Mapping Sequential IgE-Binding Epitopes on Major and Minor Egg Allergens

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Keywords

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Abstract

Introduction: Molecular studies of hen's egg allergens help define allergic phenotypes, with IgE to sequential (linear) epitopes on the ovomucoid (OVM) protein associated with a persistent disease. Epitope profiles of other egg allergens are largely unknown. The objective of this study was to construct an epitope library spanning across 7 allergens and further evaluate sequential epitope-specific (*ses*-)IgE and *ses*-IgG₄ among baked-egg reactive or tolerant children. **Methods:** A Bead-Based Epitope Assay was used to identify informative IgE epitopes from 15-mer overlapping peptides covering the entire OVM and ovalbumin (OVA) proteins in 38 egg allergic children. An amalgamation of 12 B-cell epitope prediction tools was developed using experimentally identified epitopes. This ensemble was used to predict epitopes from ovotransferrin, lysozyme, serum albumin, vitellogenin-II fragment, and vitellogenin-1 precursor. *Ses*-IgE and *ses*-IgG₄ repertoires of 135 egg allergic children (82 reactive to baked-egg, the remaining 52 tolerant), 46 atopic controls,

and 11 healthy subjects were compared. **Results:** 183 peptides from OVM and OVA were screened and used to create an aggregate algorithm, improving predictions of 12 individual tools. A final library of 65 sequential epitopes from 7 proteins was constructed. Egg allergic children had higher *ses*-IgE and lower *ses*-IgG₄ to predominantly OVM epitopes than both atopic and healthy controls. Baked-egg reactive children had similar *ses*-IgG₄ but greater *ses*-IgE than tolerant group. A combination of OVA-sIgE with *ses*-IgEs to OVM-023 and OVA-028 was the best predictor of reactive phenotype. **Conclusion:** We have created a comprehensive epitope library and showed that *ses*-IgE is a potential biomarker of baked-egg reactivity.

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Introduction

Epitope mapping – identification of immunodominant residues on allergens, has been instrumental in discovering molecular mechanisms responsible for food allergies [1, 2]. In allergic patients, IgE antibodies bind to a

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variety of epitopes, that is, sites of short amino acid (AA) sequences on an allergenic protein and activate effector functions of mast cells and basophils [3].

In hen's (*Gallus gallus*) egg allergy, ovomucoid (OVM) has been identified as the major allergenic protein [4], with OVM-depleted egg-white having diminished effect during food challenges and on serological IgE levels [5]. Egg allergic children that generate a diverse OVM epitope-specific IgE repertoire present a more severe and persistent disease phenotype [6, 7]. Three other proteins in egg-white have been determined to be allergenic in a subset of patients: ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (LYS) [8–10]. Egg yolk is generally considered less allergenic, with IgE in adults mostly associated with bird-egg syndrome – a sensitization to airborne avian antigens (e.g., feathers, droppings) [11]. Nonetheless, several immunogenic proteins in yolk have been related to egg allergy: serum albumin (sALB), vitellogenin-1 precursor (YGP42), and vitellogenin-II fragment (YGP40) [12–15]. Aside from a few studies in OVA [16, 17] and LYS [9], much less is known about epitope mapping in minor allergens and the contribution of those IgE-binding epitopes to the pathogenesis of egg allergy.

B-cell epitopes are usually 15–20 AA residues in length and consist of either conformational (discontinuous) or sequential (linear or continuous) peptides, or both [18, 19]. Conformational epitopes are AA residues located far apart within the sequence that are brought together via protein folding, that is, are dependent on the tertiary structure of the protein; while sequential epitopes are AA residues located continuously along the protein backbone structure [18]. In food allergy, high temperature (cooking) or digestion induces protein denaturation, altering the protein's tertiary structure and resulting in destruction of conformational epitopes, thus reducing the food's allergenicity [20]. About 70%–80% of children with egg allergy are able to tolerate baked-egg products [21, 22] and have a more favorable prognosis, generally outgrowing allergy by 6 years of age [23]. IgE antibodies to conformational epitopes were shown to be associated with transient egg allergy that resolves as children's intestinal permeability and enzymatic output mature, while IgE to sequential epitopes was indicative of persistent allergy, with greater OVM-specific IgE diversity associated with greater severity of allergic reactions [1, 6, 24]. It is still unknown if sequential epitopes from other egg allergens contribute to baked-egg reactivity.

