

Specific IgG₄: Possible Role in the Pathogenesis and a New Marker in the Diagnosis of *Anisakis*-associated Allergic Disease

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Abstract

IgG₄ and IgE are immunoglobulin isotypes which are mediated by the same Th2-mediated mechanism. The postulated pathogenic and protective function of IgE or IgG₄, respectively, in allergic disease is opposite in parasitic infection. The possible role of IgG₄ against recombinant major allergens on the appearance of different forms of *Anisakis simplex*-associated allergic disease was studied. Gastro-allergic anisakiasis (GAA) and *Anisakis*-sensitization-associated chronic urticaria (CU+) were compared for specific IgE, IgG₄ and the respective recognition of *Ani s 1* and *Ani s 7*. Gastro-allergic anisakiasis showed higher IgE and IgG₄ levels against crude extract and both recombinant allergens. Whereas IgE recognition of *Ani s 7* did not differ and supports both clinical entities to be associated with previous acute parasitism, the IgE recognition rates of *Ani s 1* and IgG₄ recognition of both *Ani s 1* and *Ani s 7* were higher in GAA. IgG₄ levels were associated with IgE, but also with age, time to last parasitic episode and frequency of fish intake. Logistic regression analysis showed that the presence of specific IgG₄ against *Ani s 7* was an independent marker associated with GAA. In the diagnosis of *Anisakis*-associated allergic disease phenotypes (GAA versus CU+), measurement of specific IgG₄ against recombinant allergens could be useful. Further, evaluation of specific IgE and IgG₄ facilitates more insight into the protective versus pathogenic potential of IgE and IgG₄.

Introduction

Specific IgE is the key immunoglobulin isotype associated with different forms of allergic disease: respiratory allergy, food allergy or hymenoptera allergy need specific IgE directed against the corresponding allergens. Mainly by cross-linking of the high-affinity receptor for IgE FcεRI on tissue mast-cells or circulating basophils, the degranulation of these cells promotes a cascade of events leading to the different forms of clinical allergy. In allergic disease, Th2-mediated specific IgE is thought to be pathogenic, but in different animal and human models, IgE against parasite antigens has been shown to be an evolutionary-maintained feature which helps in the clearance of parasites or maintains a low parasite burden in mammals [1–4].

In gastro-allergic anisakiasis (GAA), this Th2-type immunologic feature has been shown to be responsible for the acute hypersensitivity reactions. But this has also been postulated to prevent more severe complications by limiting the proteases-induced penetration of the live

third-stage larva of *Anisakis simplex* [5, 6]. Typical for a parasite-induced immunologic reaction, however, previous studies stated the concomitant production of other immunoglobulin isotypes, including IgG₄ [7]. Further, *A. pegreffii* has also been associated with gastro-allergic reactions [8]. Specific IgG₄ in allergy has been postulated to be protective, such as induced by immunotherapy or upon prolonged exposure to antigen [9]. Otherwise, in parasitic disease, high IgG₄ antibodies or a low IgE/IgG₄ ratio has been associated with severity of disease, parasite burden or risk of re-infection [2, 10, 11].

On the other hand, GAA cannot be considered a typical parasitic disease as it is always self-limiting, acute in its nature and chronic gastro-intestinal or extra-intestinal complications are not due to a chronic parasitism. These depend rather on immunologic response, as *A. simplex* is not adapted to humans and is not able to survive in this host for more than maximally a few days [12, 13]. This feature has been attributed to the fact that allergic reactions are frequent in acute parasitism by this nematode [11, 14]. Therefore, the presence of IgE and IgG₄ has to be

interpreted in this special context. In fact, in *Anisakis*-sensitization-associated chronic urticaria (CU+), IgG₄ is often absent. This isotype has been associated with distinct clinical occurrence of *Anisakis*-associated allergic disease [15, 16].

Whereas up to now, more than 12 allergens have been identified to be associated with IgE production against this parasite, the production of other immunoglobulin isotypes against these allergens has only scarcely been investigated [17].

We hypothesize that distinct recognition patterns of specific IgG₄ could help in the diagnosis of *Anisakis*-associated allergic disease.

The present study aimed therefore to assess the production of specific IgG₄ against two of the major allergens *Ani s 1* and *Ani s 7* in GAA and CU+ and take into account possible known factors to affect IgG₄ production as are age, time interval to the last parasitic episode and fish intake.

