edra | Issn 1764-1489 | Volume 51 N. 3/2020 – May 2020

# European Annals of Allergy and Clinical Immunology

THE OFFICIAL JOURNAL OF AAIITO | ASSOCIAZIONE ALLERGOLOGI IMMUNOLOGI ITALIANI TERRITORIALI E OSPEDALIERI
THE OFFICIAL JOURNAL OF SPAIC | SOCIEDADE PORTUGUESA DE ALERGOLOGIA E IMUNOLOGIA CLINICA

3/2020

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Ph. 0039 (0)2-88184.317
Italy subscription: 60 euro
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**Printing**

Rotomail Italia S.p.A., Strada Rivoltana (SP 14), 12/AB 20060 Vignate (MI), Italy

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Via G. Spadolini, 7
20141 Milano - Italy
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*"European Annals of Allergy and Clinical Immunology" registered at Tribunale di Milano - n. 336 on 22.10.2014*





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## *Review*



## *Original Articles*

A. Tonacci[1], E. Nettis[2], R. Asero[3], O. Rossi[4], C. Tontini[5], S. Gangemi[6]

# Omalizumab retreatment in patients with chronic spontaneous urticaria: a systematic review of published evidence

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**Key words**
*chronic urticaria; omalizumab; retreatment; therapy*

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**Doi**
10.23822/EurAnnACI.1764-1489.136

***Summary***
*A systematic review of the current literature on retreatment with omalizumab of patients with relapsing chronic spontaneous urticaria was performed. Published evidence shows that retreatment is safe and clinically effective, and that time to complete clinical response reduces as the number of retreatments increases.*

## Introduction

Chronic urticaria (CU) is a dermatological disease characterized by the rapid appearance of itchy hives, angioedema or both, lasting for 6 weeks or more (1). Approximately 0.5 -1% of the general population suffers from CU and over 60% of cases are classified as chronic spontaneous (previously termed idiopathic) urticaria (CSU), for which no obvious triggers can be identified (2,3). The average duration of CSU is generally up to 5 years, although more severe cases can last considerably longer (2,4). The EAACI/GA2/LEN/EDF/WAO urticaria guidelines suggest using daily non-sedating (second generation) H1-antihistamines as first-line treatment (1). As second-line therapy, an increase up to four times the licensed antihistamine dose may be beneficial, but around 45% of patients fail to respond (5). For these cases, omalizumab is recommended as add-on therapy, as third-line treatment option.

Omalizumab is a humanized monoclonal antibody recognizing the Fc portion of the immunoglobulin E (IgE) molecule. It is thought to reduce IgE- and FcεRI-mediated mast cell and basophil activation (6,7), with a similar outcome on both mast cells and basophils. Launched around 20 years ago to treat patients with severe asthma non responsive to standard treatment, it is currently used in several other allergic conditions including refractory CSU since 2013, displaying high efficacy and safety, especially when compared to first- and second-line therapies.

Since then, a number of studies have been published, especially in real-life settings, aimed at finding the best strategy to administer omalizumab to optimize the treatment outcome.
According to the review by Tonacci et al. (8), omalizumab 300 mg administered every 4 weeks appears to be the most effective and safe dose for the treatment of CSU, showing rapid response time. This approach displays minor adverse effects, and appears to be safe also when administered to pregnant women and their offspring. However, after the discontinuation of the drug, relapses may occur, with urticaria activity scores (UAS7) returning to pre-treatment levels in some cases, along with a poorer quality of life. In such cases, retreatment is advisable in order to increase patients' quality of life. Nevertheless, it is not clear which strategies in terms of dose and/or timing of omalizumab administration are the best to achieve good retreatment response. Within this framework, a literature review on studies about retreatment with omalizumab in CSU was carried out and results are presented and critically discussed in the present article.

## Materials and methods

We performed a literature search in PubMed until January 2020 by using logical combinations of the following terms: "urticaria, chronic", "urticaria, idiopathic", "urticaria, chronic spontaneous", "omalizumab", "anti-IgE" and "retreatment". We included reports of original data, including double-blind placebo-controlled, randomized controlled trials (DBPC-RCT), RCTs, open controlled trials, observational studies, and retrospective trials. We excluded: (1) case reports, systematic reviews, review articles, meta-analyses, as well as papers not published in English language.

## Results

According to the inclusion and exclusion criteria mentioned above, a handful of studies were retrieved. Overall literature search results are displayed in **table I**.
The works retrieved included a relatively low number of patients, mostly women, and were all concordant in stating that the first subcutaneous dose of omalizumab should be 300 mg administered every 4 weeks, with the exception of Metz et al. (9), where the initial dose and administration interval differed according to the dose and interval used to obtain a remission during the first treatment course.
Where information on dosage was available, retreatment was carried out with the same dose of omalizumab administered during the previous round. Overall, retreatment with omalizumab was safe and effective in nearly all cases, with minor side effects reported, including mild, transient local immediate skin reactions (15). Furthermore, the time to achieve complete remission decreased with subsequent treatment cycles. As observed by Nettis et al. (11), patients showed an average complete remission after 4.9 weeks during the first treatment course, dropping to 3.8 and 1.8 on the second and third retreatment courses, respectively, hence demonstrating an increased rapidity of response to treatment after multiple cycles.
Another important strategy to consider is how to choose the best timing for retreatment. From our review it is difficult to draw definite conclusions, since all the studies adopted different timing protocols and all of them obtained satisfying results. However, both the studies by Metz et al. (9), where the timing was adapted according to the occurrence of relapses, and Nettis et al. (11), where omalizumab was administered at least 8 weeks after the end of the previous course, achieved optimal results.

## Conclusions

The present review confirms the optimal efficacy and safety of omalizumab to treat refractory CSU in most cases. Overall, retreatment seems to provide the best results in terms of efficacy using the same dose as in the first cycle, usually 300 mg injected subcutaneously every 4 weeks. In any case, the interval between two subsequent treatment courses should be controlled and not exceed 8 weeks, to avoid delayed efficacy.
Future studies should address retreatment efficacy on larger samples, also trying to reduce the current gender bias by including more male subjects with CSU treated with omalizumab.

***Table I*** *- Studies retrieved in the literature search.*

| Study | No. of patients | Treatment | Retreatment | Results |
|---|---|---|---|---|
| Metz et al., 2014 (9) | 25 patients with CSU and/or CIndU (aged 18 - 74 years; 18 women) | 150 to 600 mg/month subcutaneously in 2- to 4-week intervals | Retreatment initiated after the recurrence of symptoms. All patients received the same dose of omalizumab in the same interval as the last successful treatment before discontinuation | Rapid and complete response after the first injection within the first 4 weeks of retreatment for all patients |
| Mandel et al., 2018 (10) | 18 patients with refractory CSU (aged 25 - 74 years; 14 women) | 300 mg every 4 weeks for 6 months | 3 patients retreated with the same treatment as the initial one | Complete response (UAS7 = 0) within 16 weeks; good response (UAS7 ≤ 6) within one week |
| Nettis et al., 2018 (11) | 31 patients with refractory CSU (mean age: 48.1 ± 13.4; 22 women) | 300 mg every 4 weeks for 24 weeks subcutaneously (first treatment course) | Retreatment (second and third treatment course) at least 8 weeks after the end of the previous course | First course: complete response for all patients, relapse within 5 - 20 weeks.<br>Second course: complete response in 93.5% of patients. Symptoms remission within 5 - 16 weeks after their last injection.<br>Third course: complete remission in 93.8%. ≥ 8 weeks after the administration of the last dose, 68.7% had relapse of CSU. Complete therapeutic response in 4.9 weeks (first course), 3.8 weeks (second course), 1.8 weeks (third course) |
| Nettis et al., 2018 (12) | 24 patients with refractory CSU (mean age: 48.0 ± 13.7; 14 women) | 300 mg every 4 weeks for 24 weeks (first treatment course) | 300 mg every 4 weeks for 24 weeks (second course) after 8-16-week follow-up | First course: good efficacy; relapse within 9-19 weeks. Similar efficacy during re-treatment, with slightly lower efficacy compared to the first course |
| Türk et al., 2018 (13) | 25 patients with CSU (age: 31-49; 18 women) | 300 mg/4 weeks for at least 3 months | In all patients with complete or partial response: discontinuation of omalizumab after 6 months; retreatment at the same initial dose if the recurred disease couldn't be controlled with concomitant medications | 58% had complete response at the end of treatment. At the 3rd month, 32% had complete response.<br>Eleven patients experienced relapse, omalizumab was restarted in 10 of them. After the re-initiation of omalizumab, 5 had complete response and 5 had partial response. Seven patients achieved remission after discontinuation. Time from the last omalizumab dose was 8 weeks / 18 months |
| Matucci et al., 2019 (14) | 30 patients with CSU (age: 20 - 70; 22 women) | 300 mg/4-week intervals for 6 administrations | Retreatment with the same protocol in relapsing patients; in case of a second relapse, a third treatment was performed | Cycle 1: after 6 months, 83.4% were responders, 13.3% partial responders, 3.3% did not respond. Time to achieve a partial or complete response: 5.8 ± 1 weeks. 79.1% relapsed within 12.5 ± 4.0 weeks.<br>Cycle 2: 14/15 improved their symptoms (57.1% complete remission). Mean response time: 5.0 ± 1.3 vs 6.1 ± 1.4 weeks (first cycle). 53.8% relapsed.<br>Cycle 3: 7/7 had complete remission |
| Vollono et al., 2019 (15) | 32 patients with CSU (age: 27-72; 22 women) | Subcutaneous 300 mg/ 4 weeks as add-on to H1-antihistamines for 6 months | 300 mg every 4 weeks for 5 months in case of recurrence in an 8-week treatment interruption | 13 patients completed 2 cycles of treatment, 10 patients had completed 1 cycle of treatment, 8 patients had undergone 1/2 cycle of treatment.<br>Mild, transient local skin immediate reactions observed in one patient.<br>20 patients added second-generation H1-antihistamines due to persistence of pruritus and wheals after 2-4 weeks of treatment with omalizumab monotherapy. |

**Conflict of interests**

The authors declare that they have no conflict of interests.

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M. Maleki[1], V. Mortezaee[1], M. Hassanzad[2], S. A. Mahdaviani[2], M. Poorabdollah[2], P. Mehrian[2], N. Behnampour[3], M. S. Mirenayat[4], M. Abastabar[1,5], M. Tavakoli[1], M. T. Hedayati[1,5]

# Prevalence of allergic bronchopulmonary aspergillosis in cystic fibrosis patients using two different diagnostic criteria

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**Key words**

*cystic fibrosis (CF); allergic bronchopulmonary aspergillosis (ABPA); diagnostic criteria; aspergillus sensitization; prevalence*

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**Doi**
10.23822/EurAnnACI.1764-1489.121

***Summary***

**Objective.** *There are different diagnostic criteria for the diagnosis of Allergic bronchopulmonary aspergillosis (ABPA) in CF patients. In this present study we evaluated the prevalence of ABPA in Iranian CF patients by two more usual diagnostic criteria as ISHAM working criteria (A) and CF Foundation Consensus Conference criteria (B).* **Methods.** *Eighty-six CF patients were included in the study. All CF patients underwent for Aspergillus skin prick test (AST), Aspergillus-specific IgE (sIgEAf) and Aspergillus-specific IgG (sIgGAf), total IgE. The ABPA prevalence was estimated by two diagnostic criteria, (A) and (B) and compared.* **Results.** *The frequency of positive AST, total IgE, sIgEAf and sIgGAf were 47 (54.6%), 9 (10.5%), 42 (48.8%) and 67 (77.9%), respectively. The obtained rate of ABPA prevalence (10.5%) was identical in two diagnostic criteria A and B (kappa value of 1.000).* **Conclusions.** *The applied diagnostic criteria had no significant effect on the reported rate of ABPA prevalence.*

## Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder resulting from mutations in the CF transmembrane conductance regulator (CFTR) protein (1). CF patients are susceptible to colonization by the various types of microorganisms including fungi due to abnormality in lung function such as defect in airway clearance (2). Pulmonary colonization by fungi, especially *Aspergillus* species, may lead the CF patients toward a different type of disease ranged from allergic reactions to life threatening invasive infections. Allergic bronchopulmonary aspergillosis (ABPA) is an immunological disorder caused by a hypersensitivity reaction to *Aspergillus* species allergens especially *A. fumigatus*. ABPA is a frequent event in patients with asthma and CF (3). The estimated ABPA prevalence in patients with CF was reported to be from 3% to 25% in adult patients, and 8% to 10% in children, with an overall prevalence of 8.9% (4,5).

The diagnosis of ABPA in CF patients is complex and remains problematic, because there is an overlapping between ABPA and CF in aspects of clinical symptoms, radiological, serological and microbiological features (4-7). In the diagnosis of ABPA, the evaluation of several different parameters including immediate skin test reaction to *Aspergillus* allergens, raised serum specific IgE against *Aspergillus fumigatus* (sIgE*Af*) and serum specific IgG against *Aspergillus fumigatus* (sIgG*Af*), elevated total IgE values, central bronchiectasis, infiltration in chest radiologic findings, raised peripheral eosinophil count, positive serum precipitins and sputum positive for *Aspergillus* culture have been considered. Since there is no consensus on the number of parameters needed for the ABPA diagnosis in patients with CF (8), several different diagnostic criteria were proposed which may have led to different reporting of ABPA prevalence (9). On the other hand, the occurrence of ABPA in patients with CF leads to impairment in pulmonary function and an undesirable pulmonary image, therefore, rapid diagnosis of ABPA and timely treatment is essential (10-12). In this present study we evaluated the ABPA prevalence in Iranian patients with CF using two different diagnostic criteria.

## Material and methods

*Ethics statement*

The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (code: IR.MAZUMS.REC.95.2354), and a written informed consent was completed by all patients or next of kin.

*Subjects*

Eighty-six CF patients from different parts of Iran, admitted to the referral respiratory diseases center, Masih Daneshvari hospital (Tehran, Iran), from January 2017 to February 2018 were enrolled in this study. The diagnosis of CF was confirmed by Computed Tomography scan (CT-scan), spirometry and clinical parameters including sweat chloride test and genotyping (the analysis of CFTR gene). The inclusion criteria were subjects with confirmed cystic fibrosis, without previous ABPA diagnosis. Patients with pregnancy, tuberculosis, asthma, chronic pulmonary obstructive disease were excluded from the study. All included patients were not using antifungal in time of the study. History of clinical details and demographic characteristics were evaluated in all CF patients.