Many experimental techniques have been developed for mapping sequential B-cell epitopes, including determining crystal structure of antigen-antibody complexes,

peptide library screening, and antigen perturbation by enzymatic digestion, heat, and/or hydrolysis. Laboratory methods, however, are generally costly and vary greatly (e.g., different epitope lengths) leading to the emergence of computational approaches [18, 19]. These approaches facilitate laboratory studies by quantitating how likely a given AA sequence will be immunogenic, thus reducing the need for testing all possible peptides. A combination of those tools showed promising results in identifying epitopes in shrimp [25], cockroach [26, 27], and peanut [28] allergens. In this work, we set out to construct an egg epitope library spanning across 7 egg proteins by combining both experimental and in silico techniques. We then compared sequential epitope-specific (*ses*-)IgE and *ses*-IgG₄ repertoires among egg allergic children that are either reactive or tolerant to baked-egg, and atopic and healthy controls.

Materials and Methods

Study Design

First (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000519618), overlapping peptides covering the entire length of OVM and OVA proteins were assayed in plasma or serum of 38 egg allergic children with egg-white specific (s)IgE >0.35 kU_A/L (ImmunoCAP; Uppsala, Sweden), obtained from the Food Allergy Research Initiative Biorepository at Mount Sinai. Allergy status of these patients was based on sIgE or skin prick testing and/or a convincing history, unequivocal allergic reactions following oral food challenge, or the ingestion of egg. A negative pool was included as a control and consisted of serum from 4 adults with no history of any food or environmental allergies. Based on the experimental data, immunodominant peptides, that is, epitopes were identified (step 2).

For the 3rd step, publicly available epitope prediction tools (Table 1) were used for B-cell epitope mapping for both OVM and OVA. The predictions were then compared to the experimentally identified epitopes. In step 4, predictions from those tools were combined into a single algorithm using Random Forest to optimize the prediction accuracy. After the final algorithm was established, it was applied to predict epitopes on minor egg allergens (step 5): OVT, LYS, and sALB. Sixteen epitopes were selected for testing in the subjects described above (step 6). Additionally, 8 epitopes from YGP40 and YGP42 proteins identified based on a literature search were included [15]. Protein sequences, as well as UniProt and Protein Data Bank identifiers, are provided in online supplementary Table 1.

Next (step 7), the 65 most informative epitopes were selected from all proteins, which constituted an egg epitope library for further testing. Finally (step 8), this epitope library was used to evaluate *ses*-IgE and *ses*-IgG₄ levels in egg allergic children enrolled as part of the Consortium for Food Allergy Research (CoFAR7, NCT01846208) trial [29], atopic controls with food allergies other than egg from the CoFAR2 study [30], and nonatopic (healthy) children with no medical or family history of food and environ-

Table 1. Online epitope prediction tools

Algorithm name	Prediction level, length	Algorithm type	Antibody isotype specificity	URL
Chou and Fasman beta-turn	AA (varying)	Structural	No	
Emini surface accessibility	AA (varying)	Structural	No	
Karplus and Schulz flexibility	AA (varying)	Structural	No	
Kolaskar and Tongaonkar antigenicity	AA (varying)	Structural	No	http://tools.iedb.org/bcell/
Parker hydrophilicity	AA (varying)	Structural	No	
Bepipred 2.0	Peptide (varying)	ML – random forest	No	
BCPred 1.0	Peptide (varying)	ML – string kernel	No	http://ailab.ist.psu.edu/bcpred/
ABCpred	Overlapping peptides (16-mer)	ML – neural network	No	http://crdd.osdd.net/raghava/abcpred/
BCIgeEPRED	Overlapping peptides (15-mer)	ML – random forest	Yes, IgE	https://github.com/brsaran/BCIgePred
CBTOPE	AA (varying)	ML – support vector machine	No	http://crdd.osdd.net/raghava/cbtope/
DiscoTope v2.0	AA (varying)	Structural	No	http://www.cbs.dtu.dk/services/DiscoTope/
Ellipro	Peptide (varying)	Structural	No	http://tools.iedb.org/ellipro/

Twelve open access online B-cell epitope prediction tools listed with algorithm name; type and length of the predicted sequences (sequential peptides or individual AAs); type of prediction (structural characteristics or machine learning (ML) based); immunoglobulin isotype specificity; a URL link to the web server. AA, amino acid.

mental allergies, eczema, asthma, and allergic rhinitis. Egg and baked-egg reactivity among CoFAR7 patients was determined using double-blind placebo-controlled oral food challenges with unheated egg-white protein (1,444 mg) or muffin for baked-egg (2,000 mg) [29]. The study was approved by the local Institutional Review Boards. All the study subjects provided informed consent. Methods are described in detail in the online supplementary Materials file.

