IgE sensitization to the fish parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in Italy: comparison of two immunological methods in the diagnosis of "*Anisakis*-allergy"

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SUMMARY

IgE sensitization to *Anisakis pegreffii* in Italian subjects suffering of gastro-allergic anisakiasis (GAA) with the larval detection (*N*= 5), or with history of chronic urticaria (CU+) after the fish consumption (*N*=100), was investigated. A group of 5 healthy individuals not suffering of urticaria or symptoms associated to *Anisakis* infection, nor previously infected with other parasites was also included as control group. Antibody response profiles were analyzed by western blot (WB) assay, by using both escretory/secretory products (ESP) obtained from *in vitro* culture of L3 stage of *A. pegreffii,* and larval crude extract (CE). The results obtained were compared with those achieved by the conventional immunological method for IgE-*Anisakis* allergy (i.e. ImmunoCAP: iCAP). Among the 110 subjects, 28 confirmed their IgE positivity, by both WB and iCAP methods, at the major ESP antigens/allergens. Specifically, among them, 13 resulted IgE positive to major antigens/allergens of the parasite, i.e. *Ani s 1* and *Ani s 7* and *Ani s 13;* while, 15 sera reacted only to *Ani s 7* and *Ani s 13*. Finally, 31 sera from Group 2, which resulted positive to iCAP (i.e.>0.35 kU_A/l) recognised, at the WB assay, *Anisakis* antigens/allergens *Ani s 3* (tropomyosin) and *Ani s 2* (paramyosin), obtained from *A. pegreffii* crude extract (CE). The last finding suggests that those sera resulted positive to iCAP might be a false positive reaction, due to the unspecific binding of IgE. iCAP and WB have shown a correspondence of 63.7 %: the specificity resulted of the 100% in the WB; whilst, it was around the 51.22% for the iCAP. IgE response in WB to *A. pegreffii* - *Ani s 1 plus Ani s 7* was 80% and 39.1% in GAA and CU+ patients, respectively. Whereas, WB response only to *Ani s 7* was found mainly associated to CU+ patients. IgE recognition to *A. pegreffii Ani s 13* was 100 % in GAA and in CU+ patients.

Key words: *Anisakis pegreffii*, IgE-sensitization, Italy, WB, antigens/allergens, gastro-allergic anisakiasis, chronic urticaria

INTRODUCTION

The parasites of the genus *Anisakis* are considered the most important biological hazards present in "seafood" products (1). *Anisakis* spp. larvae are indeed aetiological agents of human anisakiasis, a seafood-borne parasitic zoonosis (2). The human disease is the result of the accidental ingestion of third stage larvae of the parasite found in raw or undercooked infected seafood. Depending on the site of infection, anisakiasis can be divided into the following three types: gastric, intestinal, and ectopic anisakiasis (2, 3). Clinical symptoms, reported in acute infections, include epigastric pain, nausea and vomiting (2, 4). *Anisakis* larvae can invade the gastric and intestinal walls, causing direct tissue damage, ulcers, and, finally, eosinophilic granulomas, (2). Moreover, gastrointestinal anisakiasis may be accompanied by IgE-mediated allergic reactions (5), ranging from urticaria or angioedema to anaphylaxis (3). In this respect, Daschner et al. (6) described the gastro-allergic anisakiasis (GAA) as an acute allergic reaction with hypersensitivity symptoms appearing after the ingestion of raw and/undercooked fish also several hours after the intake of infected fish, associated to the larval penetration into the gastric mucosa (Daschner et al., 2002). In addition, the presence of IgE antibody response in individuals with no apparent symptoms was detected (7), after one month after the acute gastro-allergic anisakiasis episode (Daschner et al., 1999). It has been also observed that the allergic IgE-mediated response in the course of gastro-allergic anisakiasis due to *A. simplex (sensu lato)* involves a secondary immune Th2 type memory response and a primary immunological stimulation of Th2 and Th1 lymphocyte populations against unknowns antigens (Daschner et al., 2002).

Among the nine species of *Anisakis* so far genetically characterized (8), only two – *A. pegreffii* and *A. simplex* (*s. s.*) – have been found to cause infections in humans (9, 10, 11, 12). The species *A. pegreffii* is the most widespread zoonotic species affecting commercial fish from Italian waters

(13). It has also been reported to cause in humans gastric, intestinal and gastro-allergic anisakiasis (9, 10, 11).