*Diagnostic criteria*

Two ABPA diagnostic criteria (ISHAM working group criteria (A) (13) and The Cystic Fibrosis Foundation (CFF) Consensus Conference criteria (B) (14) were applied in this study. **Table I** shows the detailed criteria. The diagnosis of ABPA was evaluated by a team work consisting of pulmonologist, allergist-immunologist, radiologist and medical mycologist. In final the results

***Table I*** *- Criteria for diagnosis of allergic bronchopulmonary aspergillosis in cystic fibrosis patients.*

| Criteria | Evaluation parameters | |
|---|---|---|
| ISHAM working group (A) (13) | predisposing conditions | asthma, cystic fibrosis |
| | essential criteria (both must be met) | positive serum specific IgE (> 0.35 kUA/L) or immediate skin test |
| | | serum total IgE > 1000 IU/mL |
| | additional criteria (at least 2 of 3) | presence of serum specific IgG |
| | | thoracic imaging findings consistent with ABPA |
| | | peripheral blood eosinophil count > 500 cells/mL (may be historical) |
| Cystic Fibrosis Foundation Consensus Conference (B) (14) | cystic fibrosis with acute or subacute clinical deterioration | |
| | serum total IgE concentration > 1000 IU/mL unless the patient is receiving systemic corticosteroids | |
| | positive serum specific IgE (> 0.35 kUA/L) or immediate skin test | |
| | precipitating antibodies to *A. fumigatus* or serum IgG antibody to *A. fumigatus* by an in vitro test | |
| | new or recent infiltrates (or mucus plugging) on chest radiography or computed tomography that do not respond to antibiotics and standard physiotherapy | |

were compared for the differences in the prevalence of ABPA in these two diagnostic criteria.

*Pulmonary function test*

Spirometry test (Easy One NDD spirometer, Swiss) was performed for all patients and we obtained two important measurements, forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) from this test according to manufacture instructions. Values of FEV1 and FVC were recorded for study population.

*Aspergillus fumigatus skin prick test*

All subjects underwent *Aspergillus* skin prick test (SPT) with commercial *Aspergillus* allergens (Alk-Abelló, Lincoln Diagnostics, Dallas, Tx, USA) in the forearm as well as histamine 0.1 w/v served as positive control, and normal saline 0.9 w/v served as negative control. The appearance of a wheal 3 mm larger than negative control 15 minutes after exposure was considered as immediate-type hypersensitivity and positive SPT reaction.

*Aspergillus specific IgE*

We screened serum sIgE*Af* for all CF patients by using immunoCap method (an automatic immunoassay system, Phadia, Belgium) (15) according to the manufacturer's instructions. Serum sIgE*Af* level greater than 0.35 KU/L was considered as positive result.

*Aspergillus specific IgG*

Serum sIgG*Af* were measured by using a commercially available ELISA kit (IBL ELISA Kit, Hamburg, Germany) according to the manufacturer's instructions. sIgG*Af* value > 12 U/ml was considered as positive result.

*Serum total IgE measurement*

The serum total IgE (stIgE) levels was measured by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Genesis, Omega Diagnostic Group, UK) according to manufacturer's guidelines. Concentrations values were reported as IU/ml and values greater than 1000 IU/ml were considered as positive result.

*Peripheral blood eosinophil count*

The total count of white blood cells and the value of eosinophil in percentage were determined by an auto-analyser (Sysmex XT-1800i, USA) and was then recorded for each patient. Eosinophil count > 500 cells/µl was considered as positive result.

*High Resolution Computed Tomography (HRCT) and/or chest X-Ray*

All enrolled CF patients were screened by HRCT and/or chest X-Ray for evidence of bronchiectasis, centrilobular nodules/ mucoid impaction/ hyper-dense mucus. A radiologist reviewed the CT scans without knowledge of study population's clinical and paraclinical data.

*Sputum processing*

Sputum samples (once per patient) were collected from all patients who were enrolled in the present study. Each collected sputum sample was mixed in equal volume of pancreatin 0.5% for homogenization. After centrifugation, the sediment was then divided into two parts; one for fungal culture (Malt extract agar [MEA], QUELAB, Canada) and the other for direct microscopic examination mounted with 20% potassium hydroxide (KOH). All grown mold colonies were then identified at species level by molecular methods.
For molecular identification, genomic DNAs were extracted from all mold isolates, then universal primers including ITS1 and ITS4 as well as Bt2a and Bt2b (16) were used for identification of studied fungi to the species level. Herein, PCR reactions were prepared (17) and then the PCR products were analyzed by gel electrophoresis and visualized by UV illumination after safe staining. The amplified products of the ITS and β-tubulin fragments of isolated molds were conducted to automated DNA purification platform, and the purified amplicons were then sequenced. The resulting sequences were compared to the sequences deposited in the GenBank database and identified with 99-100% similarity to the corresponding ITS and β-tubulin sequences.

*Statistical analyses*

Data analyses were performed using descriptive statistics (mean ± standard deviation, frequency) by SPSS version 18 (SPSS Inc., Chicago, IL, USA). Kappa weighted test was used to find concordance between criteria A and criteria B and p-value < 0.05 was set as statistical significance.

**Results**

Out of 86 included patients, 42 (48.9%) were females and mean ± SD (range) of age was 16.14 ± 7.21 (0.7 - 34.0) years. The demographic, clinical and paraclinical data of study population (ABPA and non-ABPA) are presented in **table II**. *Aspergillus* SPT and sIgE*Af* were positive in 47 (54.7%) and 42 (48.8%) of CF patients, respectively. The overall prevalence of *Aspergillus* sensitization (positive result in *Aspergillus* SPT or sIgE*Af*) was 51 (59.3%) in study population. The mean ± SD (range)

***Table II*** *- Clinical, paraclinical and demographic data of cystic fibrosis patients with allergic bronchopulmonary aspergillosis.*

| | | non-ABPA (n = 77) | ABPA (n = 9) | total (n = 86) |
|---|---|---|---|---|
| age in year | mean ± sd (range) | 15.9 ± 7.1 (0.6-32.0) | 17.8 ± 8.4 (5.0-34.0) | 16.1 ± 7.2 (0.6-34.0) |
| gender n (%) | females | 39 (50.6) | 3 (33.3) | 42 (48.8) |
| | males | 38 (49.3) | 6 (66.7) | 44 (51.2) |
| pred-FEV1 (%) (mean ± SD) | | 47.9 ± 24.7 | 54.9 ± 22.3 | 48.6 ± 24.4 |
| pred-FVC (%) (mean ± SD) | | 49.5 ± 22.6 | 56.2 ± 19.8 | 50.2 ± 22.3 |
| history of CF in family n (%) | | 25 (32.5) | 5 (55.5) | 30 (34.9) |
| duration of CF diagnosis n (%) | | 13.6 ± 7.1 | 15.2 ± 9.7 | 13.8 ± 7.4 |
| family history of respiratory disease n (%) | | 28 (36.4) | 2 (22.2) | 30 (34.9) |
| seasonal allergies n (%) | | 3 (3.9) | 1 (11.1) | 4 (4.6) |
| nasal polyps n (%) | | 20 (26.0) | 2 (22.2) | 22 (25.6) |
| cough n (%) | | 75 (97.4) | 9 (100.0) | 84 (97.7) |
| shortness of breath n (%) | | 58 (75.3) | 8 (88.9) | 66 (76.7) |
| hospitalisation n (%) | | 70 (91.0) | 9 (100.0) | 79 (91.9) |
| previous exposure to inhaled antibiotics n (%) | | 54 (70.1) | 9 (100.0) | 63 (73.2) |
| previous use of systemic antibiotics n (%) | | 73 (94.8) | 9 (100.0) | 82 (95.3) |
| previous exposure to inhaled corticosteroids n (%) | | 70 (91.0) | 9 (100.0) | 79 (91.9) |
| previous use of oral corticosteroids n (%) | | 9 (11.7) | 7 (77.8) | 16 (18.6) |
| previous use of oral antifungal n (%) | | 9 (11.7) | 8 (88.9) | 17 (19.8) |
| haemoptysis n (%) | | 13 (16.9) | 0 | 13 (15.1) |
| increased volume of sputum n (%) | | 58 (75.3) | 8 (88.9) | 66 (76.7) |
| positive *Aspergillus* SPT (n = %) | | 38 (49.3) | 9 (100.0) | 47 (54.6) |
| *Aspergillus*-specific IgE > 0.35 KU/L | n (%) | 33 (42.8) | 9 (100.0) | 42 (48.8) |
| | mean ± sd | 2.8 ± 5.5 | 17.8 ± 13.5 | 4.4 ± 8.1 |
| | median (range) | 0.31 (0.1 - 22.4) | 16.5 (1.07 - 44.5) | 0.33 (0.1 - 44.5) |
| *Aspergillus*-specific IgG >12 U/ml | n (%) | 58 (75.3) | 9 (100.0) | 67 (77.9) |
| | mean ± sd | 55.8 ± 44.9 | 91.2 ± 50.4 | 59.5 ± 46.5 |
| | median (range) | 52.8 (0.0 - 197.7) | 83.2 (28.1 - 162.1) | 59.8 (0.0 - 197.7) |
| stIgE > 1000 IU/ml | n (%) | 0 | 9 (100.0) | 9 (10.5) |
| | mean ± sd | 290.9 ± 267.8 | 1078.2 ± 68.2 | 373.3 ± 351.2 |
| | median (range) | 212.8 (2.3 - 898.7) | 1054.0 (1008.6 - 1200.5) | 291.5 (2.3 - 1200.5) |
| current or history of bronchiectasis n (%) | | 69 (89.6) | 9 (100.0) | 78 (90.7) |
| peripheral blood eosinophil count > 500 cell/µl n (%) | | 34 (44.1) | 3 (33.3) | 37 (43.0) |

CF, cystic fibrosis; ABPA, allergic bronchopulmonary aspergillosis; SPT, skin prick test; sIgE, specific IgE; sIgG, specific IgG; stIgE, serum total IgE; FEV1, forced expiratory vol-ume in one second; FVC, forced vital capacity.

of serum sIgE*Af* was 4.3 ± 8.1 (0.1 - 44.5) KU/L. sIgG*Af* was positive in 67 (78.0%) patients with mean ± SD (range), 75.5 ± 40.1 (18 - 197.7) U/ml. stIgE value > 1000 IU/ml was reported in 9 (10.5%) of CF patients and the mean ± SD (range) of stIgE value was 373.3 ± 351.2 (2.3 - 1200.5) IU/ml.

In total, 86 sputum samples were collected from 86 CF patients of which 66 (76.7%) revealed positive fungal cultures. Two patients had more than one *Aspergillus* species isolates from the sputum samples. *Aspergillus* species (69/79, 87.3%) were the most common isolated filamentous fungi followed by *Penicil-*

*lium* spp. (5/79, 6.3%), *Scedosporium* species (3/79, 3.8%), *Alternaria alternata* (1/79, 1.3%) and *Fusarium fujikuroi* (1/79, 1.3%). Among the *Aspergillus* species, *A. flavus* (23/69, 33.3%) was the most common followed by *A. tubingensis* (19/69, 27.5%) and *A. fumigatus* (13/69, 18.8%).
In HRCT the bronchiectasis was observed in 78 (90.7%) of patients. Out of 86 patients with CF, 37 (43.0%) were presented peripheral eosinophil count greater than 500 cell/µl.

*Prevalence of ABPA*

Of 86 patients with CF, 9 (10.5%) cases were met ABPA diagnosis (classified as ABPA-central bronchiectasis), according to criteria A and B. There was no patient with diagnosed ABPA-serologic and none of them had been previously recognized as ABPA patients. Out of 9 CF patients with diagnosed ABPA, 6 (66.7%) were > 16 years old.
Concordance in positivity of SPT and sIgE*Af* was observed in all diagnosed ABPA patients as both of the criteria A and B. Baseline characteristics and the laboratory examination findings of nine diagnosed ABPA patients were summarised in **table III**. Out of 9 ABPA patients, 8 (88.9%) were positive for *Aspergillus* species growth in sputum samples of which *A. flavus* (3, 37.5%), *A. fumigatus* (2, 25%), *A. terreus* (2, 25%) and *A. tubingensis* (1, 12.5%) were identified. All patients with ABPA showed the evidence of bronchiectasis in HRCT. Three patients with ABPA (33.3%) were presented peripheral eosinophil count greater than 500 cell/µl.

## Discussion

Our results on stIgE > 1000 IU/ml, sIgE*Af*, *Aspergillus* SPT and eosinophilia was comparable with some previous studies (18,19) and in contrast with Baxter et al. (20) study who reported a rate 28.8% of SPT and 15.8% of sIgE*Af*. In this present study, positivity in *Aspergillus* SPT and sIgE*Af* were concordance in all CF patients with ABPA however out of 51 CF patients with *Aspergillus* sensitization (19.6%) had discordance in positivity of *Aspergillus* SPT and sIgE*Af*. These findings in line with some previous studies show that skin test is more sensitive and

***Table III -*** *Clinical and paraclinical data of cystic fibrosis patients with allergic bronchopulmonary aspergillosis.*

| | **P 1** | **P 2** | **P 3** | **P 4** | **P 5** | **P 6** | **P 7** | **P 8** | **P 9** |
|---|---|---|---|---|---|---|---|---|---|
| age in year | 17 | 5 | 20 | 18 | 12 | 24 | 10 | 20 | 34 |
| sex | F | F | M | M | F | M | M | M | M |
| SDM | + | + | + | + | + | + | + | + | + |
| SFC | + | + | + | + | + | + | + | + | + |
| sputum appearance | with brown plugs | normal | normal | with blackish plugs | normal | normal | normal | with black plugs | normal |
| isolated *Aspergillus* species | *A. fumigatus* | *A. flavus* | *A. terreus* | *A. flavus* | *A. flavus* | *A. terreus* | - | *A. tubingensis* | *A. fumigatus* |
| CB | + | + | + | + | + | + | + | + | + |
| MI | + | + | + | + | + | + | - | + | + |
| *Aspergillus* SPT | + | + | + | + | + | + | + | + | + |
| sIgE*Af* > 0.35 KU/L | 4.5 | 14.8 | 26.4 | 24.8 | 16.5 | 22 | 44.5 | 1.07 | 6.0 |
| sIgG*Af* > 12 U/ml | 83.9 | 160.3 | 66.6 | 60.8 | 95.6 | 162.1 | 28.1 | 33.2 | 130.4 |
| stIgE > 1000 IU/ml | 1125.6 | 1013.9 | 1010.5 | 1104.2 | 1054.0 | 1200.5 | 1142.3 | 1008.6 | 1044.7 |
| eosinophil count > 500 cells/ml | 1341 | 488 | 329 | 928 | 210 | 1236 | 375 | 241.3 | 436.8 |

CF, cystic fibrosis; SDM, sputum direct microscopy; SFC, sputum fungal culture; SPT, skin prick test; sIgE, specific IgE; sIgG, specific IgG; stIgE, serum total IgE; CB, central bronchiectasis; MI, mucoid impaction; A, *Aspergillus*.

less specific than sIgE*Af* test, may due to use of crude antigen in skin test (21,22). Therefore, the combination of sIgE*Af* test along with *Aspergillus* skin test is recommended to improve the diagnosis of ABPA (23). Among our 9 CF patients with ABPA, only 3 cases showed an eosinophil count of > 500 cells/µL. It is suggested that inhaled corticosteroids therapy before ABPA screening can reduce the eosinophil count (24).