Measurement and Quantification of Ses-IgE and Ses-IgG₄ Levels

A set of 183 overlapping peptides (15-mer, 12-mer overlap) covering the entire sequences of OVM ($n = 58$) and OVA ($n = 125$) proteins were commercially synthesized (CS Bio, Menlo Park, CA, USA). The Bead-Based Epitope Assay (BBEA) was carried out as described previously [31, 32]. A master mix of peptides coupled to LumAvidin beads (Luminex Corporation, Austin, TX, USA) was prepared in PBS-TBN buffer (1xPBS, 0.02% Tween-20, 0.1% BSA), and 100 μ L/well was added to 96-well filter plates. Plates were washed with PBS-TBN, and 100 μ L/well of 1:10 plasma dilution was incubated on a shaker for 2 h. Then plates were incubated for 30 min with 50 μ L/well of mouse antihuman secondary antibody (2 μ g/mL of IgE-phycoerythrin [PE], clone BE5; Thermo-Fisher Scientific, Rockford, IL, USA; or 0.25 μ g/mL of IgG₄-PE, clone HP6025; Southern Biotech, Birmingham, AL, USA). Plates were washed and 100 μ L/well of PBS-TBN was added. The signal was quantified as a Median Fluorescence Intensity (MFI) on the Luminex200 instrument (Luminex Corporation, Austin, TX, USA). Samples were assayed in duplicates and each plate included 2 wells with only PBS-TBN, with a plate layout generated using *PlateDesigner* [33] so that experiments are not confounded by plate and well position effects. MFIs were log₂-transformed and normalized (nMFI) by subtracting the average of the background wells using *bbeaR* R package v0.1.0 [32, 34].

Statistical Analyses

Statistical analyses were performed in R v3.5. Technical duplicates for each sample and epitope were averaged. Group comparisons were presented as ANOVA p values obtained by fitting a linear regression. Differences in sequential *ses*-IgE and *ses*-IgG₄ between egg allergic, atopic, and healthy groups were assessed by age-adjusted linear models, using the empirical Bayes method in the *limma* package v3.42, allowing estimation of the variance parameters across all epitopes [35]. The results are presented as estimated marginal means for each group; p values were adjusted for multiple testing using the Benjamini-Hochberg approach, controlling the False Discovery Rate (<0.05). Logistic regressions for each specific antibody were performed to determine their association with the baked-egg phenotype, summarized with the receiver operating characteristic (ROC) curves. A stepwise model selection was carried out to identify a best linear combination of antibodies. To obtain ROC's area under the curve (AUC) estimates, and a variability around them, logistic regressions were performed using 100 bootstrap resamples of the data using *boot* R package. This allowed for the estimation of the mean AUC and its 95% confidence interval.

Results

Epitope Mapping of OVM and OVA Proteins

Fifty-eight OVM and 126 OVA peptides were evaluated for IgE binding using BBEA in 38 egg allergic subjects with median egg-white sIgE of 50 kU_A/L and 1 control pool (negative pool, online suppl. Fig. 2). The average age of allergic patients was 6.6 years; 58% were male,

mostly of white race (75%), with >50% having atopic comorbidities, such as atopic dermatitis, asthma, and/or allergic rhinitis (Table 2). *Ses*-IgE bound to a greater number of OVM peptides and was found in more patients compared to *ses*-IgE to OVA: 46/58 (79%) of OVM peptides were detected in at least 50% of patients, while this was the case for only 14/126 (11%) of OVA peptides (Fig. 1a, b).

The nMFI values for the overlapping peptides were collapsed to the AA-level to identify immunodominant epitopes. Such epitopes were mapped to all 3 domains of OVM, with higher scores at the N-terminus (1st domain) of the protein (Fig. 1c), and in several regions of the OVA (Fig. 1e). When overlayed to the conformational structure, epitopes were located on the surface as well as buried in parts of the proteins (Fig. 1d, f). This indicates that protein breakdown or conformational change had to occur for the IgE to bind some of those residues. OVM epitopes identified with BBEA have been described in at least 2 out of 8 previous studies, while several novel IgE-binding regions were mapped for OVA (online suppl. Fig. 3).

