

# Ovomucoid (Gal d 1) specific IgE detected by microarray system predict tolerability to boiled hen's egg and an increased risk to progress to multiple environmental allergen sensitisation

C. Alessandri, D. Zennaro, E. Scala, R. Ferrara, M. Livia Bernardi, M. Santoro, P. Palazzo and A. Mari

Center for Molecular Allergology, IDI-IRCCS, Rome, Italy

## Clinical & Experimental Allergy

### Summary

**Background** Egg allergy is a very common finding in early childhood. Detecting hen's egg (HE) allergy outgrowing and reintroduction of food containing egg is a task for the allergist.

**Objective** We sought to evaluate the suitability of boiled egg food challenge compared with IgE to allergenic molecules from HE white using a microarray system.

**Method** Sixty-eight children referring to our centre by the family paediatricians for a suspected egg allergy were enrolled. Patients underwent double-blind, placebo-controlled food challenge with boiled and raw eggs. Challenge outcomes were compared with skin tests performed using egg white and yolk commercial extracts, to prick-prick test with boiled and raw egg white and yolk, total IgE, egg white specific IgE detected using ImmunoCAP and IgE to egg allergens available on the immunosolid phase allergen chip (ISAC) 103 microarray.

**Result** Nineteen subjects (28%) were reactive to both raw and boiled egg, 14 (20.5%) to raw egg only and 35 (51.4%) tolerated both boiled and raw egg. Efficiency analysis was carried out using both raw and boiled egg challenges as gold standard. Forty four of 47 Gal d 1 negative patients tolerated boiled egg (94%). Conversely, 20 of 21 Gal d 1 positive patients reacted to raw egg (95%). None of the other tests was able to discriminate patients' response to HE challenge. Furthermore, Gal d 1 positivity seems to lead to broader environmental allergen IgE sensitization.

**Conclusion and Clinical Relevance** The Gal d 1 IgE reactivity appears to be a very good predictor of HE clinical allergy. Gal d 1 positive children have a high frequency of HE allergy, whereas Gal d 1 negative children have a high frequency of tolerance to boiled egg. Multiple specific IgE detection by means of ISAC improves the diagnostic approach in HE allergic children, disclosing other food and inhalant allergic sensitizations, anyhow requiring a comprehensive clinical evaluation.

**Keywords** boiled egg, double-blind placebo-controlled food challenge, Gal d 1, Gal d 2, Gal d 3, Gal d 5, hen's egg allergy, IgE, microarray, raw egg, skin prick test

*Submitted 20 July 2011; revised 17 October 2011; accepted 21 October 2011*

### Correspondence:

Adriano Mari,  
Center for Molecular Allergology,  
IDI-IRCCS,  
Via dei Monti di Creta 104,  
00167 Rome, Italy  
E-mail: [adriano.mari@idi.it](mailto:adriano.mari@idi.it)

*Cite this as:* C. Alessandri, D. Zennaro,  
E. Scala, R. Ferrara, M. Livia Bernardi,  
M. Santoro, P. Palazzo and A. Mari,  
*Clinical & Experimental Allergy*, 2012  
(42) 441–450.

### Introduction

Hen's egg (HE) is a major source of high-quality proteins and essential nutrients, but a very common cause of food allergy in children at the same time [1, 2]. Two-thirds of food allergic children with atopic dermatitis, rhinitis, urticaria, or asthma are HE skin test or IgE positive [3, 4]. In some cases, life threatening or fatal

anaphylactic reactions can occur [5, 6], however, multiple clinical phenotypes can be observed in children with HE allergy on the basis of their reactivity [7]. In fact, positive specific IgE or skin prick tests (SPT) are signs of an allergic sensitization, rather than reflect the presence of clinical symptoms.

Ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovomucin (Gal d 3), and lysozyme (Gal d 4) have been

identified as major albumen allergens [8–10], mainly recognized by allergic children [11]. Gal d 5 [12, 13] and Gal d 6 [14] are yolk proteins more often recognized by adults [11]. Boiling eggs for at least 10 min determines the loss of more than 75% of allergenicity, whereas dry heat treatment provides a higher protein loss [15]. Heating does not completely destroy IgE-binding capacity of ovomucoid and ovalbumin but increases *in vitro* digestibility [16, 17]. This could explain why most children with egg allergy can tolerate egg in heated [18–20] and baked foods [21–23]. As cooking eggs causes fewer reactions, conformational B epitopes are deemed to play a role, mainly in ovomucoid (Gal d 1) sensitization [3, 22].

To evaluate the usefulness of the molecular diagnostic approach in children with suspected HE allergy, the response to SPT using the white boiled HE and specific IgE to HE allergens (Gal d 1, Gal d 2, Gal d 3 and Gal d 5) were compared with raw and boiled HE oral food challenge. Comparative results brought us to conclude that allergenic molecules might be helpful to better discriminate clinically allergic patients from sensitized ones.

## Methods

### Study population

The study has been carried out at the Centre for Molecular Allergology, IDI-IRCCS, Rome, Italy, during the period January 2008–September 2010. The studied population was represented by 68 children (47 male, 69.1%), age ranging from 1 to 11 years (median 4.1 years), referring to our centre by the family paediatricians for suspected HE allergy. The suspicion of HE allergy was based on reported history of reactions like asthma, rhinitis, conjunctivitis, urticaria, worsening of eczema, vomiting, other gastro-intestinal tract symptoms, anaphylaxis, after ingestion or contact with HE, and positive SPT or IgE to HE white extracts. At the time of consultation, all patients were following a HE elimination diet set before by other physicians. Forty two of them (62%) reported atopic dermatitis as the main allergic complain. All *in vivo* and *in vitro* tests performed in the present study are part of the routine diagnostic workup for HE allergic patients. Informed written consent was obtained by caregivers to perform food challenges.

Demographical data and clinical data, namely respiratory and ingestion-related symptoms on HE exposure, as well as all the *in vivo* and *in vitro* diagnostic data, were recorded for all patients by an allergy specialist and transferred from the laboratory into the InterAll software, a customized allergy electronic record for diagnostic and clinical data storing (InterAll version 3.0, Allergy Data Laboratories s.c., Latina, Italy).