To date, 14 antigens/allergens from *A. simplex (s. l.)* have been described (Allergen Nomenclature Sub-Committee, www.allergen.org). Among those, *Ani s 1* (24 kDa) and *Ani s 7* (139 kDa) are considered the most important major excretory/secretory (ESP) allergens described, as they were reported to be recognized by around 85% to 100% of infected patients, respectively. *Ani s 1* and *Ani s 7* have no homology with any other allergens that humans are known to be sensitized towards (14, 15); they are indeed also useful to diagnose *Anisakis* infections. In addition, more recently, Gonzalez-Fernandez et al. (18) described *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. Finally, *Ani s 14,* recognised by IgE in the 54% of sera from *Anisakis*-allergic patients, was recently described (Kobayashi et al., 2015). Other allergens, such as *Ani s 4* (cystatin, 9 kDa), *Ani s 6* (serine protease inhibitor, 7 kDa) and three SXP/RAL-2 family proteins, named *Ani s 5* (15 kDa), *Ani s 8* (15 kDa) and *Ani s 9* (14 kDa), are considered as minor ESP allergens recognized by less than 50% of *Anisakis*-allergic patients (16, 17). Finally, *Ani s 2* (paramyosin; 97 kDa) and *Ani s 3* (tropomyosin; 41 kDa), allergens detected in crude extract (CE) of *Anisakis* larvae, have been considered as less specific of "*Anisakis*-allergy" (16).

Anti-*Anisakis* IgE sensitization has been extensively reported in the last decades, mainly from Spain (Del Rey Moreno et al., 2006), Croatia (Mladineo et al., 2014), Morocco (Abattouy et al., 2012), and Italy (Mazzucco et.al, 2012; Guarnieri et al., 2011; Barbuzza et al., 2009). However, most of the epidemiological studies to assess anti-*Anisakis* IgE sensitization and *Anisakis*-allergy has been currently performed based on IgE-*Anisakis* reactivity detected by ImmunoCAP (iCAP) (19), (20), Mladineo et al., 2014; Abattouy et al., 2012). A high level of *Anisakis*-IgE hypersensitivity in Italian fishery products handlers, fishermen and fish consumers has been also reported, by using iCAP methodology, (21); however, in those Italian patients the larval detection

was not observed. Finally, *A. simplex (s.l.)* larvae used so far used in the diagnosis of *Anisakis*-allergy in Italy were not identified to the species level. This complicates the picture of the allergy due to the different biological species of *Anisakis* (Mattiucci & Nascetti, 2008).

The objectives of this study were to: *1)* study antibody response against both ESP and CE antigens of *A. pegreffii* investigated by the most conventional immunodiagnosis approach of *Anisakis*-allergy (i.e. immunoCAP), in comparison with the IgE immunoblotting (WB), in order to understand their significance in the diagnosis of gastro-allergic anisakiasis (GAA), and a state of IgE sensitization to the parasite allergens; *2)* detect specific IgE response to the major antigens/allergens of the species *A. pegreffii*, by immunoblotting analysis (WB) in Italian patients with gastro-allergic anisakiasis (GAA); *3)* analyse the presence of a specific IgE-sensitization to *A. pegreffii* in Italian patients suffering of chronic urticaria (CU+), without the detection of an acute parasitism.

MATERIALS AND METHODS

Serum samples

110 serum samples from patients of the "Umberto I" Teaching Hospital Rome (*N*= 59), Teaching Hospital in Pisa (*N*= 42) and "S. Spirito Hospital" in Pescara (*N*= 9), were analyzed.

They included sera from patients (*N*= 5) suffering acute allergic type I hypersensitivity symptoms such as urticaria, bronchospasm, in the 36 hours period after the ingestion of raw fish (marinated anchoives) in association with the presence of the parasite, *A. pegreffii* larva, found at the endoscopy, penetrating the stomach wall; the serum sample from four patient between 48h-72h after the parasite removal; while from one patient was taken within the 24 hours (day 1) of the onset of symptoms; the five sera they were designated as Group 1 (Table 1).

