PTX3: pentraxin 3.

*Two patients had ANCA-bispecificity, one was PR3- and BPI-ANCA positive, one was MPO- and PTX3-ANCA positive.

**Lung: includes asthma, infiltrates and other pulmonary manifestations.

vs. 89.7%, *p*=0.0015), gastrointestinal (60% *vs*. 17.6%, *p*<0.0001) and PNS involvement (80% *vs*. 48.5%, *p*<0.0001) as well as general symptoms (60% *vs*. 41.2%, *p*=0.0107) compared to PTX3-ANCA negative patients. Renal (0% *vs*. 16.2%, *p*<0.0001) and CNS involvement (0% *vs*. 13.2%, *p*=0.0002) occurred less frequently in PTX3-ANCA positive patients. PTX3-ANCA positive patients had significantly more pulmonary infiltrates (*p*<0.0001), no difference was seen in asthma or alveolar haemorrhage in comparison to PTX3-ANCA negative patients. Moreover, the PTX3-ANCA positive patients suffered significantly more frequently from polyposis nasi (*p*<0.0001) as well as rhinosinusitis (*p*=0.0003) compared to the PTX3-ANCA negative patients.
PR3-ANCA were detected in 2 patients, one presented with relapsing EGPA with multiorgan involvement, the other one was in remission. Furthermore, we found 2 OLM4-ANCA positive patients who presented with new onset and relapsing EGPA, respectively, both with multiorgan involvement (Fig. 1, Table II).

Table II. Comparison of clinical manifestations according to ANCA status.

Manifestation, n (%)	MPO-ANCA positive	MPO-ANCA negative	*p*-value
General symptoms	3 (37.5%)	28 (43.1%)	ns
ENT	6 (75%)	57 (87.7%)	0.0279
Lung*	8 (100%)	58 (89.2%)	0.0007
Kidney	1 (12.5%)	10 (15.4%)	ns
Arthralgia/arthritis	3 (37.5%)	16 (24.6%)	ns
Myalgia/myositis	1 (12.5%)	3 (4.6%)	ns
Skin	3 (37.5%)	15 (23.1%)	ns
Heart	1 (12.5%)	29 (44.6%)	<0.0001
PNS	6 (75%)	31 (47.7%)	0.0001
CNS	0 (0%)	9 (13.8%)	<0.0001
GI	1 (12.5%)	14 (21.5%)	ns
	PTX3-ANCA positive	PTX3-ANCA negative	*p*-value
General symptoms	3 (60%)	28 (41.2%)	0.0107
ENT	5 (100%)	58 (85.3%)	<0.0001
Lung*	5 (100%)	61 (89.7%)	0.0015
Kidney	0 (0%)	11 (16.2%)	<0.0001
Arthralgia/arthritis	3 (60%)	16 (23.5%)	<0.0001
Myalgia/myositis	0 (0%)	4 (5.9%)	0.0289
Skin	1 (20%)	17 (25%)	ns
Heart	2 (40%)	28 (41.2%)	ns
PNS	4 (80%)	33 (48.5%)	<0.0001
CNS	0 (0%)	9 (13.2%)	0.0002
GI	3 (60%)	12 (17.6%)	<0.0001
	PR3-ANCA positive	PR3-ANCA negative	*p*-value
General symptoms	1 (50%)	30 (42.3%)	ns
ENT	2 (100%)	61 (85.9%)	<0.0001
Lung*	1 (50%)	65 (91.5%)	<0.0001
Kidney	1 (50%)	10 (14.1%)	<0.0001
Arthralgia/arthritis	0 (0%)	19 (26.8%)	<0.0001
Myalgia/myositis	0 (0%)	4 (5.6%)	0.0289
Skin	0 (0%)	18 (25.4%)	<0.0001
Heart	0 (0%)	30 (42.3%)	<0.0001
PNS	1 (50%)	36 (50.7%)	ns
CNS	0 (0%)	9 (12.7%)	0.0002
GI	0 (0%)	15 (21.1%)	<0.0001
	OLM4-ANCA positive	OLM4-ANCA negative	*p*-value
General symptoms	2 (100%)	29 (40.8%)	<0.0001
ENT	2 (100%)	61 (85.9%)	<0.0001
Lung*	2 (100%)	64 (90.1%)	0.0015
Kidney	1 (50%)	10 (14.1%)	<0.0001
Arthralgia/arthritis	1 (50%)	18 (25.4%)	0.0004
Myalgia/myositis	0 (0%)	4 (5.6%)	0.0289
Skin	1 (50%)	17 (23.9%)	0.0002
Heart	1 (50%)	29 (40.9%)	ns
PNS	1 (50%)	34 (47.9%)	ns
CNS	0 (0%)	9 (12.7 %)	0.0002
GI	0 (0%)	15 (21.1%)	<0.0001

CNS: central nervous system; ENT: ear nose throat; GI: gastrointestinal; MPO: myeloperoxidase; OLM4: olfactomedin 4; PNS: peripheral nervous system; PR3: proteinase 3; PTX3: pentraxin 3.
*Lung: includes asthma, infiltrates and other pulmonary manifestations.

