

Ani s 1 and *Ani s 7* recombinant allergens are able to differentiate distinct *Anisakis simplex*-associated allergic clinical disorders

C. Cuéllar · A. Daschner · A. Valls · C. De Frutos ·
V. Fernández-Fígares · A. M. Anadón · E. Rodríguez ·
T. Gárate · M. Rodero · F. M. Ubeira

Received: 16 November 2011 / Revised: 14 December 2011 / Accepted: 2 January 2012
© Springer-Verlag 2012

Abstract Diagnosis in gastro-allergic anisakiasis (GAA) is straightforward, when clinical history is combined with further allergological evaluation of specific IgE by means of skin prick test and serum specific IgE. In *Anisakis simplex* sensitisation associated chronic urticaria (CU+), clinical evaluation of possible previous parasitism is difficult, and positive serum specific IgE could be due to cross-reactivity or other unknown factors. In this study, we evaluated the association between IgE seropositivity to the recombinant allergens *Ani s 1* and *Ani s 7* and several *A. simplex*-associated allergic disorders. Twenty-eight patients with GAA and 40 patients with CU+ were studied and their IgE responses were compared with a control group composed of patients with chronic urticaria not sensitized to *A. simplex* (CU−) according to the skin prick test, as well as a group of 15 healthy subjects not referring urticaria or currently *A. simplex* associated symptoms. 82.1% of GAA patients and 42.5% of CU+ patients were positive for *Ani s 1* ($P < 0.001$), while the *Ani s 7* allergen was recognized by 92.9 and 92.5% of sera from patients with GAA and CU+, respectively. The combined positivity

obtained for both allergens reached 100% in GAA, and 95% in CU+. IgE determinations to *Ani s 1* and *Ani s 7* allergens are useful to diagnose the *Anisakis* infections and to differentiate among several *A. simplex*-associated allergic disorders. The IgE responses to *Ani s 1* are mainly associated with GAA, while this molecule cannot be considered a major allergen in CU+ patients.

Keywords *Anisakis simplex* · Chronic urticaria · Gastro-allergic anisakiosis · Recombinant allergens

Abbreviations

GAA	Gastro-allergic anisakiasis
CU+	<i>Anisakis simplex</i> sensitisation associated chronic urticaria
CU−	Chronic urticaria without associated sensitisation against <i>Anisakis simplex</i>
SPT	Skin prick test

Introduction

Anisakis simplex is a parasite nematode able to produce different forms of anisakiasis (=anisakiosis). Since the late 90s, the description of different clinical allergic features associated with sensitization against this nematode has brought about a re-emergence of the awareness of this cosmopolitan fish-parasite [1, 2, 5, 8, 12, 13].

Gastro-allergic anisakiasis (GAA) has been described as a short-lived acute allergic reaction comprising urticaria/angioedema and/or anaphylaxis with or without abdominal complaints in the context of an acute parasitism by *A. simplex* [10]. In these patients, a thorough clinical history [positive skin prick tests (SPT) and the detection of specific serum IgE] underlines the suspected diagnosis,

C. Cuéllar · V. Fernández-Fígares · M. Rodero
Departamento de Parasitología, Facultad de Farmacia,
Universidad Complutense, Madrid, Spain

A. Daschner (✉) · A. Valls · C. De Frutos
Servicio de Alergia, Instituto de Investigación Sanitaria,
Hospital Universitario de La Princesa, C/Diego de León, 62,
28006 Madrid, Spain
e-mail: alvarodachner@gmail.com

A. M. Anadón · F. M. Ubeira
Laboratorio de Parasitología, Facultad de Farmacia, Universidad
de Santiago de Compostela, Santiago de Compostela, Spain

