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ABSTRACT

Gastro-allergic anisakiasis and *Anisakis* sensitisation associated chronic urticaria are diseases which differ in their IgE and IgG4 responses against both crude extract and specific allergens. *Anisakis* and *Ascaris* are closely related nematodes that usually cause problems with specificity in immunodiagnoses. In this study we measured IgE and IgG4 antibodies against *Anisakis simplex* sensu lato (s. l.) and *Ascaris suum* haemoglobins in sera of 21 gastro-allergic anisakiasis and 23 chronic urticaria patients. We used a capture ELISA with the anti-*Anisakis* haemoglobin monoclonal antibody 4E8g, which also recognises *Ascaris* haemoglobin. In addition, we determined specific IgE and IgG4 to both nematodes by indirect ELISA and immunoblotting. Anti-*A. simplex* s. l. haemoglobin IgE and IgG4 levels were higher in gastro-allergic anisakiasis than in chronic urticaria patients ($P = 0.002$ and 0.026 , respectively). Surprisingly, no patient had detectable IgE levels against *A. suum* haemoglobin. Finally, we carried out an in silico study of the B-cell epitopes of both haemoglobin molecules. Five epitopes were predicted in *Anisakis pegreffii* and four in *A. suum* haemoglobin. The epitope propensity values of *Anisakis* haemoglobin in the equivalent IgE binding region of the allergenic haemoglobin *Chi t 1* from *Chironomus thummi*, were higher those of the *Ascaris* haemoglobin. In conclusion, we describe *A. simplex* haemoglobin as a new major allergen (*Ani s 13*), being recognised by a large number (64.3%) of sensitised patients and up to 80.9% in patients with gastro-allergic anisakiasis. The presence of a specific epitope and the different values of epitope propensity between *Anisakis* and *Ascaris* haemoglobin could explain the lack of cross-reactivity between the two molecules. The absence of IgE reactivity to *Ascaris* haemoglobin in *Anisakis* patients makes *Anisakis* haemoglobin (*Ani s 13*) a potential candidate for developing more specific diagnosis tools.

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1. Introduction

Most of the parasitic anisakid nematodes use cetaceans and pinnipeds as definitive hosts, crustaceans as intermediate hosts and fish as paratenic hosts (Mattiucci and Nascetti, 2008). Live *Anisakis* L3 are able to infect humans, causing a disease known as anisakiasis (Nieuwenhuizen and Lopata, 2013). Gastro-allergic anisakiasis (GAA) was described in 2000 as a concept in which an acute allergic reaction (ranging from urticaria or angioedema to anaphylaxis) accompanies the penetration of the fish-nematode *Anisakis simplex* through the gastric mucosa (Daschner et al., 2000). The allergic reaction in GAA is simultaneous with the entrance of the nematode into the gastric mucosa. GAA, considered to be a secondary infection, gives rise to a polyclonal stimulation of all immunoglobulin

isotypes including Th1 and Th2 associated IgE, IgG, IgG4 and IgA, and in addition stimulates the production of new IgE (Daschner et al., 2002).

Haemoglobins are ubiquitous proteins, present in prokaryotes, fungi, plants and animals (Hardison, 1996). In comparison with the exhaustively studied vertebrate tetrameric human haemoglobin (64 kDa) and monomeric myoglobin (17 kDa), invertebrate haemoglobins show a wide variety of functions and structures (Weber and Vinogradov, 2001).

Although sensitisation to pork and chicken haemoglobins has been reported in a case of occupational allergy (Hilger et al., 2010), only haemoglobin of the midge *Chironomus thummi* has been characterised as an allergen in humans (Baur et al., 1982). These insects may cause severe allergic respiratory diseases in sensitised subjects (Yong et al., 1999).

Nematode haemoglobin has been investigated for its high oxygen binding affinity (De Baere et al., 1992) but nothing is

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known about its allergenicity. There is evidence of cross-reactivity between arthropods and nematodes but the role of haemoglobin is still not clear. In 1997 Pascual et al. showed cross-reacting allergens among *Chironomus*, German cockroach and *Anisakis*. These allergens were identified by immunoblotting inhibition as bands between 30 and 40 kDa (Pascual et al., 1997).

Several structures of haemoglobin are available on the www.pdb.org website. The structure of *C. thummi* haemoglobin was determined in 1979 by Steigemann and Weber (PDB: 1ECA/1ECD/1ECN/1ECO). A human IgE epitope was identified in this haemoglobin (allergen *Chi t 1.01*) in the amino acid positions 80–100 by using sera from 15 allergic patients (van Kampen et al., 2001).

Regarding nematode haemoglobins, only the crystal structure of one of the two domains of *Ascaris* haemoglobin has been elucidated (PDB: 1ASH) (Yang et al., 1995). The quaternary structure was studied by electron microscopy and showed that this molecule exists as an octamer (Darawshe and Daniel, 1991; De Baere et al., 1992; Minning and Goldberg, 1998). To date, the structure of *Anisakis* haemoglobin is unknown, although the majority of the mature amino acid sequence has been determined (Nieuwenhuizen et al., 2013).

Finally, *Anisakis* sensitisation associated chronic urticaria (CU+) is an *Anisakis*-associated allergic disease phenotype in which patients present at least twice each week with spontaneously occurring wheals, for a period of at least 6 weeks. They frequently have a previous history of fish-induced allergic and/or abdominal symptoms, together with a positive Skin Prick Test (SPT) against *A. simplex* and detectable specific IgE against *A. simplex* (Daschner et al., 2010).

GAA and CU+ differ with respect to IgE and IgG4 antibodies against crude extract and recombinant allergens such as *Ani s 1* and *Ani s 7*. A high proportion of both CU+ and GAA patients show IgE binding to *Ani s 7*, whereas IgE recognition of *Ani s 1* is significantly lower in CU+ patients. We have also observed that levels of specific IgG4 to *Anisakis* crude extract, as well as IgG4 to *Ani s 1* and *Ani s 7* recombinant allergens, were significantly higher in GAA (Cuéllar et al., 2012). Therefore, patients with different *Anisakis* sensitisation associated allergic disorders have different antigen recognition profiles.

In this study we measured IgE and IgG4 antibodies against *Anisakis* and *Ascaris* haemoglobins in sera from patients with *Anisakis* sensitisation associated with either GAA or CU+. In addition, we analysed the possible B-cell epitopes on three-dimensional models of both proteins in silico.

