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Use of a comprehensive diagnostic algorithm for Anisakis allergy in a high seroprevalence Mediterranean setting

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Summary

Background. *Diagnosis of anisakis allergy (AA) is based on the skin prick test (SPT) and specific IgE (sIgE) determination. Anyway, false positivity cases are due to cross reactivity with numerous allergens. The aim of the study was to evaluate the reliability of a comprehensive diagnostic algorithm for the AA.* **Methods.** *An observational study was conducted on a sample of consecutive subjects accessing the allergology outpatient ambulatories of two hospitals located in Western Sicily. All the recruited outpatients were tested by Skin Prick Test performed using anisakis extracts by ALK-Abelló (Madrid, Spain). Specific IgE dosage for anisakis extracts was then performed by using ImmunoCAP250 (Immunodiagnostics Uppsala, Sweden). Consequently, outpatients who tested positive to first line tests underwent sIgE testing for ascaris and tropomyosin. Lastly, outpatients positive to the first line were invited to be further tested by basophil activation test (BAT) by using Flow CAST kit and anisakis commercial extract (Bühlmann Laboratories AG, Schönenbuch, Switzerland), as confirmatory analysis.* **Results**. *One hundred and eleven outpatients with an anamnesis suggestive of sensitization to anisakis (AS) and 466 subjects with chronic urticaria (CU) were recruited in the study. Of these, 22 with AS and 41 with CU showed a sensitization to anisakis allergens. The diagnostic algorithm revealed that 8.8% of outpatients who tested positive to sIgE determination were affected by CU, while 82.5% of all the sIgE positivity was related to cross-reactivity. Overall, a genuine anisakis seroprevalence of 2.3% was documented. Within a sub-sample of 15 subjects with clinical symptoms related to AA, n. 8 showed a real positivity after BAT. A greater response to A. pegreffii allergens as compared to A. simplex was reported.* **Conclusions.** *Our preliminary findings support the high clinical specificity of BAT for AA diagnosis, suggesting implementing this method in a comprehensive diagnostic algorithm.*

Introduction

The increasing habits of eating uncooked, raw or marinated sea fish, made human exposure to *Anisakis* an issue of public health concern, so that European Food Safety Agency (EFSA, 2010) included this parasite among the most significant biological hazards in seafood (1). Particularly, the consumption of marinated or raw fish has been demonstrated to enhance the risk of sensitization to Anisakis (2). According to these widespread alimentary habits, Japan is one of the countries with the highest worldwide prevalence of Anisakiasis cases (2-3). A high Anisakis seroprevalence was reported in countries overlooking the Mediterranean Sea, such as Morocco, Spain, Croatia and Italy, where consumption of marinated or raw sea fish belongs to adept-rooted culinary tradition, especially in coastal communities (4-6). However, given the high prevalence of sea fish parasitized by *Anisakis* spp. in the Mediterranean Sea (7-9), the impact of Anisakiasis and Anisakis sensitization could be underestimated. More recently, transcriptomic studies on the characterization of Anisakidae species have identified 36 potential allergens for *A. simplex s.s.* and 29 for *A. pegreffii* (10), suggesting a possible improvement in the detection of allergenic response and in the definition of the epidemiology of Anisakis-related diseases.

Anisakiasis, in some case, is accompanied by mild or severe allergic symptoms (ranging from urticaria-angioedema to anaphylaxis), with no digestive manifestations, being in any case the gastroscopy the gold standard for its diagnosis (11). In sensitized subjects, allergic manifestations can occur even without infestation, as reported by several authors (3,11-13). Of interest, some *Anisakis* allergens have been found to be heat-stable so that cooking the fish could not prevent the allergenicity of the parasite (14-16).

To date, the diagnosis of *Anisakis* allergy has raised some criticism due to the low specificity and sensibility of the diagnostic methods based on the allergenic extract currently available. More in depth, diagnosis includes anamnesis (ingestion of fresh fish a few hours before the reaction) and in vivo skin prick test (SPT), confirmed with in vitro detection of specific IgE by serum immunoassay (2,18-19). Anyway, false positivity cases are frequent due to the cross reactivity with numerous pan allergens (2,18-21). *Anisakis* sensitization can occur by exposure to species-specific allergenic molecules such as Ani s1, Ani s4 and Ani s7, or to cross-reactive muscle proteins of other organisms such as tropomyosin and paramyosin, having a strong molecular and immunological cross-reactivity with other invertebrates, including crustaceans and dust mites (17-19,22-23). Further, cross-reactive molecules are the SXP/RAL family proteins, similar to the ones of other nematodes (18-21). Therefore, diagnosis of *Anisakis* allergy aims to discriminate between "genuine" sensitization and cross-reactivity with all of the mentioned molecules. Of interest, for SXP/RAL proteins IgE *Anisakis*: *Ascaris* (An: As)

ratio was considered a reliable tool to evaluate possible cross-reactions to other nematodes, whereas recent studies confirmed that an IgE An:As ratio ≥ 4.2 can increase the specificity of the test to 95% in subjects with specific *Ascaris*-IgE ≥ 0.35 (24). Moreover, despite the absence of clinical symptoms, healthy individuals may have high levels of specific IgE for *Anisakis* allergens and vice versa (1). Several studies indicated that 16 to 22% of blood donors had specific IgE for *Anisakis* (5,25). Another study reported that about 24% of subjects with acute urticaria showed a SPT positivity and/or specific IgE for *Anisakis*, although *Anisakis* was the real triggering cause only in 33% of cases (26). Consequently, there is a need to distinguish *Anisakis* allergy from sensitizations to other allergenic sources that are often incorrectly diagnosed. The gold standard for food allergy is the challenge with food allergens, but ethical reasons do not allow the performance of this test in case of *Anisakis* allergy suspect (27-30).