Discussion

In the present study, we aimed to determine the spectrum of ANCA antigen-specificities in a retrospective analysis of a multicentre cohort from three German vasculitis centres. Classified as AAV, EGPA has less overlap to GPA and MPA than the latter two among each other with respect to genetic, pathophysiological, clinical and therapeutic aspects. Moreover, EGPA segregates into two genetically distinct subsets based on the presence or absence of MPO-ANCA aligning with differences in clinical presentation and possibly also response to rituximab therapy (2). MPO-ANCA positive EGPA shares *HLA-DQ* association and clinical features such as glomerulonephritis and peripheral neuropathy with MPA suggestive of an autoimmune-driven pathophysiology with prominent eosinophilic features. By contrast, MPO-ANCA negative EGPA shares susceptibility variants with asthma, many of which affect mucosal barrier function such as *GPA33*, and more frequently displays disease manifestations characterised by tissue eosinophilia such as lung infiltrates and cardiomyopathy suggestive of an eosinophil-driven pathogenesis, facilitated by mucosal dysfunction and potentially triggered by environmental factors (6). Generally, about 30% of the EGPA patients are reported to be MPO-ANCA positive (2). However, it should be noted that frequencies of MPO-ANCA in EGPA vary between 10–70% depending on disease activity, methodological aspects and cohorts examined (4, 5, 8,

21, 22). Contrasting with MPA, MPO-ANCA disappear in EGPA with the reconstitution of remission (21). Consistent with earlier studies (4, 6), MPO-ANCA positive patients also more frequently displayed PNS involvement and less frequently heart involvement compared to MPO-ANCA negative patients in our study. Likewise, we found a lower prevalence of PTX3-ANCA positive patients in our cohort (5 patients, 6.8%) than previously reported (40%) (11, 23). Similar to the findings reported in those earlier studies (11, 23), we found 3 PTX3-ANCA positive patients amongst the group of patients showing no other ANCA antigen-specificities. Also consistent with the findings of those studies (11, 23), the majority of PTX3-ANCA positive patients had active EGPA in our study. Four of the five PTX3-ANCA positive patients in our cohort presented with new onset disease, whereas one PTX3-ANCA positive patient was in remission. Previous studies have shown that PTX3-ANCA levels were higher in patients with active disease compared with inactive disease (11). Thus, methodological aspects, *i.e.* differences in PTX3 preparation used, and probably the high proportion of patients in remission at the time of serum testing may have accounted for the lower prevalence of PTX3-ANCA positive patients in our cohort as compared with the earlier studies (11, 23). In AAV, PTX3-ANCA are associated with a lower prevalence of systemic, ENT and renal manifestations (12). In our study focusing on EGPA, PTX3-ANCA were associated with a higher prevalence of ENT, pulmonary, gastrointestinal and PNS involvement and a lower prevalence of renal and CNS involvement in comparison to the group of PTX3-ANCA negative patients.

The group of PR3-ANCA positive EGPA patients (n=2) was too small to allow definite conclusions on EGPA manifestations in comparison to the group of PR3-ANCA negative patients in our cohort. Noteworthy, in the new ACR/EULAR classification criteria of 2022 the detection of PR3-ANCA is given a negative score of 3 points. Scoring of at least 6 points is required for the classification as EGPA. Nevertheless, both PR3-ANCA positive patients in our study fulfilled the new 2022 ACR/EULAR classification criteria (15). Moreover, one PR3-ANCA positive patient additionally displayed BPI-ANCA positivity, the latter having not been reported in EGPA before to our knowledge. In this patient, eosinophil-rich vasculitis was shown in a skin biopsy. In this patient and the other double ANCA-positive patient (MPO- and PTX3-ANCA), there was no suspicion of drug abuse. Further causes for double ANCA-positivity such as endocarditis were excluded. In a large retrospective European multicentre study, the prevalence of PR3-ANCA positive EGPA patients was also low (2.2%) with PR3-ANCA positive EGPA patients displaying a higher prevalence of PNS involvement and a lower prevalence of skin involvement compared with MPO-ANCA positive and ANCA negative patients (7). One of the 2 patients, who were PR3-ANCA positive at diagnosis presented with relapse and was also PR3-ANCA positive at the time of serum testing, the second patient was in remission, at which time PR3-ANCA was negative, indicating that similar to MPO-ANCA (7), PR3-ANCA may disappear with the reconstitution of remission in EGPA.