2. Materials and methods

2.1. Patients and serum samples

Two groups of subjects were studied and compared: 21 patients with GAA and 23 patients with CU+ were recruited prospectively during the same time-frame. 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 *Anisakis*. The SPT was performed with *A. simplex* extract (ALK-Abelló, Madrid, Spain) using a 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 positive and negative controls, respectively. CU+ patients were included if recurrent wheals were present at least twice weekly for at least 6 weeks and they displayed a positive SPT as well as specific IgE against *Anisakis* >0.35 kU/L. Patients were excluded from this study if physical stimuli were the main eliciting agents of the urticarial reaction. Written consent was obtained

from all patients studied. The project was approved by the Ethics Committee of the University Hospital La Princesa, Madrid, Spain.

2.2. Routine laboratory determinations

Total IgE was analysed in all patients by ImmunoCAP® (Phadia, Uppsala, Sweden). Anti-*Anisakis*/*Ascaris* specific IgE and anti-*Anisakis* IgG4 were analysed by ImmunoCAP® (Phadia) against crude extract antigens.

2.3. Crude extract preparation

Anisakis simplex sensu lato (s. l.) L3 and *Ascaris suum* adults were homogenised in PBS and sonicated six times for 10 s. The homogenate was extracted in PBS at 4 °C overnight and subsequently delipidised with *n*-hexane. After centrifugation at 6700g at 4 °C, the supernatant was dialysed overnight in PBS at 4 °C (Medicell Intl. Ltd., London, UK). The protein content was quantified by the Bradford protein assay (BioRad, München, Germany).

2.4. Anti-*Anisakis*/*Ascaris* haemoglobin IgE or IgG4 determination by antigen-capture ELISA

The levels of IgE or IgG4 antibodies to haemoglobin of *A. simplex* s. l. were determined by an antigen-capture ELISA using the monoclonal antibody (mAb) 4E8g previously prepared against haemoglobin of the nematode *Anisakis pegreffii*. This mAb recognises highly immunogenic excretory-secretory haemoglobins of both *Anisakis* and *Ascaris* (Nieuwenhuizen et al., 2013). For the IgE determination, 100 µl of PBS containing 1.5 µg of the mAb 4E8g were added to each well of a 96-well ELISA plate (Costar, Corning, NY, USA) and incubated overnight at 4 °C. After washing, all wells were incubated with 200 µl of 1% BSA diluted in PBS for 1 h at 37 °C. Plates were then washed and incubated overnight at 4 °C with 100 µl of PTB (PBS plus 0.05% Tween 20 and 0.1% BSA) containing 500 µg of total protein/well from nematode crude extracts (*A. simplex* s. l. L3 or *A. suum* adult). Corresponding wells were incubated with PTB but without the extracts as control/absorbance correction. After a washing step and the addition of 100 µl/well of human sera (diluted at 1/2 with PTB), plates were incubated overnight at 4 °C. The plates were then washed repeatedly and 100 µl of a mouse anti-human-IgE mAb labelled with horseradish peroxidase (HRP) (Clone B3102E8) (SouthernBiotech, Birmingham, AL, USA) were added. After incubation at 37 °C and subsequent washing, bound Ig-HRP was detected by incubation with o-phenylenediamine (OPD; Sigma–Aldrich, Germany) in phosphate-citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 3 N sulphuric acid. The O.D. at 490 nm was calculated by subtracting the O.D. values of the same serum in the absence of antigen. Experiments were done in duplicate.

In the case of the IgG4 measurement, the sera were diluted at 1/100 with PTB and a mouse anti-human IgG4-HRP mAb (Clone HP6025) (SouthernBiotech) was used.

For data analyses, sera were considered positive if the O.D. was more than $X + 2SD$, where X and SD are the corresponding mean O.D. and the S.D. obtained with 14 *Anisakis* negative control sera.

2.5. Immunoblotting

SDS–PAGE was carried out as described by Laemmli (1970) and revised by Hames (1986) using a Mini Protean® III cell (BioRad). The gels consisted of a 4% stacking gel and 12% separating gel. Worm crude extracts (25 µg/lane) were dissolved in a sample buffer (50 mM Tris–HCl buffer, pH 8.6, containing 2% SDS, 20% glycerol and 0.02% bromophenol blue) diluted 1:1 in Tris–glycine electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3). Samples were loaded

with 5% β -mercaptoethanol and were heated for 5 min at 95 °C for reducing conditions. Electrophoresis was performed for 2 h at a constant 100 V in Tris–glycine electrode buffer. Broad range molecular mass markers (10–250 kDa Kaleidoscope®, BioRad) were incorporated into each electrophoresis run. Following the SDS–PAGE of antigens, the protein bands were transferred onto a 0.22 μ m pore size nitrocellulose membrane (Hybond® ECL, GE Healthcare, UK) in a Mini Trans-blot® Electrophoretic Transfer Cell (BioRad) with 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3. The transblot was carried out at a constant 100 V for 1 h. The membrane was blocked overnight at 4 °C with PBS containing 5% non-fat dry milk, prior to immunorecognition by incubation for 3 h with the mAb 4E8g anti-*A. pegreffii* haemoglobin (2 μ g/ml) diluted in PBS–Tween containing 1% non-fat dry milk. Each membrane was then washed with PBS–Tween 20 (3 $\times$ 5 min) and incubated for 2 h with goat anti-mouse IgG1 (γ 1), HRP conjugate (Molecular Probes Inc., Eugene, OR, USA) at 1/1000 in PBS–Tween 20 containing 1% non-fat dry milk. To visualise bands the nitrocellulose was washed with PBS–Tween 20 (3 $\times$ 5 min) and the strips were treated with a solution of 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich) with 0.05% hydrogen peroxide in PBS.

For IgG4 determination, after a blocking step, strips were incubated for 3 h with sera diluted at 1/25. After that, nitrocellulose strips were washed with PBS–Tween 20 (3 $\times$ 5 min) and they were incubated with anti-human IgG4–HRP mAb (Clone HP6025) (SouthernBiotech) at 1/500 in PBS–Tween 20 containing 1% non-fat dry milk. Finally, the strips were treated with a solution of 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich) with 0.05% hydrogen peroxide in PBS.

2.6. Anti-*Anisakis*/*Ascaris* crude extract IgG4 or IgE determination by indirect ELISA

Each well of a 96-well microtitre plates (Costar) was coated with 10 μ g/ml of *A. simplex* s. l. or *A. suum* crude extract. Duplicate dilutions of sera at 1/100 in PBS–Tween containing 0.1% BSA were added and incubated, and IgG4 quantified using mouse anti-human IgG4–HRP mAb (Clone HP6025) (SouthernBiotech).

For the IgE determination, test sera at a 1/2 dilution were added in duplicate. Samples were incubated with a murine mAb against an epsilon human IgE chain (IgG1 κ , E21A11, INGENASA, Madrid, Spain), followed by a goat anti-mouse IgG1 (γ 1), HRP conjugate (Molecular Probes Inc.).

