

Microarray evaluation of allergen-specific IgE in eosinophilic granulomatosis with polyangiitis

The pathogenesis of asthma and ear–nose–throat (ENT) manifestations in eosinophilic granulomatosis with polyangiitis (EGPA) is still poorly understood. Asthma is present in almost all patients with EGPA.¹ Severe or uncontrolled asthma occurs in more than 40% of patients and its severity correlates with serum IgE (sIgE) levels.² However, sIgE towards common allergens are detectable in less than one-third of patients with EGPA using conventional diagnostic tests.³ This suggests either that atopy is not a key pathogenic mechanism in EGPA or that uncommon antigens are involved. Our study assessed IgE specificity in EGPA using microarray technologies which have higher diagnostic reliability than traditional assays and offer a wider representation of the IgE repertoire.^{4,5} We measured sIgE towards 112 purified or biotechnologically produced allergenic molecules using the ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC) (online supplemental methods). Results are reported in ISAC standardised units (ISU). The study population comprised 29 patients with EGPA, evaluated during active and inactive disease (patients' characteristics are reported in the online supplemental table 1), 30 patients with atopic asthma, 31 with active anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (20 with granulomatosis with polyangiitis and 11 with microscopic polyangiitis) and 30 healthy controls (online supplemental methods). Positive IgE (ISU>0.3) in at least 5% of the whole study population were detected for 35 allergen components. We assessed for each of these 35 components the percentage of study subjects with low (0.3–0.9 ISU), moderate/high (1.0–14.9 ISU) and very high reactivity (≥ 15.0 ISU). The 35 allergen components were also divided based on the allergen families they belonged to, namely, food, respiratory and non-food non-respiratory ('other') allergens. The percentage of patients positive for at least one family component was calculated.

Active and inactive EGPA displayed a lower reactivity compared with the asthmatic group (figure 1A). This difference was particularly pronounced for respiratory allergens (active EGPA (55.0%) vs asthma (86.8%), $p=0.01$; inactive EGPA (38.0%) vs asthma (86.8%), $p=0.0001$) and 'other' allergens (active EGPA (13.7%) vs asthma (50.0%), $p=0.004$; inactive EGPA (17.0%) vs asthma (50.0%), $p=0.01$) (figure 1B). There were no allergen components towards which sIgE were higher in EGPA than in asthmatics. Active and inactive EGPA displayed a similar reactivity when compared with one another or to healthy controls. Patients with AAV showed a markedly lower IgE reactivity compared with patients with EGPA and asthma (figure 1A,B). This reactivity pattern did not change when we considered a threshold of 2% rather than 5% (data not shown).

No significant differences in allergen-specific sIgE positivity were found between ANCA-positive and ANCA-negative patients. Additionally, none of the EGPA symptoms typically considered 'allergic' (eg, ENT and lung involvement, urticaria) were associated with sIgE positivity (either for food, respiratory or other allergens).

Our results, obtained using a sensitive microarray technology able to detect sIgE specific for a large allergen component panel, show that patients with EGPA have a lower reactivity towards common allergens when compared with asthmatic subjects. This is apparently in contrast with the almost universal presence of asthma and 'allergic' manifestations in EGPA. However, this

corroborates previous findings obtained with traditional allergy tests, which showed that the prevalence of atopy is significantly lower in EGPA than in asthmatics. Also, patients with EGPA do not suffer from seasonal allergies as do atopic asthmatics.³