### Oral food challenge

To verify the consistency of HE allergy diagnosis, double blind placebo-controlled HE challenges were carried out using commercially available eggs. Boiled eggs were prepared by putting fresh egg into water at room temperature, bringing water to boil and going ahead boiling for 10 min. A preparation of one boiled egg, mixed with 50 mL of non-sparkling water, two teaspoons of cocoa, and one spoon of white sugar were used as verum. Centrifuged boiled egg (or the raw one) was first mixed with cocoa allowing to create a homogeneous dispersion and increase the solubility in water that was added in increasing amount up to the 50 mL volume. The same preparation, without egg, was served as placebo. Similar mixture containing one whole raw egg was used as verum for raw egg challenge, whereas placebo was the same. To ensure consistency and masking, sugarless cocoa (Cacao Amaro, Perugia, Italy), free of any nut contamination, was used for all preparations. A higher amount of cocoa and sugar was used for the placebo to obtain the same aspect as the verum.

Children were admitted to our centre in the morning, in a fasting state; administration of antihistamines, if any, was stopped at least 1 week prior to skin test and oral challenge, to avoid any effect on test outcomes. None of the patients was under steroid treatment. Double-blind, placebo-controlled food challenge (DBPCFC) was first carried out with boiled egg. Blinded active and placebo meals were randomly administered in identical doses on separate days, prepared immediately before challenge [24, 25]. Boiled HE challenge was performed first, by administering an initial dose of 0.1 mL and, in case of no reactions in the next 20 min, by progressively increasing the egg amount by a factor 5 (0.5, 2, 10, 50 mL) up to the ingestion of one egg, being the cumulative HE protein dose approximately 6 g [26]. Challenge was stopped at first appearance of symptoms accompanied by objective signs. Patients tolerating boiled HE were then challenged with raw HE in a similar way, whilst patients with positive response to boiled HE challenge were considered likely to be allergic to both boiled and raw eggs even because, after preliminary discussions with parents and caregivers, a reaction to raw eggs was considered obvious and none of them accepted the second challenge. Adverse reactions were recorded as (i) local, like not objective symptoms, restricted to perioral skin or oral mucosa directly in contact with the HE preparation, oral allergy syndrome or isolated digestive complaints, or (ii) systemic, giving objective signs and symptoms, involving organs far from the site of initial contact with the allergen, thus requiring absorption and dissemination, as urticaria, asthma, or anaphylaxis. For the purpose of the study, only reactions to DBPCFC occurring within 6 hours

from the first dose were considered. Severity of symptoms was graded according to Sampson [27].

### *Skin prick test*

Skin prick test was performed using both commercial extracts (Allergopharma, Reinbek, Germany) and fresh eggs using the prick-prick technique. Freshly prepared reagents were the white and yolk from both raw and processed boiled HE. The SPT were performed in duplicated on the volar surface of the forearm by the same investigator throughout the study, following the European Academy of Allergy and Clinical Immunology recommendations, using a 1 mm-tipped lancets (Stallergenes, Antony, France) [28]. Histamine phosphate (10 mg/mL; Allergopharma) and a glycerol-saline solution were used as positive and negative tests, respectively. The SPT results were recorded as wheal areas and expressed in mm<sup>2</sup>, by outlining the wheal with a felt-tipped pen after 15 min, and transferring it onto a recording paper sheet using transparent tape. Paper sheets were scanned and transformed into electronic images. The inner side of the drawings was digitized and the relevant area was calculated [29]. Wheal areas were stored into the InterAll patients' records (Allergy Data Laboratories). The SPT were performed before DBPCFC and considered positive when a wheal area greater than 7 mm<sup>2</sup> was recorded.

### *Total and specific immunoglobulin E determination using ImmunoCAP and immunosolid phase allergen chip*

Total and specific IgE were determined in patients' sera from venous blood samples obtained at the time of enrolment, before the first food challenge. Total and specific IgE levels for HE white and yolk were detected using ImmunoCAP (Phadia AB, Uppsala, Sweden) following manufacturer's instructions. Specific IgE detection for Gal d 1, Gal d 2, Gal d 3 and Gal d 5 were measured using ISAC 103 microarray test (PMD, Vienna, Austria); ISAC tests were performed as previously reported [11].

### *Statistics*

Descriptive statistics included frequencies for qualitative variables, and median value for most quantitative variables. Specific IgE and SPT results were expressed using median value, with interquartile ranges (25th percentile–75th percentile) (IQR). To assess test performances, receiver operating characteristic (ROC) analyses were performed on SPT, ImmunoCAP and ISAC specific IgE, using both raw and boiled egg DBPCFC as gold standards. Sensitivity and specificity, proving the diagnostic performance of each test, were estimated at the optimal cut-off

level, bordering the upper left corner in the ROC curve and optimizing both sensitivity and specificity. Significances in intra-group comparisons were evaluated by the Wilcoxon test. Serum IgE values were analysed using non-parametric Mann–Whitney *U* and Kruskal–Wallis tests for repeated independent measures. The frequencies of adverse reactions between active and placebo groups were compared by means of  $\chi^2$  test or Fisher's exact test (the latter used for two-by-two contingency tables for less than 50 observations). All tests were used with two-sided options and significance level was set at a *P* value < 0.05. Statistical analysis was performed with the SPSS package (SPSS Inc., Chicago, IL, USA) and SAS software (SAS Institute Inc., Cary, CN, USA).

## **Results**

### *Hen's egg challenge*

As no placebo reactions were recorded in the DBPCFC, HE challenge, used as gold standard, allowed us to divide the 68 patients into three groups on the basis of their immediate reactions: Group A (Allergic), including 19 patients with positive DBPCFC to boiled egg white, thus considered most likely reactive to both heated and raw eggs; Group PT (Partially Tolerant), including 14 children reacting to raw egg but tolerating boiled egg; Group ST (Sensitized but Tolerant), including 35 subjects with negative DBPCFC for both raw and boiled eggs.

Group characteristics and allergic symptoms recorded after DBPCFC are illustrated in Table 1. Patients from Group A showed more severe symptoms after DBPCFC, as one case of anaphylaxis and seven asthmatic reactions were recorded.