2.7. Statistics

Median values and interquartile ranges (IQR) were calculated for specific antibody levels and compared using a Mann–Whitney U test. The Spearman correlation coefficient was used for correlation studies. All statistics were calculated using IBM SPSS Statistics Version 20. $P < 0.01$ was considered significant.

2.8. Sequences, modelling and epitope prediction

Haemoglobin sequences were obtained from the Entrez Protein Database of the National Center for Biotechnology Information (NCBI), USA (<http://www.ncbi.nlm.nih.gov/protein>) with the following GenBank accession numbers: 1ECO_A (*C. thummi*), AFY98826.1 (*A. pegreffii*) and AAA29374.1 (*A. suum*). We then determined identity (I) and similarity (S) percentages among haemoglobin sequences: (i) *Anisakis* versus *Ascaris*: I = 65.0% and S = 79.3%; (ii) *Chironomus* versus *Anisakis*: I = 16.2% and S = 33.8%; (iii) *Chironomus* versus *Ascaris*: I = 13.2% and S = 31.6%. Three-dimensional structure of the allergenic haemoglobin *Chi t 1* was PDB: 1ECO. Using the SignalP 4.1 server (Petersen et al., 2011),

we selected the mature sequence for *A. suum* haemoglobin and deleted the amino acid positions 1–18. Using the Protein Model Portal interface (Haas et al., 2013), both haemoglobins were modelled using HHPred–MODELLER. The HHPred server identifies potential homologous proteins that can be structural templates. Firstly, this server builds an alignment of a query sequence with homologues by Position-Specific Iterative (PSI)–BLAST from the NCBI database. This alignment is annotated with a secondary structure prediction. Then, a profile Hidden Markov Model (HMM) is generated and compared with each HMM in several annotated databases of protein family alignments. Having selected the structures with the highest scores as templates, three-dimensional models were built using MODELLER software (Söding et al., 2005). For *Anisakis* haemoglobin, PDB: 1C7C, 2WTG, 1ASH, 1H97, 2DC3, 2VYW and 2C0 K were used as templates. Structural templates for complete modelling of *Ascaris* haemoglobin were PDB: 1C7C, 1ASH, 1H97, 2VHB, 2WTG, 2C0 K and 3G46. These models were evaluated using the QMEAN server, which provides a composite scoring function for the quality estimation of protein structure models based on six structural descriptors (C β interaction, all-atom interaction, solvation, torsion, agreement between the predicted secondary structure of the target sequence and the calculated secondary structure of the model, and agreement between the predicted relative solvent accessibility and the relative solvent accessibility) (Benkert et al., 2009). DiscoTope 2.0 (Kringelum et al., 2012) was used for predicting discontinuous B-cell epitopes on the haemoglobin three-dimensional structures. The DiscoTope method is based on: (i) statistical differences in amino acid composition of epitopes and non-epitopes from a dataset of experimental antibody/antigen structures; (ii) definition of surrounding amino acids of the amino acid analysed; and (iii) measurement of surface accessibility because B-cell epitopes are usually composed of exposed amino acids. The threshold value was defined as -3.7 in order to maximise the sensitivity with a high specificity of epitope detection (<http://www.cbs.dtu.dk/services/DiscoTope/instructions.php>) (Krawczyk et al., 2014). All residues with DiscoTope scores higher than -3.7 , were considered as part of an epitope. The results were visualised with PyMol (DeLano Scientific, Palo Alto, CA, USA).

3. Results

3.1. IgE and IgG4 ImmunoCAP® values

Twenty-one patients with GAA and 23 patients with CU+ were included in the study. The mean age was 50.7 ± 13.9 years and the female:male ratio was 9:12 in GAA and 20:3 in CU+ ($P = 0.001$).

As previously observed, higher values of immunoglobulins were obtained by ImmunoCAP® in the GAA group with significant differences in levels of specific IgE and IgG4 against *Anisakis* antigen ($P < 0.001$) (Fig. 1).

3.2. Specific IgE and IgG4 against *Anisakis* haemoglobin

Specific IgE and IgG4 antibodies were measured against *A. simplex* s. l. haemoglobin using antigen–capture ELISA. Levels of anti-*A. simplex* s. l. haemoglobin IgE and IgG4 were higher in the GAA group compared with the CU+ group ($P = 0.002$ and 0.026 , respectively) (Fig. 2).

IgE against *A. simplex* s. l. haemoglobin was detected in 64.3% of *Anisakis* sensitised patients. This percentage is above 50% when analysing the two *Anisakis* sensitisation associated clinical entities with cutaneous features after *Anisakis* parasitism, and thus *A. simplex* haemoglobin was considered a major allergen and named according to the rules of the International Union of Immunological Societies (IUIS) Allergen Nomenclature as *Ani s 13*