E. Rodríguez · T. Gárate
Servicio de Parasitología, Centro Nacional de Microbiología,
Majadahonda, Spain

which is important for further dietary advice, where the patients are recommended to avoid the intake of crude or undercooked fish. *A. simplex* sensitisation-associated chronic urticaria (CU+) has also been associated with the previous parasitism by this nematode in an important subset of chronic urticaria patients, mainly if they also display specific IgG₄ [13]. Pathomechanisms leading to chronic urticaria in sensitized patients is under study, and here it cannot be ruled out that positive SPT or specific serum IgE are epiphenomena or due to cross-reactivity with other invertebrate species [6, 14, 15, 17]. Whereas the clinical history is often obvious and the most important leading diagnostic feature in GAA, specific IgE detection in CU+ cannot be associated with a clear parasitic episode, and its interpretation is difficult. To evaluate whether a substantial percentage of the patients with CU+ is due to a previous parasitism by this nematode, in this study, we have evaluated the IgE antibody response to the two major allergens of *A. simplex*, *Ani s 1* and *Ani s 7*, of which at least *Ani s 7* is only released by the parasite while the infecting larvae remains alive [4, 22]. For comparison, the diagnostic value of *Ani s 1* and *Ani s 7* in GAA was also investigated.

Methods

Four groups of subjects were studied and compared: 28 patients with GAA, 40 patients with CU+, 26 chronic urticaria patients without sensitisation against *A. simplex* (CU−), and 15 healthy control subjects without history of urticaria. GAA was diagnosed when a typical history (acute urticaria/angioedema or anaphylaxis of less than 48 h duration within 48 h of raw or undercooked-fish intake) was accompanied by further positive SPT and specific IgE against *A. simplex*. Chronic urticaria patients were included if recurrent wheals were present at least twice weekly for at least 6 weeks. Patients were excluded from this study if physical stimuli were the main eliciting agents of the urticarial reaction. If patients displayed positive SPT and specific IgE against *A. simplex*, they were included in the CU+ group. CU− patients showed neither a positive SPT nor detectable serum specific antibodies against *A. simplex* (CAP-Phadia < 0.35 kU/l). Control subjects did not have any history of urticaria or known *A. simplex* sensitisation status. Written consent was obtained from all studied patients. The project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid.

Skin prick tests

Skin prick test with *A. simplex* (Lab. ALK-Abelló, Madrid, Spain) was performed by standard technique and was considered positive with a mean wheal diameter of 3 mm or

more. Histamine at 1% concentration and saline solution 0.9% (NaCl) were the positive and negative controls, respectively. Wheal diameter was measured 15 min after treatment.

Specific and total IgE

Specific IgE against *A. simplex* and total IgE were assessed by CAP-Phadia (Uppsala, Sweden). To reduce false positive results, patients were only included in the *Anisakis* sensitisation-associated groups if they displayed specific IgE > 1.5 kU/l.

Specific IgE 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 [3]. Specific anti-*Anisakis* IgE antibodies were detected by indirect ELISA, with *t-Ani s 7* or *rAni s 1* 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 µl of phosphate-buffered saline (PBS) containing *rAni s 1* at a concentration of 5 µg/ml, and wells in columns 2, 5, 8, and 11 were filled with 100 µl of 0.1 M Tris buffer, pH 10.5, containing 0.6 µg/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 nonreactive sites, 100 µl of undiluted serum was added to each well and the specific IgE was detected as previously described. Optical densities (ODs) at 492 nm were calculated by subtracting the OD value produced by the same serum in the absence of antigen.

The calculated cutoff values for *Ani s 1* and *Ani s 7* ELISA were ODs of 0.09 and 0.05, respectively, as previously established [3, 20].

Statistics

Statistical analysis was performed using SPSS ver. 15.0 for Windows. Prevalences were calculated for sex, positive *Ani s 1* and *Ani s 7* in all studied groups and compared by Chi-square-test. Mean age, *Ani s 1* and *Ani s 7* ODs were calculated. We assessed also *Ani s 1*/*Ani s 7* ratio to search a preferential recognition of allergens in different urticaria groups. These data were compared by ANOVA (normal distribution of data). For total and specific IgE, median and interquartile range were calculated and compared by Mann–Whitney. Spearman correlation coefficient was used for correlation studies.