Table 2. Demographic and clinical characteristics of 38 egg allergic subjects

	Egg allergic (n = 38)
Age, mean (SD)	6.6 (3.6)
Male, n (%)	22 (57.9)
Race, n (%)	
Asian	5 (13.2)
Other	2 (5.3)
White/Caucasian	27 (71.1)
Not Hispanic or Latino, n (%)	30 (93.8)
Asthma, n (%)	28 (73.7)
Rhinitis, n (%)	22 (57.9)
Eczema, n (%)	29 (76.3)
Egg-white sIgE, kU _A /L, median (Q1–Q3)	49.9 (22.8–90.5)

SD, standard deviation; Q1, 1st quartile; Q3, 3rd quartile.

Fig. 1. IgE epitope mapping on OVM and OVA proteins. **a, b** nMFI of 58 OVM and 125 OVA 15-mer sequential peptides measured using BBEA in 38 egg allergic children. Color shows average nMFI, peptides are arranged in the sequential order on the y-axis, and x-axis represents a proportion on patients with that peptide “present.” **c, e** Epitope score for each AA based on the average nMFI and proportion of patients with peptide “present.” Color represents score percentile across all AAs; dashed line for OVM separates 3 domains; squared shape is a glycosylation site; letters

In silico Prediction of Epitopes on Minor Egg Allergens

Using a combination of 12 B-cell epitope prediction tools, epitopes were first predicted on OVM and OVA proteins. Those epitopes were then compared to the ones mapped experimentally (Fig. 2a). A variability of predictions made it difficult to commit to a single algorithm (median positive predictive value [PPV] = 0.6). We used our experimental data to build an ensemble that optimally combined all tools into a “crowd prediction” using a Random Forest algorithm. The resultant model was tested on the remainder of the OVA protein (Fig. 2a bottom) and showed some predictive improvement (PPV = 0.7). This new algorithm was then applied to identify epitopes of minor allergens: OVT, LYS, and sALB. Sixteen epitopes from 3 minor allergens and 8 from YGP42 and YGP40 were synthesized (online suppl. Table 2). While all 12 tools contributed to the predictions, their contributions were weighted differently by the Random Forest, with BCIgEPred [36] having the highest importance index (Fig. 2b).

Experimental Testing of Epitopes from Minor Allergens and Egg Epitope Library

The 24 potential epitopes from OVT, LYS, sALB, YGP42, and YGP40 were evaluated for IgE binding using the BBEA in the same patient cohort that was used for OVM and OVA epitope mapping. As shown in Figure 3a, OVM and OVA peptides had the highest median nMFI, while IgE against minor allergens generally had lower levels. However, in a small number of patients (8/38), those IgE antibodies did show different profiles: compared to the rest of the patients, their IgE bound epitopes on OVA and minor allergens (Fig. 3b, right cluster).

To create a comprehensive egg epitope library, epitopes from all proteins were selected with more epitopes chosen from the major allergens (Fig. 3c). The selection was based on both signal intensity and variability to make sure a diverse set of epitopes was present (online suppl. Fig. 4). A few peptides with the most IgE binding from the

on a side indicate potential sites of enzymatic cleavage by P, T, and Ch. **d, f** Epitope mapping to the conformational structure of the OVM (obtained by homology modeling) and OVA (PDB: 1OVA). Epitopes are presented in different colors and correspond to the AAs from panels (**c, e**) with scores above 50th percentile. OVM, ovomucoid; AA, amino acid; PDB, Protein Data Bank; BBEA, Bead-Based Epitope Assay; nMFI, normalized Median Fluorescence Intensity; P, pepsin; T, trypsin; Ch, chymotrypsin.

(For figure see next page.)