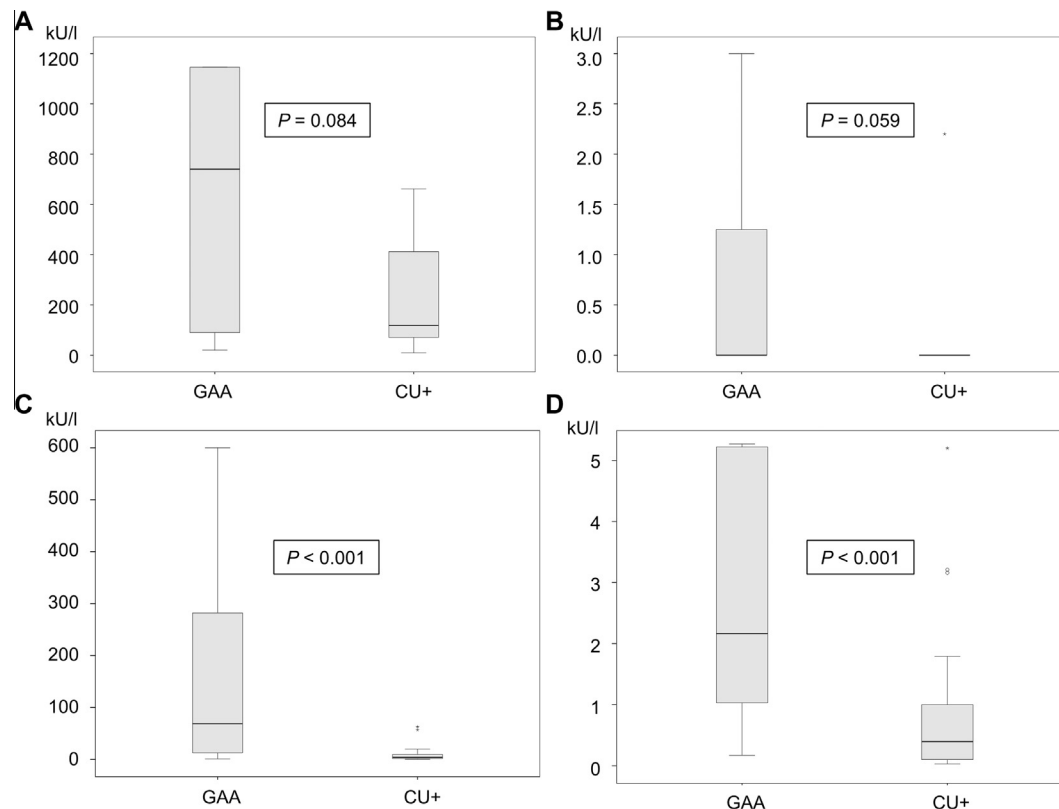


Fig. 1. Median and interquartile ranges of (A) total IgE, (B) anti-*Ascaris suum* IgE, (C) anti-*Anisakis simplex sensu lato* (s. l.) IgE and (D) anti-*A. simplex* s. l. IgG4. The results were obtained by ImmunoCAP® in patients with gastro-allergic anisakiasis and *Anisakis* sensitisation associated chronic urticaria.

(<http://www.allergen.org/viewallergen.php?aid=797>). On the other hand, when analysed separately, 80.9% of sera from the GAA group were positive versus 47.8% observed in the CU+ group. In the case of anti-*A. simplex* s. l. haemoglobin IgG4, 31.8% of 44 *Anisakis* sensitised patients were positive (47.6% of GAA versus 17.3% of CU+).

Anti-*A. simplex* s. l. haemoglobin IgE and IgG4 values were analysed for correlations with total IgE, anti-*Anisakis/Ascaris*-specific IgE and anti-*Anisakis* IgG4 measured by ImmunoCAP® against crude extract antigens. Anti-*A. simplex* s. l. haemoglobin IgE correlated significantly ($P < 0.01$) with total IgE (Rho 0.779), IgE and IgG4 specific for total *Anisakis* antigen (Rho 0.502 and 0.934, respectively) and anti-*Ascaris* IgE (Rho 0.572).

Similarly, significant correlations were observed between anti-*A. simplex* s. l. haemoglobin IgG4 and anti-*Anisakis* IgG4 and IgE measured by ImmunoCAP® (Rho 0.642 and Rho 0.484, respectively).

3.3. *Anisakis* and *Ascaris* haemoglobin detection by immunoblotting

Antigen-capture ELISA using mAbs is a valid technique for detecting and quantifying antibodies in the sera of allergic patients (Sookrung et al., 2003). However, to test that the antigen-capture ELISA gave no positive results due to the presence of haemoglobin-binding proteins or false negatives due to blocking of haemoglobin epitopes, we analysed IgG4 immuno-recognition patterns of the 44 *Anisakis* sensitised patients by immunoblotting under reducing conditions. For identification of haemoglobin recognition, we considered as positive those sera which presented the same binding pattern as the mAb 4E8g anti-*A. pegreffii* haemoglobin under reducing conditions (Fig. 3). The prevalence of anti-*Anisakis* haemoglobin IgG4 was 29.5% in the *Anisakis* sensitised patients (47.6% of GAA versus 13.0% of CU+). This prevalence was similar

to that previously obtained using the antigen-capture ELISA, in which 31.8% of 44 *Anisakis*-sensitised patients were positive (47.6% of GAA versus 17.3% of CU+). Specific IgE concentrations in serum are generally low and due to limited quantities of serum we were unfortunately unable to assess the prevalence of haemoglobin reactive IgE by immunoblotting. However, based on the results of the IgG4 ELISAs and immunoblots, the antigen-capture ELISA using mAb 4E8g seems to be a reliable measure of anti-haemoglobin antibody responses in these patients.

3.4. Study of cross-reactivity between *Anisakis* and *Ascaris* haemoglobins

The 4E8g mAb recognised both *A. simplex* s. l. and *A. suum* haemoglobins when immunoblotting experiments were carried out using crude parasite extracts under non-reducing and reducing conditions. 4E8g recognised a protein present around 37 kDa and higher molecular weight proteins in *A. simplex* s. l. L3 and *A. suum* adult somatic extracts. In both immunoblotting analyses (reduced and non-reduced) *Anisakis* haemoglobin appeared around 37 kDa (monomer), 75 kDa (dimer), 100 kDa (trimer) and 150 kDa (tetramer). Under non-reducing conditions even more haemoglobin homomers were clearly observed (Fig. 4).

We tested IgE and IgG4 antibody levels against *A. simplex* s. l. and *A. suum* crude extracts in the 44 studied serum samples (21 GAA and 23 CU+) by indirect ELISA (Table 1). Specific antibody levels were significantly higher against *Anisakis* antigen, although *Ascaris* antigen was also recognised by sera from *Anisakis* sensitised patients. This crude extract cross-reactivity was also observed by immunoblotting (Fig. 3).

None of the sera showed detectable IgE levels against *A. suum* haemoglobin (0.010 ± 0.010), in contrast to the IgE levels detected