Results

The mean age of the investigated patients was lower in CU− than in the other studied groups, but there was no

Table 1 General data of the studied groups

	GAA (n = 28)	CU+ (n = 40)	CU− (n = 26)	Control (n = 15)	P
Mean age (years)	47.1 ± 11.9	47.1 ± 17	35.5 ± 14.3	42.7 ± 9.9	0.008
Female (%)	60.7	72.5	61.6	60.0	n.s

GAA, Gastro-allergic anisakiasis; CU+, *A. simplex* sensitisation associated chronic urticaria; CU−, Chronic urticaria without sensitisation against *A. simplex*; Control, Subjects without history of urticaria or fish-intake associated symptoms

n.s not significant

difference when comparing GAA, CU+ and CU−. In addition, sex distribution was not different in all studied groups (Table 1). When comparing both groups with sensitisation against *A. simplex*, specific IgE obtained by CAP System was expectedly higher in GAA (median 21.9, IQR 5.8–69.1 kU/l) than in CU+ (median 8.5, IQR 3.7–18.7 kU/l; $P = 0.03$). Total IgE was not significantly different: GAA (median 215; IQR 75–770 kU/l); CU+ (median 145; IQR 91–310 kU/l; $P = 0.16$) (Fig. 1).

The Table 2 shows serum specific IgE values against recombinant *Ani s 1* and *Ani s 7*. Higher values were again detected in GAA than in CU+ ($P < 0.0001$ for *Ani s 1* and $P = 0.003$ for *Ani s 7*). The *Ani s 1*/*Ani s 7* ratio was also higher in GAA (68.8 ± 364.8) than in CU+ (3.3 ± 15.2 ; $P = 0.007$).

Among GAA patients, 82.1% were positive for *Ani s 1* but only 42.5% of CU+ patients recognized this allergen [$P < 0.001$ within both *A. simplex* associated groups (GAA and CU+)] (Fig. 2). In contrast, 92.9 and 92.5% of sera from patients with GAA and CU+ recognized the *Ani s 7* allergen (Fig. 3). The combined positivity obtained for both

Table 2 Mean specific IgE expressed as ODs against *Ani s 1* and *Ani s 7* recombinant allergens obtained by ELISA

	GAA	CU+	CU−	Control
<i>Ani s 1</i> ($x \pm SD$)	1.19 ± 0.94	0.44 ± 0.7	0.09 ± 0.43	0.0 ± 0.001
Significance	$P < 0.0001$			
<i>Ani s 7</i> ($x \pm SD$)	1.0 ± 0.86	0.59 ± 0.58	0.03 ± 0.13	0.02 ± 0.05
Significance	$P = 0.003$			

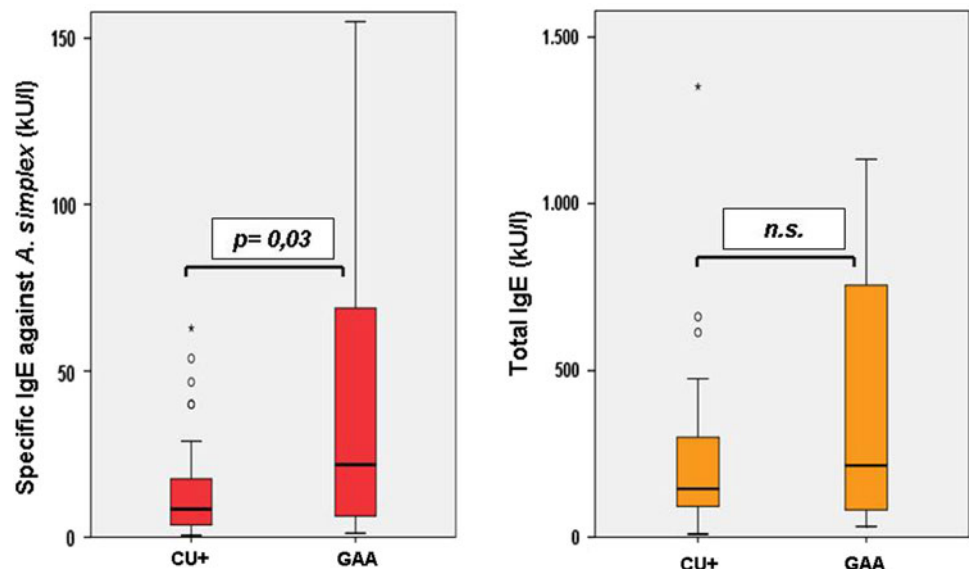
GAA, Gastro-allergic anisakiasis; CU+, *A. simplex* sensitisation associated chronic urticaria; CU−, Chronic urticaria without sensitisation against *A. simplex*. Control, Subjects without history of urticaria or fish-intake associated symptoms. Significance levels are shown for comparison between GAA and CU+