Other sera (*N*= 100) were taken from patients suffering of various allergy symptoms, such as chronic urticaria, following raw or cooked fish consumption, without the finding of larval parasite *Anisakis* in the stomach; the serum sample was taken within 7-15gg. after the occurrence of symptoms; this group of sera was designated as CU+ and here indicated as Group 2 (Table 1).

Finally, Group 3 included 5 sera samples from patients who have never suffered of urticaria after fish consumption; they were not fish consumers, never associated to *Anisakis* symptoms, nor previously infected with other parasites: they represented the negative controls.

Informed consent was obtained from all patients in the study.

Parasites

Anisakis sp. larvae were removed, manually, live, from the viscera and body cavities of fish hosts (anchovies, *Engraulis encrasicolus* and European hake, *Merluccius merluccius*) caught from the Adriatic Sea (S. Benedetto del Tronto). After their removal from the fish host, they were washed first in 2% acetic acid, then in phosphate-buffered saline (PBS). A batch of the collected larvae were transferred to the "*in vitro*" culture (see below), while another one used for the CE preparation. Each larva was cut in two parts: a part was identified to species level by genetic/molecular markers (diagnostic allozymes and sequences analysis of the mtDNA *cox2*) according to the procedures as described, in detail, elsewhere (23); while, the other one was stored at -20ºC until crude extraction (CE).

Preparation of *A. pegreffii* L3 crude extract (CE) and excretory-secretory products(ESP)

Crude extracts of L3 were prepared as following: each larval part was rinsed in physiological saline, then homogenised with PBS 10X in a mortar; the homogenate was then centrifuged at 14,000 rpm for 30 min at 4ºC; finally, the supernatant was retained.

To obtain ESP, live and active *A. pegreffii* L3, removed from the host, were washed in saline solution (0.9%) several times, then treated with 2% acetic acid at room temperature to inhibit bacterial contamination. They were then cultured *in vitro* in a sterile tissue culture dish. Five ml of PBS 1X containing penicillin (1%) and streptomycin (1%) was placed into each dish well; 60 larvae *per* well. The culture plates were then placed in a humid 37ºC incubator. Culture media were changed after 24h; the culture supernatant were collected at 96h, and stored at -20ºC until use. The larvae used for the *in vitro* culture were then removed and stored at -20ºC for their genetic identification to species level. To concentrate ESP products, each culture supernatant was mixed with 5ml 90% acetone, vortexed for one minute, and kept in ice for 15 min. It was centrifuged at 3500rpm for 10 min, and then the supernatant was discarded. After the removal of acetone by evaporation at room temperature, each pellet was dissolved in PBS, as previously described (Hwang et al., 2002).

Protein concentration from both the CE and ESP products was determined with Quick Start Bradford Protein Assay (Bio Rad), by using Bovine Serum Albumin as a standard control.

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IgE-*Anisakis* determination by iCAP

IgE against *Anisakis* sp. was first determined by the ImmunoCAP, ISAC diagnostic test (Phadia, Uppsala, Sweden). This procedure was carried out following the manufacturer's instructions. Generally, the iCAP method quantifies specific serum IgE antibodies over the range 0.01-100 kilounits of antibody per liter (kU_A/l); however, the IgE threshold level of positivity was fixed > 0.35 kU_A/l, in accordance with the manufacturer, and the relevant literature (7, 20, 22). Thus, for comparative purposes, the range 0.01-100 kilounits of antibody per liter has been converted into 6 "scores," according to the internal calibration system, as following: class 0: negative <0.35 kU_A/l; class 1: 0.35 to 0.7 kU_A/ l; class 2: 0.7 to 3.5 kU_A/l; class 3: 3.5 to 17.5 kU_A/l; class 4: 17.5 to 50 kU_A/l; class 5: 50 to 100 kU_A/l; and class 6: >100 kU_A/l.

SDS-PAGE and IgE immunoblotting (IgE-WB)

IgE immunoblotting (IgE-WB) was performed on the 110 serum samples *versus* allergens/antigens from CE and ESP. For antigen preparation, samples from ESP and CE were individually diluted 1:1 with Laemmli buffer and heated at 95ºC for 5 minutes to ensure protein denaturation.