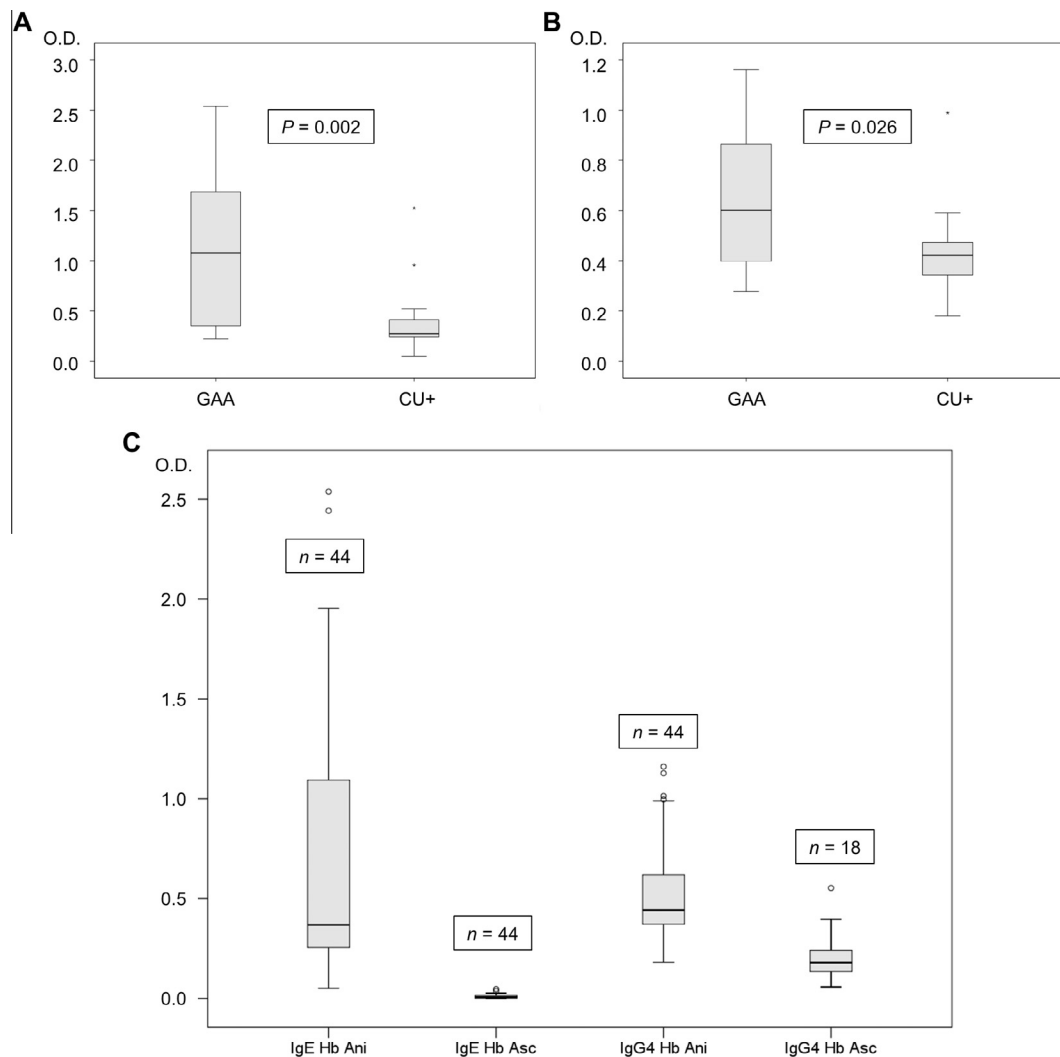


Fig. 2. Median and interquartile ranges of (A) IgE and (B) IgG4 anti-*Anisakis simplex* sensu lato (s. l.) haemoglobin. The results were obtained by capture ELISA in patients with gastro-allergic anisakiasis and *Anisakis* sensitisation associated chronic urticaria. In C no distinction was made between gastro-allergic anisakiasis and chronic urticaria. N, number of patients; IgE Hb Ani and IgG4 Hb Ani, IgE and IgG4 against *A. simplex* s. l.; IgE Hb Asc and IgG4 Hb Asc, IgE and IgG4 against *Ascaris suum* haemoglobin.

against *A. simplex* s. l. haemoglobin (0.712 ± 0.650). In the case of IgG4, the values were also higher against *A. simplex* s. l. haemoglobin (0.528 ± 0.247) although detectable levels were also observed against *A. suum* haemoglobin (0.214 ± 0.114) (Fig. 2).

3.5. Protein structure models and epitopes

The QMEAN scores of *A. pegreffii* and *A. suum* haemoglobin were 0.734 and 0.601, respectively. The QMEAN score (ranging from 0 to 1) indicates the global score of the whole model, reflecting the predicted model reliability, therefore both models are acceptable.

DiscoTope scores of each position, obtained with the DiscoTope 2.0 server, were plotted (Fig. 5). All amino acids predicted as a part of an epitope were manually coloured on the molecule. Positions were numbered considering NCBI sequences without modifications. Five epitopes were defined in *A. pegreffii* haemoglobin (Epitope 1: 16, 27, 30–56, 58 and 59; Epitope 2: 90, 92–100; Epitope 3: 239, 240, 242–251, 292–309; Epitope 4: 163–166, 169, 176, 177, 179–205, 207, 208; Epitope 5: 145–147, 150, 266, 269–271, 273–275, 278) while only four in the case of *A. suum* (Epitope 1: 49–81; Epitope 2: 116, 118–120; Epitope 3: 138, 139, 142, 144–146, 261–273, 315–338; Epitope 4: 185–187, 191, 198, 201–230).

These epitopes were mainly located above and below the haem pocket.

At Epitopes 1 and 4, similar DiscoTope score profiles were observed between both haemoglobins. However, in *A. pegreffii* haemoglobin, the equivalent human IgE binding region of *Chi t 1* (positions 80–100) was longer and showed a higher DiscoTope score than *A. suum* (Epitope 2) (Fig. 5).

Epitope 5 is a discontinuous epitope only present in *A. pegreffii* haemoglobin, not being observed in *A. suum* haemoglobin.

4. Discussion

In this work, haemoglobin has been defined as a new major allergen of *Anisakis* (*Ani s 13*) without cross-reactivity against *Ascaris* haemoglobin.

Firstly, it is noteworthy that *Anisakis* L3 and *Ascaris* adults are in different biological stages. On the one hand, *A. simplex* s. l. L3 crude extract used in the analysis was prepared from the biological stage which infects the human host (Nieuwenhuizen and Lopata, 2013). On the other hand, *Ascaris* haemoglobin is only found in adults (Blaxter, 1993; Barrett and Brophy, 2000; Hashimoto et al., 2008). Consequently, this is the only possible design for the study with regard to the source of haemoglobin.