allergens reached 100% in GAA and 95% in CU+. Figures 2 and 3 also show that serum from 1/26 patients (3.8%) among the CU− group was positive for *Ani s 1* and *Ani s 7*, and that sera from 2/15 (13.3%) patients among the control group were positive to *Ani s 7*, but not to *Ani s 1*. These were afterward shown to be SPT positive against *A. simplex*, demonstrating a proportion of positivity previously shown in our region due to past asymptomatic parasitism [9].

Discussion

This is the first study revealing the usefulness of *Ani s 1* and *Ani s 7* recombinant *Anisakis* allergens for the differential diagnosis of *A. simplex*-associated allergic diseases. Currently, there is a world-wide awareness that anisakiasis is not only due to raw-fish-eating habits but also to the different allergic features described in the last 15 years [13]. Whereas GAA has been described as a clear-cut clinical entity, which accounts for a sufficient explanation of previously

Fig. 1 Specific IgE against *A. simplex* and total IgE. GAA Gastro-allergic anisakiasis, CU+ *A. simplex* sensitisation associated chronic urticaria. n.s not significant



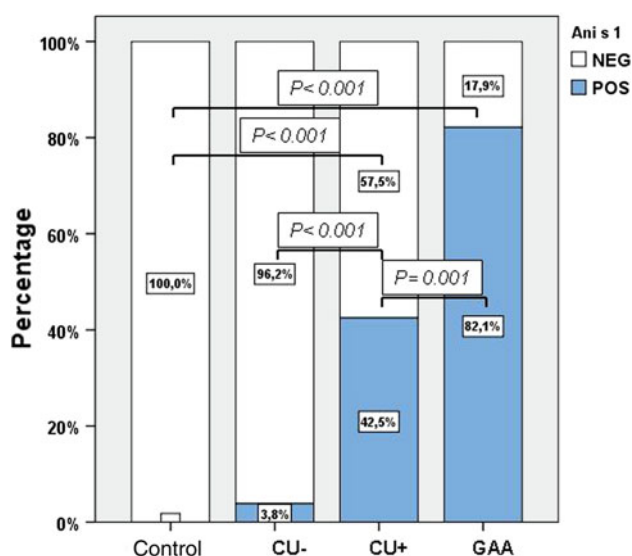


Fig. 2 Percentage of sera with positive and negative specific IgE against *Ani s 1* recombinant allergen. GAA Gastro-allergic anisakiasis, CU+ *A. simplex* sensitisation associated chronic urticaria, CU- Chronic urticaria without sensitisation against *A. simplex*. Control Subjects without history of urticaria or fish-intake associated symptoms

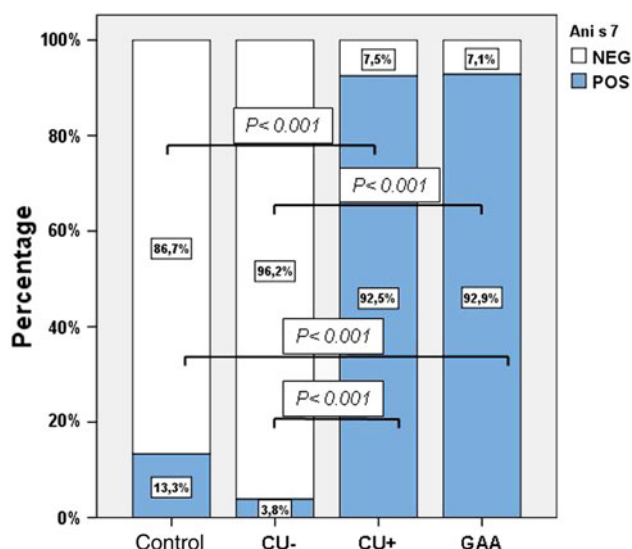


Fig. 3 Percentage of sera with positive and negative specific IgE against *Ani s 7* recombinant allergen. GAA Gastro-allergic anisakiasis, CU+ *A. simplex* sensitisation associated chronic urticaria, CU- Chronic urticaria without sensitisation against *A. simplex*. Control Subjects without history of urticaria or fish-intake associated symptoms

described *Anisakis* allergy, in regions with raw fish-eating habits, chronic urticaria is often associated with sensitization against this nematode [18].