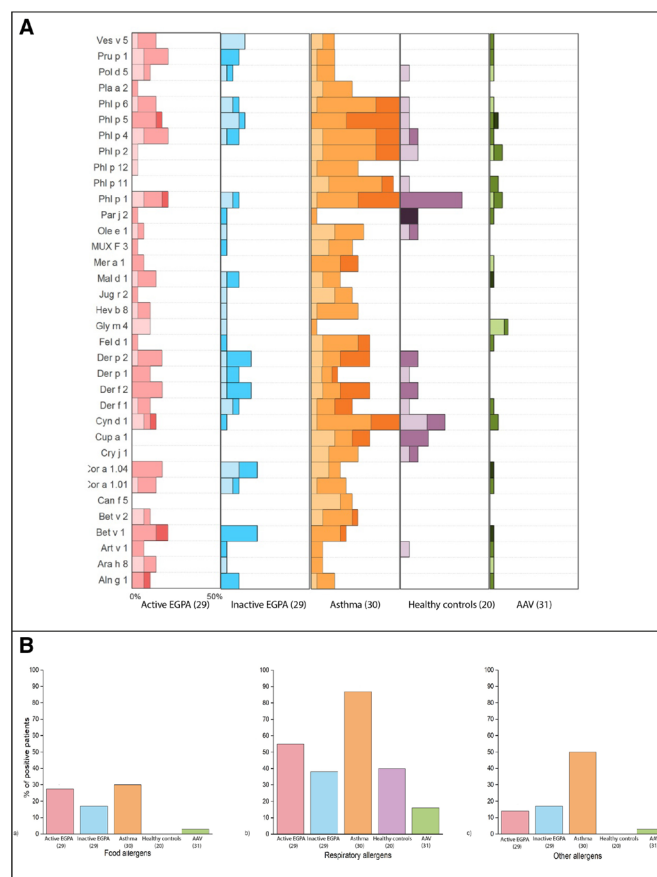


Figure 1 IgE towards common allergens in eosinophilic granulomatosis with polyangiitis (EGPA) and control groups. (A) On the Y axis, the 35 allergens for which serum IgE (sIgE) were positive in at least 5% of the study population are shown. The three shades of each colour correspond to the levels of reactivity in each study subgroup, that is, low (ISU-E 0.3–0.9), moderate/high (ISU-E 1.0–14.9) and very high (ISU-E ≥ 15.0). On the X axis, the percentages for every subgroup are expressed on a scale ranging from 0% to 50%. Allergen abbreviations are expanded in the online supplemental table 2. The number of patients per group is reported in parentheses on the X axis labels. (B) Reactivity for the different allergen families (food allergens, panel a; respiratory allergens, panel b; other allergens, panel c) in active and inactive EGPA patients (rose and blue colour, respectively) and in the control groups (orange for asthmatic patients, violet for healthy controls and green for AAV patients). Both active and inactive EGPA patients displayed lower reactivity compared with asthmatic subjects, particularly for respiratory allergens (active EGPA 55.0% vs asthma 86.8%, $p=0.01$; inactive EGPA 38.0% vs asthma 86.8%, $p=0.0001$) and 'other' allergens (active EGPA 13.7% vs asthma 50.0%, $p=0.004$; inactive EGPA 17.0% vs asthma 50.0%, $p=0.01$). Active, but not inactive, EGPA displayed higher reactivity than the other anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), particularly towards respiratory and food allergens (respiratory: active EGPA 55.0% vs AAV 16.0%, $p=0.002$; food: active EGPA vs AAV 27.5% vs 3.0%, $p=0.01$). No significant differences were found between EGPA (active and inactive) and healthy controls in terms of allergen reactivity. The number of patients per group is reported in parentheses on the X axis labels. ISU, ISAC standardised units.

This may reflect different pathogenic mechanisms underlying atopic asthma and asthma occurring in EGPA. In line with this, omalizumab (anti-IgE monoclonal antibody) demonstrated only limited efficacy in EGPA.⁶ To conclude, common allergen-induced IgE responses seem to have a marginal role in EGPA pathogenesis; therefore, further studies are needed to dissect the immunological circuits that drive 'allergic' manifestations in EGPA.

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Supplementary Methods

Patients' selection criteria

The study included 29 patients with Eosinophilic Granulomatosis with Polyangiitis (EGPA), 30 with atopic asthma, 31 with ANCA-associated vasculitis (AAV), of whom 20 had granulomatosis with polyangiitis (GPA), and 11 microscopic polyangiitis (MPA), and 30 healthy controls.

The diagnosis of EGPA had to fulfill the 1990 American College of Rheumatology (ACR) criteria¹ and/or the 2012 Chapel Hill Consensus Conference (CHCC) definitions². Disease activity (active vs inactive disease) was defined on the basis of the Birmingham Vasculitis Activity Score (BVAS)³. Persistent smouldering asthma was not considered a manifestation of active disease.

Patients with GPA and MPA had to fulfill the 1990 ACR criteria⁴ and/or the 2012 CHCC definitions². Only patients with active disease were included (BVAS > 0).

Patients with active atopic asthma had to fulfill the 2002 Global Initiative for Asthma (GINA) diagnostic criteria. Patients with extrinsic forms of asthma were excluded.

Healthy controls were free of clinically evident autoimmune or allergic diseases and were age- and sex-matched with the EGPA cases.

An Italian organisation of patients with EGPA (APACS) was involved in the dissemination of the results of the present study.

Criteria used to define organ involvement in active EGPA

Asthma was defined as the presence of respiratory symptoms such as wheezing or difficulty in breathing and presence of airflow obstruction at pulmonary function tests, reversible after administration of a bronchodilator. Ear-nose-throat (ENT) involvement was defined as the presence of one or more among nasal/paranasal polyps, chronic sinusitis, chronic rhinitis, otitis media and sensorineural hearing loss. The ENT district was studied with rhinoscopy, standard X-rays and/or computed tomography (CT) scan, audiometry and other relevant diagnostic tests. Lung involvement was