### *Skin prick test*

Skin prick test to commercial egg extracts, and prick-prick to raw and boiled egg materials performances are shown in Table 2 and Fig. 1. A statistically significant difference (*P* < 0.01) in median wheal area value was detected when comparing group A (98 mm<sup>2</sup> median wheal area for raw egg white SPT) with group PT (62 mm<sup>2</sup>) to group ST (13 mm<sup>2</sup>). The same trend was observed for boiled egg white SPT, with median wheal area significantly different from 54 mm<sup>2</sup> to 17 mm<sup>2</sup> to 0 mm<sup>2</sup> in the A, PT, and ST groups, respectively (*P* < 0.01).

### *Total and specific Immunoglobulin E determination using ImmunoCAP and immunosolid phase allergen chip*

**Total Immunoglobulin E.** Total IgE median level in the overall study group was 198 IU/L (IQR 71–423). The median value of total IgE was 285 IU/L (IQR 188–830)

**Table 1.** Group characteristics and allergic symptoms recorded after hen's egg double-blind placebo-controlled challenges

|                           | Hen's egg reactivity Groups* |      |       |
|---------------------------|------------------------------|------|-------|
|                           | A                            | PT   | ST    |
| <b>Number of subjects</b> |                              |      |       |
| Subjects                  | 19                           | 14   | 35    |
| Male/Female               | 15/4                         | 9/5  | 23/12 |
| DBPCFC                    |                              |      |       |
| Raw Egg Reactive          | Yes                          | Yes  | No    |
| Boiled egg reactive       | Yes                          | No   | No    |
| Median age                | 4.33                         | 3.17 | 4.42  |
| Atopic dermatitis         | 16                           | 5    | 8     |
| <b>Diagnostic tests°</b>  |                              |      |       |
| CAP (IU/L)                |                              |      |       |
| Total IgE                 | 285                          | 134  | 152   |
| SPT (mm <sup>2</sup> )    |                              |      |       |
| Raw egg white             | 80                           | 62.4 | 0     |
| Boiled egg white          | 50.3                         | 18   | 0     |
| Egg white commercial      | 24.5                         | 10.5 | 0     |
| Raw egg yolk              | 17                           | 24   | 0     |
| Boiled egg yolk           | 1                            | 0    | 0     |
| Egg yolk commercial       | 11.8                         | 7    | 0     |
| CAP (kU <sub>a</sub> /L)  |                              |      |       |
| Egg white                 | 7.75                         | 1.82 | 0.45  |
| Egg yolk                  | 1.64                         | 0    | 0     |
| ISAC (kU <sub>a</sub> /L) |                              |      |       |
| Gal d 1                   | 2.30                         | 0    | 0     |
| Gal d 2                   | 0.13                         | 0    | 0     |
| Gal d 3                   | 0                            | 0    | 0     |
| Gal d 5                   | 0                            | 0    | 0     |
| <b>DBPCFC symptoms°°</b>  |                              |      |       |
| Number of subjects        |                              |      |       |
| Anaphylaxis               | 1                            | 0    | 0     |
| Asthma                    | 7                            | 3    | 0     |
| Gastritis/vomiting        | 11                           | 10   | 0     |
| Enteritis                 | 2                            | 2    | 0     |
| Urticaria angioedema      | 11                           | 8    | 0     |
| Rhino-conjunctivitis      | 7                            | 6    | 0     |
| Oral allergic syndrome    | 3                            | 3    | 0     |

\*Hen's egg reactivity groups: 'A' subjects clinically allergic to raw and boiled egg; 'PT' subjects reacting to raw egg but tolerant to boiled egg; 'ST' subjects sensitized to hen's egg allergens but tolerant to raw and boiled egg.

°Median values.

°°Symptoms recorded after DBPCFC refer to boiled egg challenge for group A, and to raw egg challenge for group PT.

in group A, 134 IU/L (IQR 63–269) in group PT, and 152 IU/L (IQR 38–421) in group ST. No significant differences were detected by comparing the three groups of patients.

**ImmunoCAP.** Receiver operating characteristic curve for specific egg white IgE shows that the best criterion for sensitivity and specificity optimization is the IgE value > 1.2 kU<sub>a</sub>/L towards raw egg DBPCFC, and IgE > 2.2 kU<sub>a</sub>/L towards boiled egg DBPCFC (Fig. 1).

Statistically significant different specific IgE levels were detected in the A and PT groups when compared with the ST one: group A 7.75 kU<sub>a</sub>/L (range 2.9–16.1;  $P < 0.001$ ), group PT 1.55 kU<sub>a</sub>/L (range 1.02–4.7;  $P < 0.007$ ), group ST had 0.45 kU<sub>a</sub>/L (range 0–1.23) (Fig. 2).

**ImmunoSolid phase allergen chip.** Forty four of 47 Gal d 1 negative patients (94%) could tolerate boiled egg. Conversely, 20 of 21 Gal d 1 positive patients (95%) reacted to raw egg. Gal d 1 performance results showed 84% sensitivity and 90% specificity versus boiled egg DBPCFC (Table 2). Gal d 1 ROC curve for ISAC test shows that the best criterion for sensitivity and specificity optimization is specific IgE value  $\geq 0$  towards both raw and boiled egg DBPCFC (Fig. 1). Gal d 1 IgE in the three groups showed progressively higher levels of specific IgE comparing group ST to group PT ( $P = 0.04$ ), and a  $P < 0.001$  in all the other comparison (Fig. 3). Gal d 1 performance results showed 89% sensitivity and 92% specificity versus boiled egg DBPCFC (Table 2). None of the studied patients reacted to Gal d 5 (Table 2). The relationship between IgE to HE white detected using ImmunoCAP and IgE to Gal d 1 detected using ISAC was investigated. Statistics showed no concordance between the two tested preparations (Fisher's exact test  $P < 0.1$ ) and a significant correlation was found for  $P < 0.001$  using the Spearman correlation test, but the  $r$  value was low ( $r = 0.5$ ) (data not shown).