Basophil Activation Test (BAT) has been proposed as a reliable tool for *Anisakis* allergy, integrating standardized procedures (skin prick test and specific IgE dosage) both at diagnosis and follow-up, but evidences in support of that are scant (8,31-32). In Sicily, the largest island in the Mediterranean Sea, there is a high Anisakis seroprevalence of 15.4% which was recently reported in a sample of patients with mono-sensitization to the nematode (33).

An observational study was conducted on a sample of subjects accessing two allergology outpatient ambulatories sited in Palermo, Western Sicily, Italy, with the aims i) to assess validity and clinical specificity of a comprehensive diagnostic algorithm for *Anisakis* allergy, including SPT, IgE specific dosage for Anisakis extracts, as a first approach, followed by IgE specific for Ascaris tropomyosins and use of BAT, as confirmatory analysis, ii) to highlight any difference of sensitization between *A. pregreffii* and *A. simplex* s.s., species prevalent in fish in the Atlantic Ocean and in the Mediterranean Sea, respectively, and iii) to understand how this difference can affect the results of the diagnosis.

Materials and methods

Subjects in study

Consecutive subjects accessing the allergology outpatient ambulatories of Fatebenefratelli Buccheri la Ferla Hospital and of IBIM Research National Council of Palermo, both located in Palermo (Western Sicily, Italy), were recruited in this cross-sectional study between January 2016 and May 2017. Inclusion criteria were 1) an anamnesis suggestive of sensitization to Anisakis (AS) in individuals reporting acute clinical manifestation in the last month due to allergic reactions (asthma, rhinitis, conjunctivitis, urticaria/angioedema, abdominal pain, diarrhea, vomiting or anaphy-

laxis) after eating fresh fish or in subjects at high risk exposure to contact with sea products and abstaining from fish ingestion; 2) a > 6 weeks documented spontaneous urticaria, defined as chronic urticaria (CU), presenting with or without angioedema. To this end, a structured questionnaire was designed to collect the characteristics of the outpatients (age, sex, area of residency) together with anamnesis information, and then administered to all the subjects accessing the ambulatories during the study period. Exclusion criteria were a fish sensitization documented by diagnostic testing. Starting from the routinely diagnostic approach, the following comprehensive diagnostic algorithm (**figure 1**) was applied to the subjects included in the study according to anamnesis. As first line the outpatients were tested by SPT and IgE specific dosage for *Anisakis* extracts. Consequently, outpatients positive to first line tests underwent IgE specific testing for *Ascaris* and tropomyosins (second line) and were further checked for *Dermatophagoides pteronyssinus* (D1) IgE positivity.

Lastly, the outpatients who tested positive to the first line were invited to be further tested by BAT, as confirmatory experimental analysis. To this end, only a sub-sample of 13 outpatients answered to the call. Moreover, despite testing negative at the first line and also to a sardine prick-by-prick test, two more subjects, documenting a very suggestive clinical presentation for Anisakis allergy, were also tested by BAT. Overall, a sub-sample of 15 outpatients was tested by BAT.

Informed consent

All outpatients have read and signed an informed consent before the blood sampling and the questionnaire administration. The study was performed with the approval of the ethics committee of Policlinico Giaccone Hospital, Palermo, Italy (8/2018 - 10/09/2018) and was in agreement with the Helsinki Declaration.