defined as the presence of lung infiltrates, nodules, ground-glass opacities, pleural effusions or alveolar hemorrhage found on chest X-ray and/or high-resolution CT (HRCT) scans. Skin involvement was based on the finding of purpura, urticaria, nodules. Peripheral neuropathy comprised mononeuritis multiplex or sensorimotor polyneuropathy. Sensory and motor dysfunction was evaluated through clinical examination and nerve conduction studies. Central nervous system (CNS) involvement was defined as the presence of central neurologic manifestations that could not be attributed to other concurrent diseases. CNS involvement had to be documented by computed tomography or magnetic resonance imaging that showed focal or disseminated ischemic lesions consistent with small-vessel vasculitis. Cranial nerve palsies and meningitis were also considered as CNS manifestations. Gastro-intestinal tract involvement was defined as the presence of intestinal bleeding, bowel perforation, cholecystitis, pancreatitis, or unexplained abdominal pain, or when (even in the absence of overt clinical manifestations) endoscopic biopsies revealed tissue eosinophilia, granulomas or vasculitis. Cardiac involvement was defined as the presence of moderate-to-severe electrocardiographic and/or echocardiographic alterations (regional or global systolic dysfunction, impaired diastolic relaxation, ventricular or atrial dilation, moderate-to-severe valve abnormalities, pericarditis) in the absence of preexisting myocardial, valvular or pericardial abnormalities, or as histologically proven eosinophilic infiltration or vasculitis on an endo-myocardial biopsy.

Renal involvement was defined as the presence of proteinuria >300 mg/day, micro- or macroscopic haematuria, and otherwise unexplained renal failure (serum creatinine >1.4 mg/dL), or as the presence of histological findings consistent with EGPA-related renal involvement (pauci-immune extracapillary glomerulonephritis, focal and segmental necrotising glomerulonephritis, eosinophilic tubulo-interstitial nephritis).

Immunocap-ISAC assay characteristics

The Immunocap-ISAC microchip contained 112 allergen components, from 48 allergen sources. Allergen components were produced either as native molecules purified from natural sources or as

recombinant proteins. The allergen chip used in this study included 43 single allergens from 17 different foods, 30 single allergens from 16 different seasonal aeroallergen sources, 27 single allergens from 13 different perennial aeroallergen sources, and 12 additional single allergens from other allergen sources. Molecules in small amount were spotted in triplets on a polymer-coated solid slides. Blood samples were obtained for each patient. Serum (0.04 mL) was incubated for 60 minutes on the microchip. Slides were then washed, and labelled anti-human IgE antibodies were added and incubated for 60 minutes. Fluorescence was measured with laser scanner and evaluated with the appropriate software which quantified the fluorescence signal for each specific allergen component. The concentration of IgE in the sample was expressed in ISAC standard units (ISU-E). Values < 0.3 ISU-E were below the detection limit of the assay, values between 0.3 and 0.9 ISU-E corresponded to low-level positivity, values between 1.0 and 14.9 ISU-E to moderate-high positivity whereas values ≥ 15.0 ISU-E to very high positivity.

Statistical analysis and ethics

Statistical analysis was performed using the software STATA version 14. Categorical variables were expressed as absolute numbers and percentages. Fisher's exact test was used to compare the percentages between the different study groups. Results were considered statistically significant if p values were lower than 0.05.

The study was conducted in compliance with the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee and informed consent was obtained from all the enrolled subjects.

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Supplementary Tables

Supplementary Table 1. Demographic and clinical characteristics of the 29 patients with Eosinophilic Granulomatosis with Polyangiitis (EGPA)

Age (mean $\pm$ standard deviation) - yrs	56.6 ($\pm$ 14)
Male sex, no. (%)	20 (69.0)
ANCA-positive, no. (%)	16 (55.2)
Clinical characteristics, no. (%)	
Asthma	28 (96.6)
Peripheral neuropathy	21 (72.4)
ENT involvement	19 (65.6)
Lung involvement	19 (65.6)
Skin involvement (all kinds)	11 (37.9)
Palpable purpura	9 (31.0)
Glomerulonephritis	8 (27.6)
Heart involvement	7 (24.1)
Gastrointestinal involvement	4 (13.8)
CNS involvement	1 (3.4)

(ANCA = Anti-neutrophil cytoplasmic antibodies; ENT = Ear-Nose and Throat; CNS = Central Nervous System)

Supplementary Table 2. List of allergens reported in Figure 1

Allergenic molecule	Type of allergen
Food allergens	
Ara h 8	Peanut
Cor a 1.4	Hazelnut
Gly m 4	Soybean
Jug r 2	Walnut
Mal d 1	Apple
Pru p 1	Peach
Respiratory allergens	
Aln g 1	Alder
Art v 1	Mugwort
Bet v 1	Birch
Bet v 2	Birch
Can f 5	Dog
Cor a 1.1	Hazel pollen
Cry j 1	Japanese cedar
Cup a 1	Cypress
Cyn d 1	Bermuda grass
Der f 1	House dust mite
Der f 2	House dust mite
Der p 1	House dust mite
Der p 2	House dust mite
Fel d 1	Cat

Mer a 1	Annual Mercury
Ole e 1	Olive
Par j 2	Mugwort parietaria
Phl p 1	Timothy
Phl p 11	Timothy
Phl p 12	Timothy
Phl p 2	Timothy
Phl p 4	Timothy
Phl p 5	Timothy
Phl p 6	Timothy
Pla a 2	Plane tree
Other allergens	
Hev b 8	Latex
MUX F 3	CCD marker
Pol d 5	Paper wasp venom
Ves v 5	Common wasp venom

CCD = carbohydrate chains of bromelain