Fig. 2. IgE epitope mapping on OVM and OVA using BBEA and in silico tools. **a** Heatmap showing AA-level epitope mapping by BBEA (1st column), and epitope predictions using RF-based ensemble (2nd column), epitope prediction by at least 6 tools (2nd

column), and 12 individual algorithms. **b** Importance metric (mean decrease in Gini index) for all 12 algorithms; higher values mean higher importance. OVM, ovomucoid; AA, amino acid; BBEA, Bead-Based Epitope Assay; RF, random forest.

minor proteins were also included, since we did not want to rule out the possibility that they could still be informative in a larger patient cohort with different levels of egg allergy severity or phenotype. The final library consisted of 65 epitopes from 7 egg proteins (online suppl. Table 3).

Ses-IgE Is Associated with Baked-Egg Reactivity

The 65-plex epitope library was evaluated on a new pediatric cohort for both IgE and IgG₄ reactivity. The new cohort included 135 egg allergic patients, of whom 82 individuals were baked-egg reactive (BER) and 53 baked-egg tolerant (BET), 46 had atopic comorbidities or food allergies other than egg, and 11 were healthy subjects (Table 3). The healthy control group appeared slightly older than the atopic and egg allergic children, with median ages of 10 versus 6 versus 8 years, respectively. To account for this when comparing the *ses*-IgE and *ses*-IgG₄, the models were adjusted for age.

On average, egg allergic children had higher *ses*-IgE and lower *ses*-IgG₄ than both atopic and healthy controls; the healthy subjects had almost nondetectable *ses*-IgE levels (Fig. 4a). Differences were observed primarily in OVM and some OVA epitopes.

When egg allergic subjects were stratified by reactivity to baked-egg, higher OVM- and OVA-sIgE (Fig. 4c), and 17 *ses*-IgE (including epitopes from minor allergens) were associated with BER phenotype (Fig. 4b). While both OVM- and OVA-sIgG₄ levels were higher in BET than BER, no differences were detected in *ses*-IgG₄ levels (Fig. 4c, online suppl. Fig. 5).

To identify which individual antibodies or their combinations were most associated with the BER phenotype, a series of logistic regressions were performed. Egg-white sIgE had the greatest AUC of the ROC curve (0.68 [95% CI 0.59–0.77]), followed by *ses*-IgE to OVM-014 (AUC = 0.67 [0.57–0.77], Fig. 4d, e). We identified a linear combination

Fig. 3. Egg epitope library. **a** Median and 3rd quartile of nMFI for all OVM and OVA peptides and 24 potential epitopes from OVT, LYS, sALB, YGP42, and YGP40 proteins, measured using BBEA in 38 egg allergic children. **b** Heatmap of row-scaled nMFI for all peptides tested with BBEA; peptides are arranged by their sequential order within a protein; subjects are grouped by unsupervised hierarchical clustering with Euclidean distance and "average" ag-

glomeration algorithm. Right cluster (highlighted with pink horizontal bar) shows less IgE binding to OVM epitopes. **c** A schematic and a number of epitopes selected from each egg protein for the final 65-plex library. OVM, ovomucoid; OVT, ovotransferrin; LYS, lysozyme; sALB, serum albumin; YGP42, vitellogenin-1 precursor; YGP40, vitellogenin-II fragment; BBEA, Bead-Based Epitope Assay; nMFI, normalized Median Fluorescence Intensity.

Fig. 4. Association of *ses*-IgE and *ses*-IgG₄ with egg allergy. **a** EMmean of the nMFI for each of the 65 IgE (left) and IgG₄ (right) binding epitopes in egg allergic subjects, atopic and healthy children. Stars in the “egg allergic” column represent significant difference between allergic and atopic subjects; in the “healthy” column – allergic versus healthy subjects; in the “atopic” column – atopic versus healthy subjects. ***FDR < 0.05, **p* value < 0.05. **b** EMmean of the nMFI for each of the 65 *ses*-IgEs in BER and BET groups. Colored bars represent significant difference from BER (red if higher and blue if lower than BER) based on FDR (red/dark blue) or *p* value (pink/light blue). Stars atop of the bars represent

significant difference from healthy subjects, based on the *p* value. **c** Boxplot of IgE and IgG₄ levels specific to the whole OVM or OVA allergen extract, comparing BER and BET groups. **d** ROC curves for 5 top predictors of BER phenotype. **e** Estimates of mean AUCs and 95% CI of logistic regressions performed on 100 bootstrap resamples. A model combining several antibodies has best performance. OVM, ovomucoid; nMFI, normalized Median Fluorescence Intensity; FDR, False Discovery Rate; ROC, receiver operating characteristic; AUC, area under the curve; BER, baked-egg reactive; EMmean, estimated marginal means.