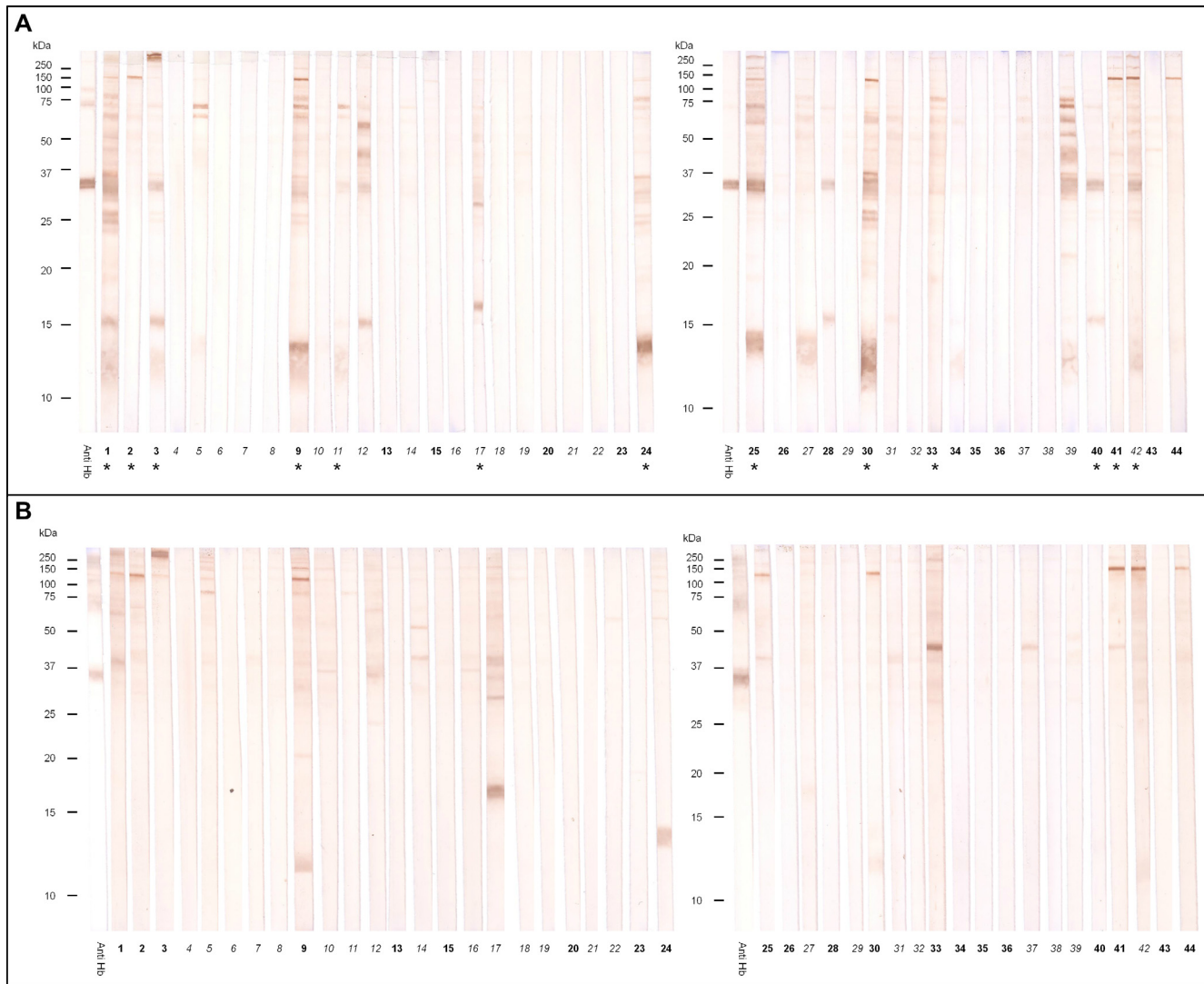


Fig. 3. Immunoblotting analysis of IgG4 binding to reduced (A) *Anisakis simplex* sensu lato L3 and (B) *Ascaris suum* adult crude extracts with sera from *Anisakis* sensitised individuals. Anti Hb, monoclonal antibody 4E8g anti-*Anisakis* haemoglobin. *Denotes positive sera. Gastro-allergic anisakiasis patients are marked in bold type. Chronic urticaria patients are marked in italic type.

We observed that GAA patients always have higher levels of *Anisakis*-specific immunoglobulins than patients with CU+. Similarly, levels of anti-*A. simplex* s. l. haemoglobin IgE and IgG4 were higher in the GAA group compared with the CU+ group. Previously, we observed that GAA patients showed higher IgE and IgG4 levels against crude extract and both *Ani s 1* and *Ani s 7* recombinant allergens. Whereas IgE recognition of *Ani s 7* did not differ between GAA and CU+ groups and suggests that both clinical entities are associated with previous acute parasitism, the IgE recognition rate of *Ani s 1* and IgG4 recognition rates of both *Ani s 1* and *Ani s 7* were higher in patients with GAA (Cuéllar et al., 2012; Daschner et al., 2014). The inclusion of haemoglobin as a new *Anisakis* allergen in the evaluation of specific IgE and IgG4 could be useful for the diagnosis of *Anisakis*-associated allergic disease phenotypes (GAA versus CU+). In addition this allergen could be of importance for sensitisation via the inhalation route including in the occupational environment (Nieuwenhuizen et al., 2006).

As *Anisakis* haemoglobin was recognised by serum IgE in more than 50% of prospectively studied patients (64.3%) with cutaneous features after *Anisakis* parasitism, this molecule can be classified as a new major allergen of *Anisakis* (*Ani s 13*). Similarly, *Ani s 1*, *Ani s 2*,

Ani s 7, as well as *Ani s 12*, have previously been characterised as major allergens (85%, 88%, 85–100% and 57%, respectively) (Moneo et al., 2000; Pérez-Pérez et al., 2000; Anadón et al., 2009; Kobayashi et al., 2011;).

The percentage of positive sera in the GAA group was higher than that observed in the CU+ group (80.9% versus 47.8%). Specific allergen *Ani s 1* showed very similar recognition percentages: 82.1% versus 42.5%. In contrast, the recognition frequencies of *Ani s 7* were almost identical between the groups: 92.9% versus 92.5% (Cuéllar et al., 2012). Our new data thus shows that patients with two different *Anisakis* sensitisation associated allergic disorders also have different haemoglobin recognition profiles. This confirms our previous hypothesis that CU+ is associated with differential recognition of *Anisakis* allergens.

With regard to the IgG4 reactivity, 31.8% of 44 *Anisakis* sensitised patients showed IgG4 positive against *A. simplex* s. l. haemoglobin. The detection of IgG4 against *Anisakis* haemoglobin could also be useful for differentiating between GAA and CU+ patients since only 17.3% of the CU+ patients had anti-*A. simplex* s. l. haemoglobin IgG4 compared with 47.6% with GAA. Differences in IgG4 production by GAA patients were also previously reported for *Ani*

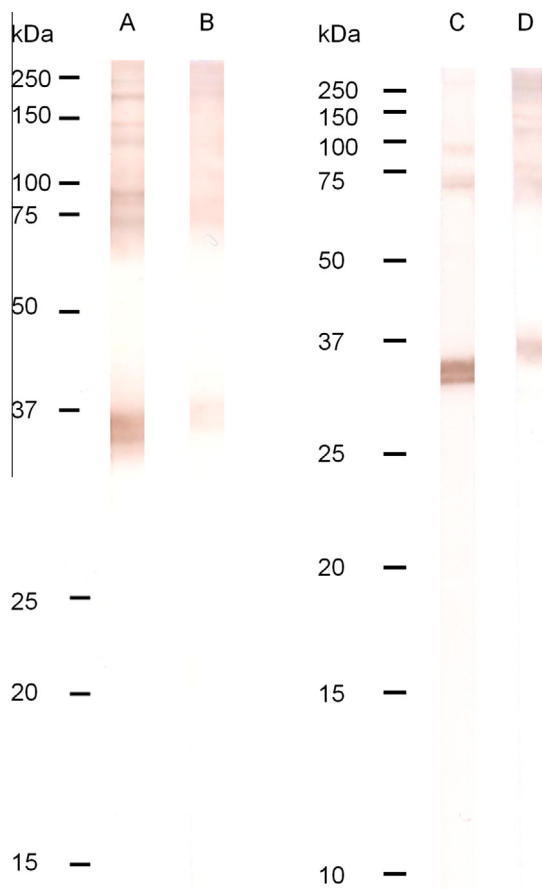


Fig. 4. Immuno-recognition patterns of monoclonal antibody 4E8g anti-*Anisakis* haemoglobin. 4E8g antibody concentration was 2 µg/ml against (A) *Anisakis simplex* sensu lato (s. l.) L3 and (B) *Ascaris suum* adult crude extracts. Lanes C and D contained *A. simplex* s. l. L3 and *A. suum* adult crude extracts, respectively, performed under reducing conditions.

s 1 (65% versus 35%) and Ani s 7 (90% versus 28%), and may protect these patients against chronic forms of skin manifestations by competing for IgE binding sites (Ozdemir et al., 2011; Daschner et al., 2014).