Figure 1 - Flow chart of the comprehensive algorithm applied for the diagnosis of *Anisakis* allergy.
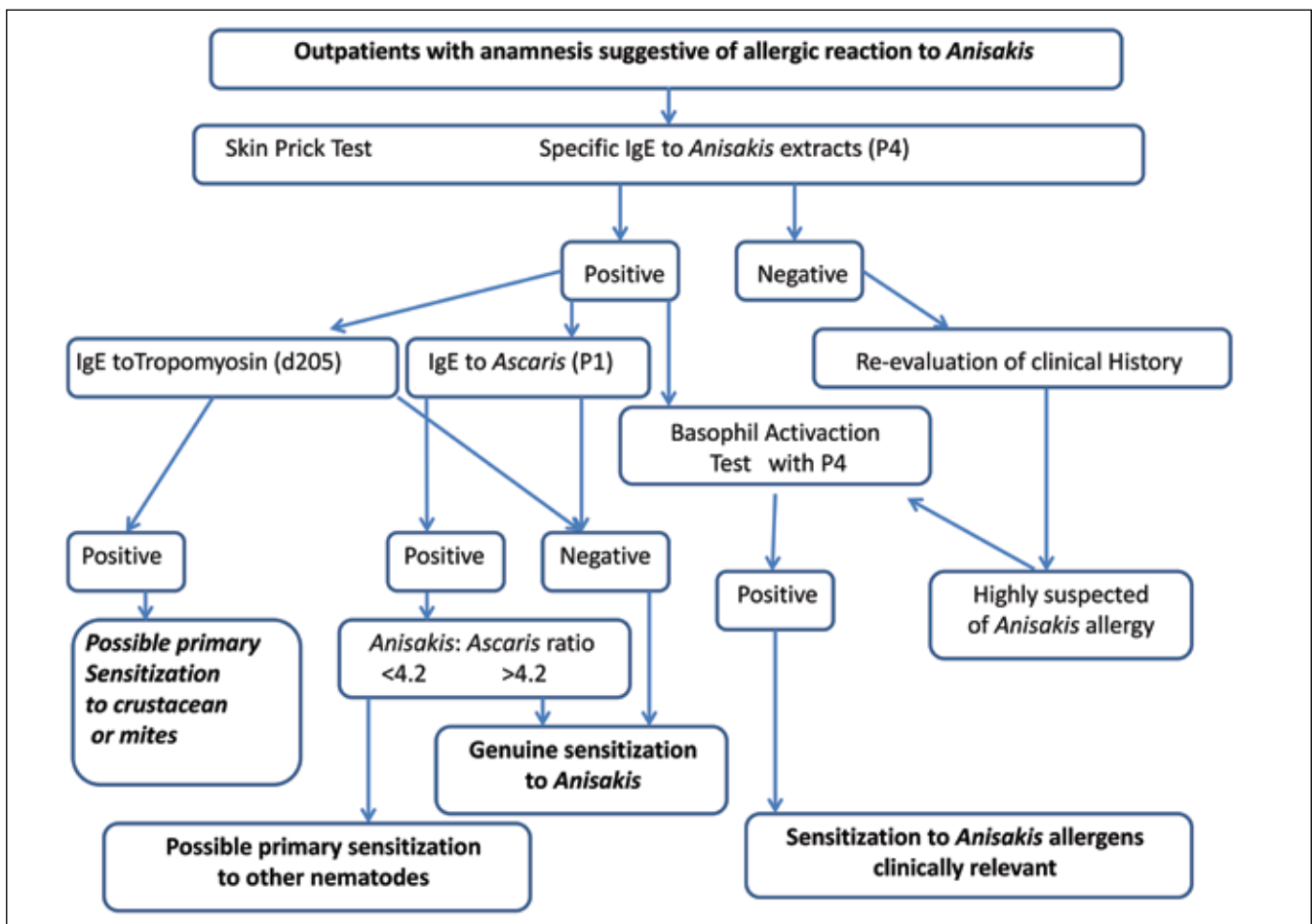
Anisakis protein extraction and species identification

Proteins' extraction from *A. pegreffii* and *A. simplex s.s.* was carried out both for SPT and BAT analysis using about 50 larvae per species collected after visual inspection from *Lepidopus caudatus* (high presence of *A. pegreffii*) and *Cluepea harengus* (high presence of *A. simplex s.s.*) fish samples (34). *Anisakis* larvae were stored overnight (o.n.) in test tubes with distilled water at -80°C and later subjected to tissues lysis. A mechanical lysis was conducted by glass potters with the addition of 1 ml of PBS (pH 7). Subsequently, the fragmented larvae were subjected to 3 sonication cycles of 30 seconds. The homogenized larvae were placed on a vertical rotor at +4 °C o.n., then centrifuged at 16,000 revolutions per minute (rpm) for 15 minutes for the supernatant collection, containing the crude extract. Protein concentration was assessed by Quibit 2.0 fluorimeter (Invitrogen, Carlsbad, California, USA). *Anisakis* larvae used for protein extraction were further analyzed for species identification by Polymerase chain reaction with Restriction Fragment Length Polymorphism (PCR-RFLP) method, according to the protocols reported in literature (10).

Current diagnostic approach

Skin Prick Test was performed using *Anisakis* extracts by ALK-Abellò (Madrid Spain). A positive result was defined by the presence of a wheal ≥ 3 mm in diameter. Specific IgE dosage (ImmunoCAP250, Immunodiagnostics Uppsala, Sweden) was then performed for *Anisakis* (p4), tropomyosin (Der p10-d205), *Ascaris* (p1) to reveal an *Anisakis* positivity, and for cod (f3), tuna (f40) and carp parvalbumin (Cyp c1-f355) allergens to diagnose a fish allergy. A specific IgE amount > 0.35 kIU/L was considered positive. In addition, a parasitological examination of the feces was carried out to verify any presence of nematodes.

Basophil activation test

Basophil activation test was performed according to the manufacturer's instructions, by using Flow CAST kit and *Anisakis* commercial extract (Bühlmann Laboratories AG, Schönenbuch, Switzerland), namely Bühlmann extract (B.e.). BAT homemade allergenic extracts were obtained from *Anisakis pegreffii* (A.p.e.) and *Anisakis simplex s.s.* (A.s.e.) as described above. For each type of allergen used in BAT, a dose response curve was implemented at the following concentrations: 112.5 ng/ml, 22.5 ng/ml, 4.5 ng/ml, 0.9 ng/ml. A threshold of 15% of activated basophils was considered positive.