Methods

Study protocol. 20 patients with GAA and 25 patients with CU+ were prospectively recruited when they met inclusion criteria. All patients had to display a positive SPT and detectable IgE antibodies against *A. simplex* at ≥ 0.7 kU/l. Besides, sensitization patients of both groups had to meet the following clinical criteria:

Gastro-allergic anisakiasis patients had an episode of acute urticaria, angioedema or anaphylaxis of less than 48-h duration within 24 h after eating raw or clearly undercooked fish and other eliciting causes have been discarded by history and allergological work-up. These criteria have previously been shown to be sufficient for a diagnosis of GAA [7, 15].

Chronic urticaria + patients were included if they had at least twice per week recurring wheals for at least the last 6 weeks. Patients were not included in this study if physical stimuli were the only eliciting agents of urticaria. Other known factors associated with CU, such as positive hepatitis serology or antithyroid antibodies, were not an exclusion factor.

All patients were studied for specific IgG₄ and IgE (crude extract), as well as specific IgG₄ against recombinant *Ani s 1* and *Ani s 7* (*Ani s 1*-IgG₄ and *Ani s 7*-IgG₄, respectively). Although detection of specific IgE against *Ani s 1* and *Ani s 7* was not the purpose of this study, measurements against these were also available in 11 GAA and 18 CU+ patients and could be used for comparisons.

Patients were asked for the number of weekly fish portions and monthly portions of anchovies in vinegar sauce, a risk factor for parasitism by *Anisakis*. The interval between the acute allergic reaction in GAA as well as the time from onset of chronic urticaria and obtaining of the serum sample was defined as time interval (TI).

Included patients gave written consent and the study protocol was approved by the University Hospital La Princesa Ethical Committee.

Specific IgE and IgG₄ against *Anisakis* crude larval antigen. Anti-*Anisakis*-specific IgE and IgG₄ were analysed by CAP-FEIA against crude extract larval antigen (Phadia, Uppsala, Sweden). Cut-off value for specific IgG₄ is 0.15 kU/l.

Specific IgE and IgG₄ against *Ani s 1* and *Ani s 7*. The recombinant allergens *t-Ani s 7* and *rAni s 1* were isolated and purified as previously described [18]. IgE seropositivity to the recombinant allergens *rAni s 1* and *t-Ani s 7* was determined by ELISA and published before [16, 18]. The calculated cut-off values for *Ani s 1* and *Ani s 7* ELISA-IgE were Optical densities (ODs) of 0.090 and 0.050, respectively, as previously established [18, 19]. Specific anti-*Anisakis* IgG₄ antibodies were detected by indirect ELISA, with *rAni s 1* or *t-Ani s 7* as the target. Wells in columns 1, 4, 7 and 10 of the 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 100 μ l of phosphate-buffered saline (PBS) containing *rAni s 1* at a concentration of 5 μ g/ml, and wells in columns 2, 5, 8 and 11 were filled with 100 μ l of 0.1 M Tris buffer, pH 10.5, containing 0.6 μ g/ml of *t-Ani s 7*. The wells in the remaining columns (controls) were filled with PBS alone. After incubation of the plates at 4 °C overnight and blocking of non-reactive sites, duplicate dilutions of sera at 1/100 in PBS-Tween, containing 0.1% BSA, were added and incubated. A mouse anti-human IgG₄-HRP (Clone HP6025) (Southern Biotech, Birmingham, AL, USA) was used. After incubating and washing, substrate (o-phenylene diamine; Sigma) was added at 0.04% in phosphate citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3N sulphuric acid and the plates were read at 490 nm. Optical densities were calculated by subtracting the OD value produced by the same serum in the absence of antigen. Values higher than the mean of the O.D. of 100 negative control sera plus four times their respective standard deviation were considered as positive. The calculated cut-off values for *Ani s 1* and *Ani s 7* ELISA-IgG₄ were ODs of 0.160 and 0.080, respectively.

Statistics. Statistical analysis was performed using SPSS ver. 15.0 for Windows.

Comparison of GAA and CU+. Mean and standard deviation were calculated for continuous variables such as age and fish intake.

Median values and interquartile ranges (IQR) were calculated for anti-*Anisakis*-specific IgE and IgG₄ as well as for values of specific IgG₄ and IgE against recombinant allergens and compared by Mann–Whitney *U*-test. Isotype ratios were calculated as IgG₄/IgE, *Ani s 1*-IgG₄/*Ani s 1*-IgE as well as *Ani s 7*-IgG₄/*Ani s 7*-IgE and also compared by Mann–Whitney *U*-test.