Protein electrophoresis was carried out in Mini-PROTEAN 3 Cell (Bio-Rad) in gradient (4-20%), following the manufacturer's instructions. First, a 10% polyacrylamide separating-gel solution was prepared and placed between two glass plates. Distilled water was added on top of the polyacrylamide solution, until the gel was polymerized. Free water was removed and a 5% polyacrylamide stacking solution was posed on top of the polymerised separating gel. Finally, each sample was loaded into an individual lane on the polyacrylamide gel. In a separate lane, a protein marker (Precision Plus Protein All Blue Standards, Biorad) was loaded to help estimate the molecular weight of any proteins detected in the (ES and CE) samples. Gels were run at 100-120 V in an electrophoresis chamber using 1X electrophoresis running buffer until the blue marker (Laemmli buffer) reached the bottom of the gel. To make proteins available for antibody detection, they were transferred from the polyacrylamide gel onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). First, the membrane was equilibrated and two fiber pads and two Whatman filter papers were soaked in pre-cooled (4ºC) transfer buffer. The polyacrylamide gel was transferred onto a soaked Whatman filter paper, gently the nitrocelluose membrane was placed on top of the gel, making sure to remove all air bubbles. This was covered with the second Whatman filter paper and the sandwich was then placed between the pre-soaked fiber pads. The gel sandwich was put into a cassette which was then introduced into the Mini Trans-Blot Cell (Biorad) apparatus. Proteins were transferred for 45 minutes at a current of 80 V at 4ºC. To confirm uniform transfer of proteins, the nitrocellulose membrane was stained in a Ponceau solution for 30s. The membrane was washed in TBS-Tween (TTBS) buffer several times to remove Ponceau dye before proceeding

to immunostaining. After blocking with 6% solution containing non-fat dry milk in TBS-Tween buffer for 3 h, the membranes were incubated overnight at 4ºC with patient's serum (1:20 dilution). After three washes, the membranes were incubated with alkaline phosphatase-labelled monoclonal anti-human IgE (Sigma-Aldrich) at 1:2000 dilution. Finally, bands detection was carried out with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) for 20 minutes. The following controls were included in each assay: negative control (human serum with specific IgE class 0 [<0.35 kU_A/L]).

Statistical analysis

Descriptive results of iCAP were expressed as mean ± standard deviation (SD). Differences between IgE response by the two immunological methods (iCAP and WB) against antigens/allergens of *A. pegreffii* in gastro-allergic-anisakiasis (GAA) and in *A. pegreffii*-sensitization chronic urticaria (CU+) patients, were assessed by χ^2 test; statistical significance was fixed at the 0.05 level.

RESULTS

Molecular identification of *Anisakis* spp. larvae

All the *Anisakis* L3 used in the present study to antigen preparation, and those removed from the endoscopy, were assigned to the species *A. pegreffii*, on the basis of those alleles diagnostic between *Anisakis* spp., as indicated in Mattiucci et al. (8). In addition, the mtDNA *cox2* sequences obtained from the larval nematodes used for the antigen preparations, showed 99% homology with those sequences previously deposited by us for *A. pegreffii* (8).

Recognition of *Anisakis*-IgE sensitization by iCAP method

The IgE antibody reaction to *Anisakis* in the patient sera are reported in Table 1. According to the the IgE threshold level of positivity, defined by an antibody level of >0.35 kU_A/l (7, 20, 22), 42 sera among those tested, were negative at the iCAP. Whereas, the average level of *Anisakis*-IgE antibody detected in the positive sera (*N*= 68) was around 29.9 kU_A/l, suggesting the presence of a high level of sensitization to *Anisakis* allergens in the examined subjects. However, a significant difference ($p< 0.01$) in the average level of IgE response to *Anisakis* between the positive sera samples, was observed. Indeed, an average level of 15.46 kU_A/l was observed in Group 2. Interestingly, a very high value (on average, iCAP = 35.12 kU_A/l) was observed also in sera samples (*N*= 43) from the Group 2 of patients, who have recorded chronic urticaria after fish intake, even if any *Anisakis* larva was detected at the endoscopy (Table 1). On the other hand, a significantly higher level ($p< 0.01$) of IgE-*Anisakis* by iCAP (mean: 97.86 kU_A/l) was found in those patients from Group 1, having symptoms of gastro-allergic anisakiasis (GAA) in association with the *A. pegreffii* L3 detection at the endoscopy. Finally, a negative level (0.01 kU_A/l) was registered in sera from Group 3 of patients (negative controls) (Table 1).