In the present study, 88.9% of CF patients with ABPA and 74.2% of CF patients without ABPA were positive for *Aspergillus* in sputum samples. The different rate of culture positivity due to *Aspergillus* in CF patients was reported (20,25,26). In our recent study on CF patients, 73.3% of the cases were positive for fungal cultures in sputum samples (27). These variations in the isolation of *Aspergillus* could be explained by various factors including environmental exposure, interactions with other CF pathogens, and therapeutic interventions (28). Interestingly, in contrast to different reports from different countries, *A. flavus* had relatively more frequency than *A. fumigatus* in CF patients with ABPA may due to geographical differences (29,30). Interestingly, *A. flavus* has also been reported as the most prevalent of *Aspergillus* species in different clinical and environmental samples in Iran (27,31).

In this present study, different *Aspergillus* species were isolated from sputum samples of nine patients with ABPA, however all of these patients showed raised sIgG*Af* and sIgE*Af*. It was noted either co-sensitization or cross-sensitization between *A. flavus* or *A. fumigatus* (32), however there is no valuable data on the correlation between culture results and skin test and in vitro antibody assays in ABPA patients.

In this present study we found an overall prevalence of 10.5% of ABPA. The obtained rate of ABPA prevalence was identical in two diagnostic criteria A and B (kappa value of 1.000). According to Rosenberg and Patterson diagnostic criteria for ABPA (33) in which a stIgE > 417 IU/ml was considered as one of the criteria for diagnosis of ABPA, some of our CF patients met the diagnosis of ABPA. According to criteria A or B on the stIgE parameter, we excluded these patients for calculation of ABPA prevalence. Recently, stIgE greater than 1,000 IU/ml was recommended as an important diagnostic indicator for ABPA diagnosis (34). stIgE level may be increased by other environmental factors in many CF and non-CF patients (29). Therefore, it has been suggested that stIgE values greater than 1000 IU/mL can be a major contributor in the prediction of ABPA (30).

Due to the use of different ABPA diagnostic criteria with a distinct sensitivity, the duration of follow-up and monitoring of the patient, the number of study population and the concentration of fungal spores in the environment, different prevalence rate of ABPA is reported by different authors (4,35,36). A 3.4% and 14.9% rate of ABPA has been reported from France (37) and Greece (38), respectively. Regardless the applied criteria, our findings were in range with different report from different countries (5,25,38-41). Two studies from Iran reported the rate 33.3% (42) and 9.0% (43) of ABPA in patients with CF. On the other hand, the numerous applied diagnostic criteria and the absence of any gold standard for the diagnosis, the comparison of the prevalence of ABPA in CF patients reported form various CF centers is very difficult (6). Considering the fact that bronchiectasis as one of the main observations in CF and in ABPA (8), all patients with ABPA showed the evidence of bronchiectasis in this present study. In the present study, the majority of patients with CF and all suspected ABPA patients were positive for bronchiectasis and sIgG*Af*. The same prevalence of ABPA was reported by two applied criteria. It should be noted that the diagnosis of ABPA in patients with CF is difficult and often delayed due to the overlapping of most ABPA pulmonary symptoms with common CF symptoms, such as bronchiectasis (39).

## Conclusions

According to our results, the prevalence rate of ABPA in Iranian CF patients in line with other previous studies from different countries was considerable. The applied diagnostic criteria had no significant effect on the reported rate of ABPA prevalence.

## Fundings

This study was supported by a research fund (No. 2354) from Invasive Fungi Research Center of Mazandaran University of Medical Sciences, Sari, Iran.

## Conflict of interests

The authors declare that they have no conflict of interests.

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ORIGINAL ARTICLE | Eur Ann Allergy Clin Immunol | Vol 52, N.3, 112-120, 2020

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# Clinical experience of a chronic urticaria referral university center

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**Key words**

*chronic urticaria; treatment; antihistamines; urticaria activity score; quality of life*

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10.23822/EurAnnACI.1764-1489.103

***Summary***

**Objective.** *Describing routine procedures, clinical profile and evolution of patients treated in a chronic urticaria (CU) reference center of a university hospital.* **Methods.** *Retrospective analysis of clinical records and database of CU patients registered between March 2011 and February 2016 in a reference center. Besides demographic characteristics, disease duration, comorbidities, angioedema, thyroid lab tests, urticaria subtypes, provocation tests, UAS and CUQ2oL scores were recorded. Patients with 3 or more visits were included in analysis regarding the first and last visits, to evaluate pharmacological treatment and differences of UAS/CUQ2oL scores, antihistamines (anti-H1) dosages and need of other medications, according urticaria subtypes.* **Results.** *During the study, 252 patients were attended, 200 with CU, including 162 women, median age 45 years (perc 25 - 75 = 27 - 58) and median duration of symptoms before diagnosis 24 months (perc 25 - 75 = 9 - 60). Regarding the etiology, 166 (83%) patients had chronic spontaneous urticaria (CSU), 34 (17%) had isolated chronic inducible urticaria (CIndU) and 66 (33%), CSU with CIndU. Among the 123 patients followed up for 3 or more visits, first prescription to 106 (86.2%) patients was monotherapy with anti-H1, and associations with other medications were prescribed to 17 (13.8%). At the last visit, 94 (76.5%) received antihistamines, and 29 (23.5%) used associations. Patients with CSU + CIndU + ASST positive need more association of anti-H1 with other medications than patients with CSU + CIndU and only CIndU ($\chi 2 = 7.998$; p 0.01). Between first and last visits, CUQ2oL mean scores changed from 35.7 (± 21.9) to 22.6 (± 21.0) ($Z = -4.833$ $p < 0.000$).* **Conclusions.** *Most of the patients presented CSU, frequently associated with CIndU. There was an improvement in the patients' quality of life during the follow-up period. All patients were treated with antihistamines and there was a great need for doses above the standardized and also for combination with other medications, especially in patients with concomitance of urticaria subtypes..*

## Introduction

Urticaria represents a heterogeneous group of diseases mediated by mast cells, in which wheals and/or angioedema occur spontaneously or induced. Chronic urticaria (CU) is defined when symptoms persist for more than six weeks, and is classified as: chronic spontaneous urticaria (CSU), with known cause or not, and chronic inducible urticaria (CIndU) in which identifiable triggering factors are responsible for the development of lesions (1).

Etiological investigation and treatment are a challenge for physicians and patients, since about 50% of patients present CSU due to unknown causes (2), leading to great frustration. First-line treatment is second-generation antihistamines (anti-H1) at licensed dose. However, response to this therapy is not always satisfactory, and further medication is often required (1).

Chronic urticaria interferes with well-being and daily life, causing a decrease in quality of life (QoL) and affecting school, work and leisure activities. Analysis of disease severity and its impact on QoL are indispensable tools in the global evaluation of these

patients (3,4). The follow-up of patients with chronic urticaria in a specialized/reference outpatient clinic enhances the diagnosis and treatment success (5).

There are few studies about chronic urticaria in Brazilian population. Information of demographic and clinical profiles as well as of therapeutic management in our country can be of great value, for researchers and for the daily practice of general practitioners and specialists (6-11).

The aim of this study was to describe the clinical profile and evolution of patients followed up in a chronic urticaria/angioedema outpatient reference clinic at a university hospital in Brazil.

## Material and methods

The study was retrospective, based on the analysis of database of patients diagnosed with chronic urticaria (CU) evaluated in the chronic urticaria outpatient clinic of Policlínica Piquet Carneiro, University of Rio de Janeiro State (PPC-UERJ), in the period of March 2011 to February 2016. This specialized unit receives patients referred by the PPC/UERJ general allergy outpatient clinic and other university centers, which are evaluated through a standardized protocol. The diagnosis of chronic urticaria was clinically defined by clinical history with occurrence of erythematous, papular, pruritic, intermittent lesions for a period of more than six weeks, with or without angioedema. Patients referred with other diagnoses as acute urticaria/angioedema, chronic pruritus and dermatitis were not included in the analysis.

After confirmation of the diagnosis, patients were submitted to CIndU provocation tests according to clinical history. Anti-H1 were stopped seven days before testing. For diagnosis of the symptomatic dermographism (SD), FricTest® is placed vertically and a cross path is performed on the volar surface of the forearm, to an extent of approximately 60 mm. A positive response to this test is considered when a pruritic palpable wheal of ≥ 3 mm width is present within 10 minutes after the challenge. For evaluation of cold and heat urticaria, respectively, an ice cube inside a plastic bag and a glass cylinder with hot water at 44 °C are applied to forearm skin for five minutes. The test responses were evaluated 10 minutes after challenge completion, and were considered positive if test site showed a palpable, visible wheal and flare-type skin. Delayed pressure urticaria was evaluated by suspension of a weight rod (diameter 1.5 cm, 2.5 Kg) over volar forearm for 15 minutes, and test response was assessed 6 hours after the end of provocation testing. The presence of a red palpable swelling at the application site was considered positive (12).

Autologous serum skin test (ASST) and autologous plasma skin test (APST) were indicated in patients with urticaria refractory to treatment with standardized dose anti-H1, and that tolerated discontinuation these drugs use 7 days before testing. Venous blood was collected into sterile glass tubes, without accelerator or anticoagulant for serum, and with sodium citrate for plasma. Blood was allowed to clot at room temperature for 30 minutes before separation, which is done with a bench centrifuge at relative centrifugal force of 500 g for 10 minutes. The ASST and APST were performed by intradermal injection of 0.05 ml of serum, plasma and sterile physiological normal saline (NS) and a positive histamine control by skin prick testing (10 mg/ml) in volar forearm. After 30 minutes, the mean of the maximum perpendicular diameters of any red wheal reactions to the ASST, APST and the NS control skin test were calculated. ASST/APST were positive if ASST/APST mean wheal - NS mean wheal ≥ 1.5 mm (13).

The disease severity was assessed by the physician in-clinic urticaria activity score (UAS), a commonly used Patient Report Outcome measure that assesses the key sign (wheals) and symptom (itch) of CSU, in all medical visits. This score was recorded by the patient and evaluated both number of wheals (0 = none; 1 = 1-20 wheals; 2 = 21-50; 3 = > 50) and intensity of itch (0 = none; 1 = mild; 2 = moderate; 3 = intense) in the last 24 hours, on a scale of 0 to 6. The 0 score corresponds to the controlled disease, while 6 score to great intensity disease (1).

Qol was also assessed in all medical visits, through Chronic Urticaria Quality of Life Questionnaire ($CUQ_2oL$). This tool comprises 23 items, which in the original in Italian are divided into six domains and, in the Portuguese validated version (Brazilian culture), into three: I, sleep / mental state / feeding; II, pruritus / impact on activities; and III, edema / limitations / appearance. (8) The patient should respond taking account into the last two weeks, indicating in a five-point Likert scale the intensity of each item separately, ranging from 1 = "nothing" to 5 = "very much". For each of the three dimensions a score is calculated, and then a total score is given for all dimensions. The score ranges from a minimum of 23 to a maximum of 115, indicating a better and worse overall quality of life, respectively. In order to make scores more meaningful and to permit comparisons between different populations of patients, linear transformations of raw scores indicating the percent of maximum possible score were performed. Thus, the minimum possible score is defined as 0 and the maximum possible score is defined as 100 (8,14).

In addition, thyroid laboratory tests (free T4, TSH, and thyroid autoantibodies [TAA] as thyroid peroxidase antibody [anti-TPO] and thyroglobulin antibody [anti-TG]), which are routinely collected on the suspicion of CU in our service, were requested for all patients.

Besides socio-demographic characteristics as age and gender, the following clinical data was recorded: presence of angioedema, urticaria subtypes (CSU, CIndU), comorbidities (atopic, cardiovascular, psychiatric, rheumatologic, endocrinological and oncological diseases), time between the onset of symptoms and the first medical visit, results of provocation tests, ASST/APST,

thyroid laboratory tests, UAS ratings (scores < 4 and ≥ 4) and $CUQ_2oL$ scores at first consult of all patients with CU attended at the outpatient clinic during the study period. The distribution of results of thyroid autoantibodies and ASST was evaluated, as well as $CUQ_2oL$ scores according to UAS ratings.

Patients with ≥ 3 visits to the urticaria outpatient clinic were included in analysis regarding the first and last visits, to evaluate pharmacological treatment and differences of $CUQ_2oL$ scores, UAS ratings, anti-H1 dosages (on demand and single dose versus up to four times the standard dose) and need for medications associated with anti-H1, according urticaria subtypes (isolated CIndU; CSU + CIndU; CSU + CIndU + ASST positive). We also analyzed frequency of patients who were discharged from the outpatient clinic, who interrupted follow-up, and those who were being followed up in February 2016, and analyzed UAS/$CUQ_2oL$ scores in the first and last visit between patients in follow-up, and who interrupted follow-up, with the objective of evaluating whether severity and impact on quality of life are related to follow-up abandonment.

Descriptive statistics were reported by frequency and means ± standard deviation (SD) and medians (interquartile range [IQR]). Prevalence rates are shown as percentages. The chi-square and McNemar's tests were used to study the relationship between qualitative variables. Non-parametric (Wilcoxon, U-Mann Whitney or Kruskal-Wallis) tests were used to study the relationship between continuous variables. Significance was achieved with $p < 0.05$. Statistical analysis was performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

*Ethical aspects*

This study followed the principles of the Declaration of Helsinki and was approved by the Research Ethics Committees of the Institute of Public Health of the University of the State of Rio de Janeiro (Process no. 1.675.616/2017). Confidentiality of data was ensured throughout the study.

## Results

During the study period, 252 patients were attended in the chronic urticaria outpatient clinic, 52 of whom had no chronic urticaria (UC). The most frequent diagnoses among these group were chronic pruritus (13), acute urticaria (13), acute angioedema (10), atopic dermatitis (2), contact dermatitis (5) and others (9).

From the 200 CU patients seen at the first evaluation with median age 45 years (IQR 27 - 58 years; range 5 - 82 years), 162 (81%) were female and 29 (14.5%), children. The median time between the onset of urticaria and the first evaluation was 24 months (IQR 9 - 60 months; range 2 - 564 months), 82 (41.0%) had symptoms for less than 1 year, 21 (16.0%) less than 2 years, 40 (20.0%) for 2-5 years and 46 (23.0%) for more than five years.