In the present study, we further detected OLM4-ANCA as novel ANCA antigen-specificity in 2 patients with new onset and relapsing EGPA with multiorgan involvement, respectively. Both patients displayed no other ANCA antigen-specificity. So far, detection of OLM4-ANCA has only been reported in 2 patients with non-vasculitic inflammatory symptoms (23). Of note, the glycoprotein OLM4, an anti-apoptotic factor, and PTX3, an acute phase protein and pattern recognition receptor, are stored in specific granules in a subset of human neutrophils. OLM4 and PTX3 can be translocated to the neutrophil cell surface-membrane and released upon activation, *e.g.* during infections, thereby potentially becoming both targets and effectors of the autoimmune process similar to MPO and PR3 stored in azurophilic granules and translocated to the neutrophil cell surface-membrane in AAV (2, 11, 12, 23). The proportion of OLM4 positive neutrophils varies between individuals (1-70% of neutrophils) but shows little variation within an individual over time. Its function in human neutrophils and potential differential functions of the OLM4 positive neutrophil subset however has not been sufficiently determined yet (24).

In our cohort, most patients were treated with conventional synthetic disease-modifying drugs (csDMARD) for the induction or maintenance of remission in EGPA. However, 2 patients were treated with rituximab. One of them was PR3-ANCA positive, the other ANCA negative. In these 2 relapsing patients, rituximab induced remission. Rituximab therapy results in a decrease of inflammatory activity in the majority of EGPA patients, but implications of the ANCA status on the response remain to be addressed by future prospective studies (6, 25-27). The disease course of those 2 patients treated with rituximab in our cohort allows no definite conclusion with respect to the efficacy of rituximab in EGPA and the impact of ANCA.

Our study has several limitations, one being its retrospective design. Another limitation is the high proportion of patients in remission at the time of serum testing. Therefore, it is unclear whether the number of patients with PTX3- and OLM4-ANCA would have been higher, if all patients had been tested at the time of diagnosis. Further prospective studies to validate the spectrum of ANCA target antigen-specificities in EGPA are needed.

In conclusion, our study shows detection of ANCA with various antigen-specificities other than MPO discloses a higher total prevalence of ANCA in EGPA. Clinical manifestations differ between EGPA patients with distinct ANCA antigen-specificities and ANCA negative patients. Thus, ANCA with antigen-specificities other than MPO such as PTX3 and OLM4 may emerge as potential biomarkers defining further EGPA subsets within the group of MPO-ANCA negative EGPA patients.