Molecular and morphological differences between *A. simplex* and *A. pegreffii* L3 have been reported (Quiazon et al., 2008). Nevertheless, some allergens such as Ani s 1, Ani s 2, Ani s 9 and Ani s 12 have been compared between both *Anisakis* spp. and show high similarities in the amino acid sequences (Quiazon et al., 2013). Consequently, *A. simplex* and *A. pegreffii* haemoglobins are likely to share the majority of their amino acid sequences.

We showed by immunoblotting that the 4E8g mAb recognised both *A. simplex* s. l. L3 and *A. suum* adult haemoglobins.

Table 1

Median and interquartile ranges of IgE and IgG4 immunoglobulin levels by ELISA against *Anisakis simplex* sensu lato L3 (Ani) and *Ascaris suum* adult (Asc) crude extracts in patients with gastro-allergic anisakiasis and *Anisakis* sensitisation associated chronic urticaria. Values are shown as O.D. at 490 nm.

Group	IQR	IgE-Ani	IgG4-Ani	IgE-Asc	IgG4-Asc
GAA	25	0.168	0.084	0.043	0.014
	50	0.782	0.224	0.061	0.026
	75	1.170	0.776	0.075	0.100
CU+	25	0.105	0.005	0.038	0.003
	50	0.210	0.032	0.052	0.007
	75	0.316	0.154	0.069	0.016
GAA and CU+	25	0.133	0.014	0.043	0.005
	50	0.253	0.107	0.059	0.015
	75	0.824	0.325	0.070	0.059

Nieuwenhuizen et al. (2013) previously observed that 4E8g bound to a 37 kDa protein present in somatic and excretory-secretory *A. pegreffii* products and a 40 kDa protein present in *Ascaris lumbricoides* adult extract, as well, a dimeric 80 kDa protein that we detected in our experiments. Binding to this excretory-secretory protein was also demonstrated by immunohistochemistry using both nematodes (Nieuwenhuizen et al., 2013). For this reason, we decided to use the 4E8g mAb to capture *A. suum* haemoglobin, in order to measure, by ELISA, the presence of serum anti-*A. suum* haemoglobin IgE in the same manner in which anti-*Anisakis* immunoglobulins were tested. Serodiagnostic methods using excretory-secretory antigens are more sensitive and specific, as was indicated in the case of visceral larva migrans diagnosis (Magnaval et al., 1992).

Although the rates of specific antibodies were significantly higher against homologous *Anisakis* antigen, the heterologous *Ascaris* one was also recognised by sera from *Anisakis* sensitised patients, showing cross-reactivity between both crude antigens. By means of immunoblotting, Nieuwenhuizen et al. (2013) observed that sera from mice experimentally infected with live larvae contained both specific IgG and IgE to *A. pegreffii* haemoglobin. However, cross-reaction with *A. lumbricoides* was only observed for IgG antibodies, suggesting that *Anisakis* but not *Ascaris* haemoglobin could be an IgE-binding protein.

Despite having confirmed the cross-reactivity between *Anisakis* and *Ascaris* crude extracts, none of the *Anisakis* sensitised patients demonstrated IgE against *Ascaris* haemoglobin by capture ELISA. In order to confirm the lack of IgE recognition of *Ascaris* haemoglobin it would be necessary to compare *Ascaris* and *Anisakis* sensitised subjects, but our results to date suggest the absence of cross-reactivity in *Anisakis* sensitised patients.

Anti-*A. simplex* s. l. haemoglobin IgE correlated with both IgE specific for *Anisakis* and *Ascaris* total antigens. The patients do not seem to have IgE against *Ascaris* haemoglobin, but perhaps some of their other anti-*Anisakis* antibodies are cross-reactive.

We observed that the only *Anisakis* haemoglobin sequence ((*A. pegreffii*) GenBank accession number AFY98826.1) is considerably similar to *Ascaris* haemoglobin ((*A. suum*) GenBank accession number AAA29374.1). There are 201 exact matches between *Anisakis* and *Ascaris* haemoglobin sequences (309 and 320 total aminoacids without signal peptides, respectively). However, based on the models generated with HHPred-MODELLER, we observed that the proteins share neither the same surface nor electrostatic potential (data not shown), which is important for antibody binding (van Oss, 1995).

DiscoTope scores of Epitopes 1 and 4 (one in each globin domain) of both haemoglobins seem to be similar and could be involved in IgG binding because the equivalent region (peptide 41–60) in *Chi t 1* was recognised by a rabbit antiserum and mouse monoclonal IgG antibodies (van Kampen et al., 2001). The C-terminal tail and the central region of the sequence of *Ascaris* makes Epitope 3 of *Ascaris* haemoglobin sufficiently different to Epitope 3 of Ani s 13. The most important epitopes in Ani s 13 for IgE binding

