To the Editor,

Allergy to hen's eggs is among the most common hypersensitivity in children (1) whereas, allergy to eggs from other species' is less frequent and usually observed in patients already allergic to hen's eggs (2). So far, only few reports suggest the possibility of selective immunoreactivity to proteins from eggs of different birds (2,3).
The principal egg white allergens' reported in the literature are: ovomucoid (Gal d 1), ovoalbumin (Gal d 2), lysozyme (Gal d 4) and ovotrasferrin (Gal d 3) (4).

The yolk shows generally a minor allergenic potential and the main protein involved is the alpha-livetin (Gal d 5), responsible for the bird-egg syndrome (5). The role of others egg yolk allergens, including protein YGP-42 (Gal d 6), vitellenin (apovitellenin I) and apoprotein B (apovitellenin VI), (5).

Heat treatments commonly lead to the loss of allergenic potential; this is the case of white egg proteins (6). Some children suffering from egg allergy tolerate cooked egg better than the raw one (7).

In June 2017, a 14-year-old girl was referred to the Allergy Unit of Anna Meyer Children Hospital because of the appearance of itching of the throat, vomiting and facial urticarial plus oedema, 30 minutes after the consumption of goose's egg, as an omelet. She had eaten goose's egg only once before without any clinical reaction and tolerated chicken eggs. Parents signed an informed consent.

Skin Prick Tests (SPTs) with commercially available extract (ALK-Abelló, Madrid, Spain) of common inhaled allergens and of hen's egg proteins (ovalbumin and ovomucoid) and Prick-By-Prick test (PPT) with raw egg white and yolk from: duck (*Anas domesticus*), goose (*Anser domesticus*), hen (*Gallus domesticus*), turkey (*Meleagris gallopavo*) and Caliphornia quail (*Callipepla californica*), were performed. As a positive and negative controls histamine dichlorohydrate at 10 mg/mL and saline solution at 0.9% concentration were respectively used. The SPTs and PPT results were considered positive if the diameter of the wheal was at least 3 mm, after fifteen minutes. The patient was positive to grass pollen and dust mite and negative to hen's egg proteins. PPT was positive only for goose's

egg yolk (7 mm) and white (7 mm).

Blood was drown for total and specific serum IgEs measurement with Enzyme-immunoassay using Pharmacia CAP System uniCAP® (Pharmacia Diagnostics, Uppsala, Sweden) following the manufacturer's instructions. Specific IgE levels for chicken's egg ovoalbumin, ovomucoid, and egg white were negative (< 0.1 kUI/L). Total serum IgEs were 196 kUI/L. The results of skin tests and serum specific IgE confirmed the selective sensitization to goose's eggs.

Residual serum was used in immunoblotting. In order to evaluate the impact of heating treatment on the protein immunoreactivity, samples of eggs from hen and goose were analysed as raw and cooked (omelet) samples. Egg white, yolk and raw and cooked omelet samples were freeze-dried and solubilized in Sample Buffer (0.125 Tris HCl pH 6.8, 1% Sodium Dodecyl Sulphate, 2.5% β-mercaptoethanol, 3.75% glycerol, containing bromophenol blue as a run marker) at the final concentration of 5, 10 and 5 mg/mL, respectively. Sample proteins were separated using SDS-PAGE in a gradient gel (12-22% acrylamide). After the electrophoretic run (90 V at room temperature, for approximately 4 h) gels were dyed with Coomassie Brilliant Blue G-250. All materials and instruments were ~~purchased~~ from Sigma Aldrich (Milan, Italy).

The pattern of specific IgE binding to egg proteins was evaluated by immunoblotting (8). After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Merck Millipore, Darmstadt, Germany) by western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad) using 25 mM Tris, 193 mM glycine and 10% methanol transfer buffer, and incubated with the serum from the patient. Briefly, the membrane was dry at room temperature for 2 hours and washed three times with 0.25% gelatin solution (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton-X). The membrane was then immersed in 10 mL of 0.25% gelatin solution containing 0.3 mL of serum from the patient. Antigen-IgE complex was detected using goat anti-human IgE antibodies (Sigma Aldrich, Milan, Italy) labeled with alkaline phosphatase. After incubation for 4 h at room temperature, the membrane was washed and incubated with bromochloroindolyl phosphate/nitroblue tetrazolium solution till colour development.

The electrophoretic patterns of raw and cooked eggs are shown in **Figure 1**. The use of pre-stained molecular weight standard solution allowed the identification of the principal egg allergens.

The profile of egg white proteins shows three main proteins having molecular weights (MW) ranging from 6 and 81 kDa; ovalbumin (40-48 kDa) and ovotransferrin (66-77 kDa) were easily identified in all white samples. Ovoalbumin appeared as a wide diffuse band from 27 to 46 kDa and was characterized by different electrophoretic mobility between the various species, as previously described (3). A band corresponding to lysozyme (14 kDa) was observed only in hen's egg white sample.

Several protein bands, between 6 and 200 kDa, were present in the yolk samples, being mostly correlated with livetins (9).