ImmunoCAP inhibition assay

In order to assess the cross reactivity in the A.p.e. and A.s.e. extracts, a specific pool was derived from the sera of the 15 in-

dividuals tested by BAT and then used to perform CAP-inhibition at -20 °C, according to the method described by Savi et al (35). Two 100 mL aliquots per sera were incubated separately for 12 h at 4°C with 200 ml of A.p.e. and A.s.e. extracts at increasing dilutions (0 µg/ml; 25 µg/ml; 50 µg/ml; 100 µg/ml; 200 µg/ml). Then, sIgE against Anisakis were determined and the inhibition effect was computed using the following formula: % inhibition = 100 - (IgE inhibited sample (kU/l) × 100/IgE anti-*Anisakis* (kU/l) at zero concentration of larvae extracts).

Statistical analysis

Absolute and relative frequencies for qualitative variables and mean ± standard deviation (SD) for continuous variables were calculated as descriptive statistics of the subject's characteristics. Chi-square test was used to compare the two outpatients' groups (*Anisakis* allergy suspected versus chronic urticaria) for categorical variables, while t-test was performed to make comparisons for continuous variables.

In order to describe the distribution of percentages of BAT using B.e., in the sub-sample of 15 outpatients, a violin plot with box-wishers and individual values was drawn. To further compare in the 15 outpatients the two homemade allergenic extracts (A.p.e versus A.s.e.) by different concentration values, and their 95% confidence intervals, the non-parametric Kruskal-Wallis rank sum test was performed. Iteration between types of extracts and concentrations was tested as well. A significance level p-value < 0.05 was considered for statistical analysis. Descriptive statistical analysis, Chi-square and Student's t tests were performed by MedCalc® software. The non-parametric analysis of variance and the violin plot were performed using RStudio (version 1.1.383) [RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/] for the statistical software R (version 3.4.3) [R Core Team (2017)]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Results

In **table I** are summarized the characteristics of the 577 outpatients (n. 433, 75.0% females), mean age 37.6 (SD ± 20), recruited in the study. Of these, 111 (19.2%), mean age 36.4 (SD ± 15), documented an anamnesis suggestive of AS, while n. 466 (80.8%), mean age 38.2 (SD ± 20), were affected by a documented CU. There was no statistically significant difference between the two groups by age (p-value > 0.05) nor gender (p-value > 0.05). Outpatients with an anamnesis suggestive of AS were more frequently residents in coastal areas (26; 22.5%) as compared to ones affected by CU (43; 9.2%) (p-value 0.0002). A positive SPT was documented in 59 (10.2%)

Table I - *Characteristic of 577 outpatients enrolled in the study to investigate for Anisakis sensitization status.*

Characteristic	Total	Anamnesis suggestive for Anisakis sensitization	Chronic urticaria	p-value
n. (%)	577 (100%)	111 (19.2%)	466 (80.8%)	
age (mean ± SD)	37.6 (± 20)	36.4 (± 15)	38.2 (± 20)	> 0.05
female n. (%)	433 (75.0%)	84 (75.7%)	349 (74.9%)	> 0.05
male n. (%)	144 (25.0%)	27 (24.3%)	117 (25.1%)	
area of residence n. (%)				
urban	442 (76.6%)	74 (66.7%)	368 (78.9%)	
inland	66 (11.4%)	11 (9.9%)	55 (11.8%)	0.0002
coastal	69 (11.9%)	26 (22.5%)	43 (9.2%)	
skin prick test n. (%)				
positive	59 (10.2%)	20 (18.1%)	39 (8.4%)	0.01
negative	518 (89.8%)	91 (81.9%)	427(91.6%)	
Total	577 (100%)	111 (100%)	466 (100%)	
specific IgE n. (%)				
positive	63 (10.9%)	22 (19.8%)	41 (8.8%)	0.001
negative	514 (89.1%)	89 (90.2%)	425 (91.2%)	
Total	577 (100%)	111 (100%)	466 (100%)	

of the 577 recruited outpatients, while an IgE positivity was detected in 63 (10.9%) subjects of the study sample. SPT positivity resulted significantly higher in subjects with an anamnesis suggestive of AS (20; 18.1%) than in the ones affected by CU (39; 8.4%) (p-value 0.01). Along the same lines, an IgE positivity was more frequently documented in outpatients suspected to be sensitized to *Anisakis* (22; 19.8%) as compared to CU outpatients (41; 8.8%) (p-value 0.001). Furthermore, all of the SPT positive patients tested positive to *Anisakis* IgE, while 36 outpatients resulted positive to F40, F3 and F355.

In **Table 2** is reported the distribution of IgE specific positivity by tropomyosin, *Ascaris* and *Anisakis* only in outpatients with an anamnesis suggestive for *Anisakis* sensitization as compared to outpatients with a documented chronic urticaria. No statistically significant difference between the two groups was reported for tropomyosin IgE positivity (p-value 0.07) and *Ascaris* IgE positivity (p-value 0.123). Instead, a statistical difference was highlighted in the distribution of *Anisakis only* IgE positivity (p-value 0.012). Overall, a genuine *Anisakis* seroprevalence of 2.3% was documented in the study sample (**table II**).

In **table III** are shown the results of the application of the comprehensive diagnostic algorithm on the sub-sample of 15 outpatients, 9 from the group of subjects with an anamnesis suggestive of AS and 6 from the CU group. Within the first group,

7 subjects (A, B, C, D, G, H, I) were positive to the first line diagnosis, while of the two negative outpatients with an anamnesis highly suggestive of *Anisakis* allergy one (E) tested negative to SPT and IgE but tested positive to BAT only, and the other subject (F) tested negative to all the tests. *Ascaris* IgE was positive in 3 outpatients (A, D, G) and the IgE An:As ratio resulted higher than 4.2. None of the tested subjects from the CU group resulted positive to BAT. Within this group, *Ascaris* IgE were positive in 2 outpatients (N, Q), with one (N) showing an IgE An:As ratio equal to 9.96, while the other one (Q) documented an IgE An:As ratio of 0.63. Overall, 8 outpatients (A, B, C, D, E, G, H, I) tested positive to BAT.