Testing 103 allergenic molecules for IgE using the microarray approach allowed us to detect sensitizations also to other important allergenic sources as shown in Fig. 4. Allergenic molecules were considered either as markers of allergen group sensitization (Fig. 4, Panel A) or as markers of sensitization to a given allergenic source (Fig. 4, Panel B–C; i.e. Bos d 4, 5, 8 for milk allergy, Phl p 1, 2, 4, 5, 6, 11 for grass pollen allergy). Additional details on allergens are given in Fig. 4 legend. Thirteen patients had no additional IgE detectable sensitizations on the ISAC103. Ten were in the ST group, three in the PT group, and none in the A group. Evaluating the 54 subjects being positive for at least one non-HE allergen, there is a general trend to record higher sensitization prevalence to other food and non-food allergens in group A, with some exception. Within allergenic molecule groups (Fig. 4, Panel A) an increasing sensitization prevalence has been recorded for LTP and profilin groups, no differences for parvalbumin, inverted sensitization prevalence for Bet v 1-like molecules, inconclusive for tropomyosin. Within food allergenic sources (Fig. 4, Panel B), cow's milk represented the most frequent sensitizer in all studied groups with the same decreasing trend among the three clinical groups, showing statistically significant differences (42.1% group A, 35.7% group PT, 22.9% group ST;

Table 2. Overall test performance results as obtained by receiver operating characteristic analysis comparing all diagnostic tests performed in the study and using raw and boiled hen's egg double-blind placebo-controlled food challenge (DBPCFC) as gold standards

| Gold standard | Test                   | Allergen preparations | Optimal cut-off point | Sensitivity (%) | Sensitivity 95% CI | Specificity (%) | Specificity 95% CI | +LH | -LH  | PPV (%) | NPV (%) |
|---------------|------------------------|-----------------------|-----------------------|-----------------|--------------------|-----------------|--------------------|-----|------|---------|---------|
| Boiled egg    | SPT (mm <sup>2</sup> ) | Egg white extract     | 8                     | 84              | 67.2–94.7          | 77              | 59.9–89.6          | 4   | 0.2  | 77      | 84      |
|               |                        | Raw egg white         | 48                    | 88              | 71.8–96.6          | 80              | 63.1–91.6          | 4   | 0    | 81      | 88      |
|               |                        | Boiled egg white      | 11.6                  | 85              | 68.1–94.9          | 94              | 80.8–99.3          | 15  | 0.2  | 93      | 87      |
|               |                        | Egg yolk extract      | 0                     | 70              | 51.3–84.4          | 83              | 66.4–93.4          | 4   | 0.3  | 79      | 74      |
|               |                        | Raw egg yolk          | 8.4                   | 79              | 61.1–91            | 80              | 63.1–91.6          | 4   | 0.3  | 79      | 80      |
|               | CAP (kUa/L)            | Boiled egg yolk       | 4.3                   | 45              | 28.1–63.6          | 94              | 80.8–99.3          | 8   | 0.6  | 88      | 65      |
|               |                        | Egg white             | 1.23                  | 79              | 61.1–91.0          | 71              | 53.7–85.4          | 3   | 0.3  | 72      | 78      |
|               |                        | Egg yolk              | 0.11                  | 64              | 45.1–79.6          | 88              | 72.5–96.7          | 5   | 0.4  | 84      | 71      |
|               | ISAC (kUa/L)           | Gal d 1               | 0                     | 61              | 42.1–77.1          | 97              | 85.1–99.9          | 21  | 0.4  | 95      | 72      |
|               |                        | Gal d 2               | 0.21                  | 40              | 22.9–57.9          | 94              | 80.8–99.3          | 7   | 0.6  | 87      | 62      |
| Raw egg       | SPT (mm <sup>2</sup> ) | Gal d 3               | 0.06                  | 18              | 7.0–35.5           | 100             | 90–100             |     | 0.82 | 100     | 57      |
|               |                        | Gal d 5               | NA                    | NA              | NA                 | NA              | NA                 | NA  | NA   | NA      | NA      |
|               |                        | Egg white extract     | 11                    | 89              | 66.9–98.7          | 73              | 58.9–85.1          | 3   | 0.1  | 57      | 95      |
|               |                        | Raw egg white         | 71.2                  | 79              | 54.4–93.9          | 90              | 77.8–96.6          | 8   | 0.2  | 75      | 91      |
|               |                        | Boiled egg white      | 23.3                  | 95              | 74.0–99.9          | 86              | 72.8–94.1          | 7   | 0    | 72      | 98      |
|               | CAP (kUa/L)            | Egg yolk extract      | 10.7                  | 58              | 33.5–79.7          | 86              | 72.8–94.1          | 4   | 0.4  | 61      | 84      |
|               |                        | Raw egg yolk          | 8.4                   | 68              | 43.4–87.4          | 59              | 44.2–73            | 2   | 0.5  | 39      | 66      |
|               |                        | Boiled egg yolk       | 4                     | 53              | 28.9–75.6          | 86              | 72.8–94.1          | 4   | 0.5  | 59      | 82      |
|               | ISAC (kUa/L)           | Egg white             | 2.25                  | 84              | 60.4–96.6          | 75              | 61.1–86.7          | 3   | 0.21 | 57      | 92      |
|               |                        | Egg yolk              | 0.11                  | 84              | 60.4–96.6          | 81              | 67.4–91.1          | 4   | 0.19 | 64      | 92      |
| DBPCFC        | SPT (mm <sup>2</sup> ) | Gal d 1               | 0                     | 84              | 60.4–96.6          | 90              | 80.4–97.7          | 8   | 0.18 | 76      | 94      |
|               |                        | Gal d 2               | 0                     | 53              | 28.9–75.6          | 84              | 70.3–92.7          | 3   | 0.51 | 57      | 82      |
|               | CAP (kUa/L)            | Gal d 3               | 0.41                  | 21              | 6.1–45.6           | 98              | 89.1–99.7          | 10  | 0.81 | 80      | 76      |
|               |                        | Gal d 5               | NA                    | NA              | NA                 | NA              | NA                 | NA  | NA   | NA      | NA      |
|               | ISAC (kUa/L)           | Gal d 5               | NA                    | NA              | NA                 | NA              | NA                 | NA  | NA   | NA      | NA      |

CAP, IgE detection using ImmunoCAP system (Phadia, Sweden); CI, Confidence interval; ISAC, IgE detection using ISAC 103 microarray system (PMD, Austria); +LR, positive likelihood ratio; –LR, negative likelihood ratio; NA, not assessed; NPV, negative predictive value; PPV, positive predictive value; SPT, skin prick test.