Table 3. Demographic and clinical characteristics of egg allergic children, atopic, and healthy controls

	BER (n = 82)	BET (n = 53)	Atopic (n = 46)	Healthy (n = 11)	p value (all groups) [§]	p value (BER vs. tolerant) ^{§§}
Age, years (mean, SD)	8.4 (2.5)	8.3 (3.2)	6.6 (2.7)	9.9 (4.2)	0.001	0.796
Male sex, n (%)	44 (53.7)	34 (64.2)	28 (60.9)	7 (63.6)	0.635	0.304
Race, n (%)						
Asian	7 (8.5)	4 (7.5)	6 (13.6)	0 (0.0)		
Black/African American	2 (2.4)	3 (5.7)	4 (9.1)	2 (66.7)		
White	63 (76.8)	43 (81.1)	33 (75.0)	1 (33.3)	0.001	0.485
Other	10 (12.2)	3 (5.7)	1 (2.3)	0 (0.0)		
Asthma history, n (%)	50 (61.0)	32 (60.4)	21 (47.7)	0 (0.0)	0.099	0.999
Allergic rhinitis history, n (%)	68 (82.9)	45 (84.9)	26 (59.1)	0 (0.0)	<0.001	0.948
Atopic dermatitis history, n (%)	52 (63.4)	34 (64.2)	24 (54.5)	0 (0.0)	0.001	0.999
Baked-egg OFC, mg (median, Q1–Q3)	550 (100–780)	2,000 (2,000–2,000)				<0.001
Egg-white OFC, mg (median, Q1–Q3)		144 (44–444)				
Serology (median, Q1–Q3)						
Total IgE, kU/L	862 (392–1,677)	873 (304–1,741)	357 (151–1,001)	74.8 (28.9–98.5)	<0.001	0.968
Egg IgE, kU _A /L	24.2 (12.7–65.0)	10.8 (6.7–27.1)	0.1 (0.1–0.5)			<0.001
OVA IgE, kU _A /L	14.7 (6.0–29.6)	5.8 (3.4–17.4)				0.001
OVM IgE, kU _A /L	15.1 (6.1–38.1)	7.0 (2.5–13.4)				0.001
Egg IgG, mg/mL	5.2 (2.9–8.5)	7.5 (3.8–13.1)				0.027
Egg IgG ₄ , mg/mL	0.5 (0.2–1.1)	0.8 (0.2–3.1)				0.044
OVA IgG ₄ , mg/mL	0.4 (0.1–1.0)	0.6 (0.1–1.9)				0.134
OVM IgG ₄ , mg/mL	0.1 (0.0–0.3)	0.2 (0.0–1.4)				0.091

SD, standard deviation; Q1, 1st quartile; Q3, 3rd quartile; OVA, ovomucoid; OVM, ovomucin; BER, baked-egg reactive; BET, baked-egg tolerant. [§]ANOVA p values for the difference among 4 groups obtained by fitting a simple linear regression model. ^{§§}p values for the comparison of the baked-egg reactive and tolerant groups using t test for normally distributed variables, Wilcoxon-Mann-Whitney test for non-normal variables, and χ^2 test for categorical variables.

of OVA-sIgE and *ses*-IgEs to OVM-023 and OVA-28 that together was more predictive of the BER phenotype (AUC = 0.72 [0.63–0.76]) than any individual antibody.

Discussion

B-cells are capable of generating a specific antibody response against up to 10^{15} unique molecules [37], making epitope mapping an arduous task. Many experimental methods can be used for functional epitope identification, including competition, antigen fragmentation or modification, and peptide library assays [19]. Screening peptide libraries is a common approach in food allergy research since it allows simultaneous evaluation of many epitope targets. Common techniques include PEPSCAN, SPOT membranes, peptide microarrays, bead-based, or phage immunoprecipitation assays. Over the years, our group has moved from SPOT membranes to microarrays to BBEA technology, due to the higher sensitivity, reproducibility, rapid throughput, and simpler laboratory protocols of the BBEA [31, 32]. Using this assay, we screened all sequential peptides along the OVM and OVA backbone – the 2 most allergenic proteins of egg-white. We were able to confirm epitopes on OVM that have been identified previously [4, 6, 7, 38–42], and detect several OVA epitopes. Since we expected a small number of patients to have detectable IgE to minor allergens – OVT, LYS, sALB, YGP42, and YGP40 – and given that combined those allergens would constitute >450 peptides, we first conducted an epitope screening *in silico*.