The number of positive IgG₄ and IgE (total and recombinant allergens) was compared by chi-square test.

Separately for GAA and CU+, we performed nonparametric correlation analysis (Spearman Rank test) between *Ani s 1*-IgG₄, *Ani s 7*-IgG₄ and other studied immunoglobulins, time interval and fish intake.

Further three linear regression models were performed for specific IgG₄ and both recombinant allergens as dependent variable. We analysed specific IgE, TI, age, fish intake and belonging to CU+ or GAA group as possible explaining variables with stepwise exclusion of non-significant variables. We further performed a logistic regression model with GAA versus CU+ as dependent variable with inclusion of specific IgE, IgG₄ against *Anisakis* larval antigen and the respective recombinant allergens, *Ani s 1* and *Ani s 7*.

Results

Mean age was 51.1 ± 12.0 in GAA and 51.1 ± 15.4 years in CU+ (n.s.). Eighty eight percent were female in CU+ and 40% were female in GAA ($P = 0.001$). Median TI in GAA was 4 months (IQR 2–6 months), and median duration of chronic urticaria in CU+ was 12 (IQR 6–36 months).

Comparing the antibody levels in patients with GAA and CU+, we have observed that specific IgE and IgG₄ to *Anisakis* whole antigen, as well as IgE and IgG₄ to *Ani s 1* and *Ani s 7* recombinant allergens, were significantly higher in the former (Table 1). We have also observed that the IgG₄/IgE ratios showed high variability. While the IgG₄/IgE-*Ani s 7* was near significantly higher in GAA than in CU+ ($P = 0.06$), we have not observed differences in the IgG₄/IgE ratios induced by *Ani s 1* or by total larval *Anisakis* antigen (Fig. 1). Likewise, IgG₄ antibodies were more often positive in GAA for both *Ani s 1* and *Ani s 7* ($P = 0.05$ and $P < 0.001$, respectively; Fig. 2).

Figure 3 shows positivity patterns when analysing patients for simultaneous IgE and IgG₄ antibodies against recombinant allergens. We can state that in both GAA and CU+, *Ani s 1*-IgG₄ is only positive when IgE against the same allergen is positive.

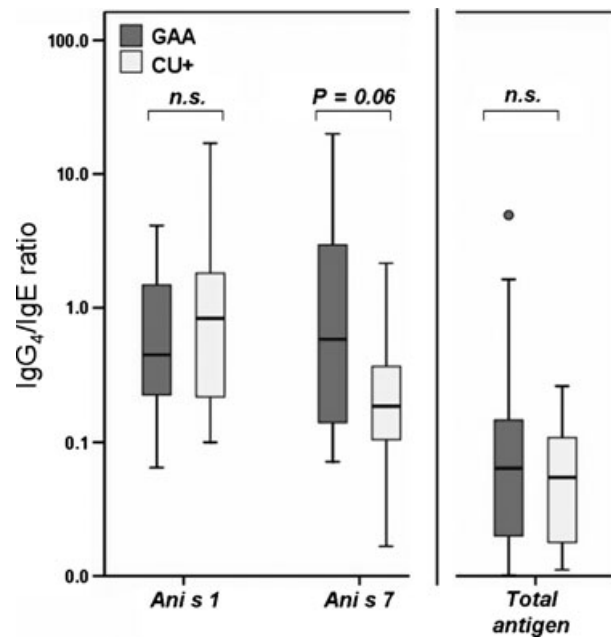


Figure 1 IgG₄/IgE ratios for *Ani s 1*, *Ani s 7* and *Anisakis* total larval antigen. This chart compares immunoglobulin isotype ratios between gastro-allergic anisakiasis (GAA) and *Anisakis*-sensitization-associated chronic urticaria (CU+), for total antigen, as well as recombinant *Ani s 1* and *Ani s 7*.

Considering only GAA patients, the main significant correlations were observed between *Ani s 1*-IgG₄ and total specific IgG₄ ($R = 0.66$; $P = 0.02$) and between *Ani s 7*-IgG₄ and total specific IgG₄ ($R = 0.77$; $P < 0.001$). In contrast, the main significant correlations in CU+ patients were between *Ani s 1*-IgG₄ and *Ani s 1*-IgE ($R = 0.64$; $P = 0.004$) as well as specific IgG₄ ($R = 0.4$; $P < 0.05$).