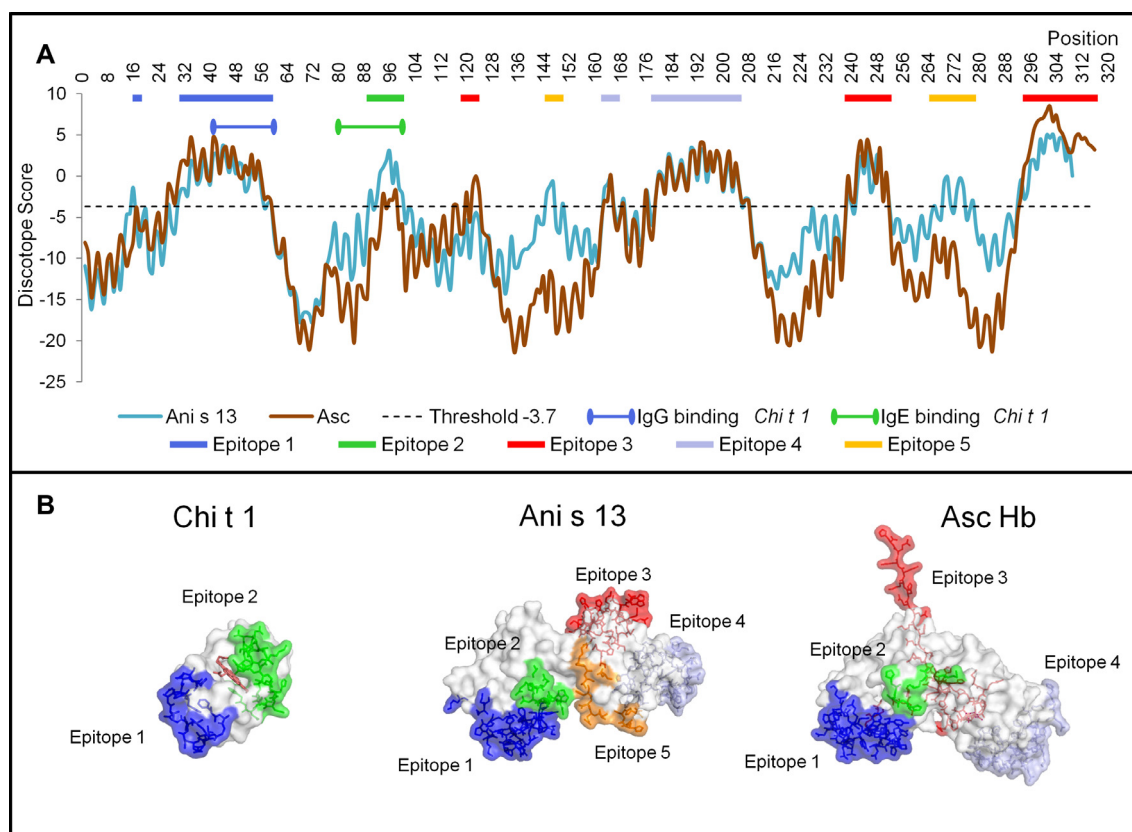


Fig. 5. DiscoTope scores of (A) *Anisakis pegreffii* and *Ascaris suum* haemoglobin models and (B) a three-dimensional representation of *Chi t 1.01* (PDB: 1ECO), *A. pegreffii* (*Ani s 13*) and *A. suum* (*Asc Hb*) haemoglobin models. *Chi t 1.01* positions 41–60 did not bind human IgE (only rabbit antiserum and mouse monoclonal antibody IgG), whereas 80–100 is the major human IgE binding epitope (van Kampen et al., 2001).

and specificity might be Epitope 2 and Epitope 5, respectively. Epitope 2 in *Ani s 13* has more extension and more propensity to be an epitope than in *Ascaris* haemoglobin. Our results suggest that *Anisakis* haemoglobin could be allergenic because the surface of the Epitope 2 binds IgE similarly to the equivalent region 80–100 in *Chi t 1.01*. Van Kampen et al. (2001) showed that in a bank of 15 *Chi t 1* allergic patients, 13 had similar IgE levels against peptide 80–100 of *Chi t 1*.

Epitope 5 is an *Anisakis* conformational epitope not present in *Ascaris* haemoglobin. Van Kampen et al. (2001) claimed that not only linear peptides (peptide 80–100) are involved in IgE binding, but also conformational epitopes for IgE exist on *Chi t 1*. Two out of the above-mentioned 15 patients recognised only the whole molecule of *Chi t 1* and not the peptides. Furthermore, Epitope 5 could be responsible for the specificity of the IgE from *Anisakis*-positive patients.

Some studies which used modelling/immunobioinformatics approaches (with the DiscoTope server) compared the predictions with experimental results. For example, DiscoTope correctly predicted B and T cell epitopes of a novel malarial antigen in about 55% of cases (Bergmann-Leitner et al., 2013). New methods of conformational epitope prediction did not show significant improvements over DiscoTope and other previous methods. In another study, different in silico epitope prediction programs were evaluated with foot-and-mouth disease virus (FMDV) (Borley et al., 2013). DiscoTope performed the best among the programs studied, identifying 61% of the previously described epitopes for all serotypes of FMDV, although it performed poorly in terms of precision. In the same article, the authors tried to use a consensus approach to identify novel epitopes by a consensus of all three programs (Epitopia, Ellipro and DiscoTope). However, this strategy did not improve precision or accuracy over any single program.

Other possibilities could explain the allergenicity of *Ani s 13*: dimerisation, peptidase cleavage and/or glycosylations. Dimerisation processes are essential for the cross-linking of the B-cell receptors by allergens (Ghosh and Gupta-Bhattacharya, 2008; Rouvinen et al., 2010), but a dimerisation could hide or show new epitopes. *Chi t 1* has been demonstrated as monomer and dimer (Weber and Vinogradov, 2001), however *Ascaris* haemoglobin and other nematode haemoglobins are known to be stable as octamers although we still do not have a crystal structure of the intact octamer (Goldberg, 1999). We do not know yet whether *Ani s 13* is stable as a dimer or as octamer. The theoretical molecular weight of *Anisakis* haemoglobin is 36.7 kDa. If “n” is the number of monomers: n = 2, 73.4 kDa; n = 3, 110.1 kDa; n = 4, 146.8 kDa; n = 5, 183.5 kDa; n = 6, 220.2 kDa; n = 7, 256.9 kDa; n = 8, 293.6 kDa; our immunoblotting analyses suggest that *Ani s 13* is present in solution in all forms, from monomer to octamer because of the wide range of bands from 37 kDa to more than 250 kDa which is more clearly observed under non-reducing conditions.

All allergens may be affected by a protease cleavage process. For example, the absence of allergenicity of vertebrate tropomyosin could be due to an enzymatic cleavage in smaller peptides than in the case of invertebrate tropomyosins. These shorter peptides could not fold correctly, and would not be recognised by IgE (Mikita and Padlan, 2007).

Finally, glycosylations are essential for the epitope mapping of a molecule because they modify the surface and consequently the epitopes. In addition, a limitation of DiscoTope 2.0 is that it does not consider glycosylation sites for predicting epitopes. In the case of *Ascaris* haemoglobin, two N-glycosylations were experimentally determined in the positions 19 (N-terminal residue of the mature protein) and 216; one in each globin domain (De Baere et al., 1992).

To summarise, as happens with other allergens of this parasite, anti-*A. simplex* s. l. haemoglobin immunoglobulin levels were higher in the GAA group compared with the CU+ group and therefore could improve the identification of *Anisakis* associated allergic disease phenotypes. Some of the structural differences between *Anisakis* and *Ascaris* haemoglobin molecules might be responsible for the absence of IgE cross-reactivity observed in this study. *Anisakis* haemoglobin is a major and specific allergen, being recognised by a large number of sensitised patients, and its use could improve tools for the early diagnosis of *Anisakis* allergy.

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