Heating determines a significant reduction of the protein abundance and immunoreactivity in hen's egg, while goose's egg maintains a profile similar to raw samples, preserving its immunoreactivity. Heating can strongly influence the structure of proteins with the production of polymers or aggregates having high MW. These aggregates are normally characterized by covalent bounds that are mantained during denaturation with SDS sample buffer. When the MW is particularly high, aggregates cannot enter the gel pores and the abundance of protein bands appears reduced.

Circulating IgEs from the patient's serum was challenged versus both raw and cooked omelet (**Figure 1**).

Immunoblotting shows a mild IgE interaction with proteins present in the egg white samples, while the patient presented strong reaction to all yolk and raw omelet samples. Proteins recognized by the serum had a molecular weight of approximately 60-115 kDa.

In this case, serum IgE from the patient recognized proteins with MW between 60-115 kDa. Among these proteins, band A (MW 70 kDa) was identified as the known allergen alpha-livetin.

A strong reactivity was observed against two proteins having MW of 95 and 110

kDa, previously identified by Guilmineau et al. (2005) as an apovitellenin and an apovitellin, respectively. The second one (band B) was the most immunoreactive component. Apovitellins are the apoproteins of lipovitellins (high-density lipoproteins of egg yolk) and their MWs, determined by electrophoretic analysis, are the only information at disposal from the scientific literature. Apovitellins consist of five major polypeptides between 31-110 kDa; the latter is the most abundant and has the same MW of the reactive band B (10).

This study underlines the different behaviour of yolk proteins from hen and goose at heating processes , which could explain the patient's tolerance to hen's eggs despite her allergy to egg from goose.

This means that a patient allergic to hen egg may tolerate eggs from other birds, but this could be also associated with the way of cooking eggs. Heating process may influence the tolerability by reducing or even enhancing the allergenicity of eggs from different birds.

In conclusion, to our knowledge, is the first time that an apovitellin has been reported as a possible egg allergen; in addition this study highlights the different proteins thermal resistance among avian species, and its role in modulating allergenicity.

References

1. Nwaru BI, Hickstein L, Panesar SS et al. Prevalence of common food allergies in Europe: A systematic review and meta-analysis. *Allergy Eur J Allergy Clin Immunol* 2014: **69**:992–1007.
2. Contreras FJC, Muñoz MTG, Mateos MAM et al. Allergy to quail ' s egg without allergy to chicken ' s egg . *Case report*. 2008: **36**:234–237.
3. Añíbarro B, Seoane FJ, Vila C et al. Allergy to eggs from duck and goose without sensitization to hen egg proteins. *J Allergy Clin Immunol* 2000: **105**:834–836.
4. Mine Y, Yang M. Recent Advances in the Understanding of Egg Allergens: Basic, Industrial, and Clinical Perspectives. *J Agric Food Chem* 2008: **56**:4874–4900.
5. Caubet J-C, Wang J. Current understanding of egg allergy. *Pediatr Clin North Am* 2011: **58**:427–443.
6. David NA, Penumarti A, Burks AW et al. Food allergen extracts to diagnose food-induced allergic diseases: How they are made. *Ann Allergy, Asthma Immunol* 2017: **119**:101–107.
7. Leonard SA, Nowak-Wegrzyn AH. Baked Milk and Egg Diets for Milk and Egg Allergy Management. *Immunol Allergy Clin North Am* 2016: **36**:147–159.
8. Cudowska B, Kaczmarski M, Restani P. Immunoblotting in the diagnosis of cross-reactivity in children allergic to birch. Rocz Akad Med Bialymst 2005: **50**: 268-73.
9. Chalamaiah M, Esparza Y, Temelli F et al. Physicochemical and functional properties of livetins fraction from hen egg yolk. *Food Biosci* 2017: **18**:38–45.
10. Guilmineau F, Krause I, Kulozik U. Efficient analysis of egg yolk proteins and their thermal sensitivity using sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and nonreducing conditions. *J Agric Food Chem* 2005: **53**:9329–9336.

Figure legend

Figure 1 – SDS-PAGE (upper panel) and Immunoblotting (lower panel) of raw and cooked egg samples

W–egg white; Y–yolk; Ob–omelet before cooking; Oa–omelet after cooking; MK–Molecular weight marker solution; α-liv–α-livetin; OVT–ovotransferrin; OVA–ovalbumin; LYS–lysozyme

Characteristic of the gradient gel:

Gradient running gel: 12-22% acrylamide; 0.11-0.20% bis-acrylamide; 0.36 M TRIS-HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.06% ammonium persulfate; and 0.30% N,N,N',N'- tetramethylenediamine (TEMED).

Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS-HCl buffer pH 6.8; 0.1% SDS; 0.12% ammonium persulfate; and 0.25% (TEMED).

Running buffer: 25 mM TRIS, 0.19 M glycine and 0.1% SDS (w/v), pH 8.8.

Molecular Weight Marker Solution (prestained broad range, Bio-Rad): myosin (199.3 kDa), β-galactosidase (114.2 kDa), bovine serum albumin (81.4 kDa), ovalbumin (46.6 kDa), carbonic anhydrase (33.9 kDa), soybean trypsin inhibitor (27.6 kDa), lysozyme (17.8 kDa) and aprotinine (6.1 kDa).