In GAA, specific IgG₄ to whole *Anisakis* antigens and *Ani s 7* correlated with age ($R > 0.5$; $P < 0.03$), whereas in CU+, *Ani s 1*-IgG₄ correlated negatively with TI ($R = \text{between } 0.63$; $P = 0.001$) and both *Ani s 1*-IgG₄ and *Ani s 7*-IgG₄ correlated positively with fish intake ($R = \text{between } 0.45 \text{ and } 0.6$; $P < 0.02$).

With respect to the linear regression analysis, we have also observed that (1) the specific IgG₄ response to *Anisakis*

Table 1 Median and interquartile range (IQR) of studied immunoglobulin levels in gastro-allergic anisakiasis (GAA) and *Anisakis simplex*-sensitization-associated chronic urticaria (CU+).

Group	IQR	<i>Ani s 1</i> -IgE	<i>Ani s 7</i> -IgE	<i>Ani s 1</i> -IgG ₄	<i>Ani s 7</i> -IgG ₄	Specific IgE	Specific IgG ₄
GAA	25	1.12	0.09	0.08	0.16	16.4	985
	50	2.00	1.93	0.78	0.45	68.9	2585
	75	2.28	2.20	2.87	1.39	335.0	5245
CU+	25	0.01	0.06	0.01	0.00	1.6	92
	50	0.02	0.27	0.08	0.03	4.9	395
	75	0.30	0.58	0.45	0.16	11.9	1157
$P =$		<0.001	0.01	0.01	<0.001	<0.001	<0.001

Significance values are given for comparison between GAA and CU+. Specific IgE and IgG₄ against total larval antigen in kU/l. IgE and IgG₄ antibodies against recombinant *Ani s 1* and *Ani s 7* are given in OD values by ELISA.

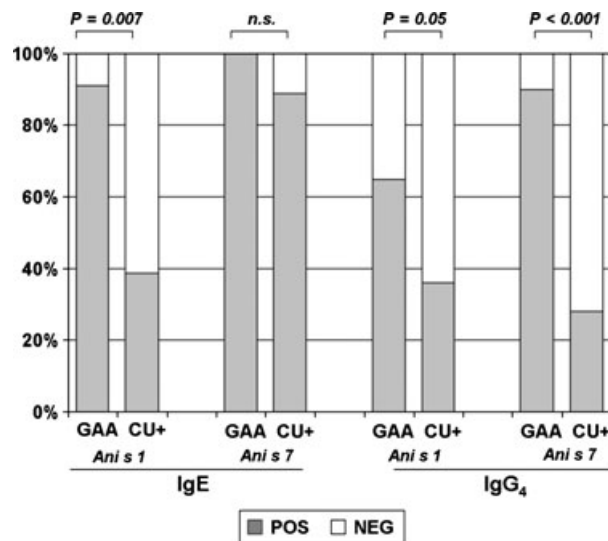


Figure 2 Frequency of occurrence of positive (POS) or negative (NEG) *Ani s 1* and *Ani s 7* in gastro-allergic anisakiasis (GAA) and *Anisakis simplex*-sensitization-associated chronic urticaria (CU+). Cut-off values for IgE and IgG₄ were 0.090/0.05 and 0.160/0.080 for *Ani s 1* and *Ani s 7*, respectively.

whole antigen was significantly and independently influenced by age ($B = 2.2$; $P = 0.03$) and specific IgE ($B = 3.1$; $P = 0.004$) as explaining variables; (2) the *Ani s 1*-IgG₄ response was independently influenced by specific IgE ($B = 2.5$; $P = 0.02$) and a tendency for time interval ($B = -1.8$; $P = 0.08$) as explaining variables; and (3) the *Ani s 7*-IgG₄ response was significantly and independently influenced by specific IgE ($B = 3.7$; $P \leq 0.001$) and belonging to either CU+ or GAA groups ($B = -2.2$; $P = 0.03$) as explaining variables, which confirmed that the frequency of anti-*Ani s 7*-IgG₄ antibodies is higher in GAA than in CU+.

Finally, the logistic regression analysis with group-belonging as dependent variable showed that *Anisakis*-specific IgE, as well as anti-*Ani s 7*-IgG₄, were significant and independent explaining variables (Fig. 4).