In **Table IV** is reported the comparison of basophil activation test performed between B.e. (commercial extract) and A.s.e. and A.p.e. (homemade extracts) in the sub-sample. Four outpatients (A, B, G, I) tested positive to all the extracts, three (C, E, H) were reactive to B.e., while only one (D) documented a positivity to A.p.e. Moreover, three BAT positives subjects (G, H, I) didn't document any clinical sign or symptom after ingestion of sea products. Of the remaining individuals, seven outpatients tested negative to BAT, one (F) belonged to the AS group, while 6 subjects (M, N, O, P, Q, L), clinically negative to fish products allergy, were from the CU group: 3 simple chronic urticaria (M, N, Q), 2 chronic urticaria with associated atopic dermatitis (O,

Table II - *Distribution of IgE specific positivity by* **a)** *Tropomyosin,* **b)** *Ascaris and* **c)** *Anisakis in 111 outpatients with an anamnesis suggestive for Anisakis sensitization compared to 466 outpatients with a documented chronic urticaria.*

	Specific IgE	Total	Anamnesis suggestive for Anisakis sensitization n. (%)	Chronic urticaria n. (%)	p-value
a	positive for tropomyosin	32 (5.5%)	10[1] (9.0%)	22[2] (4.7%)	0.07
	negative for *Anisakis* and *Ascaris*	545 (94.5%)	101[3] (91.0%)	444[4] (95.3%)	
	Total	577 (100.0%)	111 (100.0%)	466 (100.0%)	
b	positive for *Ascaris*	18 (3.1%)	6 (5.4%)	12 (2.6%)	0.123
	negative for *Anisakis* and tropomyosin	559 (96.9%)	105 (94.6)	454 (97.4%)	
	Total	577 (100.0%)	111 (100.0%)	466 (100.0%)	
c	positive for *Anisakis* only	13 (2.3%)	6 (5.4%)	7 (1.5%)	0.012
	negative for *Ascaris* and tropomyosin	564 (97.7%)	105 (94.6%)	459 (98.5%)	
	Total	577 (100.0%)	111 (100.0%)	466 (100.0%)	

Dermatophagoides pteronyssinus (D1)
[1]D1 = n. 10/10 (100%); [2]D1 = n. 22/22 (100%); p-value > 0.05;
[3]D1 = n. 36/101 (35.6%); [4]D1 = n. 192/444 (43.2%); p-value 0.16;

Table III - *Application of the comprehensive diagnostic algorithm (experimental lab analysis) on the sub-sample of 15 outpatients.*

Subject	anamnesis	age	sex	SPT	IgE1 Anisakis kU/L	IgE1 tropomyosin kU/L	IgE1 Ascaris kU/L	Anisakis / Ascaris IgE ratio	BAT
A	urticaria angioedema	69	f	+	98.2	0.0	8.96	10.95	p
B	anaphylaxes 3° grade	52	f	+	6.87	0.0	0.0	-	p
C	angioedema	23	f	+	6.75	0.02	0.08	84.37	p
D	diarrhoea and urticaria	10	m	+	0.6	3.14	1.32	0.45	p
E	urticaria angioedema	68	m	-	0.0	0.0	0.0	-	p
F	urticaria angioedema	41	f	+	0.01	0.01	0.01	-	n
G	no symptoms	45	m	+	> 100	0.01	0.3	> 100	p
H	no symptoms	27	m	+	1.09	0.02	0.0	-	p
I	no symptoms	43	f	+	7.9	0.0	0.0	-	p
L	chronic urticaria with atopic dermatitis	55	f	+	0.8	-	0.1	8.0	n

Table III - *(continued)*

Subject	anamnesis	age	sex	SPT	IgE1 Anisakis kU/L	IgE1 tropomyosin kU/L	IgE1 Ascaris kU/L	Anisakis / Ascaris IgE ratio	BAT
M	chronic urticaria	74	f	+	0.76	0.0	0.04	19.0	n
N	chronic urticaria	64	f	+	54.5	0.0	5.47	9.96	n
O	chronic urticaria with atopic dermatitis	30	f	+	0.35	0.10	0.13	2.7	n
P	urticaria angioedema and idiopathic anaphylaxes	46	f	+	0.03	0.0	0.0	0.0	n
Q	chronic urticaria	33	f	+	0.35	0.0	0.55	0.63	n

[1]The diagnostic cut-off of the specific IgE is 0.35 kU/L.
SPT, skin prick test; BAT, basophil activation test; P, positive; N, negative.