IgE immunoblotting (IgE-WB)

IgE antibody reactivity of those sera samples resulted positive to the iCAP method was tested against ESP and CE of *A. pegreffii* in WB, in order to discriminate between specific IgE response in GAA and IgE-sensitized subjects (CU+) against *A. pegreffii* allergens, from a IgE cross-reactivity with allergens other than A. *pegreffii*.

Generally, all the positive sera at the iCAP, showed at the IgE-WB analysis a higher number of protein bands when they were blotted against both ESP and CE (Fig. 1A). However, in 4 sera from the Group 1, a specific IgE-antibody reaction to those bands at 24 kDa and 139 kDa , i.e. *Ani s 1* and *Ani s 7,* respectively, considered as major *Anisakis*-antigens/allergens, was observed (Fig. 1A and Table 2). In addition, some sera (*N*= 9) among Group 2, which showed a high level of iCAP,

exhibited a similar blotting profile to those observed in the sera from Group 1 (Fig. 1B and Table 2). However, in 14 sera among the Group 2 *plus* one serum from Group 1, the detection of IgE-antibody reaction only *versus* the *Ani s 7* was recorded (Table 2). Finally, four sera from Group 1 (GAA patients) and thirteen from Group 2 (CU+ patients), among those positive to *Ani s 1* and/or *Ani s 7*, showed reactivity also at the 37 kDa band, which corresponds to *Ani s 13* (haemoglobin) (Fig. 1A and B).

In contrast, 31 sera from the Group 2 samples that were found positive at the iCAP (i.e. values comprised between iCAP>0.7- 50 kU_A/l) showed IgE-antibody reaction *versus A. pegreffii* CE at 41 kDa and 97 kDa, corresponding to *Ani s 3* (tropomyosin) and *Ani s 2* (paramyosin), respectively (Fig. 1C, Table 2). Finally, the remaining negative cases at the iCAP (*N*= 46) included in the Group 2, *plus* the 5 negative controls, demonstrated no apparent reactivity bands to any of CE and ESP antigens from *A. pegreffii* (Fig. 1D).

WB *versus* iCAP diagnostic performance

Out of the sera samples analyzed in the present study, 61.82% resulted positive with iCAP. Among those, Group 1 (GAA) detected the three major allergens recognised in *Anisakis*, except in one case in which IgE-antibody reaction *versus Ani s 1* antigen was not detected (Table 2 and Fig. 1); the samples from Group 2 (13.3 %) recognised the antigens *Ani s 1*, *Ani s 7* and *Ani s 13* with WB; instead, 20.6% recognised only the *Ani s 7* and *Ani s 13*. The highest values of iCAP were observed only in those sera which had IgE response only to *Ani s 7,* or in combination with *Ani s 1* and *Ani s 13* (Table 2).Therefore, only the 25.5% of the sera samples, here tested, were positive, when using both WB and iCAP.

A statistically significant difference ($p< 0.001$) in the positivity to the WB method was found between the sera samples included in the classes of IgE-antibody levels 1 and 2 detected by the iCAP analysis (Table 2), *versus* those in the classes no. 3, 4, 5, and 6 of kU_A/l positivity (Table 2).

DISCUSSION

The present study represents the first detection of species-specific IgE-mediated sensitivity to *A. pegreffii*, zoonotic agent of gastric, intestinal and gastro-allergic human anisakiasis, in Italy (11). So far, several cases of *Anisakis*-IgE sensitivity in Italian patients have been generically reported (21; Guarneri et al., 2011); however, the specific antigens of *A. pegreffii* recognised by IgE-antibody responses, were not characterised in those studies. Whereas, in the present study, *Ani s1 , Ani s 7* and *Ani s 13* were detected, for the first time, in *A. pegreffii* ES products, and recognised as those antigens/allergens responsible for IgE-*A. pegreffii* sensitivity in the human sera here tested.