About 112 (55.0%) patients also complained of angioedema episodes. The most common comorbidities were arterial hypertension in 57 (28.5%) patients, allergic rhinitis in 50 (25.0%), asthma in 19 (9.5%), hypothyroidism in 17 (8.5%), rheumatological diseases in 11 (5.5%), oncological diseases in 8 (4.0%), psychiatric diseases in 7 (3.5%) and atopic dermatitis in 3 (1.5%) (**table I**). Non-steroidal anti-inflammatory drugs triggered urticaria in 29 (14.5%) patients, antibiotics in 7 (3.5%), whereas ACE-inhibitors and dexchlorpheniramine maleate in only one patient each.

Regarding the etiology, 166 (83.0%) patients had CSU and 34 (17.0%) had isolated CindU. Sixty-six (33.0%) patients with CSU presented CindU (**table I**).

All patients underwent provocation tests for dermographism with 86 (43.0%) positive tests, 79 for cold with 8 (10.1%), 78 for heat with 3 (3.8%), 64 for delayed pressure with 7 (10.9%), 76 for ASST with 41 (53.9%), and 72 and for APST with 28 (38.8%).

Thyroid laboratory tests were requested for all patients, but only 146 performed thyroid hormone serum levels (T4 and TSH) and 121 thyroid autoantibodies measurements (anti-TPO and anti-TG). Of these, 15 (10.2%) presented alterations in hormonal levels (8 patients T4 normal and TSH high, 4 patients T4 normal and TSH low, 3 patients T4 high and TSH normal) and 22 (18.2%) positive thyroid autoantibodies (TAA). In a subset of 54 patients submitted to ASST and TAA measurements, it was observed: 30 ASST positive 8 (26.7%) patients with increased TAA serum levels, and 22 (73.3%) normal, and 24 ASST negative, two (8.3%) positive patients with increased TAA serum levels, and 22 (91.7%) normal.

The $CUQ_2oL$ median scores (0 - 100) on the first visit was 26.2 (IQR 13.35 - 44.10; range 0 - 78.62). Physician in-clinic UAS scores < 4 were observed in 171 (85.5%) patients and ≥ 4 in 28 (14.5%), with 88 (44.0%) patients that presented pruritus, and only 48 (24.0%) that had wheals at the time of the first evaluation. The $CUQ_2oL$ scores are high (i.e. worse) in patients with UAS scores ≥ 4 (U 832.000; $p < 0.000$) (**table I**).

*Follow-up data*

The clinical characteristics of the 123 patients followed by 3 or more medical visits are very similar to the patients seen at least once, as seen in **table I**. Among these patients, 22 were followed-up for less than one year, 50 for one, 23 for two and 28 for three to five years, with median follow-up time of 14 months (IQR 7 - 27 months; range 2 - 58).

Only eleven patients (9%) were discharged due to disease remission, 42 (34%) interrupted the follow-up and 70 (57%) were still under follow-up in February 2016. Patients in remission

***Table I*** *- Sample general and clinical characteristics.*

| Characteristics | CU patients (n = 200) | CU patients' follow-up group[1] (n = 123) |
|---|---|---|
| Sex, n (%) | | |
| male | 38 (19.0) | 19 (15.5) |
| female | 162 (81.0) | 104 (84.5) |
| Age, y (median / range) | 45 (27 - 58) / 5 - 82 | 43 (28 - 58)/ 6 - 82 |
| children, n (%) | 29 (14.5) | 18 (14.6) |
| adults | 171 (85.5) | 105 (85.4) |
| Time of disease in first visit (m) median (IRQ) / range | 24 (9 - 60) / 2 - 564 | 24 (9 - 72) / 2 - 360 |
| < 1 y, n (%) | 82 (41.0) | 47 (38.2) |
| 1-2 y | 32 (16.0) | 18 (14.6) |
| 2-5 y | 40 (20.0) | 25 (20.2) |
| > 5 y | 46 (23.0) | 32 (26.0) |
| Angioedema, n (%) | 112 (56.0) | 69 (56.0) |
| Urticaria subtypes, n (%) | | |
| CSU (isolated) | 100 (50.0) | 53 (43.0) |
| CSU + CIndU | 66 (33.0) | 47 (38.2) |
| CIndU (isolated) | 34 (17.0) | 23 (18.8) |
| Autoimmunity, n / total n (%) | | |
| ASST | 41 / 76 (53.9) | 38 / 63 (60.3) |
| APST | 28 / 72 (38.8) | 24 / 60 (40.0) |
| Thyroid autoantibodies | 22/ 121 (18.2) | 18 / 94 (19.1) |
| Comorbidities | | |
| arterial hypertension | 57 (28.5) | 35 (28.4) |
| allergic rhinitis | 50 (25.0) | 28 (22.7) |
| asthma | 19 (9.5) | 15 (12.1) |
| hypothyroidism | 17 (8.5) | 17 (13.8) |
| rheumatological diseases | 11 (5.5) | 9 (7.3) |
| oncological diseases | 8 (4.0) | 4 (3.2) |
| psychiatric diseases | 7 (3.5) | 2 (1.6) |
| atopic dermatitis | 3 (1.5) | 1 (0.8) |
| Physician in-clinic UAS in first visit, n (%) | | |
| scores < 4 | 171 (85.5) | 101 (82.1) |
| scores ≥ 4 | 28 (14.5) | 22 (17.9) |
| $CUQ_2oL$ mean (SD) | n = 160 | n = 102 |
| Total | 28.6 (20.6) | 35.7 (21.9) |
| UAS scores < 4[2] | 25.9 (19.7)[3] | 32.4 (21.1)[4] |
| UAS scores ≥ 4[2] | 44.0 (19.0)[3] | 49.0 (20.8)[4] |

CSU, chronic spontaneous urticaria; CIndU, chronic inducible urticaria; ASST, autologous serum skin test; APST, autologous plasma skin test; UAS, urticaria activity score (0 - 6); $CUQ_2oL$, Chronic Urticaria Quality of Life Questionnaire (0 - 100); m, months; y, years; SD, standard deviation; IRQ, interquartile range. [1]123 CU patients followed for at least 3 visits. [2]U-Mann Whitney test to evaluated $CUQ_2oL$ scores between patients with UAS scores < 4 and ≥ 4. [3]p < 0.000. [4]p = 0.004.

presented median time of disease progression at the first evaluation of 48 months (IQR 6 - 60 months), follow-up time in our clinic of 21 months (IQR 12 - 30) and disease time at discharge of 72 months (IQR 31 - 83). Among those who remained in follow-up in February 2016, medians were respectively 25 months (IQR 12 - 84), 16 months (IQR 9 - 34) and 54 months (IQR 31 - 101) in the last clinic consultation.

Evaluation of UAS groups (scores < 4; ≥ 4) and CUQ2oL scores between patients who interrupted follow-up and still in follow-up showed no difference between UAS groups and a lower impact on the quality of life at last visit in the patients who interrupted follow-up, (CUQ2oL scores mean at last visit in follow-up group: 28.1 (± 2.9) and in interrupted follow-up group: 15.7 (± 18.4) (U 932.500; p 0.001)

Between first and last visits CU-$Q_2$oL mean scores changed from 35.7 (± 21.9) to 22.6 (± 21.0) (Z -4.833; p < 0.000), although the physician in-clinic UAS scores demonstrated a less significant change between visits (p 0.04) (**table II**).

On the first visit, patients were treated with anti-H1, 106 (86.2%) as monotherapy and 17 (13.8%) with combination with other medications. In 16 patients (13.0%) the dosage of anti-H1 was on demand, in 56 (45,5%) the maintenance dose was standardized and 51 (41,5%) received up to four times the standard dose (**table III**).

On the other hand, the therapeutic regimen used in the last visit of these patients was anti-H1 monotherapy for 94 patients (76.5%), while 61.8% of them used twofold to fourfold doses, with relevant difference between the two assessments (p 0.008; p < 0.000 respectively) **(table III).** The most frequently prescribed anti-H1 were, in their respective order, cetirizine, hydroxyzine and fexofenadine. Associations with other drugs were necessary in 29 (23.5%) patients, being the most common doxepin (17), followed by oral corticosteroids in short courses for exacerbations (13), montelukast (3), anti-IgE (3) and cyclosporin (2). Seven patients needed association with two or more medications (**figure 1**).

Treatment with anti-IgE and cyclosporin was necessary in five patients, all women with associated angioedema; two had hypothyroidism; one rheumatoid arthritis, and two ASST positivity.

CUQ2oL scores, UAS groups, need of anti-H1 association with other medications and anti-H1 posology in first and last visit according CU subtypes (only CIndU, CSU + CIndU, CSU + CIndU + ASST positive) were evaluated. A tendency to better quality of life in patients with CIndU at the first visit (p 0.07) was observed. In the last visit was observed major association of anti-H1 with other medications in patients with CSU + CIndU +ASST positive ($\chi 2$ 7.998; p 0.01), and a trend not statistically proven for the use of doses above the standard doses in this group of patients ($\chi 2$ 5.558; p 0.06) (**table IV**).

***Table II** - Activity and quality of life evaluation between first and last visit.*

| | first visit | last visit | p value |
|---|---|---|---|
| $CUQ_2$oL, mean (SD), n = 102 | 35.7 (21.9) | 22.6 (21.0) | < 0.000[1] |
| physician in-clinic UAS, n (%) n = 123 | | | |
| scores < 4 | 101 (82.0) | 112 (91.0) | 0.04[2] |
| scores ≥ 4 | 22 (18.0) | 11 (9.0) | |

UAS, urticaria activity score (0 - 6); $CUQ_2$oL, Chronic Urticaria Quality of Life Questionnaire (0 - 100); SD, standard deviation. [1]Wilcoxon test. [2]McNemar test.

## Discussion

In this study, clinical profile and evolution of CU patients followed-up at a subspecialized university outpatient clinic was described. The patients were admitted with two years of average time of disease and after being treated by several specialists and submitted to many treatments (as about 30% presented symptoms for at least five years), demonstrating the difficulty in appropriate diagnosis and management of this disease (3).

Most of the evaluated patients had CSU, of which 33% associated with CIndU. Maurer et al. (3) evaluated the prevalence and

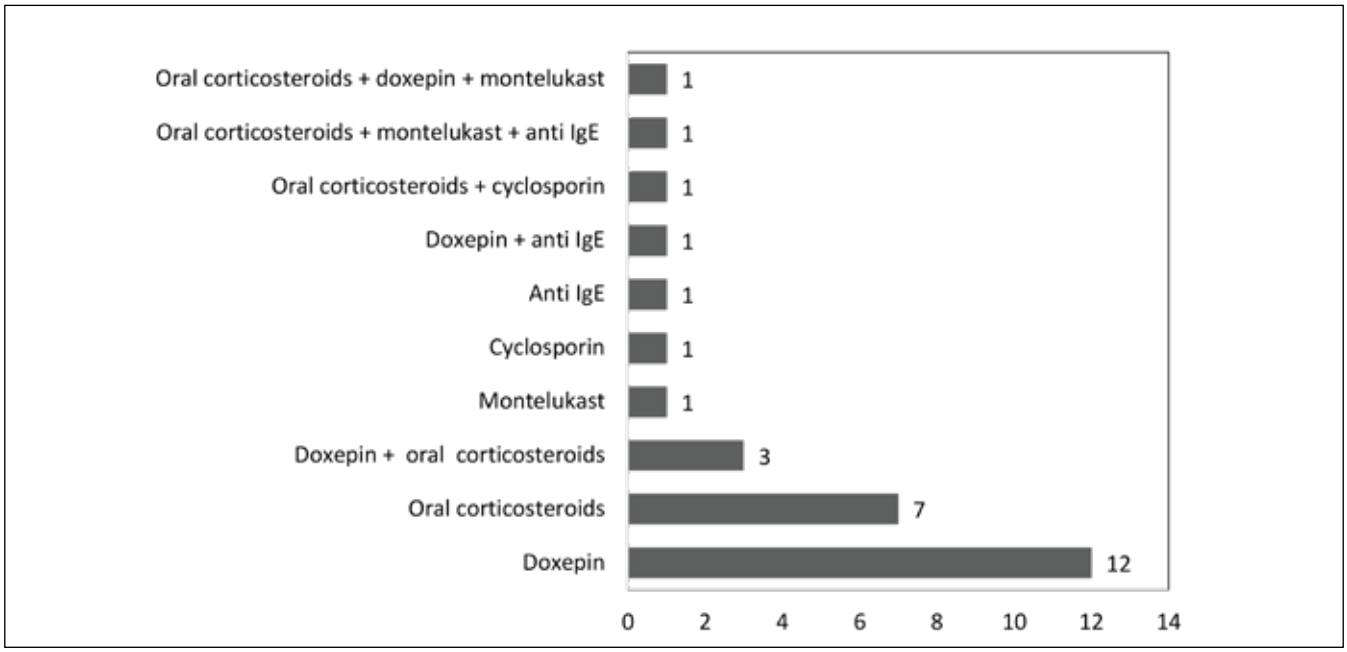
***Table III** - Urticaria pharmacologic treatment at the initial and last visit of follow-up.*

| Treatment (n = 123) | first visit | last visit |
|---|---|---|
| Medications, n (%)[1] | | |
| anti-H1 only | 106 (86.2) | 94 (76.5) |
| ant-H1 + others | 17 (13.8) | 29 (23.5) |
| Type of medications, n (%) | | |
| anti-H1 | | |
| cetirizine | 81 (65.8) | 103 (83.7) |
| fexofenadine | 23 (18.7) | 30 (24.3) |
| hidroxizine | 20 (16.2) | 33 (26.8) |
| bilastine | 9 (7.3) | 24 (19.5) |
| loratadine | 3 (2.4) | 11 (8.9) |
| dexclorfeniramine | 0 | 6 (4.8) |
| desloratadine | 2 (1.6) | 0 |
| levocetirizine | 1 (0.8) | 0 |
| ebastine | 1 (0.8) | 0 |
| others | | |
| doxepin | 8 (6.5) | 17 (13.8) |
| oral corticosteroid | 11 (8.9) | 13 (10.5) |
| montelukast | 1 (0.8) | 3 (2.4) |
| anti-IgE | 0 | 3 (2.4) |
| cyclosporin | 1 (0.8) | 2 (1.6) |
| Anti-H1 dosage, n (%) | | |
| on demand | 16 (13.0) | 9 (7.5) |
| single dose | 56 (45.5) | 38 (30.8) |
| twofold dose | 37 (30.0) | 33 (26.8) |
| threefold dose | 5 (4.0) | 19 (15.4) |
| fourfold dose | 9 (7.5) | 24 (19.5) |
| Anti-H1 dosage, n (%)[2] | | |
| on demand + single dose | 72 (58.5) | 47 (38.2) |
| twofold to fourfold dose | 51 (41.4) | 76 (61.8) |

Urticaria pharmacologic treatment at the initial and last visit of follow-up, in 123 CU patients followed for 14 months (perc 25 - 75 = 7 - 27 months; range: 2 - 58) at least 3 visits.