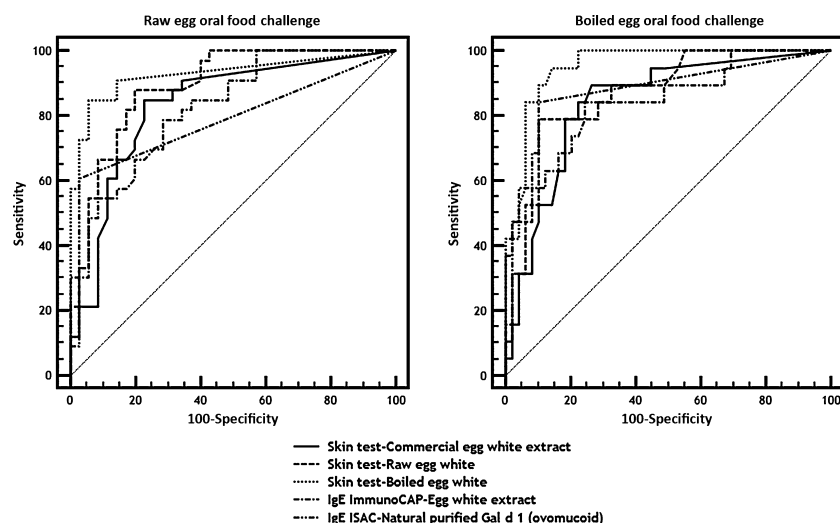


Fig. 1. Receiver operating characteristic (ROC) analyses performed on Skin Test, ImmunoCAP and immunosolid-phase allergen chip (ISAC) specific IgE, using both raw (left panel) and boiled (right panel) egg double-blind placebo-controlled food challenge as gold standards.

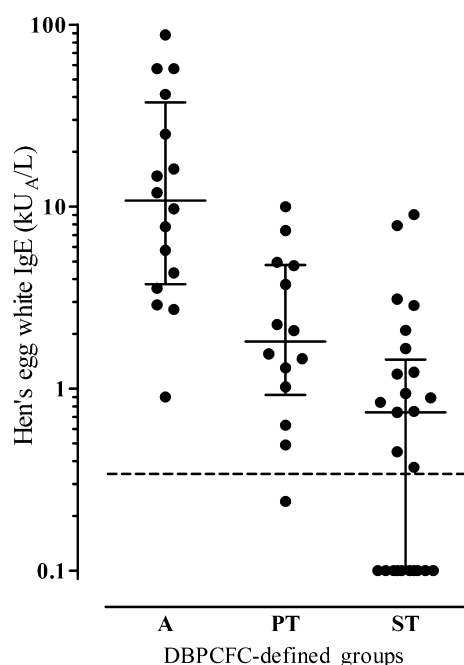


Fig. 2. Hen's egg white specific IgE detected by the ImmunoCAP system in the three study groups. Groups: 'A' subjects clinically allergic to raw and boiled egg; 'PT' subjects reacting to raw egg but tolerant to boiled egg; 'ST' subjects sensitized but tolerant to raw and boiled egg. Median and interquartile range are shown. Kruskal–Wallis test showed statistically significant differences for  $P < 0.0001$ .

$P < 0.01$ ). Within allergenic sources causing symptoms by inhalation (Fig. 4, Panel C), patients belonging to group A had the highest prevalence of inhalant allergenic molecule sensitization rate, being grass allergens the most frequent sensitizers (68.4% group A, 43% group PT, 40% group ST;  $P < 0.01$ ), followed by olive pollen, mites, cypress pollen, and mammal epithelia.

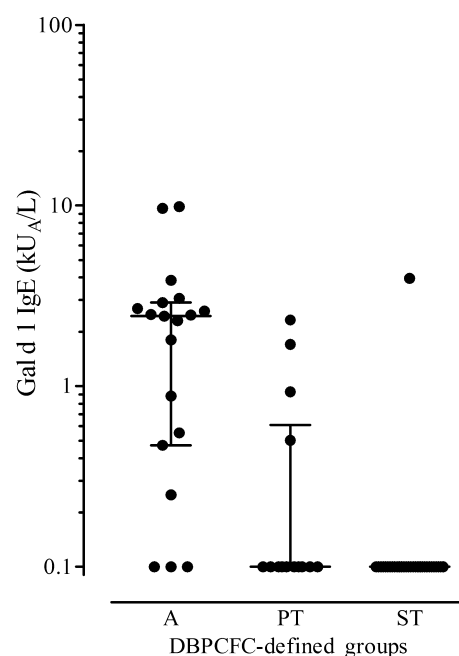


Fig. 3. Specific IgE to Gal d 1 detected using the ISAC103 microarray system in the three hen's egg sensitized groups: A (Allergic), PT (Partially Tolerant), ST (Sensitized but Tolerant). Median and interquartile ranges are reported. Kruskal–Wallis test showed statistically significant differences for  $P < 0.0001$ .