Discussion

IgE production is mainly associated with a pathogenic potential in allergic disease, but it is thought to have

evolved at least partially in relationship with a protective immune response in parasitic disease. IgE production is, together with mast cell activation and eosinophilia, elicited by a Th2-driven response. On the other side, IgG₄ has been reported to recognize identical antigenic structures [20]. Sequential switching from IgM to IgG₄ to IgE has been explained and the fact that IgE inducing antigens are also good inducers of IgG₄ production [21, 22].

In *Anisakis*-associated allergic disease, we have an interesting model, where allergic features and a parasite-induced immune response come together. Gastro-allergic anisakiasis is a clinical entity where the simultaneous production of several immunoglobulin isotypes has been verified, including IgE and IgG₄ [7]. On the other side, in CU+, not only circulating levels of IgE but also of IgG₄ are lower and frequently undetectable in the latter [15]. It has been shown that CU+ behaves clinically different from other forms of CU without sensitization against *Anisakis*. Here, immune phenomena, such as cytokine production, are also different [15, 16, 23]. Chronic urticaria has a multifactorial origin, associated or triggered in some cases with infectious disease [24]. This has also been postulated in CU+, although the pathogenesis has not yet been elucidated. In fact, the presence of IgE in this entity has to be interpreted as evidence of a previous parasitism by *A. simplex*, but a direct role of this immunoglobulin is uncertain [16]. In a recent publication where *Anisakis* hypersensitivity has also been associated with chronic urticaria, the authors even prompted this sensitization to be the cause of CU [25]. Whereas the acute urticaria or anaphylaxis in GAA is elicited by IgE molecules recognizing allergens of the invading live larva when penetrating the gastro-intestinal mucosa, the role of other isotypes of immunoglobulins in the pathogenesis of CU+ cannot be ruled out.

In this sense, compared with GAA, we expectedly demonstrated lower levels of specific IgE and IgG₄ against total antigen as well as the studied recombinant allergens in CU+. The occurrence of IgG₄ positivity to *Ani s 1* and *Ani s 7*, as well as IgE to *Ani s 1*, was also lower in CU+, whereas no difference in the occurrence of IgE positivity was found for *Ani s 7*, a fact previously described. This indicates further a previous contact with live larva [16, 18]. Although as a whole, all immunoglobulin levels were lower



Figure 3 Simultaneous positivities for IgE and IgG₄ against *Ani s 1* and *Ani s 7* in gastro-allergic anisakiasis (GAA) and *Anisakis*-sensitization-associated chronic urticaria (CU+). In this analysis, only patients were included, in whom both IgE and IgG₄ were available.

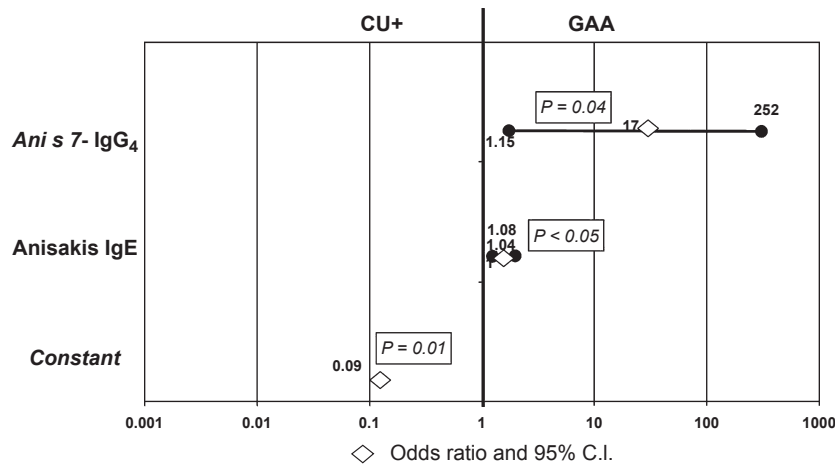


Figure 4 Significant explaining variables after logistic regression for belonging to gastro-allergic anisakiasis (GAA) or *Anisakis*-sensitization-associated chronic urticaria (CU+). IgE and IgG₄ against total antigen as well as against *Ani s 1* and *Ani s 7* were initially included with stepwise exclusion of non-significant variables.

in CU+, the near-significant lower IgG₄/IgE ratio in CU+ when analysing immunoreactivity against *Ani s 7* is interesting and could be one of the main immunoglobulin-associated features differentiating GAA from CU+, which will be further assessed below.