Table IV - *Comparison of Basophil Activation Test results between Bühlmann extract and homemade extracts.*

Subject	anamnesis	BAT1 (%)								
		B.e. 22.5 ng/ml	A.p.e. 112.5 ng/ml	A.p.e. 22.5 ng/ml	A.p.e. 4.5 ng/ml	A.p.e. 0.9 ng/ml	A.s.e. 112.5 ng/ml	A.s.e. 22.5 ng/ml	A.s.e. 4.5 ng/ml	A.s.e. 0.9 ng/ml
A	urticaria angioedema	84.4	75.5	79.9	75.1	53.3	84.5	80.0	72.3	32.4
B	anaphylaxes 3° grade	70.1	83.6	75.4	58.6	20.8	43.6	28.0	3.5	0.0
C	angioedema	15.2	8.4	3.4	2.1	0.7	5.5	2.2	0.9	0.5
D	diarrhoea and urticaria	0.8	43.8	9.0	1.2	0.6	0.5	0.9	0.5	0.0
E	urticaria angioedema	39.5	0.8	0.6	0.4	0.0	2.2	1.3	0.5	5.7
F	urticaria angioedema	1.2	0.74	0.81	0.0	0.0	0.0	0.39	0.0	0.0
G	clinical symptoms without eating fish	55.2	84.1	75.1	61.0	16.2	44.5	10.0	1.1	0.7
H	clinical symptoms without eating fish	59.1	3.4	0.8	0.0	0.0	0.0	0.0	0.0	0.0
I	clinical symptoms without eating fish	48.8	26.7	54.3	33.7	12.2	36.1	7.0	0.7	1.2
L	chronic urticaria with atopic dermatitis	0.8	1.7	0.2	0.0	0.0	0.3	1.8	0.2	0.0
M	chronic urticaria	0.8	0.0	0.6	0.0	0.6	0.6	0.0	0.0	0.9
N	chronic urticaria	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
O	atopic	4.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P	urticaria angioedema and idiopathic anaphylaxes	4.7	2.8	0.0	0.0	0.0	8.8	3.9	0.0	0.0
Q	chronic urticaria	0.0	0.0	1.9	0.0	0.0	0.9	1.1	0.0	0.0

[1]The diagnostic cut off of the BAT is 15% of activated basophiles.
BAT, basophil activation test; B.e., Bühlmann extract; A.p.e., *Anisakis pegreffii* extracts; A.s.e., *Anisakis simplex* sensu stricto extracts.

L), and 1 with urticaria angioedema and idiopathic anaphylaxes (P). Furthermore, according to our findings, B.e. tends to act as a greater basophil activator compared to the homemade extracts at the concentration of 22.5 ng/ml.

The violin plot depicts the distribution of detected basophiles activation percentages, tested by Bühlmann extract, in the sub-sample of 15 outpatients (**figure 2**). The empirical kernel density estimate clearly shows the diversity in the distribution between positive and negative values. Furthermore, within the positive outpatients, except for one subject being slightly over the diagnostic cut-off of 15%, percentages of detected basophiles activation were consistently high.

Lastly, an homologous inhibition higher than 70% was determined by A.p.e and A.s.e at 50 µg/ml, 100 µg/ml and 200 µg/ml concentrations, but at 25 µg/ml the A.p.e. shows an higher inhibition than the A.s.e. (**figure 3**).

Discussion

We conducted a cross-sectional study with the aims to assess the reliability of a comprehensive diagnostic algorithm for *Anisakis* allergy, including BAT as confirmatory analysis, and to evaluate if the exposure to the different Mediterranean Sea (*A. pregreffii*) and Atlantic Ocean (*A. simplex* s.s.) species could affect the re-

sults of the test. For this purpose, a sample of consecutive subjects accessing two outpatient allergology ambulatories located in Western Sicily, one of a general hospital and another from a research center, was recruited in the study.

The proposed diagnostic algorithm is based on cost-effective tests, commercially available, including specific IgE to investigate any possible cross reaction plus an "in vitro" simulation of allergenic challenge by BAT. We haven't used the commercially available microarray ISAC (Immunodiagnostics Uppsala, Sweden), that contains Ani s1 (specific of *Anisakis* spp.) and Ani s3 (tropomyosin), because this assay is very expensive and no data on its diagnostic accuracy have been reported. By contrast, other tropomyosins, presenting a high analytical accuracy and about 70% of sequence homology, are available for Immuno-CAP platform at cheaper prices (17-18). Therefore, we cannot exclude a residual misdiagnosis. In particular, it was conceived to overcome three issues related to the diagnosis of *Anisakis* allergy: 1) the molecular allergenic expression of this nematode ranges from specific epitopes to several cross reactive proteins that causes a lack of specificity in routinely testing; 2) few and insufficient allergenic molecules are commercially available to perform a more accurate diagnosis: 3) the double blind placebo controlled food challenge (DPFCC), gold standard in food allergy diagnosis, is not applicable. Applying the commonly used