Anti-H1, antihistamines. [1]p = 0.008 was obtained by comparison between patients treated with only anti-H1 and anti- H1 with other medications in first and last visits. McNemar test. [2]p < 0.000 was obtained by comparison between patients treated with anti-H1 on demand and single doses versus and anti- H1 treated with doses above the standardized. McNemar test.

***Figure 1*** *- Medications associated with antihistamines in last visit.*




distribution of chronic urticaria in several countries and found that in patients presenting with nonacute urticaria, 66 to 93% had CSU, from 4 to 33% had CindU, and cholinergic urticaria diagnosis varied from 1 to 7%, being the combination of CSU with CindU common. The frequency of CU subtypes in the Brazilian population is still little known. In a study carried out in São Paulo in 2011 with a sample of 62 patients, authors found a frequency of 32.3% of CSU, 27.4% of CindU alone

***Table IV*** *- Chronic urticaria subtypes and evaluation of disease activity/quality of life and pharmacological treatment.*

| | first visit | | | | last visit | | | |
|---|---|---|---|---|---|---|---|---|
| | CIndU (n = 23) | CSU + CIndU (n = 24) | CSU + CIndU + ASST (n = 23) | p value | CIndU (n = 23) | CSU + CIndU (n = 24) | CSU + CIndU + ASST (n = 23) | p value |
| $CUQ_2oL$ (mean/SD) | 23.7 ± 15.0 | 40.0 ± 23.7 | 38.6 ± 24.2 | 0.07[1] | 16.4 ± 12.6 | 25.5 ± 22.2 | 21.0 ± 22.2 | 0.51[1] |
| physician in-clinic UAS, n (%) | | | | | | | | |
| scores < 4 | 21 (91.3) | 20 (83.3) | 17 (73.9) | 0.29[2] | 21 (91.3) | 21 (87.5) | 20 (73.9) | 0.88[2] |
| scores ≥ 4 | 2 (8.7) | 4 (16.7) | 6 (26.1) | | 2 (8.7) | 3 (12.5) | 3 (26.1) | |
| Medications, n (%) | | | | | | | | |
| anti H1 | 23 (100) | 22 (91.7) | 19 (82.6) | 0.10[2] | 20 (87.0) | 22 (91.7) | 14 (60.9) | **0.01**[2] |
| anti H1 + other drugs | 0 (0) | 2 (8.3) | 4 (17.4) | | 3 (13.0) | 2 (8.3) | 9 (39.1) | |
| Anti-H1 dosage, n (%) | | | | | | | | |
| on demand + single dose | 16 (69.5) | 13 (54.2) | 11 (47.8) | 0.30[2] | 13 (56.5) | 17 (29.2) | 17 (26.1) | 0.06[2] |
| twofold to fourfold dose | 7 (30.5) | 11 (45.8) | 12 (52.2) | | 10 (43.5) | 11 (60.8) | 12 (73.9) | |

CSU, chronic spontaneous urticaria; CIndU, chronic inducible urticaria; ASST, autologous serum skin test; UAS, urticaria activity score; $CUQ_2oL$: Chronic Urticaria Quality of Life Questionnaire (0 - 100); anti-H1, antihistamines. [1]Kruskal-Wallis test; [2]Chi-square test.

and 40.3% of CSU/CindU association (10). Another study published in the same year in Rio de Janeiro, with 112 patients, showed that 36% of patients presented CSU, 24% isolated CindU and 44% associated CSU/CIndU (8).

Urticaria symptoms are brought about by activated skin mast cells and their subsequent release of histamine and other proinflammatory mediators. The underlying causes and the mechanisms of mast cell activation in most types of urticaria are unknown, and remain to be identified. The presence of IgG autoantibodies against IgE receptors, or IgE and IgE anti-autoantigens as thyroid peroxidase (TPO) on the membrane of basophils and cutaneous mast cells, shows an association between CU and autoimmunity (13,15). ASST/APST are in vivo tests that evaluate autoreactivity, but do not define the diagnosis of chronic autoimmune urticaria. They indicate, when positive, that there may be autoantibodies or other soluble factors potentially involved in the degranulation of cutaneous mast cells. This method should be complemented, if possible, with basophil histamine releasing test and specific IgG autoantibodies against FcεRIa and/or anti-IgE immunoassay to demonstrate antibody specificity (7,13).

Asero et al. have reported that the autologous plasma skin test (APST) is more sensitive than ASST, which was not confirmed by other authors (16-18). In the urticaria outpatient clinic we routinely performed ASST and APST to evaluate the two tests. In the descriptive analysis of this sample we found 53.9% of positivity for ASST and 38.8% for APST. The comparison between the two methods is not the objective of the present research, but a higher positivity of the ASST is observed in our series.

Considering that autoimmune factors may be common features of both thyroid autoimmunity and urticaria, it is likely that both may coexist within the same patient. We found elevated thyroid autoantibodies serum levels in 18.3% patients, that were present in 26.7% of those ASST positive and 8.3% in negative ones. In a systematic review about CSU and autoimmune thyroid diseases, the authors found the frequency of elevated thyroid autoantibodies varying from 3.7% to 37.1% (19).

Among patients followed up in the service for at least three visits, just a small portion was free of disease (less than 10%), with disease time at discharge of 72 months. Van der Valk et al. in a retrospective study with 372 adults identified remission after 5 and 10 years in 29% and 44% of patients, respectively (20). In another retrospective study, Kulthanan et al. revealed that in 337 adults with CSU, 34.5% had remission after 1 year (21). Kozel et al. prospectively measured 220 adults for 3 years and found that 35% of CU patients after 1 year did not present any more symptoms (22). In our study, the disease presented a longer course, which may have occurred because it is a referral service in a tertiary hospital, where the CU duration may be greater.

About 34% patients abandoned treatment, which draws attention to this high dropout rate. Nevertheless, this group had a lower impact on quality of life at the last visit to the service than the patients still in follow-up, which may suggest that the abandonment of the follow-up may be partially related to the improvement or remission of the disease.

The pillars of chronic urticaria management are avoiding triggering factors and pharmacotherapy. The first line treatment is modern second generation anti-H1 in a standardized dose (1), but symptoms improve in less than 50% of patients using this dose (3). Doses above the standard were prescribed for 41.4% of our follow-up group already in the first consultation, since they were previously treated without adequate control, which is a frequent finding in referral services like ours. Anti-H1 monotherapy was instituted in 86.2% of the patients at the first consultation in the follow-up sample, being cetirizine the anti-H1 of choice because of its low cost in our country. As it is available free of charge in public health system, hydroxyzine, a first generation anti-H1 with sedative action, was the second most prescribed antihistamine. Doxepin is a tricyclic antidepressant, which acts through the mixed inhibition of serotonin and norepinephrine recapture, presenting antihistaminic and sedative properties was used in 13.8% of patients (23). In 2014, the AAAAI recommended the use of first-generation anti-H1 or doxepin at night as a third-line treatment of chronic urticaria (24,25). However, in most recent 2017 EAACI/GA²LEN/EDF/WAO guideline, the recommendation for CU treatment is up dosing second generation anti-H1 up to fourfold in patients unresponsive to second generation anti-H1 usual dose. If there is no improvement, it is recommend adding on omalizumab, now their third line treatment proposal.

Regarding treatment in the last medical visit, it was observed that there was a need to increase the standardized dose of anti-H1 in 61.8% of patients and to association with other drugs in 23.5% to achieve control. Patients who present CSU + CIndU + ASST positive need more association of other medication to anti-H1 and also a trend to use doses above the standard doses than patients with only CIndU or CSU + CIndU, showing that patients with ASST positive are more refractory to anti-H1. However, several studies, support that a positive ASST is linked to severe disease, and in our study, we did not find this association (26,27).

Only three patients used anti-IgE until February 2016, since it is available for clinical use in Brazil only since December 2015. Anti IgE access is still a problem in our country and patients frequent request it judicially from the State government or health insurance, because it is not provided by public health services. Currently, 16 patients with refractory CU are on treatment with anti IgE in our Unit.

The use of UAS and $CUQ_2oL$ helps to monitor the evolution of the disease and the efficacy of treatment. The UAS and

$CUQ_2oL$ scores at the first visit were low, due to the heterogeneity of the patients with only 88 (44.0%) patients that presented pruritus, and 48 (24.0%) that had wheals at the first evaluation. The majority of patients' sample analyzed presented mild urticaria activity with low quality of life impairment. However, the evaluation of the $CUQ_2oL$ scores according to UAS showed that patients with higher disease activity have a worse impact on QoL. A significant decrease of $CUQ_2oL$ scores was observed in the follow-up of the patients, which suggests a better control of the disease (28). The analysis of $CUQ_2oL$and UAS according to diagnostic subtypes showed no relevant differences except for a tendency to improve quality of life in patients with CIndU at the first visit. However, it should be mentioned that UAS and $CUQ_2oL$ do not evaluate adequately patients with CIndU, since their questions are not specific for this type of urticaria (29).

The best method for assessing activity is UAS7, since this instrument evaluates the seven days prior to the consultation, evaluating more broadly a disease that has a fluctuating course. Its limitations are the inability to perform at the first consultation and dependence on patient compliance. If UAS7 had been used in the evaluation of the last visit, we would probably have a more accurate analysis of the disease activity. We did not use UAS7 routinely during all period of this study. Only 17 patients answered UAS7 at the last medical visit. Some patients do not understand, and other forget to fill the diary and bring it. About two years ago we regularized the UAS7 use, but the rate of return of this tool has been low, and only patients with more severe disease tend to use UAS7 adequately. Measures to increase adherence to this tool were implemented, and currently the rate of compliance is improving.

Our study had some limitations that must be considered. This study was performed in a single tertiary center; therefore, selection bias might have occurred. It should be considered that is a retrospective study, with analysis of patients' database, and some information as results of thyroid lab tests were not available for all patients. There might be patients with hypo or hyperthyroidism and positive autoantibodies that were not evaluated. Another limitation already mentioned above was a low use of UAS7, which possibly would be a more sensitive tool than physician in-clinic UAS in assessing disease activity. Despite these limitations, we provide useful information regarding the natural course time of CU, disease severity, quality of life and pharmacological treatment prior to the introduction of anti-IgE in chronic urticaria therapy in our country.

## Conclusions

Most of our patients presented CSU to which CIndU was frequently associated. All patients were treated with antihistamines and there was a great need for doses above standardized, and also for combination with other medications. We have difficulties in the access to immunobiological therapy, which costs are still a barrier to its use in most of our patients. The disease has a prolonged course and at the time of discharge many patients had symptoms for more than five years. The use of standardized questionnaires for CU have been shown to be important tools to optimize the follow-up and treatment of this challenging disease, which has impact on patients' quality of life. Measures to reduce patients withdraw from treatment and to recover these patients are required in an outpatient clinic specializing in CU.

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# Prevalence of allergic rhinitis in children with otitis media with effusion

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**Key words**

*allergic rhinitis; allergens; deafness; hearing loss; otitis media with effusion; prevalence; risk factors*

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**Doi**
10.23822/EurAnnACI.1764-1489.119

***Summary***

**Introduction.** *The prevalence of allergic rhinitis in children with persistent otitis media with effusion in different countries varies between 82% to 93%. Many risk factors of otitis media with effusion has been studied and proven. However, its association with allergic rhinitis remains controversial.* **Objective.** *The main objective of this study is to determine the prevalence of allergic rhinitis in children with persistent otitis media with effusion. This study is also aimed to identify the risk factors of otitis media with effusion, common allergens associated with allergic rhinitis and determine the hearing threshold of children with otitis media with effusion.* **Methods.** *A hundred and thirty children were recruited. History taking, physical examination and hearing assessment were done in the first visit. Those with allergic rhinitis underwent skin*
*prick test and treated with intranasal corticosteroid and antihistamine. A second examination and hearing assessment were then repeated after 3 months.* **Results.** *The prevalence of allergic rhinitis in children with persistent otitis media with effusion in this study was noted to be 80.3%. Among these children, dust mites appeared to be the most common allergen (87.7%). Another risk factor appeared to be families with more than 4 members per-household (96%). It is noted that that otitis media with effusion caused a hearing loss up to 33 dB. However, there was a statistically significant improvement of the hearing threshold during second visit after commencement of allergy treatment. It was also noted that the hearing threshold in allergic rhinitis group was significantly impaired compared to the non-allergic rhinitis group.* **Conclusions.** *Allergic rhinitis and larger family household appeared to be common risk factors in children with persistent otitis media with effusion. There is significant hearing loss noted in children suffering from otitis media with effusion and allergic rhinitis. The hearing threshold improved remarkably with medical therapy. This study hence clarifies the controversy on the association between allergic rhinitis and otitis media with effusion.*

## Introduction

Otitis media with effusion (OME) is defined as inflammation of the mucous membrane with fluid collection in the middle ear in the absence of acute infection (1). The pathogenesis of OME can be multifactorial, it involves infection of the tubotympanum, eustachian tube dysfunction and allergy (2).

Prevalence of OME among children in different countries varies between 6.5% to 10.9% (3). A local study done by Saim A et al. in 1997 reported the overall prevalence of OME among the preschool children to be 13.8%. The prevalence is noted to be higher in Kuala Lumpur (17.9%) compared to the district of Kuala Selangor (9.5%) (4). National Hearing Survey for hearing and ear disorder in Malaysia showed the prevalence of OME to be 5.1% in the general population, 2.9% in children below 10 years old and 2.6% in children aged 10 to 19 years (5). The prevalence of persistent OME on the other hand was reported as high as 8.7% in Turkey (3). However, similar study has yet to be reported locally or regionally (3).

There are several factors that influence the development of OME. Non-medical risk factors include age, large family size, history of OME in a sibling, short duration or no breastfeeding

and passive smoking. Medical risk factors include the history of acute otitis media (AOM), allergic rhinitis (AR), history of acute tonsillitis and craniofacial anomalies (6).

The association between AR and OME has been a controversial issue with many contradicting results. In a study conducted by Fernadez and McGovern on 113 children with OME, 83% were found to be have allergy based on history and 92% had positive skin prick test (7). Alles R et al. studied on the prevalence of atopic disorders in 209 children with OME and they found 89% had AR (8). Passali D et al. reported that children with persistent OME and AR were found to have eustachian tube (ET) dysfunction (9). Allergic rhinitis can cause swelling of nasal mucosa resulting in eustachian tube dysfunction and subsequently leading to OME (10).