## Discussion

In the present study, we compared 'classical' diagnostic approaches in HE allergy, by means of DBPCFC and specific IgE detection, with an allergenic molecule-based microarray IgE testing. We herein report how Gal d 1 IgE reactivity let us to recognize boiled HE allergic patients, thus supporting the advantage of using boiled

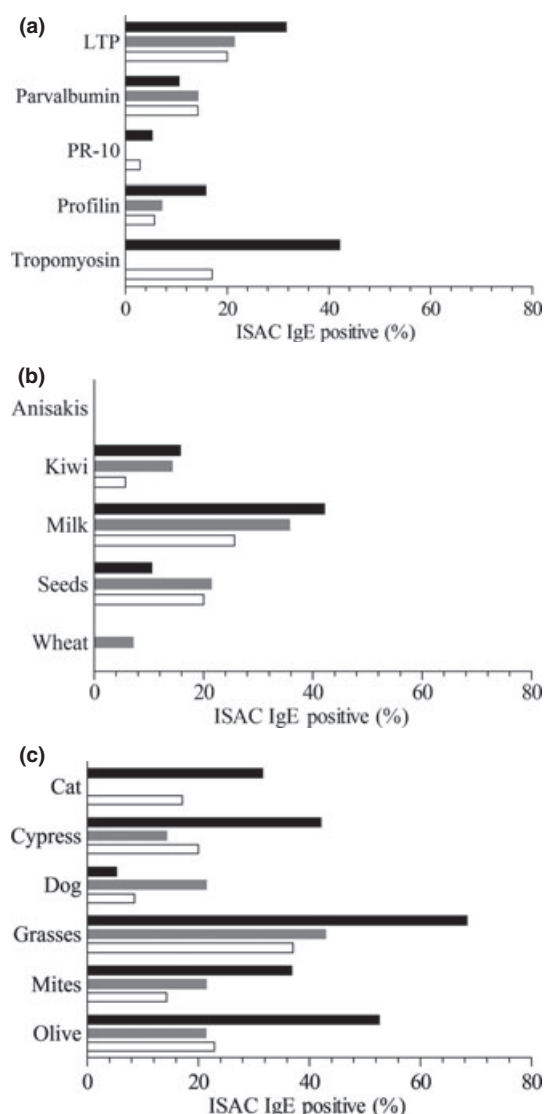


Fig. 4. Additional allergen sensitizations detecting IgE using the immunosolid-phase allergen chip (ISAC)103 microarray system in the three hen's egg sensitized groups: A (Allergic; black bar), PT (Partially Tolerant; grey bar), ST (Sensitized but Tolerant; white bar). At least one positive IgE result for one of the following allergens has been evaluated for grouping by allergenic molecule groups and allergenic sources: Panel A – LTP: Cor a 8, Pru p 3; Parvalbumin: Cyp c 1, Gad c 1; PR-10: Act d 11, Api g 1, Ara h 8, Bet v 1, Cor a 1.0101, Cora 1.0401, Dau c 1, Mald 1, Pru p 1, Gly m 4; Profilin: Bet v 2, Hev b 8, Mer a 1, Ole e 2, Phl p 12; Tropomyosin: Der p 10, Ani s 3, Pen a 1, Pen i 1, Pen m 1; Panel B – Anisakis: Ani s 1; Kiwi: Act d 1, Act d 2, Act d 5; Cow's milk: Bos d 4, Bos d 5, Bos d 8; Seeds: Ana o 2, Ara h 1, Ara h 2, Ara h 3, Ber e 1, Cor a 9, Gly m 5, Gly m 6, Ses i 1; Wheat: Tri a 18, Tri a 19, Tri a Gliadin; Panel C – Cat: Fel d 1, Cypress: Cry j 1, Cup a 1; Dog: Can f 1, Can f 3; Grasses: Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 11; Mites: Der p 1, Der p 2, Der f 1, Der f 2, Eur m 2; Olive: Ole e 1. Detailed information about the nature of additional IgE positive allergens detected using ISAC microarray system can be obtained from <http://www.allergome.org>.

HE DBPCFC as the first step in the diagnostic workup of HE allergic patients and Gal d 1 as marker of full HE reactivity.

Up to now, food allergy diagnosis is based on patient history [30], in combination with SPT and/or measurement of allergen-specific IgE [31, 32]. In the case of HE allergy, a significant percentage of patients have specific IgE levels towards HE white or HE proteins below the suggested positive decision point [18, 23, 33, 34], and DBPCFC remains the gold standard to confirm the suspected food allergy [30]. Egg white IgE levels < 2.5 kU/L and a skin test wheal diameter of egg white commercial extract equal or smaller than 3 mm was associated with a 50% pass rate during the supervised oral egg challenge [32].

In our study 51% patients were sensitized to HE but tolerated HE even if they had positive SPT or specific HE IgE.

Raw eggs (fresh or lyophilized) have been employed in most of DBPCFC published in literature, [2, 35], but few data are available on cooked HE challenge [2, 23]. In our study, DBPCFC was performed not only for raw HE, but also for boiled HE, letting us to identify a distinct group of 'partially tolerant' patients, able to eat boiled but not raw HE. Furthermore, our study suggest that a different approach could be adopted for HE cooking, much shorter than in Ando's study [23] where 60 min at 90°C were used in the their study protocol, compared to 10 min at 100°C in the present study [15]. Gal d 1 is considered as the main HE allergen [36] because of the presence of disulphide bonds allowing its tertiary structure to remain unaltered, and let it retain its allergenicity after heating and proteolytic digestion. As from reports in the literature, we argue that our HE processing reduces but does not eliminate the Gal d 1 IgE binding capacity [18, 37–39]. We can thus speculate that a different IgE epitope recognition pattern characterizes patients belonging to our study groups. Overall, our heat processing might either destroy some IgE epitopes or facilitate the following gastrointestinal digestion of Gal d 1. We cannot exclude that the two phenomena could be combined and generate the non-responsiveness of test positive patients.

Recently published results, obtained in a different population of HE allergic patients, showed that yolk negative patients were by trend younger, that IgE reactivity to Gal d 5 works as marker for yolk sensitization, and that in the course of the allergic disease children raise additional IgE antibodies first against other egg white allergens (i.e. Gal d 3 and Gal d 4) and then to yolk allergens [26]. These findings are confirmed by the present study and by our previous survey on a huge allergic population [11]. Although additional yolk

sensitization does not seem to cause more severe symptoms, it can worsen the quality of life because it expands dietary restrictions.

As for any diagnostic test sensitivity and specificity are the main performance parameters.

The best performance on the analysed ROC curve, using as gold standard the raw and boiled egg DBPCFC, was provided by the boiled white SPT (Fig. 1), with a criterion set at  $>11.6 \text{ mm}^2$ .

Gal d 1 negative children (specific IgE = 0 kU<sub>a</sub>/L) showed a high frequency of tolerance to boiled egg (94%). Conversely, Gal d 1 positive children showed a high frequency of raw HE allergy versus a criterion set at  $>0 \text{ kU}_a/\text{L}$ . Therefore Gal d 1 IgE positive results might be considered as the marker of raw and boiled egg clinical reactivity.