Interestingly, as shown in Fig. 3, IgG₄ reactivity can only be evidenced when there is also simultaneous IgE reactivity to the same allergen. The fact that some of the patients with CU+ did not react against any of both IgE as well as IgG₄ allergens can be due to antibodies being produced against other not studied allergens. Otherwise we cannot rule out that due to the time dependency of the immunoglobulin levels after the parasitic contact, these could have reached very low and thus undetectable levels. The question arises if lower IgG₄ positivity rates in both entities are due to a different velocity of fall for IgE and IgG₄ over time after the last parasitic contact or could really reflect a differential immune response in GAA compared with CU+. Correlation studies as well as regression models show clearly a positive association of IgG₄ with IgE, but even if IgE has been previously shown to fall continuously after a peak (4–6 weeks) of the acute parasitic episode, especially for IgG₄-*Ani s 1*, there seems to be a further independent negative association. Therefore, if IgE were really produced sequentially after IgG₄, the latter isotype would drop more rapidly than IgE after an acute episode of parasitism. IgG₄ has been postulated to reflect extended exposure in a modified Th2 response, prolonged infection by parasites or repeated hymenoptera stings [2, 26, 27]. Further, IgG₄ declines after antiparasitic chemotherapy [28, 29]. How can IgG₄ be interpreted in our model?

Fish-eating habits and the high prevalence of *Anisakis* infection in consumed fish could be able to maintain an immunologic response due to repeated contact with at least

some of the Th2-driving antigens [30]. This is supported by the positive correlation of both IgG₄-*Ani s 1* and *Ani s 7* with fish intake. Otherwise repeated contact with live larva and thus excretory–secretory proteins could find a similar IgG₄ driving model in repeated hymenoptera stings.

Different studies have shown that raw fish consumption habits are associated with sensitization against *A. simplex* [8, 31, 32]. Further, a higher rate of parasitic episodes led to acute urticaria in GAA, and a lower probability of parasitic episodes was associated with CU+ [30]. Taken into account the different IgG₄/IgE ratios and the higher rate of IgG₄ positivities of *Ani s 1* and *Ani s 7* in GAA, the present study therefore indicates IgG₄ to be produced after repeated parasitism, with acute urticaria being a timely limited clinical feature of GAA. The positive correlation of age with IgG₄ and IgG₄-*Ani s 7* in GAA and specific IgE, as well as age being independent explaining variables in regression for IgG₄, indicates IgG₄ to be produced in relationship with a higher probability of repeated parasitism. This leads to an atypical age pattern of allergic phenomena due to *A. simplex* sensitization, compared with classical atopic allergies [5, 32].

On the contrary, in CU+ patients, the absence of ‘protective’ IgG₄ leads to a chronic form of skin manifestation. In fact, intermediate IgG₄ levels have been associated with a prolonged acute urticaria [33].

It is interesting to denote that mainly IgG₄-*Ani s 7* differs in both clinical entities. *Ani s 7* is therefore not only a major allergen, but a dominant antigen, which in GAA, but rarely in CU+ also induces specific IgG₄ antibodies. Logistic regression showed therefore that not only specific IgE is higher in GAA as previously shown, but emphasizing also IgG₄-*Ani s 7* levels to be independently higher differentiating GAA from CU+. Previous studies with *Ascaris* infection showed absent correlation of unfraction-

ated antigen preparation when analysing IgG₄ and IgE, but clear association when using a recombinant ABA-1A protein, highlighting the usefulness of studying the dominant-specific allergens [34].

A limitation of this study is the low sample size dictated by the availability of patients, which should be considered when interpreting our results. Otherwise the obtained data are in accordance with known immunologic features of acute parasitic disease and thus should help to broaden diagnostic possibilities to the IgG₄ isotype.

Together, we show that *Anisakis*-associated allergic disease can be considered a human model between allergic and parasitic disease, facilitating more insight into the protective versus pathogenic potential of IgE and IgG₄. Especially *Ani s 7*-IgG₄ production seems to be associated with GAA, being not only a marker differentiating this entity from CU⁺, but also a factor that could have its correlate in protecting from chronic urticaria.

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Conflict of interest

The authors declare no conflict of interest.

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