References

1. JENNETTE JC, FALK RJ, BACON PA *et al*.: 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum* 2013; 65(1): 1-11. https://doi.org/10.1002/art.37715
2. KITCHING AR, ANDERS HJ, BASU N *et al*.: ANCA-associated vasculitis. *Nat Rev Dis Primers* 2020; 6(1): 71. https://doi.org/10.1038/s41572-020-0204-y
3. JAKES RW, KWON N, NORDSTROM B *et al*.: Burden of illness associated with eosinophilic granulomatosis with polyangiitis: a systematic literature review and meta-analysis. *Clin Rheumatol* 2021; 40(12): 4829-36. https://doi.org/10.1007/s10067-021-05783-8
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(1): 270-81. https://doi.org/10.1002/art.37721
5. 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(9): 2926-35. https://doi.org/10.1002/art.21250
6. 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(1): 5120. https://doi.org/10.1038/s41467-019-12515-9
7. PAPO M, SINICO RA, TEIXEIRA V *et al*.: Significance of PR3-ANCA positivity in eosinophilic granulomatosis with polyangiitis (Churg-Strauss). *Rheumatology* (Oxford) 2021; 60(9): 4355-4360. https://doi.org/10.1093/rheumatology/keaa805
8. SCHÖNERMARCK U, LAMPRECHT P, CSERNOK E, GROSS WL: Prevalence and spectrum of rheumatic diseases associated with proteinase 3-antineutrophil cytoplasmic antibodies (ANCA) and myeloperoxidase-ANCA. *Rheumatology* (Oxford) 2001; 40(2): 178-84. https://doi.org/10.1093/rheumatology/40.2.178
9. LASKARI K, HELLMICH B, ADAMUS G, CSERNOK E: Autoantibody profile in eosinophilic granulomatosis and polyangiitis: predominance of anti-alpha-enolase antibodies. *Clin Exp Rheumatol* 2021; 39 (Suppl. 129) S:83-7. https://doi.org/10.55563/clinexprheumatol/08k9af
10. SHIDA H, NAKAZAWA D, TATEYAMA Y *et al*.: The presence of anti-lactoferrin antibodies in a subgroup of eosinophilic granulomatosis with polyangiitis patients and their possible contribution to enhancement of neutrophil extracellular trap formation. *Front Immunol* 2016; 7: 636. https://doi.org/10.3389/fimmu.2016.00636
11. SIMON A, SUBRA JF, GUILPAIN P *et al*.: Detection of anti-pentraxin-3 autoantibodies in ANCA-associated vasculitis. *PLoS One* 2016; 11(1): e0147091. https://doi.org/10.1371/journal.pone.0147091
12. PADOAN R, GATTO M, GHIRARDELLO A *et al*.: IgG anti-Pentraxin 3 antibodies are a novel biomarker of ANCA-associated vasculitis and better identify patients with eosinophilic granulomatosis with polyangiitis. *J Autoimmun* 2021; 124: 102725. https://doi.org/10.1016/j.jaut.2021.102725
13. TALOR MV, STONE JH, STEBBING J, BARIN J, ROSE NR, BUREK CL: Antibodies to selected minor target antigens in patients with antineutrophil cytoplasmic antibodies (ANCA). *Clin Exp Immunol* 2007; 150(1): 42-8. https://doi.org/10.1111/j.1365-2249.2007.03453.x
14. 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(8): 1094-100. https://doi.org/10.1002/art.1780330806
15. GRAYSON PC, PONTE C, SUPPIAH R *et al*.: 2022 American College of Rheumatology/European Alliance of Associations for Rheumatology Classification Criteria for Eosinophilic Granulomatosis with Polyangiitis. *Ann Rheum Dis* 2022; 81(3): 309-314. https://doi.org/10.1136/annrheumdis-2021-221794
16. WECHSLER ME, AKUTHOTA P, JAYNE D *et al*.: Mepolizumab or placebo for eosinophilic granulomatosis with polyangiitis. *N Engl J Med* 2017; 376(20): 1921-32. https://doi.org/10.1056/NEJMoa1702079
17. MUKHTYAR C, LEE R, BROWN D *et al*.: Modification and validation of the Birmingham Vasculitis Activity Score (version 3). *Ann Rheum Dis* 2009; 68(12): 1827-32. https://doi.org/10.1136/ard.2008.101279
18. EXLEY AR, BACON PA, LUQMANI RA *et al*.: Development and initial validation of the Vasculitis Damage Index for the standardized clinical assessment of damage in the systemic vasculitides. *Arthritis Rheum* 1997; 40(2): 371-80. https://doi.org/10.1002/art.1780400222
19. GOLETZ S, GIURDANELLA F, HOLTSCHE MM *et al*.: Comparison of two diagnostic assays for anti-laminin 332 mucous membrane pemphigoid. *Front Immunol* 2021; 12: 773720. https://doi.org/10.3389/fimmu.2021.773720
20. WATERS PJ, KOMOROWSKI L, WOODHALL M *et al*.: A multicenter comparison of MOG-IgG cell-based assays. *Neurology* 2019; 92(11): e1250-e1255. https://doi.org/10.1212/wnl.0000000000007096
21. 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(6): 1011-7. https://doi.org/10.1136/annrheumdis-2012-201531
22. KEOGH KA, SPECKS U: Churg-Strauss syndrome: clinical presentation, antineutrophil cytoplasmic antibodies, and leukotriene receptor antagonists. *Am J Med* 2003; 115(4): 284-90. https://doi.org/10.1016/s0002-9343(03)00359-0
23. AMIRBEAGI F, THULIN P, PULLERITS R *et al*.: Olfactomedin-4 autoantibodies give unusual c-ANCA staining patterns with reactivity to a subpopulation of neutrophils. *J Leukoc Biol* 2015; 97(1): 181-9. https://doi.org/10.1189/jlb.5A0614-311R
24. LUNDQUIST H, ANDERSSON H, CHEW MS, DAS J, TURKINA MV, WELIN A: The olfactomedin-4-defined human neutrophil subsets differ in proteomic profile in healthy individuals and patients with septic shock. *J Innate Immun* 2022: 1-14. https://doi.org/10.1159/000527649
25. MENDITTO VG, ROSSETTI G, OLIVARI D *et al*.: Rituximab for eosinophilic granulomatosis with polyangiitis: a systematic review of observational studies. *Rheumatology* (Oxford) 2021; 60(4): 1640-50. https://doi.org/10.1093/rheumatology/keab046
26. TEIXEIRA V, MOHAMMAD AJ, JONES RB, SMITH R, JAYNE D: Efficacy and safety of rituximab in the treatment of eosinophilic granulomatosis with polyangiitis. *RMD Open* 2019; 5(1): e000905. https://doi.org/10.1136/rmdopen-2019-000905
27. MOHAMMAD AJ, HOT A, ARNDT F *et al*.: Rituximab for the treatment of eosinophilic granulomatosis with polyangiitis (Churg-Strauss). *Ann Rheum Dis* 2016; 75(2): 396-401. https://doi.org/10.1136/annrheumdis-2014-206095