Ani s 1 and *Ani s 7* were found in *A. pegreffii* having the same molecular weight as those reported in *A. simplex* (s.l.). However, a comprehensive comparative molecular characterization of the major allergens (i.e. *Ani s 7* and *Ani s 1*) between the two zoonotic *Anisakis* species (*A. simplex* (s. s.) and *A. pegreffii*) has not yet been carried out. So far, only two variable sites (out of 163) in the *Ani s 1* amino acid sequence analysis, have been detected between *A. simplex* (s. s.) and *A. pegreffii* (24).

The present study has shown that the detection of specific IgE-antibody reaction to *A. pegreffii* is best achieved by WB, rather than by the iCAP method. In comparing the sensitivity and specificity of the WB and iCAP methods, a serum sample was considered to be truly positive when detected by WB at *Ani s 1*, *Ani s 7* and *Anis 13* antigens. This criterion was based on the following aspects: *(i) Ani s 7*, *Ani s 1,* and, probably, *Anis 13* are ES antigens released and recognized by the host immune system only in *Anisakis* infections (17); *(ii)* WB appears to be the more specific one of the two assays. WB carried out with ES products as antigens has revealed positivity only in 41.1% of the examined patients who had been deemed positive by iCAP. However, positivity by WB for specific IgE to *A. pegreffii Ani s 1* and *Ani s 7* antigens was recorded when iCAP returns a “high positivity”

level (>3.5 kU_A/l). Thus, our findings support the evidence that the iCAP value >3.5 kU_A/l as a threshold of positivity. It was suggested that, likely, the combined use of the *Ani s 1* and *Ani s 7* antigens increase the individual sensitivity of the iCAP assay, because *Ani s 1* and *Ani s 7* increase the number of epitopes available to bind to IgE antibodies in serum (20).

While there is concordance between the two methods when the iCAP value is lower than 0.35 kU_A/l, the discordance between the two methods when the iCAP values are higher than 0.35 kU_A/l (Table 3). This could be related to the fact that iCAP also recognises several nonspecific antigens/allergens, such as *Ani s 2* (paramyosin; 97 kDa) and/or *Ani s 3* (tropomyosin; 41 kDa). They correspond to somatic allergens detected in *Anisakis* (16); they are thought to be primarily responsible for cross-reactivity between *Anisakis* and other invertebrates, such as prawns and the house dust mite (22, 25, 26). On the other hand, it has been demonstrated that the tropomyosin of *A. simplex* (s. l.) shares with the same protein from other nematodes, such as *Ascaris lumbricoides,* and mites, at least 70-80% of the amino acid sequence, which includes the IgE binding epitopes (27). Both those antigens (i.e. tropomyosin and paramyosin) are abundant in the CE of *A. pegreffii* as shown in the present study (Fig. 1C). Analogously, a high sensitivity, but low specificity, was previously observed, by the iCAP assay, when employing *A. simplex* larvae CE as target antigens (20). That finding was related to cross-reactivity with other nematodes, and invertebrates (22, 28). According to WB results here presented, most of the iCAP positive results obtained in the present study were likely false positive due to cross-reactivity to those aspecific antigenic structures. Thus, if the iCAP should be the only method employed, a misinterpretation in the serodiagnosis of the zoonotic manifestation known as "*Anisakis*-allergy" has been resulted also in those patients here analysed. Further, serological methods using CE obtained from *A. pegreffii* larvae, as target antigens, are less specific due to the presence of cross-reacting allergens.

On the contrary, also in the present study, *Ani s 1* and *Ani s 7*, appear to be the major ESP antigens of *A. pegreffii*, having no homology with other antigens/allergens to which humans are

known to be sensitized (8, 14, 15). Since *Ani s 13* has been recognised also in the present study, by a large number of patients, and cross-reaction with other nematode hemoglobin has been not documented (18), this allergen could be also considered a potential candidate for the specific diagnosis of allergic anisakiasis. Thus, the three antigens/allergens are those so far reported to be currently the most specific and sensitive ones to be used in serodiagnostic tests for human anisakiasis (18, 29). In the sera samples recorded positive to *Anisakis* allergy in the present study, *Ani s 1* was responsible for the 46.4% of sensitivity, while it was 100% for *Ani s 7* and *Ani s 13*. Furthermore, we observed that the IgE recognition rate of *Ani s 1* and *Ani s 7* in GAA patients were, respectively, 80% and 100%, in accordance with previous studies (18, 30). However, one serum sample among those collected from GAA patients, did not react against *A. pegreffii* - *Ani s 1* antigen; that finding could be related to the fact that the serum sample was collected after 24 hours from the larval removal. Indeed, it has been reported that, in GAA patients, specific as well as total IgE and other specific immunoglobulin isotype levels, depend upon the time interval elapsed (TI) between the acute parasitic episode and the obtaining of the serum sample (Cuellàr et al., 2013).