It is worth noting that, in terms of sensitivity, ISAC test showed a rather low sensitivity. The possible explanation is that egg contains several allergens, and patients reacting to raw egg challenge and negative to a specific HE allergen on ISAC test are most likely sensitized to some of the other egg allergens rather than Gal d 1. On the other hand, all egg allergens but Gal d 1 are destroyed using heat, and this explains the high sensitivity of Gal d 1 ISAC test against boiled egg DBPCFC. Nevertheless, it is important to underline that the majority of the subjects tested negative for HE allergens on ISAC were in the PT and ST groups, where a partial or total tolerance has been achieved by patients. It could be argued that as reported in literature, outgrowing clinical allergy leads to negativity of tests later on, and ISAC IgE detection reaches such condition before all other tests. This suggestion requires anyhow additional studies before it can be taken as a useful index in our hands, mainly using allergenic molecules on different diagnostic systems as for ImmunoCAP and ISAC in the present study.

Recently Clark et al suggested that guidelines for reintroduction of cooked egg should be modified using IgE levels to HE components, after definition of some biomarkers [40]. We would add the suggestion to include specific IgE detection to Gal d 1 on the ISAC microarray as a further good marker for the same purpose.

Whether regular ingestion of cooked egg might allow the development of raw egg tolerance remains to be demonstrated [41]. Even if oral food challenge remains the standard for the diagnosis of egg allergy [2], in case of Gal d 1 negative patients, a DBPCFC with well-cooked eggs can avoid elimination diets and expensive and potentially dangerous protocols of raw egg oral

induction of tolerance in highly supervised settings [42]. It is worth noting that if well-cooked egg is tolerated, then cooked egg in a wheat matrix can be even better tolerated [43]. It is however mandatory to alert patients about the risk of ingesting raw or not well done egg [19].

The evaluation of 103 allergenic molecules by means of the ISAC microarray approach allowed us to detect significant clinical sensitizations to other important allergenic sources confirming that HE sensitization is often associated with other sensitizations to food and inhalants [44]. Several additional food and non-food allergen were detected positive in all three groups, but the Gal d 1 positive/boiled egg allergic showed the highest trend to other sensitization, having all subjects who already got a sensitization to another allergenic source. The extended IgE evaluation using ISAC is highly relevant for an early intervention and start of appropriate treatments without additional blood drawing and large amount of serum required [45]. It remains that detecting IgE does not mean clinical allergy hence a careful clinical evaluation for each positive test is required much focusing on any clinical tolerance status for food allergens and any pre-clinical status for inhalant allergens.

## Conclusion

IgE detection to Gal d 1 on the ISAC microarray system, and possibly by any other system where the whole panel of HE allergens are available, in patients tested positive for HE extracts seems to be a useful tool to predict oral tolerance to boiled eggs. DBPCFC in egg allergic children should be performed first with boiled eggs to introduce at least well cooked eggs in HE allergic children's diet. Moreover, multiple specific IgE evaluation by means of ISAC improves the diagnostic approach in HE allergic children, whose sensitization to other food and inhalant allergens would otherwise remain unknown.

## Acknowledgements

We are grateful to Silvia Monti and Giorgio Perotti for their timely support in patients' data management, and to Debora Pomponi, Marina Liso, and Chiara Rafaiani for their routine work on ISAC IgE testing.

The study has been supported by the Italian Ministry of Health, Programma Ricerca Corrente 2008–2010.

*Conflict of interest:* The other authors declare no conflict of interest.