The Malaysian Clinical Practice Guidelines on Management of OME in children recommends surgical intervention to be considered after three months of OME with hearing loss of more than 25 dB (average frequency of three) and/or structural changes to the tympanic membrane or middle ear. Myringotomy with ventilation tube insertion is recommended as the surgical treatment of choice (6).

Persistent fluid in the middle ear cavity may form a barrier for sound conduction affects the threshold acuity (11). If left untreated, OME may be complicated with hearing loss especially conductive type, vestibular problem, poor school performance, behavioral problems, recurrent acute otitis media and reduce the quality of life (12).

## Objectives

This study aimed to determine the prevalence of allergic rhinitis (AR) in children with persistent OME in Universiti Kebangsaan Malaysia, Medical Centre (UKMMC). It is also conducted to identify the risk factors, common allergens and hearing threshold in children with OME.

## Materials and methods

*Study Instruments*

***Otoscope***

An otoscope is used to visualize the tympanic membrane to identify middle ear effusion as defined by a dull appearance of the tympanic membrane with the presence of air-fluid level or air bubbles.

***Conventional (226 Hz) tympanometry***

This test was carried out by an audiologist and a researcher. A tympanometer was used to measure the compliance of the tympanic membrane. It contained an earpiece which was introduced into the ear canal. A miniature loudspeaker generated a 226 Hz probe tone which was reflected off the tympanic membrane. The data was generated in a graph which was interpreted as normal (type A), flat or decreased maximum tympanic membrane compliance (type B) or of negative middle ear pressure (types C). Decreased mobility of the tympanic membrane is due to effusion in the middle ear and it is the most common cause of type B tympanogram. Tympanograms were labeled as type C if there is a presence of a significant negative pressure which indicate eustachian tube dysfunction. For this study, type B tympanogram with normal ear canal volume was considered as otitis media with effusion.

***Hearing assessment***

Play audiometry and pure tone audiometry were the hearing assessment used depending on the ability of the child to understand the task. Hearing assessment for pure tone audiometry was done at 250 Hz, 500 Hz, 1 kHz, 2 kHZ, 4 kHz, and 8 kHz. The level of severity of hearing loss was defined as follows, mild hearing loss 26 - 40 dBHL, moderate hearing loss 41 - 70 dBHL, severe hearing loss 71 - 90 dBHL, and profound hearing loss was > 90 dBHL.

***Skin prick test (SPT)***

Skin prick test was performed on all subjects with AR by an otorhinolaryngology doctor. The skin area on the inner forearm was labeled with codes to identify tested allergens. A drop of the allergen solution was placed on the skin. The skin was then pricked through the drop using the tip of a lancet. Reading on the result was done after 20 minutes and the size of the wheals was outlined with a felt tip pen and transferred to the recording sheet by adhesive transparent tape. A positive result was considered if the size of the wheal was 3 mm or more. Common aeroallergen and food allergens were done according to a previous study done in UKMMC (13).

***Rigid nasal endoscopy / flexible nasopharyngolaryngoscopy***

Rigid nasal endoscopy or flexible nasopharyngolaryngoscopy was performed by the researcher or trained medical officer. Nasopharynx was assessed for adenoid hypertrophy. Adenoid hypertrophy was graded based on the grading system described by Cassano et al. (14), as follows:

Grade 1: the adenoid occupying upper segment of the nasopharynx (< 25% of choana);

Grade 2: adenoid tissue occupying the upper half of the nasopharynx (≥ 25 to < 50%);

Grade 3: adenoid extending over nasopharynx with obstruction choana and partially the tube (≥ 50 to < 75%);

Grade 4: total choanal obstruction (≥ 75 to 100%).

*Methodology*

This study is a prospective cross-sectional study. The permission to carry out the study was obtained from the ethics and

research committee of UKMMC with a code number of FF-2016-380. Doctors and audiologists had been explained regarding the methodology prior to obtaining patients for the study. All subjects who fulfilled the inclusion and exclusion criteria for OME were identified. The research had been explained to patients or their guardians and informed consent was obtained. Information sheets with details on the study, available in Malay or English languages was provided to the parents or guardians
In this study, data was extracted from the first 2 hospital visits. During the first visit, a thorough history on AR was obtained based on the Allergic Rhinitis and Its Impact on Asthma (ARIA) Guidelines 2010. Antihistamine and intranasal steroid were given to patient with AR according to the guidelines The AR symptoms were classified into 4 groups which are intermittent mild AR, intermittent moderate-severe AR, persistent mild AR, persistent moderate-severe AR (15).
During physical examination, patients' ear canals were examined with an otoscope. Patients with dull tympanic membrane or presence of air bubbles were considered to have abnormal otoscopic examination. These patients were subsequently tested for tympanometry and unilateral or bilateral type B tympanometry patients with normal ear canal volume were diagnosed to have OME. Subsequently, they underwent hearing assessment and nasal endoscopy to evaluate the size of adenoids. All patients diagnosed with AR underwent skin prick test.
After 3 months, patients were re-examined with an otoscope and tympanometry. During this follow-up, patients who had resolved OME were discharged. Hearing assessment was performed for patients with persistent type B. Courtesy phone calls were made during the period of 3 months until second visit to ensure the subjects and to reinforce on the compliance of medications.

**Results**

*Patient population*

One-hundred and fifty children with OME were identified during study duration of 10 months. However, due to default in follow up only 130 were recruited for this study. Mean age at presentation was 8.79 years old with (standard deviation ± 3.76 years). Minimal age was 4 years old and the maximum age was 18 years old. Fifty-three percent of children with OME (70/130) were 4-8 years old, 33.8% (44/130) were 9-13 years old and 12.3% (16/130) aged 14-18 years old (**figure 1**). Otitis media with effusion was significantly higher in 4-8 years old group compared to the older children. Sixty-seven children were male (51.5%) and 63 children (48.5%) were female (**figure 2**). The male to female ratio was 1.06:1 but the difference was not statistically significant.
In our study, the prevalence of AR in OME children was 52.3% (68/130). Among 68 OME children with AR, 13.2% (9/68) had intermittent mild AR, 25.0% (17/68) had intermittent moderate-severe AR, 29.4% (20/68) had persistent mild AR and 33.8% (23/68) had persistent moderate-severe AR (**figure 3**).
After 3 months, among 130 children with OME, 71/130 (54.6%) had persistent OME and 59/130 (45.4%) had resolved OME. We found that among the persistent OME group, 57/71 (80.3%) of children had AR. This study showed that AR is a statistically significant risk factor for developing persistent OME ($p < 0.0001$) (**table II**).
The most significant presenting complaint among children with persistent OME was reduced hearing 81.7% compared to 52.5%

***Figure 1*** *- Age distribution of children with OME.*

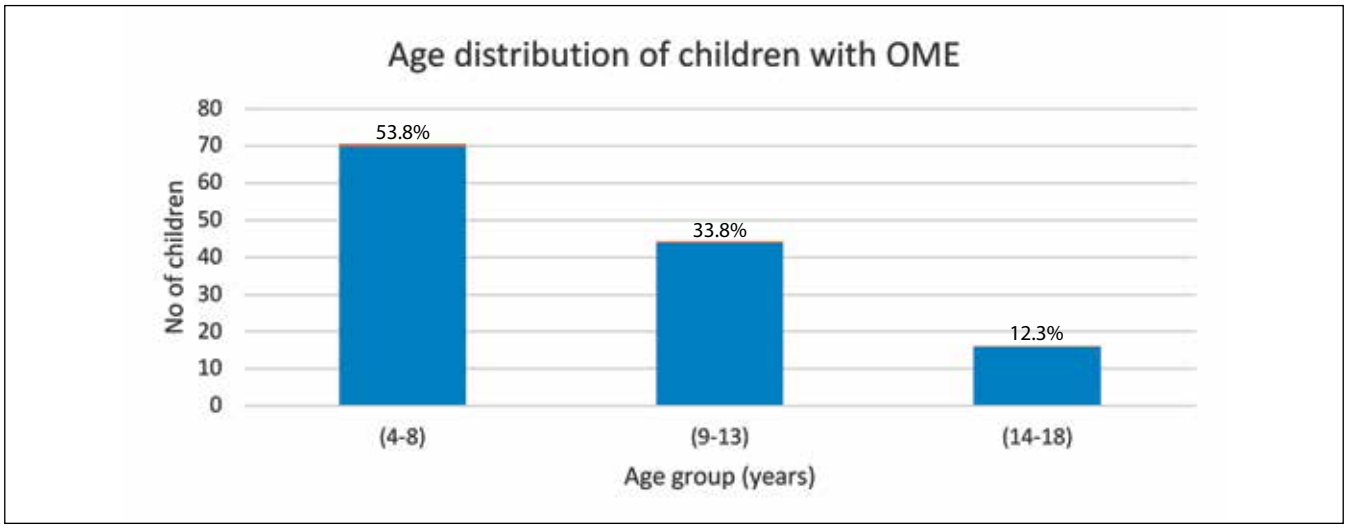


***Figure 2*** *- Gender distribution in children with OME.*

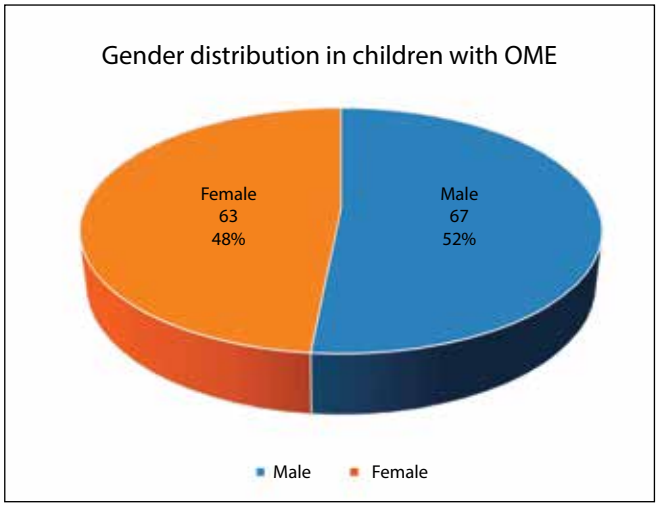
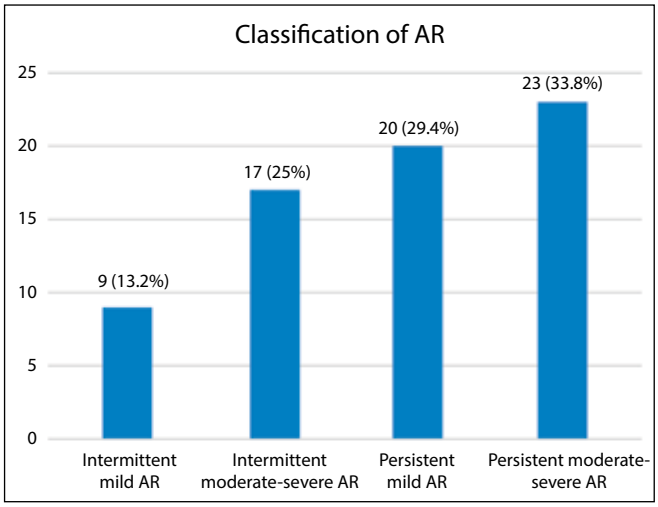



***Figure 3*** *- Classification of Allergic Rhinitis.*




of children with non-persistent OME ($p < 0.0001$) (**table I**). Family size was statistically significant influence on developing persistent OME. It was less common in the small families (< 3 family members) than in the bigger families. Ninety-six percent (68/71) of children with persistent OME had more than 4 family members in the household. The history of OME in siblings proved a significant risk factor on developing persistent OME. The results showed that 25% of those with persistent OME had siblings with history of OME compared to 11.9% of children with non-persistent OME ($p = 0.04$) (**table II**).

In our study, the history of tonsillitis was not a significant risk factor for developing persistent OME. Twenty-five percent of those with persistent OME had the history of tonsillitis compared to 16.9% of children with non-persistent OME and this was not statistically significant ($p = 0.24$). Regarding the history of AOM infection, 8.5% of children in both groups had a

***Table I*** *- Symptoms of OME in Children.*

| Variables (n=65) | Non-Persistent | Persistent | Total | χ2 | p-value |
|---|---|---|---|---|---|
| **Reduce Hearing** | | | | | |
| Yes | 31 (52.5%) | 58 (81.7%) | 89 (68.5%) | | |
| No | 28 (47.5%) | 13 (18.3%) | 41 (31.5%) | | |
| **Otalgia** | | | | | |
| Yes | 9 (15.3%) | 12 (16.9%) | 21 (16.2%) | 0.065 | 0.799 |
| No | 50 (84.7%) | 59 (83.1%) | 109 (83.8%) | | |
| **URTI** | | | | | |
| Yes | 36 (61.0%) | 42 (59.2%) | 78 (60.0%) | 0.047 | 0.829 |
| No | 23 (39.0%) | 29 (40.8%) | 52 (40.0%) | | |
| **AOM** | | | | | |
| Yes | 5 (8.5%) | 7 (9.9%) | 12 (9.2%) | 0.074 | 0.786 |
| No | 54 (91.5%) | 64 (90.1% | 118 (90.8%) | | |
| **Tonsilitis** | | | | | |
| Yes | 16 (27.1%) | 20 (28.2%) | 36 (27.7%) | 0.018 | 0.894 |
| No | 43 (72.9%) | 51 (71.8%) | 94 (72.3%) | | |