To date, the most optimistic performances among a range of structure- and sequence-based *in silico* methods are at best modest, with an area under the curve metric usually below 0.70 [19, 43]. Since epitope databases used in building these methods are derived from an aggregate of bacterial, viral, fungal, environmental, and autoantigens, which usually encounter the immune system in an intact form [19], the applicability to predicting epitopes on food proteins, which can be subject to physiological denaturing, is even less optimistic. In our study, we observed great variability among the 12 algorithms with an average PPV of only 0.6. Recently, the principle of “wisdom of crowds” that utilizes best aspects of multiple algorithms has been recognized as a more robust approach [44], and when combined, the PPV of those tools increased to 0.7. Additionally, in allergy, the antibody of interest is IgE, which develops its high affinity to antigens over multiple rounds of receptor maturation [45]. However, most *in silico* tools provide isotype-agnostic predic-

tions, which are generally derived from data dominated by the IgG subclass [46]. Not surprising, in our ensemble method, the most important predictor was BCiGEpred, which was created specifically for IgE-binding epitopes [36]. Using a combination of experimental and computational approaches, we proposed a library of 65 potentially informative epitopes and showed that varying levels of IgE to these epitopes can be detected among egg allergic children.

Based on the previous experience, we opted for use of 15-mer peptides, which is an average length of a B-cell epitope [18, 19, 47]. While there is a possibility that shorter or longer peptides could have better IgE binding, as observed in a study using peptide microarrays [41], our goal was to create an epitope library that is standardized and experimentally viable. We focused on sequential epitopes, since currently there is no reliable way of testing conformational epitopes, other than using whole antigens that contain both epitope types and competing out the overall binding effect of sequential epitopes. Additionally, it has been shown that persistent egg and milk allergy are associated with both type and number of sequential epitopes [6, 24, 48, 49].

The persistence of egg allergy is usually associated with reactivity to baked egg. In fact, ~70%–80% of egg allergic children are able to tolerate heat-denatured, baked-egg products [20–22, 50] and most of these will tolerate all forms of egg by 6 years of age [23], if baked-egg is incorporated in their diet [51–53]. Thus, knowing epitope profiles that trigger pathogenic IgE could provide useful diagnostic information and inform therapeutic decisions. Additionally, increases in egg-specific IgG₄ antibodies have been observed in patients desensitized by immunotherapy [54–56], and their epitope-specific profiles could be associated with allergic phenotypes. Using our novel epitope library, we compared *ses*-IgE and *ses*-IgG₄ levels in BER and tolerant children, as well as atopic and healthy subjects. We observed that egg allergic subjects had higher levels of *ses*-IgE, especially to OVM epitopes, than the control groups, but lower *ses*-IgG₄ levels. Atopic and healthy children had comparable *ses*-IgG₄, likely reflecting egg consumption by children in those groups [57]. When egg allergic children were stratified by baked-egg reactivity, the BET group had lower levels of 17 *ses*-IgEs than the reactive children. The ability of antigen- and epitope-specific IgEs to differentiate between BET and BER phenotypes is considered “moderate,” with AUCs of 0.66–0.68. A combination of 3 antibodies (OVA-sIgE, OVM-023 *ses*-IgE, and OVA-028 *ses*-IgE) improved the model performance, with AUC of 0.72. These results sug-

gest that combining antigen- and epitope-specific IgEs helps differentiate between the 2 phenotypes, but additional mechanisms must be contributing to disease pathogenesis. Berin et al. [24] previously showed that in addition to sIgE to egg-white, OVM and OVA, the BER phenotype is associated with basophil responses, but not egg-specific IgG or egg reactive T-cells [24]. In subsequent work, we are evaluating if the addition of other epitope-specific antibody isotypes (IgM, IgD, IgG_{1,2,3,4}, and IgA_{1,2}) will provide better differentiation between BER and BET subjects.