Figure 2 - *Distribution of detected basophils activation percentages, tested by Bühlmann extract, in the sub-sample of n. 15 outpatients.*
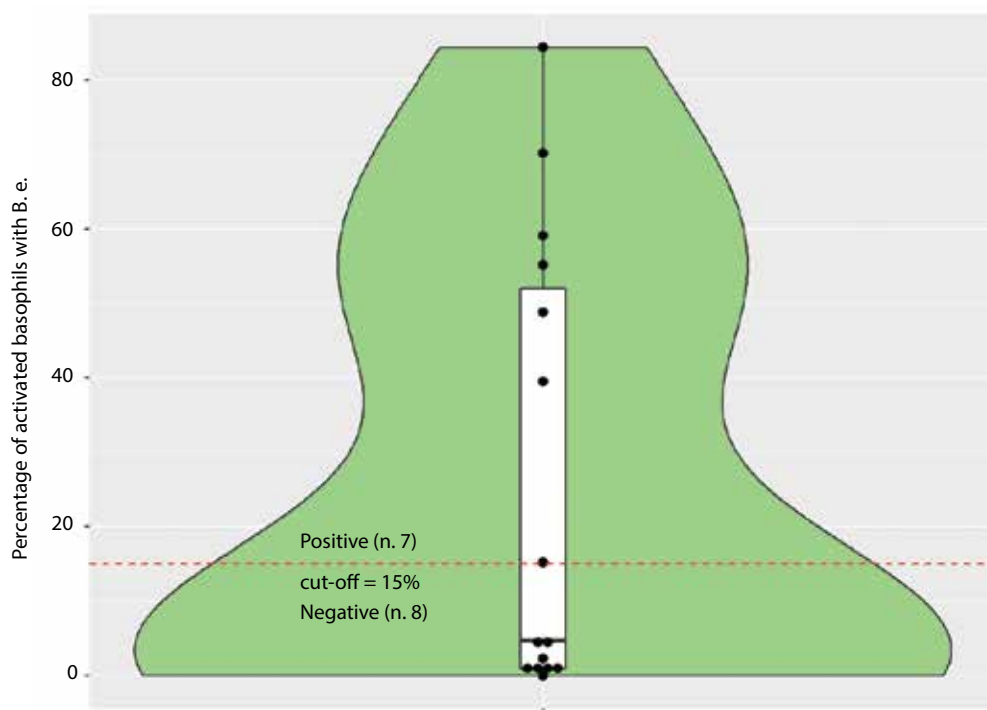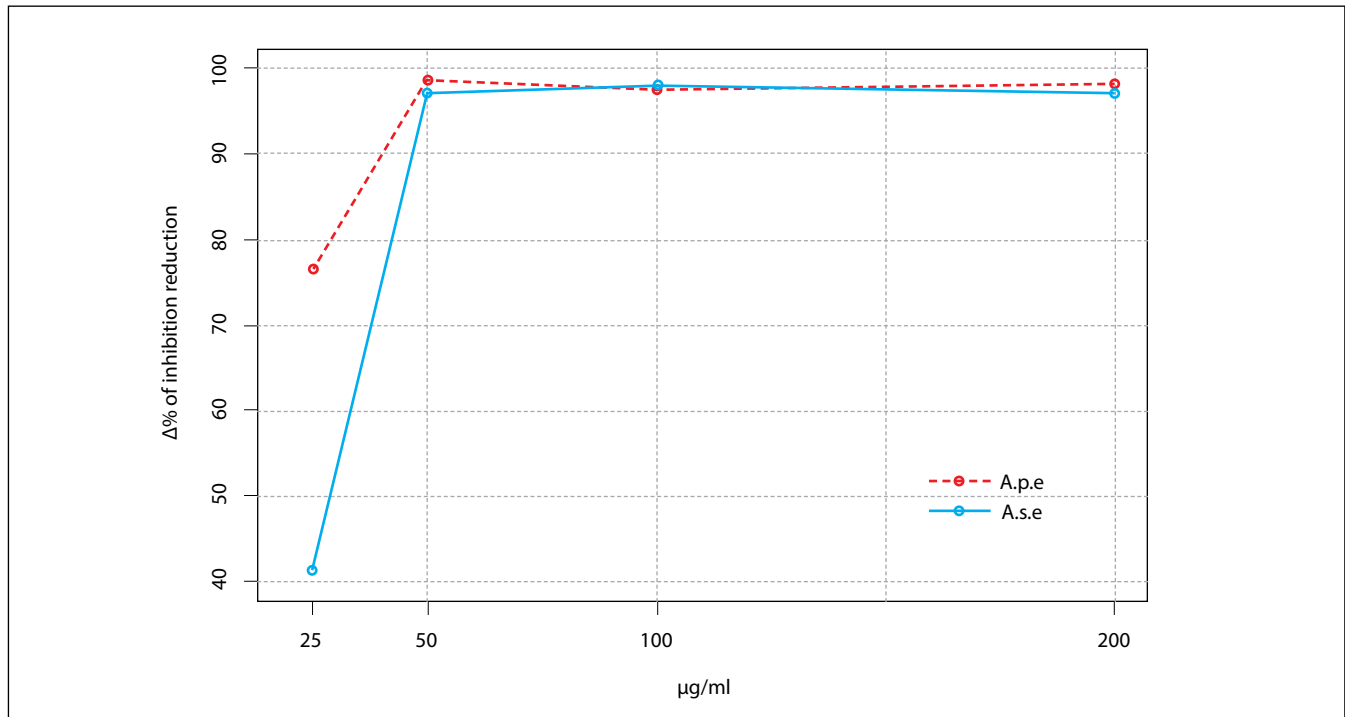
Figure 3 - *Cross-reactivity of Anisakis whole extracts. IgE reactivity to Anisakis ImmunoCAP (P4) competitively inhibited by pre-incubation of serum with various concentrations of A.p.e and A.s.e extracts.*