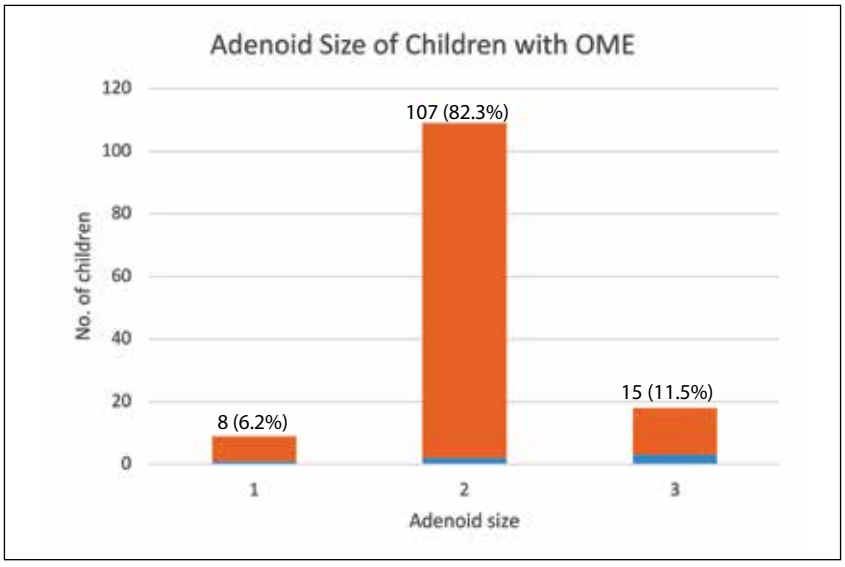
***Table II*** *- Risk factors of Children with OME.*

| Variables (n=65) | Non-Persistent | Persistent | Total | χ2 | p-value |
|---|---|---|---|---|---|
| **History of OME Siblings** | | | | | |
| Yes | 7 (11.9%) | 18 (25.4%) | 25 (19.2%) | 3.774 | 0.052 |
| No | 52 (88.1%) | 53 (74.6%) | 105 (80.8%) | | |
| **Allergic Rhinitis** | | | | | |
| Yes | 48 (81.4) | 14 (19.7) | 62 (47.7) | 49.073 | *<0.0001 |
| No | 11 (18.6) | 57 (80.3) | 68 (52.3) | | |
| **Passive Smoking** | | | | | |
| Yes | 20 (33.9%) | 30 (42.3%) | 50 (38.5%) | 0.95 | 0.33 |
| No | 39 (66.1%) | 41 (57.7%) | 80 (61.5%) | | |
| **History of Tonsillitis** | | | | | |
| Yes | 10 (16.9%) | 18 (25.4%) | 28 (21.5%) | 1.346 | 0.246 |
| No | 49 (83.1%) | 53 (74.6%) | 102 (78.5%) | | |
| **Breast Feeding** | 44 (74.6%) | 51 (71.8%) | 95 (73.1%) | | |
| < 6 months | 15 (25.4%) | 20 (28.2%) | 35 (26.9%) | 0.123 | 0.725 |
| ≥ 6months | | | | | |
| **Family Size** | | | | | |
| ≤ 3 people | 10 (16.9%) | 3 (4.2%) | 13 (10.0%) | Fisher's exact test | ***0.020** |
| > 3 people | 49 (83.1%) | 68 (95.8%) | 117 (90.0%) | | |
| **History of AOM** | | | | | |
| Yes | 5 (8.5%) | 6 (8.5%) | 11 (8.5%) | Fisher's exact test | 1 |
| No | 54 (91.5%) | 65 (91.5%) | 119 (91.5%) | | |

history of AOM infection and this was not statistically significant (p = 1.00) (**table II**). History of exposure to cigarette smokes or passive smoker showed no significant association between two groups (p = 0.33), 42.3% of children with persistent OME has been exposed to smokes compared to 33.9% of children with non-persistent OME. Duration of breastfeeding was also not statistically significant in developing OME in both persistent and non-persistent group (p = 0.725) (**table II**).

In this study, we did not include the OME children with grade 4 adenoid as it is one of the confounding factors in developing persistent OME. Among the 130 children recruited, 8 (6.2%) children had adenoid grade 1, 107 (82.3%) had grade 2 and 15 (11.5%) had grade 3 (**figure 4**).

***Figure 4*** *- Adenoid Size of Children with OME.*



Sixty-five out of 68 children with AR underwent skin prick test to evaluate the sensitivity to the common allergens. From this test, the 3 most common allergens were *Dermatophagoides pteronyssinus* (DP) 87.7%, *Dermatophagoides farinae (*DF) 86.2% and *Blomia tropicalis* 63.1% which are all aeroallergens. For food allergy, it is noted that 40% were allergic to prawn and crab, followed by 33.8% to squid, 16.9% to fish and 15.4% to chicken meat as shown in **figure 5**.

*Hearing Thresholds*

Two hundred thirty ears of children with OME were assessed with pure tone audiometry. The mean of hearing threshold for visit 1 and visit 2 were analyzed. In visit 1, the mean frequency and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the right ear were 33.38 dB (12.96), 33.50 dB (12.22), 32.74 dB (12.86), 29.36 dB (12.20), 28.76 dB (13.80), 28.68 dB (12.34) respectively. The mean frequency and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the left ear were 33.32 dB (13.10), 33.75 dB (12.84), 33.41 dB (13.05), 30.00 dB (12.54), 29.47 dB (13.45) and 28.76 dB (12.53), respectively. There were no significant differences across almost all frequencies between the right and left ears (**table III**).

After 3 months (Visit 2), the mean of every frequency was analyzed again. The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the right ear were 28.50 dB (12.87), 29.79 dB (12.97), 28.29 dB (13.17), 25.00 dB (12.46), 25.98 dB (13.02), and 24.70 dB (12.26), respectively. The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k

***Figure 5*** *- Positive Skin Prick Test for Allergens.*

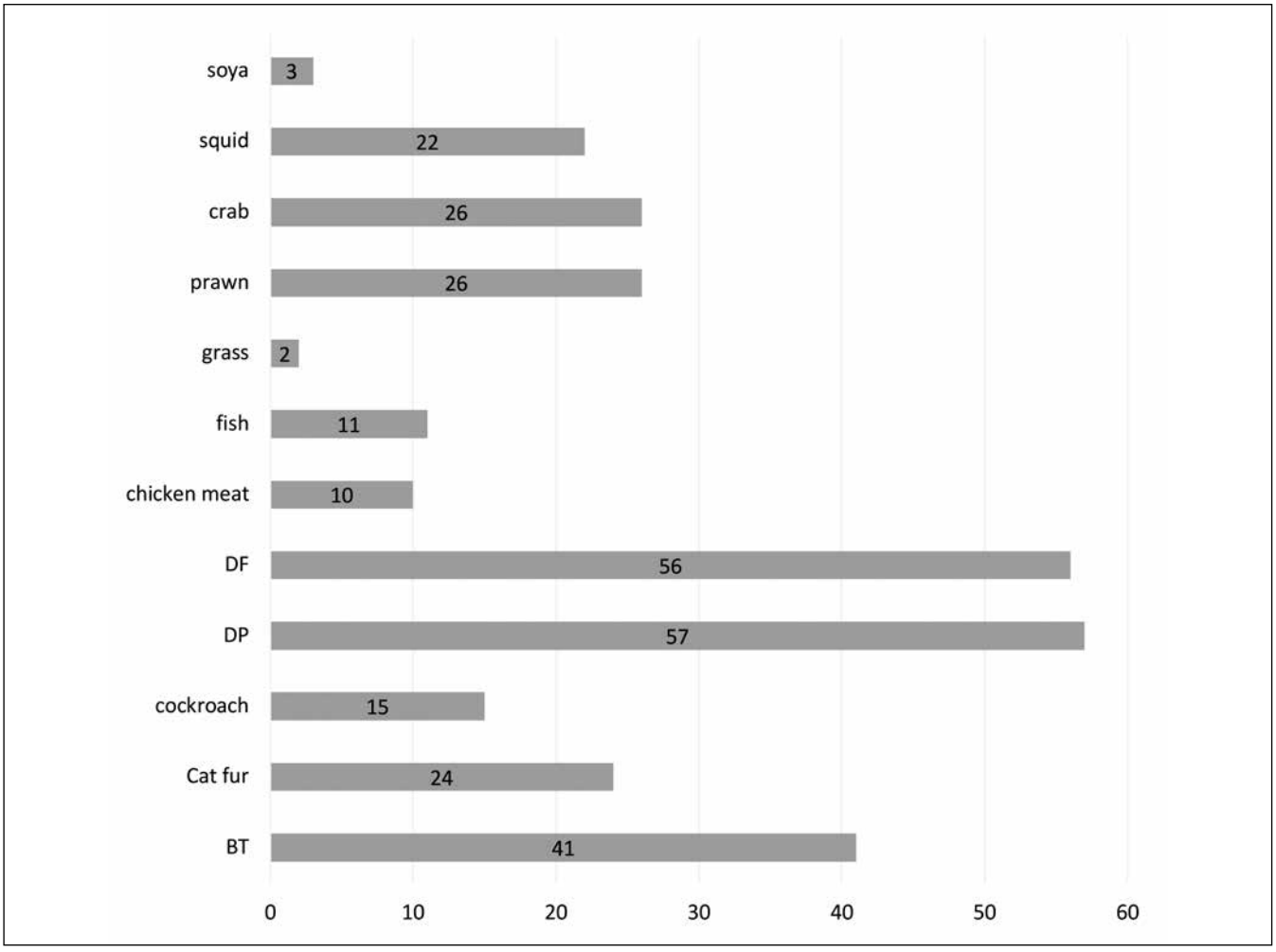


***Table III*** *- Hearing threshold in Visit 1 and Visit 2.*

| Descriptive | | | | Wilcoxon signed-rank test | |
|---|---|---|---|---|---|
| Frequency (Hz) | Right (n=117) Left (n=113) | Mean (SD) | | | |
| | | Visit 1 | Visit 2 | Mean Rank | p-value |
| 250 | R | 33.38 (12.96) | 28.50 (12.87) | 42.23 | **<0.0001** |
| | L | 33.32 (13.10) | 29.03 (13.62) | 47.20 | **<0.0001** |
| 500 | R | 33.50 (12.22) | 29.79 (12.97) | 46.89 | **<0.0001** |
| | L | 33.75 (12.84) | 30.31 (13.25) | 44.73 | **0.001** |
| 1000 | R | 32.74 (12.86) | 28.29 (13.17) | 43.00 | **<0.0001** |
| | L | 33.41 (13.05) | 30.66 (14.28) | 38.85 | **<0.0001** |
| 2000 | R | 29.36 (12.20) | 25.00 (12.46) | 45.06 | **<0.0001** |
| | L | 30.00 (12.54) | 27.39 (13.11) | 39.50 | **0.021** |
| 4000 | R | 28.76 (13.80) | 25.98 (13.02) | 44.59 | **0.029** |
| | L | 29.47 (13.45) | 26.73 (14.09) | 43.66 | **0.007** |
| 8000 | R | 28.68 (12.34) | 24.70 (12.16) | 47.84 | **0.001** |
| | L | 28.76 (12.53) | 25.97 (14.03) | 46.09 | **0.002** |

in the left ear were 29.03 dB (13.62), 30.31 dB (13.25), 30.66 dB (14.28), 27.39 dB (13.11), 26.73 dB (14.09) and 25.97 dB (14.03), respectively. There were significant differences for mean hearing threshold across tested frequency for right and left ears in Visit 1 and Visit 2. It showed that the hearing level significantly improved during Visit 2. From our study, we found that 500 Hz was the most vulnerable frequency which showed high hearing level of 33.50 dB ($p < 0.0001$). The low frequency hearing threshold was significantly affected compared to high frequency hearing threshold (**table III**).

The hearing threshold of children with OME with AR and non-AR was analyzed. The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the right ear of AR children were 36.35 (13.08), 35.63 (12.45), 35.24 (13.08), 31.51 (12.36), 31.51 (12.78). The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the left ear of AR children were 34.92 (13.59), 35.75 (12.87), 35.46 (13.85), 32.08 (12.40), 31.46 (14.51), 31.08 (12.88) (**table IV**). The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the right ear of non-AR children were 29.91 (12.03), 31.02 (11.54), 29.81 (12.05), 26.85 (11.62), 25.56 (13.37), 25.37 (11.02). The mean and standard deviation of 250 Hz, 500 Hz, 1 k, 2 k, 4 k and 8 k in the left ear of non-AR children were 31.15 (12.21), 31.04 (12.41), 30.63 (11.42), 27.19 (12.28), 26.77 (11.26), 25.63 (11.42). We found that there were significant differences between these two groups and conclude that children with OME and AR had a significant higher hearing threshold compared to non-AR (**table IV**).

## Discussion

Otitis media with effusion is an inflammatory process in the middle ear, which can occur as acute or chronic with a collection of non-purulent fluid behind the intact tympanic membrane. It is the most frequent cause of hearing loss in children and the most common reason for surgery (16).

In our study, the highest prevalence of OME was among children between the ages 4-8 (53%) and it tends to decrease in later ages. Our result was consistent with other studies, in which age was a risk factor for OME. Al-Humaid reported in their multivariate regression model, there was a statistically significant correlation between OME and age less than 8 years ($p < 0.0001$; OR = 5.052, 95% CI: 3.289 - 7.762) (17).

A number of studies reported a high prevalence of AR in children with OME. The prevalence of AR in OME children in our study was 52.3%. In a study conducted by Jordan et al, he reported that 74% (91/123) children with OME had allergic rhinitis (18). Lecks et al tested 82 children with OME using skin prick test and found that 72 (88%) of children had positive results. Alles et al reported 89% prevalence of AR in the OME children (8). In a recent study done by Pau and Ng, 7.5% of the AR group were found to have OME compared with 1.6% in the non-AR group ($p = 0.016$). Their study showed a significant difference in the prevalence of OME between AR and non-AR subjects (10).

The eustachian tube dysfunction is the most probable mechanism that can cause middle ear effusion. In children with AR, inflammation over the eustachian tube lining results in a

***Table IV*** *- Comparison of Hearing Level between OME Children with AR and non-AR.*

| Frequency (Hz) | Right (n=117) Left (n=113) | Descriptive | | | | Mann-Whitney U test | |
|---|---|---|---|---|---|---|---|
| | | AR | | Non-AR | | U-value | p-value |
| | | N | Mean rank | N | Mean rank | | |
| 250 | R | 63 | 66.48 | 54 | 50.27 | 1229.5 | **0.009** |
| | L | 65 | 60.83 | 48 | 51.81 | 1335.5 | **0.044** |
| 500 | R | 63 | 64.80 | 54 | 52.23 | 1317.0 | **0.034** |
| | L | 65 | 62.46 | 48 | 49.60 | 1320.5 | **0.036** |
| 1000 | R | 63 | 65.10 | 54 | 51.89 | 1342.5 | **0.048** |
| | L | 65 | 61.71 | 48 | 50.63 | 1261.5 | **0.015** |
| 2000 | R | 63 | 65.04 | 54 | 51.95 | 1311.0 | 0.145 |
| | L | 65 | 62.59 | 48 | 49.43 | 1205.0 | **0.037** |
| 4000 | R | 63 | 64.69 | 54 | 52.36 | 1254.0 | 0.073 |
| | L | 65 | 61.98 | 48 | 50.26 | 1196.5 | **0.033** |
| 8000 | R | 63 | 65.98 | 54 | 50.86 | 1236.5 | 0.058 |
| | L | 65 | 63.42 | 48 | 48.31 | 1143.0 | **0.015** |

negative middle ear pressure. Cytokines were assumed to be responsible among the inflammatory regulators of the middle ear inflammation of chronic OME (19). The role of allergy in persistent OME has been proposed following several mechanisms: 1, inflammation of the middle ear mucosa; 2, the inflammatory process of the eustachian tube; 3, nasal obstruction due to the inflammatory process; 4, aspiration of bacteria-laden allergic nasopharyngeal secretion into the middle ear cavity (20).