In the initial testing for the epitope library selection, we were able to detect low levels of *ses*-IgE to minor allergens in a small number of subjects. When evaluated on BER and tolerant subjects, most differences were observed for the epitopes on OVM and OVA allergens. While IgE sensitization to proteins in yolk was shown to be helpful in prognosis of allergy persistence [58] or low egg-white to egg-yolk IgE ratios could predict children who are able to consume hard-boiled eggs [14], there was a large heterogeneity among sIgE levels and skin prick test positivity. It is possible that IgE to minor allergens could be more informative when measured against whole antigens, preserving conformational epitopes; or there may be other sequential epitopes that are not represented in our epitope library.

This work is a first attempt to define a comprehensive egg epitope library. We used epitope prediction tools and exclusively considered 15-mer peptides; the ensemble method was developed using only 2 egg allergens. Development of epitope prediction tools tailored to food allergens and IgE isotype, accounting for protein denaturing, is still needed for robust epitope mapping.

Overall, we have created an epitope library with experimentally derived epitopes from major OVM and OVA allergens and included a set of computationally derived epitopes from minor allergens – OVT, LYS, sALB, YGP42, and YGP40. We showed that *ses*-IgE is associated with BER phenotype, and in future work, we will evaluate epitope-specific antibody repertoires in response to therapeutic interventions, for example, baked-egg therapy or oral immunotherapy, and across different antibody isotypes.

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Statement of Ethics

A Data Safety Monitoring Board of the Division of Allergy, Immunology and Transplantation of the National Institute of Allergy and Infectious Diseases, and local Institutional Review Boards approved study procedures. The CoFAR7 study was registered on clinicaltrials.gov (NCT01846208: CoFAR7 Baked egg or egg oral immunotherapy for children with egg allergy). All study subjects (or their parents/guardians) provided written informed consent.

Conflict of Interest Statement

Dr. Suprun, Ms. Grishina, Dr. Dunkin, and Dr. Leung have nothing to disclose. Dr. Sicherer reports grants from Food Allergy Research and Education, grants from HAL Allergy, and NIH-NIAID, personal fees from American Academy of Allergy, Asthma and Immunology, UpToDate, Johns Hopkins University Press. Dr. Wood reports grants from NIH-NIAID, Astellas, DBV, Aimmune, Regeneron, Sanofi, other from Up To Date. Dr. Jones reports grants from NIH-NIAID; personal fees from Food Allergy Research and Education, Aimmune Therapeutics, EMMES Corporation; grants DBV Technologies, Astellas, Inc., FARE, NIH-NIAID, Sanofi, Regeneron, and Genentech, Inc. Dr. Burks reports personal fees from Aimmune Therapeutics, Inc., Astella Pharma Global Development, Consortia TX, Inc., DBV Technologies, Intrimmune Therapeutics, Prota Therapeutics, N-Fold, LLC, Aravax, Hycor Biomedical, AllerGenis, kaléo, UKKO, Inc.; grants from NIH, Johns Hopkins/NIH, FARE, stock from Allertein, Mastcell Pharmaceuticals, and UpToDate royalties. Dr. Burks has patents US#7879977, US#6835824, US#6486311, US#6441142, US#5973121, US#5558869 with royalties paid. Dr. Getts and Mr. Witmer are employees of Genisphere LLC. Dr. Getts is a scientific consultant of AllerGenis LLC and has a patent PCT/US15/020,715 (WO) pending. Dr. Suárez-Fariñas received research funding to Mount Sinai by a grant from AllerGenis LLC. Dr. Sampson reports grants from Immune Tolerance Network, and NIH-NIAID, consulting fees from DBV Technologies, N-Fold Therapeutics, and Siolta Therapeutics; Mount Sinai has licensed the technology for a BBEA for food-allergen epitope analysis to AllerGenis LLC, where Dr. Sampson serves as an unpaid Board of Directors member and advisor.

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Author Contributions

The experiments were conceived and designed by M.S., M.SF., and H.S. Sample acquisition and experimental procedures were performed by M.S., G.G., M.W., and D.D. B.G. provided technical

information and advice on the components of the BBEA. Data were analyzed by M.S. and M.SF.; and M.S., M.SF., and H.S. drafted the paper. S.H.S., R.A.W., S.M.J., D.Y.M.L., and A.W.B. were site PIs for CoFAR2 and CoFAR7 and provided information and advice regarding participant characterization. All authors provided critical review and approval of the manuscript.

Data Availability Statement

The data that support the findings of this study consist of multiple data sources and are available from the first author (M.S.) or the corresponding author (H.S.) upon reasonable request.

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