In the last years, recombinant allergens are being proposed for the diagnosis of allergy, not only searching for different sensitisation patterns but also for distinct clinical phenotypes [21, 23]. Twelve *A. simplex* allergens have been

characterized (<http://www.allergome.org>) and some of them have been claimed to be useful for *A. simplex* allergy [7, 19], but frequently, clinically differentiated patient groups are not taken into account. Furthermore, *A. simplex* allergens should not be regarded as food-allergens, as has been recently discussed [11]. In our analysis, we were able to show that patients with different allergic disorders associated with *A. simplex* sensitisation (i.e., GAA and CU+) have a different recognition profile of *Ani s 1*.

The main findings of the present study can be summarized as three major results: we confirm the previously demonstrated high-sensitivity and specificity of the studied recombinant allergens, whereas new findings are (1) the high proportion of positive *Ani s 7* in CU+ and GAA, and (2) the lower proportion of positive *Ani s 1* in CU+ with respect to the GAA group. The concept of major allergen is applied when the frequency of recognition of an allergen among allergic patients is above 50%. The latter results allow us to challenge the definition of major allergen for *Ani s 1*, while confirming again the reported immunodominance of *Ani s 7* in the context of *A. simplex* allergens. Moreover, our data also confirmed that the combined use of *Ani s 1* and *Ani s 7* determinations is currently the best choice for serodiagnosis of human anisakiasis in terms of specificity and sensitivity, as 100% of sensitivity could be stated for GAA patients using this method. The complementarity of *Ani s 1* data with *Ani s 7* determinations was also previously established [3, 4]. Our data with *Ani s 1* and *Ani s 7* allergens also revealed that one patient in the CU- group was unexpectedly positive for both allergens, and that two patients in the control group were positive for *Ani s 7*. The results are not expected to be false positive but due to past asymptomatic infections by the parasite in these subjects. In fact, a similar result was previously obtained [3] where 2.1% of *Ani s 7* positive sera were detected among allergic patients with *Anisakis* CAP values below 1.5 kU/l.

The results of this study also underpin the hypothesis that in most patients with CU+ in our studied region, sensitisation is really caused by a previous parasitic contact with *A. simplex*. *Ani s 7* has previously been shown to be recognized by specific IgE of Wistar rats intraperitoneally inoculated with live *Anisakis* L3 larvae [4]. This recognition during the acute phase of infection is also confirmed by our current study in the patients with a clear history of GAA. The fact that nearly all CU+ patients also recognized *Ani s 7* confirms that the detection of specific IgE is really associated with a previous active parasitism, and possible cross-reactivity seems not to be a causal factor [14]. It is noteworthy that patients were recruited from a geographical zone where house-dust-mite sensitization is not frequent due to mainly dry climatic conditions. Interestingly, even if specific IgE is lower in CU+ patients, *Ani s 7* is still recognized

in most patients. These results could also be useful when assessing other *Anisakis*-associated diseases. In a previous study of prolonged urticaria-induced acute parasitism, defined as an acute urticarial reaction of less than 6 weeks, but more than 72 h, serological features were similar to CU+ with lower serum specific and total IgE values [12]. Thus, even if CU+ or prolonged urticaria are associated with lower immunoglobulin production, *Ani s 7* is not only a major allergen but also recognized independently of the amount of specific IgE production. Further, these results could also be applied for the interpretation of future studies addressing positive SPT or serum specific IgE in subjects without urticaria or a history of fish-induced symptoms. In this sense, the high prevalence of positive serum IgE for *A. simplex* in some regions has been interpreted as previous subclinical parasitism and not as cross-reactivity [18, 24].