## References

- 1 Eggesbo M, Botten G, Halvorsen R, Magnus P. The prevalence of allergy to egg: a population-based study in young children. *Allergy* 2001; 56:403–11.
- 2 Caubet JC, Wang J. Current understanding of egg allergy. *Pediatr Clin North Am* 2011; 58:427–43.
- 3 Cooke SK, Sampson HA. Allergenic properties of ovomucoid in man. *J Immunol* 1997; 159:2026–32.
- 4 Heine RG, Laske N, Hill DJ. The Diagnosis and Management of Egg Allergy. *Curr Allergy Asthma Rep* 2006; 6:145–52.
- 5 Sampson HA, Mendelson L, Rosen JP. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N Engl J Med* 1992; 327:380–4.
- 6 Allen CW, Campbell DE, Kemp AS. Egg allergy: are all childhood food allergies the same? *J Paediatr Child Health* 2007; 43:214–8.
- 7 Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA. Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy* 2007; 62:758–65.
- 8 Langeland T. A clinical and immunological study of allergy to hen's egg white. III. Allergens in hen's egg white studied by crossed radio-immuno-electrophoresis (CRIE). *Allergy* 1982; 37:521–30.
- 9 Hoffman DR. Immunochemical identification of the allergens in egg white. *J Allergy Clin Immunol* 1983; 71:481–6.
- 10 Holen E, Elsayed S. Characterization of four major allergens of hen egg-white by IEF/SDS-PAGE combined with electrophoretic transfer and IgE-immunoautoradiography. *Int Arch Allergy Appl Immunol* 1990; 91:136–41.
- 11 Scala E, Alessandri C, Bernardi ML et al. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy* 2010; 40:911–21.
- 12 Sansosti A, Negro Alvarez JM, Pagan Aleman JA, Martinez Quesada J, Postigo I. Bird-egg syndrome. *Arch Allergy Immunol Clin* 2009; 40:95–6.
- 13 Mandallaz MM, De Weck AL, Dahinden CA. Bird-egg syndrome. Cross-reactivity between bird antigens and egg-yolk livetins in IgE-mediated hypersensitivity. *Int Arch Allergy Appl Immunol* 1988; 87:143–50.
- 14 Amo A, Rodriguez-Perez R, Blanco J et al. Gal d 6 is the second allergen characterized from egg yolk. *J Agric Food Chem* 2010; 58:7453–7.
- 15 Fu TJ, Maks N, Banaszewski K. Effect of heat treatment on the quantitative detection of egg protein residues by commercial enzyme-linked immunosorbent assay test kits. *J Agric Food Chem* 2010; 58:4831–8.
- 16 Martos G, Lopez-Exposito I, Benchari-tiwong R, Cecilia Berin M, Nowak-Wegrzyn A. Mechanisms underlying differential food allergy response to heated egg. *J Allergy Clin Immunol* 2011; 127:990–7.
- 17 Ouahidi I, El Hamsas AE, Aarab L. Modulation of egg white protein allergenicity under physical and chemical treatments. *Food Agric Immunol* 2011; 22:57–68.
- 18 Urisu A, Ando H, Morita Y et al. Allergenic activity of heated and ovomucoid-depleted egg white. *J Allergy Clin Immunol* 1997; 100:171–6.
- 19 Eigenmann PA. Anaphylactic reactions to raw eggs after negative challenges with cooked eggs. *J Allergy Clin Immunol* 2000; 105:587–8.
- 20 Des Roches A, Nguyen M, Paradis L, Primeau MN, Singer S. Tolerance to cooked egg in an egg allergic population. *Allergy* 2006; 61:900–1.
- 21 Konstantinou GN, Giavi S, Kalobatsou A et al. Consumption of heat-treated egg by children allergic or sensitized to egg can affect the natural course of egg allergy: hypothesis-generating observations. *J Allergy Clin Immunol* 2008; 122:414–5.
- 22 Lemon-Mule H, Sampson HA, Sicherer SH, Shreffler WG, Noone S, Nowak-Wegrzyn A. Immunologic changes in children with egg allergy ingesting extensively heated egg. *J Allergy Clin Immunol* 2008; 122:977–83.
- 23 Ando H, Moverare R, Kondo Y et al. Utility of ovomucoid-specific IgE concentrations in predicting symptomatic egg allergy. *J Allergy Clin Immunol* 2008; 122:583–8.
- 24 Bindslev-Jensen C, Ballmer-Weber BK, Bengtsson U, Blanco C et al. Standardization of food challenges in patients with immediate reactions to foods – position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 2004; 59:690–7.
- 25 Nowak-Wegrzyn A, Assa'ad AH, Bahna SL, Bock SA, Sicherer SH, Teuber SS. Work Group report: oral food challenge testing. *J Allergy Clin Immunol* 2009; 123:S365–83.
- 26 Egger M, Alessandri C, Wallner M et al. Is aboriginal food less allergenic? Comparing IgE-reactivity of eggs from modern and ancient chicken breeds in a cohort of allergic children. *PLoS ONE* 2011; 6:e19062.
- 27 Sampson HA. Anaphylaxis and emergency treatment. *Pediatrics* 2003; 111:1601–8.
- 28 Dreborg S, Frew A. Position paper: allergen standardization and skin tests. *Allergy* 1993; 48:49–53.
- 29 Mari A, Di Felice G, Afferni C et al. Assessment of skin prick test and serum specific IgE detection in the diagnosis of Cupressaceae pollinosis. *J Allergy Clin Immunol* 1996; 98:21–31.
- 30 Sampson HA. Update on food allergy. *J Allergy Clin Immunol* 2004; 113:805–19.
- 31 Host A, Andrae S, Charkin S et al. Allergy testing in children: why, who, when and how? *Allergy* 2003; 58:559–69.
- 32 Knight AK, Shreffler WG, Sampson HA et al. Skin prick test to egg white provides additional diagnostic utility to serum egg white-specific IgE antibody concentration in children. *J Allergy Clin Immunol* 2006; 117:842–7.
- 33 Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* 2001; 107:891–6.
- 34 Celik-Bilgili S, Mehl A, Verstege A et al. The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. *Clin Exp Allergy* 2005; 35:268–73.
- 35 Tripodi S, Di Rienzo Businco A, Alessandri C, Panetta V, Restani P, Matri-cardi PM. Predicting the outcome of oral food challenges with hen's egg through skin test end-point titration. *Clin Exp Allergy* 2009; 39:1225–33.
- 36 Bernhisel-Broadbent J, Dintzis HM, Dintzis RZ, Sampson HA. Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice. *J Allergy Clin Immunol* 1994; 93:1047–59.

- 37 Matsuda T, Tsuruta K, Nakabe Y, Nakamura R. Reduction of ovomucoid immunogenic activity on peptic fragmentation and heat denaturation. *Agric Biol Chem* 1985; **49**:2237–41.
- 38 Fu TJ, Abbott UR, Hatzos C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid—a comparative study. *J Agric Food Chem* 2002; **50**:7154–60.
- 39 Urisu A, Yamada K, Tokuda R *et al*. Clinical significance of IgE-binding activity to enzymatic digests of ovomucoid in the diagnosis and the prediction of the outgrowing of egg white hypersensitivity. *Int Arch Allergy Immunol* 1999; **120**:192–8.
- 40 Clark A, Islam S, King Y *et al*. A longitudinal study of resolution of allergy to well-cooked and uncooked egg. *Clin Exp Allergy* 2011; **41**:706–12.
- 41 Allen CW, Kemp AS, Campbell DE. Dietary advice, dietary adherence and the acquisition of tolerance in egg-allergic children: a 5-yr follow-up. *Pediatr Allergy Immunol* 2009; **20**: 213–8.
- 42 Garcia Rodriguez R, Urrea JM, Feo-Brito F *et al*. Oral rush desensitization to egg: efficacy and safety. *Clin Exp Allergy* 2011; **41**:1289–96.
- 43 Kato Y, Oozawa E, Matsuda T. Decrease in antigenic and allergenic potentials of ovomucoid by heating in the presence of wheat flour: dependence on wheat variety and intermolecular disulfide bridges. *J Agric Food Chem* 2001; **49**:3661–5.
- 44 Kulig M, Bergmann R, Niggemann B, Burow G, Wahn U. Prediction of sensitization to inhalant allergens in childhood: evaluating family history, atopic dermatitis and sensitization to food allergens. The MAS Study Group. Multicentre Allergy Study. *Clin Exp Allergy* 1998; **28**:1397–403.
- 45 Soderstrom L, Lilja G, Borres MP, Nilsson C. An explorative study of low levels of allergen-specific IgE and clinical allergy symptoms during early childhood. *Allergy* 2011; **66**:1058–64.