In this prospective study, we followed up 130 children with OME in 3 months duration. We found that 71 out of 130 (54.6%) children had persistent OME and 59 (45.4%) children had resolved OME. Pau and Ng reported, 85.7% of OME children showed resolution at 3 months follow up. However, in their study, the number of subjects at follow-up was too small to show any statistical differences in the rate of OME resolution (10). Rosenfeld found that OME of unknown duration had 28% spontaneous resolution by 3 months (95%, CI 14 - 41%), rising to 42% by 6 months (21). A higher percentage of resolution in our study was due to the use of intranasal corticosteroid and oral antihistamine in children with AR. Furthermore, the patients and guardian were counseled on the compliance of the medications.

We also found that 57 out of 71 (80.3%) children with persistent OME had allergic rhinitis. This indicates that allergy probably plays an important role in chronic or persistent OME rather than uncomplicated OME. Tracy et al reported the incident of atopy was 24% in a study of 59 children with persistent OME (22). Species of house dust mites that triggers atopy differs according to georgraphical location It is also influenced by the temperature and humidity of the country. The top 3 commonest dust mites reported in our study: *Dermatophagoides pteronyssinus (*DP), *Dermatophagoides farinae (*DF) and *Blomia tropicalis* are also the most common house dust mite species in Southeast Asia. Studies reported that they are sensitizing allergens among Malaysians and Singaporeans (13).

The studied risk factors on developing persistent OME is a large family household. Our study supported the findings of other studies in which children with persistent OME significantly had a larger number of family members in the household compared to non-persistent OME children (p = 0.04). Al-Humaid reported from their multivariate regression model, they found that family size of more than 4 members is one of the strongest predictors of OME (OR = 4.192, 95% CI: 2.033 - 8.643) (17). In Australia, Jacoby, found a higher incidence of persistent OME in Aboriginal children and they highlighted the need to reduce the crowding in the Aboriginal household (23). Other risk factors such as the history of AOM, tonsillitis, exposure to smoking or passive smoking, duration of breastfeeding and gender were not significant in this study.

Otitis media with effusion related hearing loss is the most important issues in childhood. Although the degree of hearing loss has been repeatedly labeled as “mild to moderate”, a specific frequency has seldom been reported. In our study, we studied the full-range frequency specific pure tone audiometry. The average hearing thresholds calculated based on 250 Hz, 500 Hz, 1 kHz, 2 kHz, 4 kHz, and 8 kHz are shown in **table III**. We found that OME produced a hearing loss up to 33 dB (mild hearing loss).

We also found that configuration of hearing loss is much more affected at low frequency. Previous experimental studies found that the primary mechanism in hearing loss at low frequencies is due to a reduction of the admittance of the middle-ear airspace due to the displacement of air with fluid (24).

In this study, there was a statistically significant in the improvement of the hearing threshold after 3 months of treatment. We found that, even though the OME had not fully resolved, the hearing level in these children were improved. Thus, we recommend that we should give longer watchful waiting time for the OME children with AR in view of there is a chance for improvement in the subsequent visit. Unnecessary surgery in this group of children can be avoided with optimum medical treatment.

Our study is different from others as we treated and we advised on the compliance of medications and we followed them up closely for 3 months to see the progression. Regarding the hearing assessment, we tested throughout all the frequency of hearing level and not just labelled "mild to moderate hearing loss", as we might miss the undetected improvement of the hearing level in a certain frequency. We also excluded the children with grade 4 adenoid enlargement as this is the confounding factor for persistent OME, so that we can prove the persistent OME is purely due to AR.

## Conclusions

There is a high prevalence rate of AR among children with OME which clarifies their association as intended by one of the objectives of this study. Allergic rhinitis children with OME had significant hearing loss compared to non-AR children with OM and the threshold improved significantly with medical therapy.

*Study implications*

We should optimize and counsel the patients and parents regarding the compliance of medical treatment for OME with AR to avoid. Children with persistent moderate to severe AR should be screened for OME and hearing assessment should be performed as they have higher tendency for developing hearing impairments. We should give longer watchful waiting time for OME children with AR before we embark on surgery.

*Recommendation*

We can improve our study by investigating the speech perception in noise as well as in quite using standardized test procedure to give improved "real world" understanding of the effects of OME on hearing abilities.

*Study limitation*

This is a prospective study which required follow up period of 3 months. In view of that, there was a high dropout rate in this study. We should recruit the control group (non OME children) to have a better comparison. Due to the budget limitation, we were unable to perform SPT in all OME children hence it was only done in AR children.

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Eur Ann Allergy Clin Immunol Vol 52, N.3, 131-141, 2020

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

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**Key words**

*anisakis; food allergy; diagnosis of anisakis sensitization; basophil activation test; Immunocap*

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**Doi**
10.23822/EurAnnACI.1764-1489.118

***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-Abellò (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-

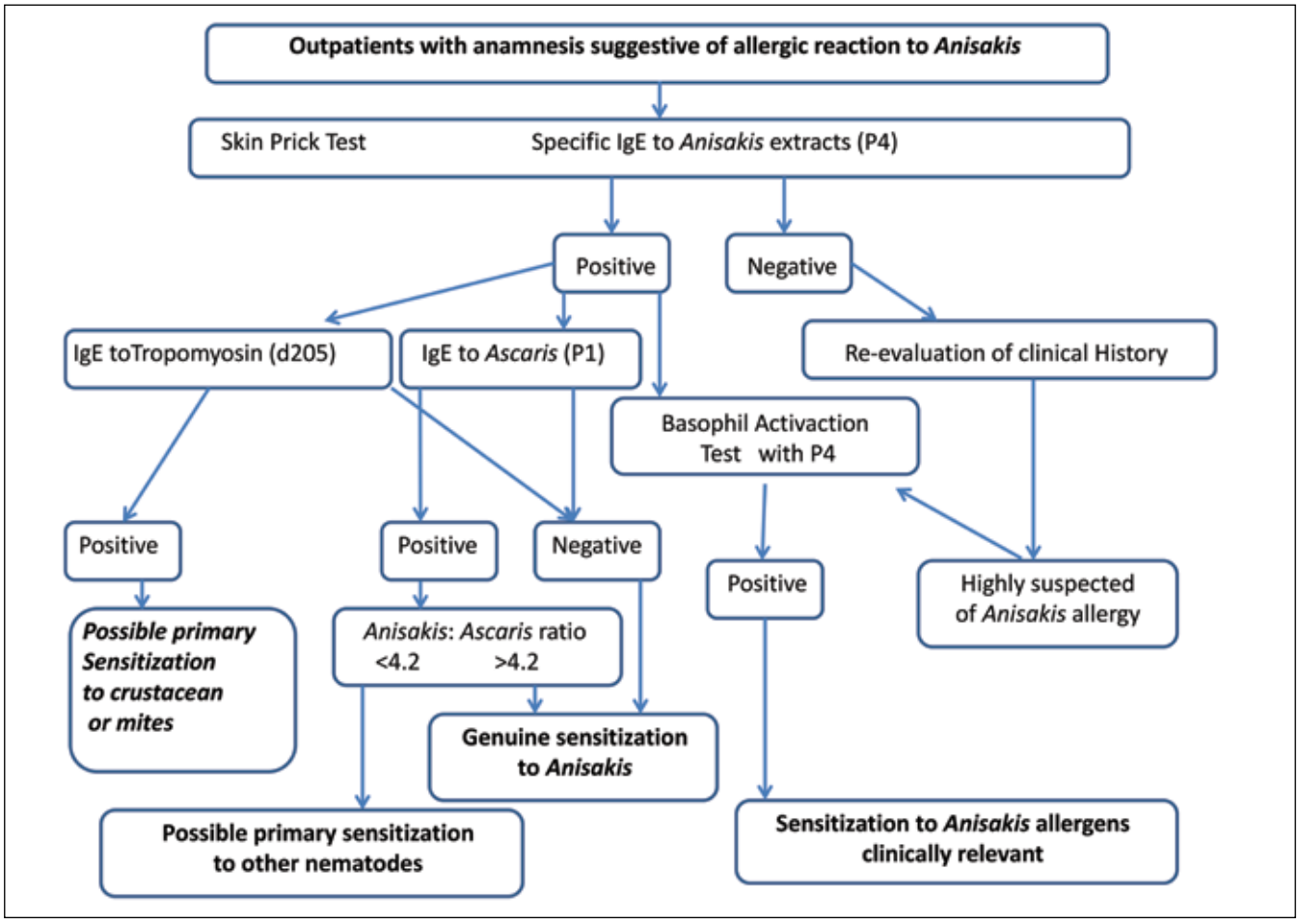
laxis) after eating fresh fish or in subjects at high risk exposure to contact with sea products and abstaining from fish ingestion; 2) a > 6 weeks documented spontaneous urticaria, defined as chronic urticaria (CU), presenting with or without angioedema. To this end, a structured questionnaire was designed 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 individuals 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%) | 0.0002 |
| inland | 66 (11.4%) | 11 (9.9%) | 55 (11.8%) | |
| 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 results 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 ImmunoCAP 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.*

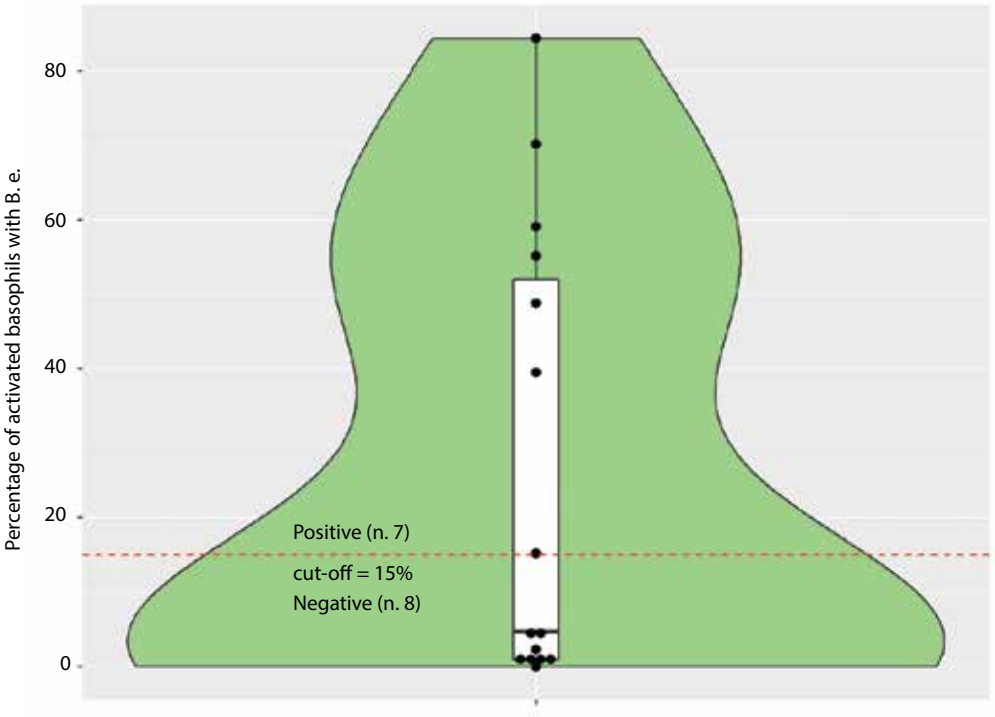
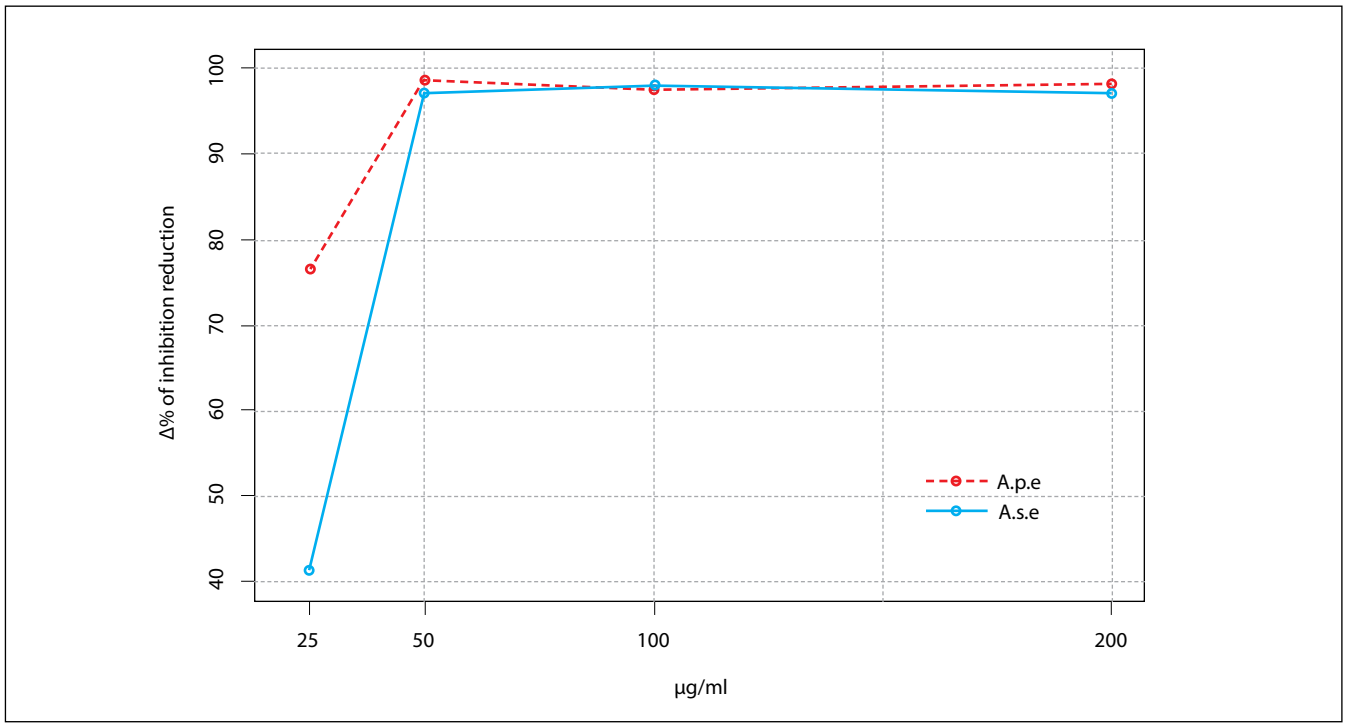


***Figure 3*** *- Cross-reactivity of Anisakis whole extracts. IgE reactivity to Anisakis ImmunoCAP (P4) competitively inhibited by pre-incubation of serum with various concentrations of A.p.e and A.s.e extracts.*




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. pegreffii* 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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