Differing results are obtained for *Ani s 1*, the first of all described *A. simplex* allergens, denominated major allergen [16]. Whereas GAA patients recognized this allergen in the same percentage as *Ani s 7*, in CU+, only 42.5% of patients showed specific IgE against this recombinant allergen. This allergen, used in conjunction with *Ani s 7*, is thus able to differentiate in a high proportion both studied *A. simplex* associated allergic diseases. In CU+, we can not know the time when acute parasitism would have taken place, but low specific immunoglobulin levels have been interpreted as an indicator of a longer elapsed time. As a previous study showed that the response to *Ani s 1* was more prolonged in time than to *Ani s 7* in patients who presented allergic symptoms compatible with fish-related allergies where urticaria and/or angioedema was the most frequent allergic manifestation observed [3], our results are thus in concordance with the hypothesis that CU+ is associated not only with lower specific IgE levels but also with a differential recognition of allergens.

A further feature of our results is the interpretation of major allergen as an allergen recognized by more than 50% of allergic patients. Whereas there is no doubt for *Ani s 7* to be still a major allergen in both studied groups, only in GAA can *Ani s 1* still be the denominated major allergen when patients with sensitisation are assessed. This highlights the necessity of carefully evaluating the clinical history of patients, whose sera are analyzed if studies with various recombinant or purified allergens are performed.

In conclusion, the recombinant *Ani s 1* and *Ani s 7* have been proven useful for the diagnosis not only of GAA but also for CU+, combining specificity and sensitivity and open new ways of studying possible mechanism when studying causes or pathophysiology of CU+. Our results also prove that *Ani s 7* is a stronger marker of previous *A. simplex* infections. Further, sensitization against *A. simplex* in most patients with CU+ seems to be due to previous parasite infection.

Acknowledgments The study was funded by grants from Fundación Sociedad Española de Alergología e Inmunología Clínica (SEAIC) 2009 and Fundación Mutua Madrileña 2009. We thank Laura Fernández Gámez for technical support.

References

1. Alonso A, Daschner A, Moreno-Ancillo A (1997) Anaphylaxis with *Anisakis simplex* in the gastric mucosa. *N Engl J Med* 337:350–351
2. Alonso-Gómez A, Moreno-Ancillo A, López-Serrano M, Suarez-de-Parga J, Daschner A, Caballero M, Barranco P, Cabañas R (2004) *Anisakis simplex* only provokes allergic symptoms when the worm parasitises the gastrointestinal tract. *Parasitol Res* 93:378–384
3. Anadón AM, Rodríguez E, Gárate MT, Cuéllar C, Romarís F, Chivato T, Rodero M, González-Díaz H, Ubeira FM (2010) Diagnosing human anisakiasis: recombinant *Ani s 1* and *Ani s 7* allergens versus the UniCAP 100 fluorescence enzyme immunoassay. *Clin Vaccine Immunol* 17:496–502
4. Anadón AM, Romarís F, Escalante M, Rodríguez E, Gárate T, Cuéllar C, Ubeira FM (2009) The *Anisakis simplex Ani s 7* major allergen as an indicator of true *Anisakis* infections. *Clin Exp Immunol* 156:471–478
5. Audicana M, Fernández de Corres L, Muñoz D, Fernández E, Navarro J, del Pozo M (1995) Recurrent anaphylaxis caused by *Anisakis simplex* parasitizing fish. *J Allergy Clin Immunol* 96:558–560
6. Bernardini R, Mistrello G, Novembre E, Roncarolo D, Zanotta S, Lombardi E, Cianferoni A, Pucci N, De Martino M, Vierucci A (2005) Cross-reactivity between IgE-binding proteins from *Anisakis simplex* and *Dermatophagoides pteronyssinus*. *Int J Immunopathol Pharmacol* 18:671–675
7. Caballero M, Moneo I, Gómez-Aguado F, Corcuera M, Casado I, Rodríguez-Pérez R (2008) Isolation of *Ani s 5*, an excretory-secretory and highly heat-resistant allergen useful for the diagnosis of *Anisakis* larvae sensitization. *Parasitol Res* 103:1231–1233
8. Consortium A-IA (2011) *Anisakis* hypersensitivity in Italy: prevalence and clinical features: a multicenter study. *Allergy* 66:1563–1569
9. Daschner A, Alonso-Gómez A, Caballero T, Barranco P, Suarez-De-Parga J, López-Serrano M (1998) Gastric anisakiasis: an underestimated cause of acute urticaria and angio-oedema? *Br J Dermatol* 139:822–828
10. Daschner A, Alonso-Gómez A, Cabañas R, Suarez-de-Parga J, López-Serrano M (2000) Gastroallergic anisakiasis: borderline between food allergy and parasitic disease-clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by *Anisakis simplex*. *J Allergy Clin Immunol* 105:176–181
11. Daschner A, Cuéllar C, Rodero M (2012) The *Anisakis* allergy debate: does an evolutionary approach help? *Trends Parasitol* 28:9–15
12. Daschner A, De Frutos C, Valls A, de la Osada FV (2010) Different clinical presentation of *Anisakis simplex* associated urticaria is dependent on the frequency of raw fish intake. *Allergol Immunopathol (Madr)* 38:166–167
13. Daschner A, Pascual C (2005) *Anisakis simplex*: sensitization and clinical allergy. *Curr Opin Allergy Clin Immunol* 5:281–285
14. Johansson E, Aponno M, Lundberg M, van Hage-Hamsten M (2001) Allergenic cross-reactivity between the nematode *Anisakis simplex* and the dust mites *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Dermatophagoides pteronyssinus*. *Allergy* 56:660–666
15. Lorenzo S, Romarís F, Iglesias R, Audicana M, Alonso J, Leiro J, Ubeira F (2000) O-glycans as a source of cross-reactivity in