Finally, in our study, in CU+ patients, WB response to *A. pegreffii* antigens/allergens (i.e. *Ani s 1, Ani s 7* and *Ani s 13)* was around the 39.0%.

It is notable that most *Anisakis* IgE sensitization has been reported from Mediterranean countries (7, 31, 32), Japan (33), or South Korea (34); while, it seems to be rare or absent in North-European countries, such as Norway, despite the large amount of fish consumed there (22). On the other hand, *Anisakis* sensitization has been associated (35) with high consumption levels of raw or uncooked fish and prior infection with live larvae has been implicated in sensitization to the parasite allergens (30). This differential IgE-*Anisakis* sensitization finding could be related to the contrast between the high consumption of raw, undercooked or marinated fish in the Mediterranean countries (i.e marinated anchoives, spanish bouquerones, etc.), as opposed to the lower levels generally consumed in the North-European countries.

However, another possible reason for the high *Anisakis*-sensitization found so far in the Mediterranean populations, may be explained by a possible human genetic susceptibility to *Anisakis* antigens/allergens. Indeed, a significant association between sensitization to *Anisakis* sp. and the alleles found at the human leucocyte antigen system (HLA, the name of the major histocompatibility complex) loci DRB1*1502-DQB1*0601, has been shown in some Spanish populations (36). Generally, human populations from Mediterranean countries have experienced a long history against geohelminths, such as for instance, *Ascaris lumbricoides*. As a consequence, a certain genetic variability in those HLA alleles related to IgE binding epitops against antigens of *A. lumbricoides* - thus protective *versus* those parasites - could have been promoted, by natural selection. However, among those, some IgE - binding proteins, such as, for instance, tropomyosin has been postulated to induce cross-reactive IgE antibody response also against *A. simplex sensu lato* (Arruda and Santos, 2005). It has also postulated that those human populations who have not experienced geohelminth infection could have lead to activation of allergic response against similar antigens (such as tropomyosin), with an exaggerated IgE-mediated immune response (Reddy and Fried, 2008). On the other hand, a number of epidemiological studies have suggested that the increase in the prevalence of allergic disorders that has occurred over the past few decades is attributable to the reduced microbial and parasitic burden during childhood, as a consequence of the westernized life-style (Romagnani, 2004). This hypothesis needs further investigation over a larger number of Mediterranean populations, including those from Italy.

CONCLUSION

Our findings about WB analysis *versus A. pegreffii* antigens, suggest that most of the sera deemed positive to iCAP might be false-positive due to the nonspecific binding of IgE. Our suggestion is to consider sera showing iCAP values lower than 0.35 kU_A/l, as truly negative; it means they are not requiring further analysis with WB. On the contrary, the positivity of sera with higher iCAP values

should be confirmed with a WB, or by ELISA method employing ESP antigens, rather than CE, especially in the presence of allergic signs and symptoms following consumption of fish. The results achieved in the present study also indicate that WB assay represents a standard method in the diagnosis of *Anisakis*-allergy, as previously suggested (20).

However, other investigation and methodological approach are required in the understanding of the "*Anisakis*-allergy" manifestation in humans.

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Legend to Figure

Figure 1 Western blot analysis with L3 excretory-secretory antigens (ESP) and crude extract (CE) of *Anisakis pegreffii* using sera from Group 1 (GAA patients) (A) showing bands at 24 kDa (*Ani s 1*), 37 kDa (*Ani s 13*) and 139 kDa (*Ani s 7*); from patients of Group 2 (B) showing bands at 24 kDa (*Ani s 1*), 37 kDa (*Ani s 13*) and 139 kDa (*Ani s 7*); and from sera of Group 2 (C) showing bands of 97 kDa (*Ani s 2*), 41 kDa (*Ani s 3*). Negative sera (D) showed no reaction *versus* allergens/antigens of both CE and ES of *A. pegreffii*. The molecular weight markers are indicated on the left.