diagnostic approach, we documented an *Anisakis* sensitization prevalence of 10.9%, being slightly lower than seroprevalence reported in previous studies conducted in Spain and Italy, ranging from 12.7% to 15.4% (9,31,36). Of interest, studies conducted in Italy showed a higher prevalence of sensitization to *Anisakis* in coastal areas and in large cities, probably due to culinary traditions and imported food habits. This is particularly true in the island of Sicily, where the main cities overlook the sea and the gastronomic tradition includes an intake of raw fish products (marinated anchovies or salted sardines), considered to be a potential cause of sensitization to *Anisakis*. This evidence was confirmed by our findings as recruited outpatients with an anamnesis suggestive of AS were more frequently residents in coastal areas as compared to ones affected by CU. Another documented risk factor to be taken into account in these areas at very high vocation to sea fish industry and commercial distribution is the occupational exposure, particularly involving anglers, fishermen and fishmongers (37).
Recent studies have found a significant association between chronic urticaria and positivity to *Anisakis* diagnosed with currently used tests (31,38-40), suggesting the contribution of *Anisakis* hypersensitivity in individuals with CU, with a significant clinical improvement after a fish-free diet (31,38-41).

Our results showed a lower prevalence of *Anisakis* positivity in individuals with CU as compared to the mentioned studies, being these differences probably related to the eating habits of the populations in the study, as previously pointed out.
The prevalence of Anisakis sensitization obtained with the classic approach did not match with the real clinical prevalence, since 8.8% of those positive to specific IgE determination consisted of subjects with CU and 79.4% (n. 50/63) of all the IgE positivity were related to cross-reactivity events (positivity to tropomyosin or to *Ascaris* allergens). Therefore, an overall genuine positivity was found in 2.3% of all the outpatients recruited in our study, with a higher significative prevalence in AS group as compared to CU group. Moreover, we haven't found any statistically significant difference between AS and CU outpatients with regard to cross reactive molecular sensitization, giving consistence to the previous result.
Preliminary studies supported in vitro use of *Anisakis*-related allergens for BAT, arguing that this method may replace the challenge test in vivo (8,31-32) given its high specificity. Nevertheless, these studies provided data obtained with *Anisakis simplex* extract without specifying the different species (8,10,31-32). Another limitation of those studied was due to the experimental designs, involving healthy patients as controls, which could

represent a control group with a small statistical significance according to *Anisakis* seroprevalence in the general population. Our experimental analysis confirmed the high clinical specificity of BAT even in subjects with spontaneous CU not related to fish ingestion. More in depth, BAT confirmed the sensitization to *Anisakis* allergens in 8 subjects. Of these, 5 outpatients showed urticaria-angioedema or 3rd degree anaphylaxis after the ingestion of fish or cephalopods and 3 presented a clear medical history, even if they did not consume fish products. Of interest, one outpatient sensitized to tropomyosin showed positivity to A.p.e also but at the highest allergen concentrations (112.5 ng/ml), while another one, negative both to SPT and specific IgE, tested positive to BAT. This diagnostic aspect has already been described with regard to other food allergens (40-41). Furthermore, despite the fact that the BAT positive subjects showed a higher percentage of basophils activated with *A. pegreffii* allergens than with *A. simplex* s.s., the sensitivity of the test was not affected. On the other hand the ImmunoCAP inhibition test highlighted a lower blockage determined by A.s.e, suggesting that the *A. pregreffii* was the main source of the primary sensitization in the population studied. Lastly, we are not able to explain our finding documenting that B.e. tents to be a greater basophil activator as compared to the homemade extracts at some specific concentrations because we do not have any information about the *Anisakis* species and the molecular pattern of the preparation. At present, there are no clear guidelines on dietary restrictions for patients with *Anisakis* hypersensitivity. Several allergens of *Anisakis* are heath stable proteins (14-18), although some authors have reported a clinical improvement in patients sensitized to *Anisakis* after avoiding fish or consuming only frozen or well-cooked fish products during the follow-up (31,43). Therefore, the preliminary data provided by the present work could be useful for the development of clinical guidelines and to address future studies to provide more affordable evidences in support of public health strategies to be implemented in order to reduce the health risk related to *Anisakis* exposure (44).

In conclusion, our preliminary findings confirm the high specificity of BAT in the detection of *Anisakis* sensitization, supporting at the same time the opportunity to implement a comprehensive diagnostic algorithm for *Anisakis* allergy, including anamnesis, SPT and the determination of specific IgE for *Anisakis*, *Ascaris* and tropomyosin, as a first approach, followed by the use of BAT as confirmatory analysis. Moreover, the BAT should be performed in patients highly suspected of *Anisakis* allergy as well, despite testing negative to both SPT and specific IgE. Nevertheless, the not automatized execution and the relative high cost of this test suggest not to apply the BAT for every suspected *Anisakis* allergy case, then supporting the use of the proposed diagnostic comprehensive algorithm.

Last but not least, to the best of our knowledge, the findings of this preliminary study documented for the first time a difference in the prevalence of sensitivity in favor of *A. pegreffii* than *A. simplex* s.s. that could be related to a higher consumption of fish from the Mediterranean Sea, where this species of parasite is the most represented. This evidence should be taken into account when using the proposed diagnosis algorithm.

However, further studies on more consistent samples should be performed in order to confirm all the evidences provided and, particularly, to validate the proposed comprehensive diagnostic algorithm.

Conflict of interests

The authors declare that they have no conflict of interests.

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