- determinations of human serum antibodies to *Anisakis simplex* antigens. *Clin Exp Allergy* 30:551–559
16. Moneo I, Caballero M, Gómez F, Ortega E, Alonso M (2000) Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J Allergy Clin Immunol* 106:177–182
 17. Pascual C, Crespo J, Martín SS, Ornia N, Ortega N, Caballero T, Muñoz-Pereira M, Martín-Esteban M (1997) Cross-reactivity between IgE-binding proteins from *Anisakis*, German cockroach, and chironomids. *Allergy* 52:514–520
 18. Puente P, Anadón A, Rodero M, Romarís F, Ubeira F, Cuéllar C (2008) *Anisakis simplex*: the high prevalence in Madrid (Spain) and its relation with fish consumption. *Exp Parasitol* 118:271–274
 19. Rodríguez-Mahillo A, González-Muñoz M, Gómez-Aguado F, Rodríguez-Pérez R, Corcuera M, Caballero M, Moneo I (2007) Cloning and characterisation of the *Anisakis simplex* allergen Ani s 4 as a cysteine-protease inhibitor. *Int J Parasitol* 37:907–917
 20. Rodríguez E, Anadón AM, García-Bodas E, Romarís F, Iglesias R, Gárate T, Ubeira FM (2008) Novel sequences and epitopes of diagnostic value derived from the *Anisakis simplex* Ani s 7 major allergen. *Allergy* 63:219–225
 21. Twardosz-Kropfmüller A, Singh MB, Niederberger V, Horak F, Kraft D, Spitzauer S, Valenta R, Swoboda I (2010) Association of allergic patients' phenotypes with IgE reactivity to recombinant pollen marker allergens. *Allergy* 65:296–303
 22. Ubeira FM, Anadón AM, Salgado A, Carvajal A, Ortega S, Aguirre C, López-Goikoetxea MJ, Ibanez L, Figueiras A (2011) Synergism between prior *Anisakis simplex* infections and intake of NSAIDs, on the risk of upper digestive bleeding: a case-control study. *PLoS Negl Trop Dis* 5:e1214
 23. Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H (1999) The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 29:896–904
 24. Valiñas B, Lorenzo S, Eiras A, Figueiras A, Sanmartín M, Ubeira F (2001) Prevalence of and risk factors for IgE sensitization to *Anisakis simplex* in a Spanish population. *Allergy* 56:667–671